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Purification of *Thermus aquaticus* DNA Polymerase Expressed in *Escherichia coli*

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DNA polymerase from *Thermus aquaticus* has become a common reagent in molecular biology because of its utility in DNA amplification and DNA sequencing protocols. A simplified method is described here for isolating the recombinant *Taq* enzyme after overproduction in *Escherichia coli*. Purification requires 8 to 10 h and entails heat treating and clearing the *E. coli* lysate, followed by precipitation of the enzyme with polyethyleneimine and elution from Bio Rex 70 ion exchange resin in a single salt step. The resulting enzyme preparation contains a single, nearly homogeneous protein consistent with the previously established size of the *Taq* DNA polymerase in a yield of 40–50 mg of protein per liter of cell culture.

In recent years experimental possibilities have been radically affected by the advent of selective DNA segment amplification *in vitro* using the polymerase chain reaction [PCR, (1)][2]. Central to the wide dissemination of these techniques has been the introduction of heat stable DNA polymerases, primarily from *Thermus aquaticus* (*Taq*), so that the process can be automated using a thermal cycler without continuous addition of fresh DNA polymerase (2). Methods for cloning, direct sequencing, clinical diagnosis, and myriad other uses (3) have proliferated with the ability to acquire microgram quantities of particular DNA fragments from mixtures as complex as whole genomes and present in quantities as low as the DNA or RNA from a single cell.

Since these combined applications often result in large expenditures of enzyme, it is of some interest to have a method by which individual laboratories can easily purify large quantities of the *Taq* DNA polymerase for routine use. We describe here the use of an overproducing plasmid construct of the *Taq* DNA polymerase gene with a simple 1-day purification scheme to yield approximately 1 g of nearly homogenous enzyme from a 12-liter culture of *Escherichia coli*.

MATERIALS AND METHODS

Strains and culture media. *T. aquaticus* strain YT-1 was the gift of D. Hunt and N. Pace (Indiana University) and was grown at 55–60°C on solid medium containing 1.5% agar and nutrients described previously (4). *E. coli* strain DH1, used as a host for recombinant plasmids to overproduce the *Taq* DNA polymerase, was grown in LB medium supplemented with 80 μg/ml ampicillin to propagate plasmids and indicated concentrations of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to induce production of the enzyme.

Activity and protein assays. Protein concentrations were determined by measuring absorbance ratios at 280 and 260 nm. Protein content was visualized by electrophoresis through denaturing polyacrylamide gels [5% stacking gel, 10% separating gel, (5)] and staining with Coomassie brilliant blue R. DNA polymerase assays during purification were performed by monitoring incorporation of radiolabeled dATP into high molecular weight DNA using a primed M13 viral template. Briefly, 1 μl of serially diluted enzyme fraction was assayed in a final volume of 5 μl containing 50 mM Hepes (pH 7.9) (*N*-2 hydroxyethylpiperizine-*N*′-2-ethanesulfonic acid), 1.5 mM $MgCl_2$, 50 mM KCl, 100 μM each dGTP, dCTP, and dTTP, 10 μM dATP including 1 μCi [α-^{32}P]dATP, 1 μg M13mp18 single-stranded DNA, and 0.1 μg M13 universal primer 17mer. Reactions were incubated for 1 min at 37°C and then for 3 min at 65°C. Deionized formamide (10 μl) containing 0.1% xylene cyanol was added to terminate the reactions and the samples were heated

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[2] Abbreviations used: PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PMSF, phenylmethylsulfonyl fluoride; PEI, polyethyleneimine.

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TABLE 1

Taq DNA Polymerase Purification Summary

Enzyme fraction	Protein (mg)	Nucleic acid (mg)	Activity[a]
Cleared lysate	8460	1020	7×10^6
PEI eluate	2640	48	7×10^6
BioRex 70 eluate	1140	nd[b]	6×10^6

Note. Protein, nucleic acid and enzyme activity content is given for major fractions of the isolation. Assay procedures are described under Materials and Methods.

[a] Enzyme activity approximated by comparison to commercial enzyme in both primed M13 and PCR assays.

[b] None detectable.

before analysis of 5 μl aliquots by electrophoresis through denaturing 8% polyacrylamide gels (6) and autoradiography for 5–60 min using DuPont Cronex 4 film. Titration of enzyme fractions using polymerase chain reaction amplification from human genomic DNA was performed in 50-μl reactions with 1 μl of diluted enzyme added immediately before overlaying with mineral oil and beginning temperature cycling. Reactions contained 50 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 50 mM KCl, 100 μM dATP, dGTP, dCTP, and dTTP, 0.6 μg human total DNA, and 0.1 μg each of two 35mer primers (5′-CTTCAGCGAGGCACAGTCCAAAGGGGACATCCTGC-3′ and 5′-CATGACGTCCACAAGGCTCAGCAAATGGGCTTTCT-3′) that amplify a 2100-base pair fragment of the human von Willebrand factor genomic DNA including portions of exons 28–30 (8,9). Reactions were cycled 35 times for 1.25 min at 94°C and 3 min at 72°C. Aliquots of 5 μl were analyzed by electrophoresis through 1.2% agarose gels in the presence of TBE buffer [90 mM Tris–HCl, pH 8.3, 90 mM borate, 25 mM EDTA (ethylenediaminetetracetate)] and 0.3 μg/ml ethidium bromide using λ phage DNA cleaved with *Bst*EII (Bethesda Research Laboratories) as size markers. Since the most common practical uses for this enzyme are primer extension sequencing and PCR, enzyme activity was determined by multiple titrations with both the primed M13 assay and the PCR assay relative to commercial *Taq* DNA polymerase (Cetus Corp.). Enzyme activity given in Table 1 is expressed as previously defined units (7).

Construction of overproducing clone. *T. aquaticus* DNA was isolated from duplicate individual colonies of strain YT-1 as described (7) except that the colonies were suspended in only 200 μl of lysis buffer and the preparation was scaled down proportionately. A 2500-base pair fragment containing the *Taq* DNA polymerase gene was prepared by PCR amplification from 0.1, 0.2, or 0.5 μg of this DNA template (either uncut or after cleavage with *Bst*EII) and 0.2 μg each of two primers that annealled to opposite ends of the coding region. The 5′ (amino terminal) primer sequence was 5′-CACGAATTCGGGGATGCTGCCCCTCTTTGAGCCCAAG and the 3′ (carboxyl terminal) primer was 5′-GTGAGATCTATCACTCCTTGGCGGAGAGCCAGTC, with the first 9 nucleotides in each case being extrinsic to the *Taq* DNA polymerase gene and creating the underlined unique *Eco*RI and *Bgl*II restriction endonuclease sites, respectively, at the two ends of the amplified DNA fragment. Thirty-five-cycle PCR reactions were performed under standard buffer conditions (3) with alternating incubations of 94°C for 1 min and 72°C for 3 min, ending with one 10-min incubation at 72°C. The resulting amplification products were cleaved with *Eco*RI and *Bgl*II and DNA fragments of approximately 2500 bp were isolated from low melting point agarose gels. The fragments were ligated into expression vector pTTQ18 [Amersham, (10)] that had been cleaved with *Eco*RI and *Bam*HI, giving a fusion that uses the tac promoter, ribosome binding site, and transcription terminator from the vector. The native *Taq* translation terminator is present, but the fusion uses the initiator methionine of the vector, replacing the first two amino acids encoded for the native enzyme (Met–Arg–· · ·) with Met–Asn–Ser–· · · (7). Recombinant plasmids were transformed into *E. coli* strain DH1 and plated on LB media with ampicillin. Correct plasmid constructions were initially identified by restriction endonuclease analysis of plasmid minipreps (11) and confirmed by inducing logarithmic 1-ml cultures for 4 h with 2 mM IPTG and testing lysates (small scale version of clarified lysates described below) for *Taq* DNA polymerase. Induced overproduction of the expected 94-kDa protein (7) was monitored with denaturing polyacrylamide gel electrophoresis (5) and activity in diluted lysates was measured with the primed M13 template assay.

Purification of Taq DNA polymerase. Large scale preparations of the *Taq* DNA polymerase were done from 12-liter batches of cells. Preparation of cell lysates was performed by minor modifications of a previously described method (7). LB media plus ampicillin was inoculated with 1 ml of a saturated overnight culture of DH1 containing the recombinant plasmid and grown at 37°C with good aeration to early exponential phase ($A_{600\,nm}$ approximately 0.2). IPTG was added to a final concentration of 0.5 mM and the cultures were allowed to continue growing for 16–20 h. IPTG titrations and time courses of induction showed no difference in the level of accumulated *Taq* protein with concentrations as low as 0.25 mM IPTG and induction times of 6 to 24 h (data not shown). Cells were harvested, resuspended in 2400 ml buffer A (50 mM Tris–HCl, pH 7.9, 50 mM dextrose, 1 mM EDTA), collected by centrifugation, resuspended in 480 ml buffer A containing 4 mg/ml lysozyme (Sigma), and incubated at room temperature for 15 min. Buffer B (480 ml) was then added [10 mM Tris–HCl, pH

7.9, 50 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Tween 20, 0.5% Nonidet-P40) and the mixture was incubated in a 75°C water bath for 60 min in 180-ml aliquots to allow more rapid temperature equilibration. Cell debris and denatured protein was removed by centrifugation at 4°C for 15 min in a Sorvall GSA rotor at 8000 rpm. Subsequent operations were performed at 4 to 8°C.

Taq DNA polymerase was recovered from this clarified lysate by precipitation with polyethyleneimine (PEI). Because the required PEI concentration was slightly variable and too much PEI inhibited precipitation, preliminary titrations were normally performed. Neutralized 10% PEI (Miles) was added to 200-μl aliquots of lysate to a final concentration of 0.05 to 0.8% and the mixture was vortexed and incubated for 5 min at room temperature. The precipitate was recovered by spinning for 5 min in a microcentrifuge and resuspended in 200 μl buffer C (20 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM PMSF, 0.5% Tween 20, 0.5% Nonident-P40) containing 0.25 M KCl to elute the DNA polymerase. Residual precipitate was removed by recentrifugation and activity in dilutions of the supernatant was determined by the primed M13 assay. After titration, neutralized 10% PEI was added dropwise to the remaining lysate to the optimum concentration (usually 0.15%), incubated 10 min, and the precipitate collected by centrifugation for 20 min at 8000 rpm in a Sorvall GSA rotor. The pellet was washed once by resuspension in 240 ml buffer C containing 0.025 M KCl and the precipitate was recovered by centrifugation. The *Taq* DNA polymerase was eluted from the precipitate by resuspension in 240 ml buffer C containing 0.15 M KCl and the residual precipitate was removed by centrifugation. Resuspensions of the PEI precipitates were routinely done with several strokes in a Dounce homogenizer with a loose pestle to ensure an even suspension.

The PEI eluate was diluted to 50 mM KCl with buffer C and applied to a 150-ml column of BioRex 70 ion exchanger (Bio-Rad) that had been preequilibrated in buffer C containing 50 mM KCl. The column was then washed with 6 column volumes of the same buffer and the enzyme was eluted in a single step with buffer C containing 200 mM KCl. The peak of protein from this step was pooled and dialyzed for 12 h against two changes of 1 liter each storage buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 1 mM dithiothreitol, 50% glycerol). No gelatin is added to this buffer (7) because the DNA polymerase is sufficiently concentrated for storage (10–20 mg/ml). This preparation is stable for at least 10 min at 94°C, at least 24 h at room temperature, and at least 6 rounds of freezing and thawing (data not shown). Aliquots of 100 μl are kept at −80°C except for working stocks, which are stable for at least several months when stored at −10°C.

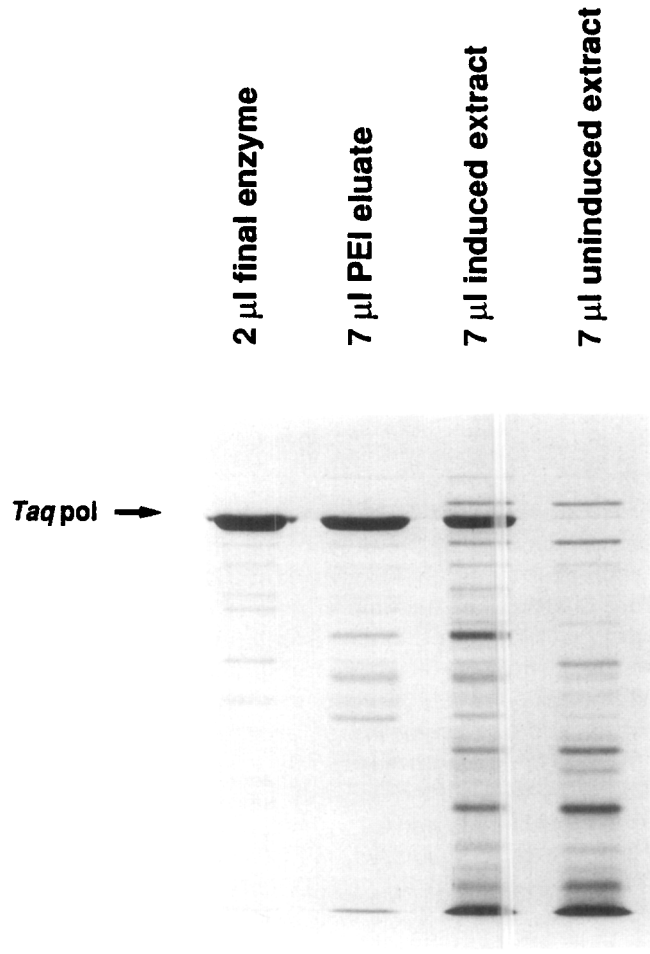

FIG. 1. Analysis of protein content. Denaturing polyacrylamide gel electrophoresis with Coomassie staining was performed as described under Materials and Methods to visualize the protein in aliquots from several stages of the purification. The two right-hand lanes contained clarified lysate from DH1 cells containing the overproducer plasmid either with or without IPTG induction of *Taq* DNA polymerase synthesis. The left-hand two lanes show the PEI eluate and final enzyme fraction (BioRex 70 eluate), and have been deliberately overloaded with 40 μg of protein per lane. The expected position of full-length *Taq* DNA polymerase (94 kDa) relative to standard markers (not shown) is indicated to the left.

RESULTS AND DISCUSSION

Purification of *Taq* DNA polymerase is relatively straightforward when it is produced in *E. coli* because the enzyme appears to accumulate stably on long-term induction and because the initial steps of heat treating and clarifying the cell lysate denature and remove most contaminating protein. A summary of the recovery of protein and DNA polymerase activity is presented in Table 1 and denaturing polyacrylamide gel visualization of protein fractions from the isolation procedure is shown in Fig. 1. Comparison of the induced and uninduced pattern of proteins in the clarified lysates shows

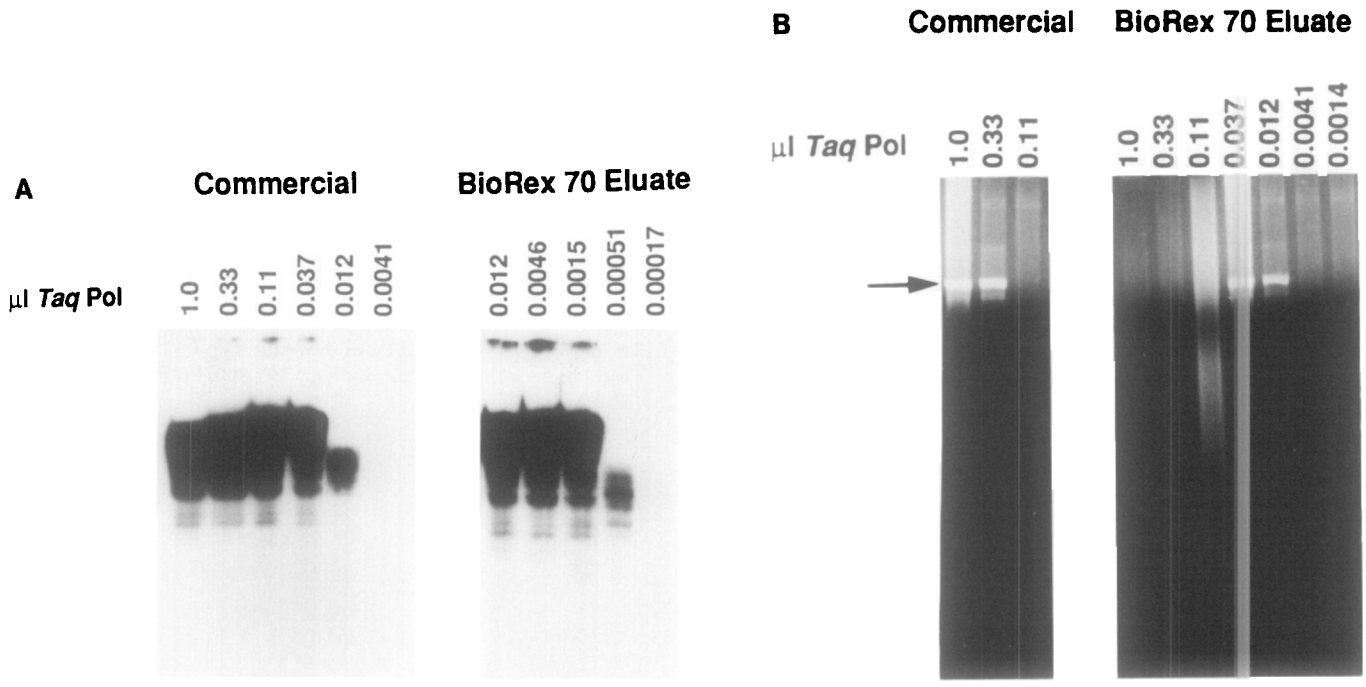

FIG. 2. *Taq* DNA polymerase assays. Activity of the isolated *Taq* DNA polymerase was titrated relative to a commercial preparation using both single-round primer extension assays and polymerase chain reaction amplifications. (A) One microgram of M13mp18 viral DNA was primed with the universal forward primer and the incorporation of [α-^{32}P]dATP into high molecular weight primer extension products with dilutions of the indicated enzymes was monitored by denaturing polyacrylamide gel electrophoresis and autoradiography. (B) Thirty-five cycles of PCR were performed with total human DNA and the primers described under Materials and Methods using the same dilutions of enzymes as in A. The position of the expected amplified DNA fragment relative to *Bst*EII-cleaved λ phage DNA markers is indicated by an arrow. Disappearance of correctly amplified fragment at high enzyme concentrations is discussed in the text.

that a significant proportion of the protein in lysates from induced cells appears as a band corresponding to the full-length *Taq* DNA polymerase (94 kDa) and that PEI precipitation of the enzyme removes most contaminants. The final BioRex 70 chromatography step removes slightly more extraneous protein, but more importantly removes residual PEI that can interfere with DNA polymerase function. The final enzyme fraction still contains trace contamination from *E. coli* proteins (Fig. 1). Residual *E. coli* nucleic acids are undetectable by absorbance at 260 nm but can be detected by more sensitive assays at levels consistent with contaminations in commercial preparations (Norman Pace, personal communication).

Although the *Taq* DNA polymerase produced by these methods has performed well in a wide variety of applications, it should be emphasized that it is absolutely necessary to carefully titrate the correct dilution of the enzyme, especially for use in PCR. Figure 2 shows titrations of the final enzyme fraction for both an M13 template extension of the universal primer (A) and a PCR amplification of a single copy sequence from human DNA (B). Parallel titrations of a less concentrated commercial preparation of *Taq* DNA polymerase are shown for comparison. The M13 reactions show a slight tendency to produce shorter primer extension products when a vast excess of enzyme is used, but it is the PCR reactions that show the narrowest permissible range of enzyme concentration. As DNA polymerase concentration is increased as little as fourfold past the optimum, a smear of products first accumulates encompassing both high and low molecular weight species. When the enzyme concentration is increased still further the product becomes exclusively an intense band of very small molecular weight that has migrated off the bottom of the gel in Fig. 2B. This behavior appears to be native to the *Taq* DNA polymerase, since it has been observed in enzyme preparations from varied purification schemes and is equally stable to incubation at 94°C (data not shown). Once a pooled and dialyzed preparation has been titrated, however, the stored aliquots appear to perform consistently at the determined dilution indefinitely.

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