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A simple and efficient method for extraction of Taq DNA polymerase

Sique Chen, Xiujuan Zheng, Hongrui Cao, Linghui Jiang, Fangqian Liu, Xinli Sun *

Key Laboratory of Ministry of Education for Genetics, Breeding and Multiple Utilization of Crops, Fujian Agricultural & Forestry University, Fuzhou 350002, China

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ABSTRACT

Background: Thermostable DNA polymerase (Taq Pol I) from *Thermus aquaticus* has been widely used in PCR, which was usually extracted with Pluthero's method. The method used ammonium sulfate to precipitate the enzyme, and it saved effort and money but not time. Moreover, we found that 30–40% activity of Taq Pol I was lost at the ammonium sulfate precipitation step, and the product contained a small amount of DNA.
Results: We provided a novel, simplified and low-cost method to purify the Taq Pol I after overproduction of the enzyme in *Escherichia coli*, which used ethanol instead of ammonium sulfate to precipitate the enzyme. The precipitate can be directly dissolved in the storage buffer without dialysis. In addition, DNA and RNA contamination was removed with DNase I and RNase A before precipitation, and the extraction procedure was optimized. Our improvements increase recovery rate and specific activity of the enzyme, and save labor, time, and cost.
Conclusions: Our method uses ethanol, DNase I, and RNase A to purify the Taq Pol I, and simplifies the operation, and increases the enzyme recovery rate and quality.

1. Introduction

PCR is a most widely-used technique in biology, which utilizes thermostable DNA polymerase from *Thermus aquaticus* (Taq) [1,2]. The gene that encoded the Taq DNA polymerase had been cloned and expressed in *Escherichia coli* efficiently [3]. This has greatly facilitated the production and reduced the price of the enzyme. Even so, the expense spent on the enzyme is also very great to some labs, for example, some genetics labs which need to do a large number of PCRs to detect polymorphism of molecular markers (SSR, RAPD, AFLP, and CAPS etc.). Thus, many labs made the enzyme by themselves to save money.

The full-length Taq DNA polymerase (Taq Pol I) gene encodes a 94 kD native protein with 832 amino acids [3]. Subsequently, a 61 kD Taq Pol I, termed 'Stoffel fragment' with 544 amino acids, was obtained. Comparing with the full-length Taq Pol I, the deletion version lost 5′ to 3′ exonuclease activity [4].

Lawyer et al. [3] firstly expressed the gene in the *E. coli* and isolated the enzyme with its thermostable property [3]. Engelke et al. [5] further purified the enzyme by precipitation with polyethyleneimine, and then refined through the column of BioRex 70 ion exchanger after incubating it in a 75°C water bath. Their product exhibited a strong Taq Pol I band and a few weak bands on SDS-PAGE gel. However, it takes at least one day to complete the extraction with a busy working [5]. Lawyer et al. [4] developed another scheme to obtain the enzyme without nucleic acid contamination [4]. Polyethyleneimine and ammonium sulfate were used to precipitate the A260 absorbing material, and phenyl-sepharose CL-4B was used to remove the residual nucleic acids, but the method was more complicated than Engelke's. Actually, *E. coli* proteins cannot affect the activity of Taq Pol I [6], but the DNA contamination may influence PCR, and some short DNA fragments may be as primers to produce false positive bands, especially using *E. coli* DNA as substrate. Pluthero [7] simplified Engelke's method by precipitating the enzyme with ammonium sulfate and then dialyzing against two changes of the storage buffer [7]. This method was widely used for homemade Taq Pol I, and it saved effort and money but not time comparing with Engelke's method. However, we found that 30–40% activity of Taq Pol I was lost at the ammonium sulfate precipitation step, and the product contained a small amount of DNA.

Stoffel fragment was more thermostable than Taq Pol I [4], and this property was used to quickly extract Stoffel fragment within boiling lysis of bacterial expressed culture, and the DNA was removed with dibasic phosphate and ethanol extraction [8,9]. We tried the method and found that it was not suitable to Taq Pol I. However, we discovered by accident that ethanol was able to effectively precipitate the enzyme and less affected its activity. Thus, we attempted to improve Pluthero's method with the feature.

* Corresponding author.
E-mail address: xinlisun@hotmail.com (X. Sun).
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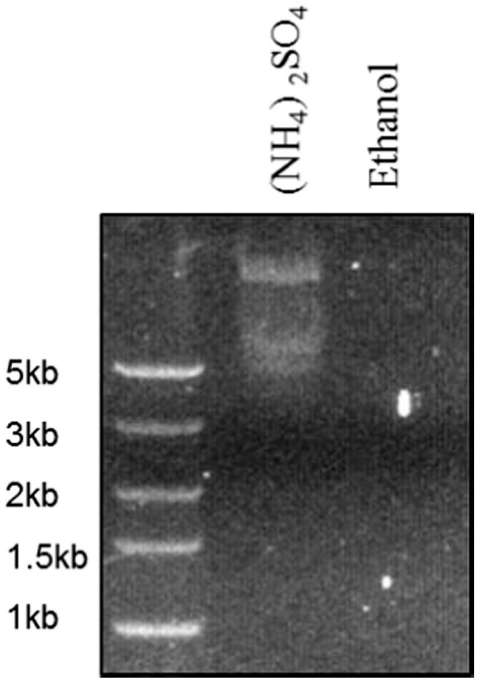

Fig. 1. The detection of the DNA in the Taq Pol I enzyme extracted with Pluthero's ($(NH_4)_2SO_4$) and our methods (Ethanol). Ten microliter enzyme was added for the electrophoresis analysis.

2. Materials and methods

2.1. Extraction and purification of Taq Pol I

The *E. coli* strain (DH1) with Taq Pol I gene was a gift from Dr. Bo Ding and Dr. Guoliang Wang in the Ohio State University. The gene expression was induced by IPTG.

The *E. coli* cell culture and inducible expression of the *Taq Pol* I gene referred to Pluthero's method [7]. After 12 h of induction the cells were harvested by centrifugation, and washed in 50 mL of buffer A (50 mM of Tris-HCl, pH 7.9; 50 mM of dextrose, 1 mM of EDTA, 1 mM of PMSF) per liter of original culture volume. The cell pellet was re-obtained by centrifugation, and re-suspended in 50 mL of buffer A. After twice freezing in liquid nitrogen and thawing in room temperature, 1 mL of lysozyme (100 mg/mL) was added, and the mixture was kept at room temperature for 15 min. An equal volume of lysis buffer was added (10 mM of Tris-HCl, pH 7.9; 50 mM of KCl, 1 mM of EDTA, 1 mM of DTT, 1 mM of PMSF, 0.5% of Tween 20, 0.5% of Nonidet P40). The lysis mixture was incubated at 75°C for 30 min, and centrifuged at 16,000 × g for 10 min at 4°C to remove the cell debris. One percent of the digestive solution (10 mM of Tris-HCl, pH 7.5; 15 mM of NaCl, 2 mM of CaCl2, 100 mM of MgCl2, 50% of glycerol, 0.5 mg/mL of DNase I, 10 mg/mL of RNase A) with PMSF (Final concentration 0.5 mM) was added in the mixture to remove DNA and RNA, after the

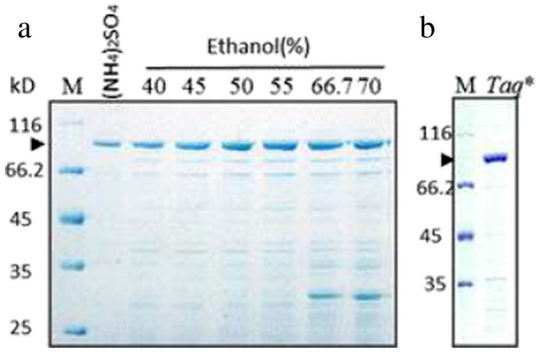

Fig. 3. The purities of the enzymes were detected with SDS-PAGE, and 10 μL enzyme was added for the analysis. a: The enzymes were precipitated by ethanol of different concentrations, and Sangon Biotech DNase I (DD0099) was used to remove DNA. b: The enzyme was precipitated by 55% ethanol, and Thermo Scientific™ DNase I (RNase-free, EN0521) was used to remove DNA. *Taq Pol I.

mixture temperature drops to room temperature. Five min later, the mixture was treated for 30 min in a 75°C water bath again to denature DNase I, and centrifuged at 16,000 × g for 10 min at 4°C. The supernatant was transferred to other plastic bottles, and added ethanol to the final concentration of 55%, and centrifuged at 16,000 × g for 15 min at 4°C to recover Taq Pol I. The precipitate was dissolved in 25 mL of storage buffer (50 mM of Tris-HCl, pH 7.9; 50 mM of KCl, 0.1 mM of EDTA, 1 mM of DTT, 0.5 mM of PMSF, 50% of glycerol) and then stored at -20°C.

2.2. Activity and protein assays

The enzyme activity was determined by multiple titrations with the PCR assay relative to commercial Taq DNA polymerase (Sangon Biotech). Protein concentrations were determined with Bradford method. Protein content was visualized by electrophoresis through denaturing polyacrylamide gels (4% stacking gel, 12% separating gel) and staining with Coomassie brilliant blue R.

2.3. PCR and real-time PCR

PCR reactions were carried out in a total volume of 20 μL using GeneAmp PCR system 9700 or Eppendorf Mastercycler, and the mixture contained 20 mM of Tris-HCl, pH 8.4; 1.5 mM of $MgCl_2$, 20 mM of KCl, 10 mM of $(NH_4)_2SO_4$, 0.1% of Triton × 100, 0.2 mM of dNTP, 0.1 μM of primers, 50 ng of rice DNA and 0.2 μL of Taq Pol I. Real-time PCR was performed in a total volume of 20 μL using ABI 7500 Real time PCR system, and the SYBR green (final concentration: 1/20,000) and ROX (final concentration: 1/400) were purchased from Invitrogen. They were added in the PCR mixture with 0.5 μL of enzyme and other materials. All comparative experiments were performed with the same enzyme activities.

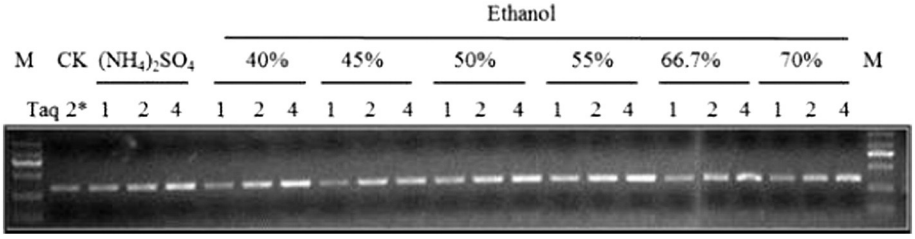

Fig. 2. The activities of the Taq Pol I enzymes extracted with different methods were compared with electrophoresis analysis. *The microliters of the enzymes used for PCR, which were diluted from 1 μL to 20 μL with PCR buffer. M, DNA marker (from upper to lower band, 1500, 1000, 750, 500, 250 and 100 bp); CK, Commercial enzyme from Sangon Biotech as a positive control; $(NH_4)_2SO_4$, extracted with Pluthero's method; Ethanol, extracted with ethanol of different concentrations. Rice *actin1* gene (342 bp) was amplified.

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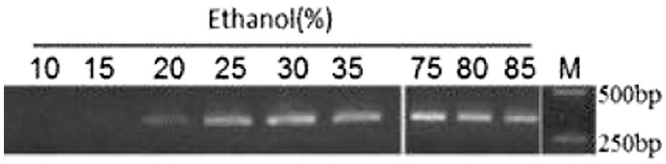

Fig. 4. The activities of the enzymes extracted with ethanol of lower or higher concentrations were detected. Rice *actin1* gene (342 bp) was amplified.

3. Results

Yang's method was used to quickly extract the Stoffel fragment without DNA contamination [9]. We tried to use the method to extract Taq Pol I, but found that boiling water was not able to effectively disrupt the *E. coli* cells. We used freezing and thawing treatment and lysozyme to lyse the cells, and obtained the enzyme according to Yang's method. The activity of fresh Taq Pol I was fine, but its activity was lost after two d of storage at -20°C. Sediment was found in the storage. We re-dissolved the sediment with our storage buffer to detect its activity, and found that no activity remained in the precipitate. These suggested that the method was not suitable to Taq Pol I. We fortunately discovered that two volumes of ethanol were able to effectively precipitate the protein when we tried the upper method. So we thought that the feature should be used to purify the enzyme, but we must find a way to remove DNA and RNA contamination before the attempt.

We used DNase I and RNase A to digest DNA and RNA contamination. Lysis mixture was treated in water bath at 75°C for 30 min, and centrifuged to remove most of *E. coli* proteins and DNA. DNase I and RNase A were added in the supernatant at this step. The mixture was kept at room temperature for 5 min, and then treated at 75°C for another 30 min to denature DNase I and *E. coli* protein residues. DNA and RNA in the purified enzyme were checked with electrophoresis, and no contamination was found in our enzyme, but DNA was revealed in the enzyme extracted with Pluthero's method (Fig. 1).

To find the optimal ethanol concentration, we detected the activities and purities of these enzymes separated out with different ethanol concentrations (Figs. 2 and 3a). The result showed that there were no obvious differences among enzyme activities (Fig. 2), but the enzyme precipitated with 55% ethanol is a little better than others. The sediments in 66.7% and 70% ethanol contained a strong contaminated protein band (Fig. 3a). Thus, we think that the best ethanol concentration is 55% according to the present result. To reveal the lower and upper limits of the concentration, the enzyme activities extracted with ethanol of lower or higher concentrations were detected (Fig. 4). The result showed that 20% ethanol is the lower limit, but no upper limit was found. Higher ethanol concentration decreased the activity of the enzyme (Fig. 4).

We found the quality of DNase I strongly influenced the recovery rate of the enzyme. Some DNases contain a little protease contamination that was not easy to be removed and digested Taq Pol I protein. We firstly used the DNase I (Sangon Biotech, DD0099), and found it decreased the recovery rate of Taq Pol I to 50% (Table 1), which is lower than Pluthero's method (Table 1). Moreover, a long time treatment and more DNase I would destroy all enzymes. We tried another DNase I (Thermo Scientific™ DNase I, RNase-free, EN0521), and found that this change increased the recovery rate of Taq Pol I (93.3%) and the specific activity (Table 1), and improved the enzyme purity (Fig. 3b). The purified enzyme was able to conserve at -20°C for several months without obvious activity losing.

To examine whether the enzyme extracted with our method can be applied to a variety of experiments like the Pluthero's extracted. We amplified rice DNA fragments of different lengths and Wrky10 gene with high GC content using the enzymes extracted by two methods, and obtained a similar result (Fig. 5a and b). We also used the enzyme in real-time PCR to analyze rice actin1 gene, and the result indicated that both of the enzymes extracted with our and Pluthero's method can be applied to real-time PCR (Fig. 5c). At the present time, most PCRs have been done with this enzyme in our lab.

4. Discussion

Since proteins differ markedly in their solubilities at high ionic strength, salting-out is the most common method used to precipitate a target protein. Ammonium sulfate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed. The ammonium sulfate concentration is increased stepwise, and the specific protein can be recovered at a certain stage [10,11]. The method is usually used in the early step of protein purification to remove most of undesired protein. Because Taq Pol I is a high thermostable protein, most of unspecific proteins were removed with heating, and Taq Pol I was left in the supernatant and then was precipitated with ammonium sulfate (30 g/100 mL lysate) [7]. However, we found that the concentration of ammonium sulfate cannot make all of Taq Pol I salting-out, and we tried to increase the concentration, but the effect of salting-out was not better (data not shown). In addition, adding ammonium sulfate will change the pH value that could affect the enzyme activity and stability, and will increase the density of the solution that makes the operation difficult because the protein of salting-out is not on the bottom of centrifuge tube after centrifuged. The high concentration ions in the protein must be removed with dialysis, which increases labor, time, cost, and loss of the enzyme activity. These imply the salting-out with ammonium sulfate is not a desired method to purify the enzyme.

Many organic solvents were also used to purify the protein, and they can decrease solvation layer around the protein, and then increase attractive electrostatic and dipole forces that make protein aggregation and precipitation [10,11]. On the other hand, organic solvents can influence protein stability, and even make it denaturation [12]. The selection of organic solvents to purify a certain protein is very difficult, because different proteins show different tolerances and solubilities to the same or distinct organic reagents, and these need to be determined by experiments [10,11]. That is why the protein precipitation with organic solvents is not commonly used in the purification comparing with salting-out. We occasionally discovered that ethanol can efficiently precipitate Taq Pol I and scarcely affected its activity, and tested the enzyme's tolerance and solubility in different ethanol concentrations. We also established an extraction method of the enzyme with the character, which made the operation easy and

Table 1
Taq DNA polymerase purification summary.

	Pluthero's method			Our method with DNase I (DD0099)			Our method with DNase I (EN0521)		
Enzyme fraction	Protein (mg)	Activity (U)	specific Activity (U/mg)[c]	Protein (mg)	Activity (U)	Specific activity (U/mg)	Protein (mg)	Activity (U)	Specific activity (U/mg)
Cleared lysate[a]	10.49	100,000	9532	10.88	90,000	8272	0.51	5400	9310
Treated[b]				9.279	60,000	6466	0.494	5400	10,931
Purified	2.76	62,500	22,644	2.23	45,000	20,215	0.092	5040	54,782

[a] The lysis mixture was incubated at 75°C for 60 min to Pluthero's method, and 30 min to our method, and then centrifuged to get cleared lysate.
[b] The cleared lysate was treated with DNase I, and incubated at 75°C for another 30 min, and then centrifuged. The enzyme activity of the supernatant was tested.
[c] Is the activity of an enzyme per milligram of total protein.

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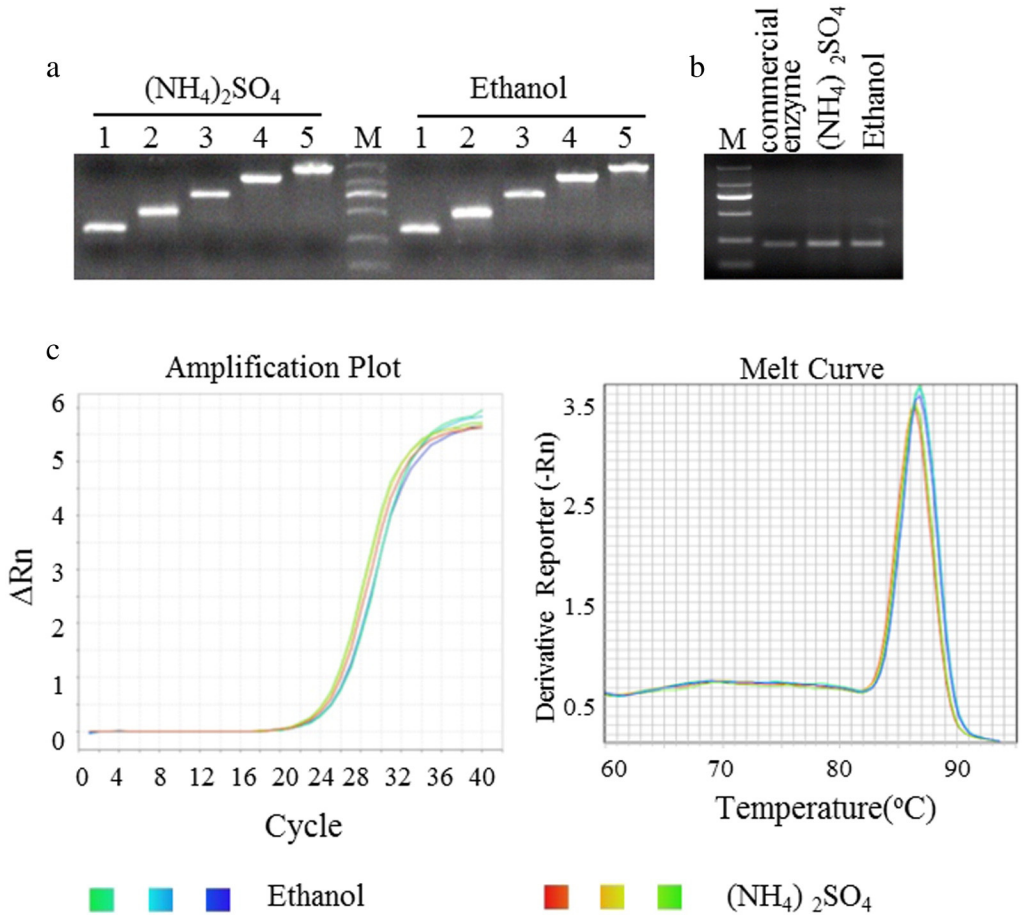

Fig. 5. a: The enzymes were used to amplify different size DNA fragments in rice. 1, 342 bp; 2, 508 bp; 3, 759 bp; 4, 1172 bp; 5, 1515 bp. M, DNA marker (from upper to lower band, 1500, 1000, 750, 500, 250, and 100 bp). b: Rice *Wrky10* gene with high GC content (69.4%) was amplified with different Taq DNA polymerases, and 3% DMSO was added in the PCR mixture. c: The enzymes extracted with our and Pluthero's method were used to analyze the actin1 gene in rice with qPCR, and each enzyme was repeated three times. The amplification plot and melt curve were shown.

saved time and cost. This method may be used to extract other thermostable DNA polymerase from thermophilic bacteria and archaea, such as *pfu* DNA polymerase.

Though Taq DNA polymerase has been cloned since 25 years ago [3] and is not expensive now, the expense on the enzyme is also astonishing to some labs that need to do large amount of PCRs to analyze the polymorphism of molecular markers for construction of a genetic map, positional cloning, and evolutionary analysis etc. We invented a novel method to extract and purify Taq Pol I, which can be easily used for large scale extraction in many labs and companies without a need of expensive equipment and skilled biochemical techniques. This can interest many laboratories and companies that hope to make their own Taq DNA polymerase for their PCR assays.

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Competing interests

The authors declare no competing interests.

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