Heat-Mediated Activation of Affinity-Immobilized *Taq* DNA Polymerase

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ABSTRACT

A novel strategy for heat-mediated activation of recombinant Taq *DNA polymerase is described. A serum albumin binding protein tag is used to affinity-immobilize an* E. coli*-expressed* Taq *DNA polymerase fusion protein onto a solid support coated with human serum albumin (HSA). Analysis of heat-mediated elution showed that elevated temperatures (>70°C) were required to significantly release the fusion protein from the solid support. A primer-extension assay showed that immobilization of the fusion protein resulted in little or no extension product. In contrast, fusion protein released from the HSA ligand by heat showed high polymerase activity. Thus, a heat-mediated release and reactivation of the* Taq *DNA polymerase fusion protein from the solid support can be obtained to allow for hot-start PCR with improved amplification performance.*

INTRODUCTION

The polymerase chain reaction (PCR) technique (25) has become an important and widespread tool in molecular biology. However, for applications where only a few target molecules are present in complex biological samples, mispriming can occur yielding nonspecific amplification products, including primer oligomerization (3,17). This problem may be circumvented by the design of nested primer systems (1). Alternatively, so-called "hot-start PCR" procedures have been used, designed to eliminate polymerase activity at low, non-stringent temperatures before the first amplification cycle. Hot-start PCR procedures have been shown to circumvent some of the mispriming reactions and to result in PCR amplifications with higher sensitivity, specificity and yield (3). The original description of hot-start processes included the opening and reclosing of the reaction tubes at 60°–80°C to add a critical missing component to initiate polymerization (7,18,19). However, various approaches have since been described to avoid the manual intervention, e.g., by replacing mineral oil with wax or jelly-like compounds to create an initial two-compartment system (3,9,13) or by adding a heat-labile antibody directed towards the *Taq* DNA polymerase (14,28). Recently, it has also been shown that a hot-start procedure not only facilitates the detection of low-copy-number targets, but also improves various other PCR applications such as reverse-transcription (RT) PCR, multiplex PCR, in situ PCR and long range PCR (2,21,27,29,30).

Immobilization of enzymes has proven to be a useful procedure for many applications because of the altered characteristics of the immobilized enzyme compared to its soluble counterpart (4). Preliminary results using a truncated *Taq* DNA polymerase fusion protein showed that immobilized polymerase has little or no polymerase activity (J. Lundeberg, unpublished). This prompted us to investigate whether a heat-mediated elution from a solid support could be used to achieve a temperature-induced reactivation of an immobilized *Taq* DNA polymerase fusion protein, which would allow for the design of convenient hot-start procedures for PCR, without the need to open the tubes and using two-phase systems or antibodies.

MATERIALS AND METHODS

Bacterial Strains and Plasmid Vectors

E. coli strains RRIΔM15 (24) and BL21(DE3)pLysS (Novagen, Madison, WI, USA) were used as bacterial hosts for cloning and gene expression, respectively. *Thermus aquaticus* strain YT-1 (No. 25104; ATCC, Rockville, MD, USA) was used as source for the gene-encoding *Taq* DNA polymerase I. Plasmid pGEM®-T (Promega, Madison, WI, USA) and pAff2c (20) were used as vectors. The latter vector is adapted for fusion of target proteins to the tripartite affinity tag Bio-His_6-ABP, consisting of a recognition sequence for in vivo biotinylation (26), a His_6 peptide (8) and parts (residues 146–

266) of the serum albumin binding protein (ABP) region from streptococcal protein G (22).

DNA Constructions

Genomic DNA was isolated from *T. aquaticus* essentially as described previously (16). The gene encoding *Taq* DNA polymerase I was amplified from genomic DNA by PCR, essentially according to Engelke et al. (6), but with an alternative 3′ PCR primer (carboxyl terminal) (5′-CAC GCG TCG ACC TCC TTG GCG GAG AGC CAG TCC TC-3′) and with formamide (5% final concentration) in the PCR mixture. The PCR fragment was ligated into pGEM-T according to the manufacturer's instructions. Positive clones were selected by plasmid restriction mapping. By using the restriction sites introduced by the PCR primers, *Eco*RI (upstream) and *Sal*I (downstream), the *Taq* DNA polymerase I gene was isolated and introduced into *Eco*RI- and *Sal*I-digested pAff2c by ligation. To create an in-frame termination codon (TAA), a linker (5′-TCG ACT AAC TGC AGG CAT GCA-3′ [sense]; 5′-AGC TTG CAT GCC TGC AGT TAG-3′ [antisense]) was inserted between the *Sal*I and *Hin*dIII sites of pAff2c. Thus, the resulting plasmid pAff2-Taq encodes the Bio-His_6-ABP tag, followed by *Taq* DNA polymerase I (residues 3-832) (16), designated TagTaq.

Gene Expression, Fusion Protein Purification and Immobilization

E. coli cells harboring the plasmid pAff2-Taq were grown overnight at 37°C in a shaker flask containing 50 mL of tryptic soy broth (30 g/L; Difco, Detroit, MI, USA) supplemented with 5 g/L of yeast extract (Difco), 100 µg/mL of ampicillin and 34 µg/mL of chloroamphenicol. On the following morning, the culture was diluted in a ratio of 1:40 into shaker flasks containing the same media as above but without chloroamphenicol and grown at 30°C. Isopropyl-β-D-thiogalactoside (IPTG) and d-biotin were added to final concentrations of 1 and 0.1 mM, respectively, when the optical density (A_{580} nm) of the culture was approximately 1. Cells were grown for 5 h, harvested by centrifugation, resuspended in 30 mL of washing buffer (50 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 0.05% Tween® 20 and 1 mM EDTA) and stored at -20°C. Thawed cells were sonicated, centrifuged at 30 000× *g* for 20 min, followed by filtration (0.45-µm filter) of the supernatant. The solution was immediately applied onto a 5-mL human serum albumin (HSA)-Sepharose® column at room temperature as previously described (22). After loading, the column was washed with 200 mL of washing buffer, followed by 60 mL of PCR buffer (10 mM Tris-HCl, pH 8.5, 50 mM KCl, 2 mM $MgCl_2$ and 0.1% Tween 20). The TagTaq fusion protein immobilized to HSA-Sepharose was stored at 4°C in PCR buffer or eluted with 0.5 M HAc, pH 2.8, after a prewash with 60 mL of 10 mM NH_4Ac, pH 5.5. Relevant HAc-eluted fractions were pooled and loaded on PD-10 columns (Pharmacia Biotech, Uppsala, Sweden) for buffer exchange to 2× storage buffer (40 mM Tris-HCl, pH 8.0, 200 mM KCl, 0.2 mM EDTA, 2 mM dithiothreitol, 1% Tween 20 and 1% Nonidet® P-40) and stored at 4°C. The amount of affinity-purified fusion protein was estimated from absorbance measurement of the HAc eluate using an extinction coefficient of 1 cm^2/mg.

Heat Elution of Affinity-Immobilized Fusion Protein

Equal volumes of settled HSA-Sepharose beads with affinity-immobilized TagTaq fusion protein and PCR buffer were mixed, after which 50-µL aliquots (ca. 25 µg of immobilized TagTaq fusion protein) were transferred to individual tubes. The tubes were incubated in a GeneAmp® PCR System 2400 Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA) for 5 min at different temperatures (45°, 50°, 55°, 60°, 65°, 70°, 75°, 80°, 85°, 90° and 95°C). Immediately after heat treatment, the beads were resuspended, sedimented by centrifugation and the supernatant was transferred to a new tube. As a control, one tube was not heat-treated. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed using 8%–25% gradient gels and the Phast-System™ (Pharmacia Biotech), followed by staining with Coomassie® Blue R-350 (Pharmacia Biotech). The samples were prepared by mixing 8 µL of heat-eluted material with 2 µL of 5× loading buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 12.5 mg/mL SDS, 25% 2-mercaptoethanol and 0.5 mg/mL bromophenol blue). The reference sample was prepared by taking 8 µL of suspended beads and mixing them with 2 µL of 5× loading buffer.

DNA Polymerase Activity Assay

The polymerase activity was investigated for TagTaq fusion protein affinity-immobilized to HSA-Sepharose beads (Figure 2A) by extension at different temperatures of a fluorescein-labeled primer (FITC-USPEXT [5′-fluorescein-CAG TCA CGA CGT TGT AAA ACG GCC AGT-3′]), annealed to a linear (248-nucleotide [nt]) single-stranded (ss) DNA fragment prepared in the following manner: multiple PCRs were carried out in total volumes of 50 µL of PCR buffer containing 0.2 mM dNTPs, 5 pmol of each primer RIT27 (non-labeled) and RIT30 (biotin-labeled) (11), approximately 10 pg pRIT28 (10) plasmid target DNA and 1 U of AmpliTaq® DNA Polymerase (Perkin-Elmer). The PCR program used was 95°C for 5 min, followed by 35 cycles of 95°C for 30 s and 70°C for 2 min. A final extension was at 70°C for 7 min. Immobilization of the pooled biotinylated PCR products (400 µL) was performed by mixing with 2 mg streptavidin-coated paramagnetic beads (Dynabeads® M-280 Streptavidin; Dynal A.S, Oslo, Norway) according to

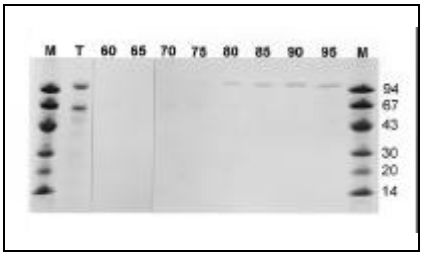

Figure 1. Results of the heat-mediated elution. Analysis by SDS-PAGE of material eluted from HSA-Sepharose at different temperatures; lane T: total proteins (starting material) on the HSA-Sepharose beads after affinity-immobilization of the TagTaq fusion protein directly from an *E. coli* lysate; lanes 65–95: material eluted after a 5-min incubation of the starting material at temperatures 65°, 70°, 75°, 80°, 85°, 90° or 95°C, respectively; lane M: marker proteins (sizes in kDa).

Hultman et al. (10). The nonbiotinylated strand was eluted by treating the beads with 100 µL 0.1 M NaOH for 5 min at room temperature. The supernatant containing the eluted strands was transferred to a separate tube and neutralized with 60 µL 0.1667 M HCl. The extension templates were prepared in batch by mixing 17 µL FITC-USPEXT primer (1 pmol/µL) with 153 µL of the single-stranded complementary DNA and heating to 95°C for 30 s, followed by cooling to room temperature.

For the following extension experiments, separate tubes were arranged containing 5 µL 10× PCR buffer, 5 µL 20 mM dNTPs, 28.5 µL H_2O and 1.5 µL suspended HSA-Sepharose containing approximately 40 ng affinity-immobilized TagTaq fusion protein. The samples were processed by two alternative routes (Figure 2A), of which one involved a preincubation step at 95°C for 5 min. Ten microliters of extension template solution were added at room temperature to both heat-treated and non-heat-treated samples, which were incubated in pairs at different temperatures. After 5 min, the reactions were stopped by the addition of 50 µL of loading dye (100% deionized formamide and 5 mg/mL of Dextran Blue 2000; Pharmacia Biotech) supplemented with 20 mg/mL SDS and 10 mM EDTA, and cooled to 4°C in the PCR block. The tubes were stored at -20°C. The temperatures investigated were 45°, 50°, 55°, 60°, 65°, 70° and 75°C. In a separate experiment imitating the first two steps in a PCR cycle, two samples in their respective routes containing the extension substrate were incubated at 95°C for 5 min and then extended at 70°C. The PCR 2400 Thermal Cycler was used for heat incubations and PCRs.

Evaluation of Activity Assay

The extension products from the activity assay were analyzed on a 6% polyacrylamide gel mounted in an ALF DNA Sequencer™ (Pharmacia Biotech). An external marker (Sizer™ Size Marker 50–500; Pharmacia Biotech) was used for subsequent fragment length estimations, and an internal standard (Sizer 150; Pharmacia Biotech) was used for product quantification. To allow for analysis within the dynamic range of the fluorescence-based instrument, two dilutions of the samples were prepared by mixing 1 µL of sample, 2 µL of internal standard and 7 µL of loading dye or by mixing 4.5 µL of sample, 2 µL of internal standard and 9 µL of loading dye, followed by heating to 95°C for 5 min and cooling on ice.

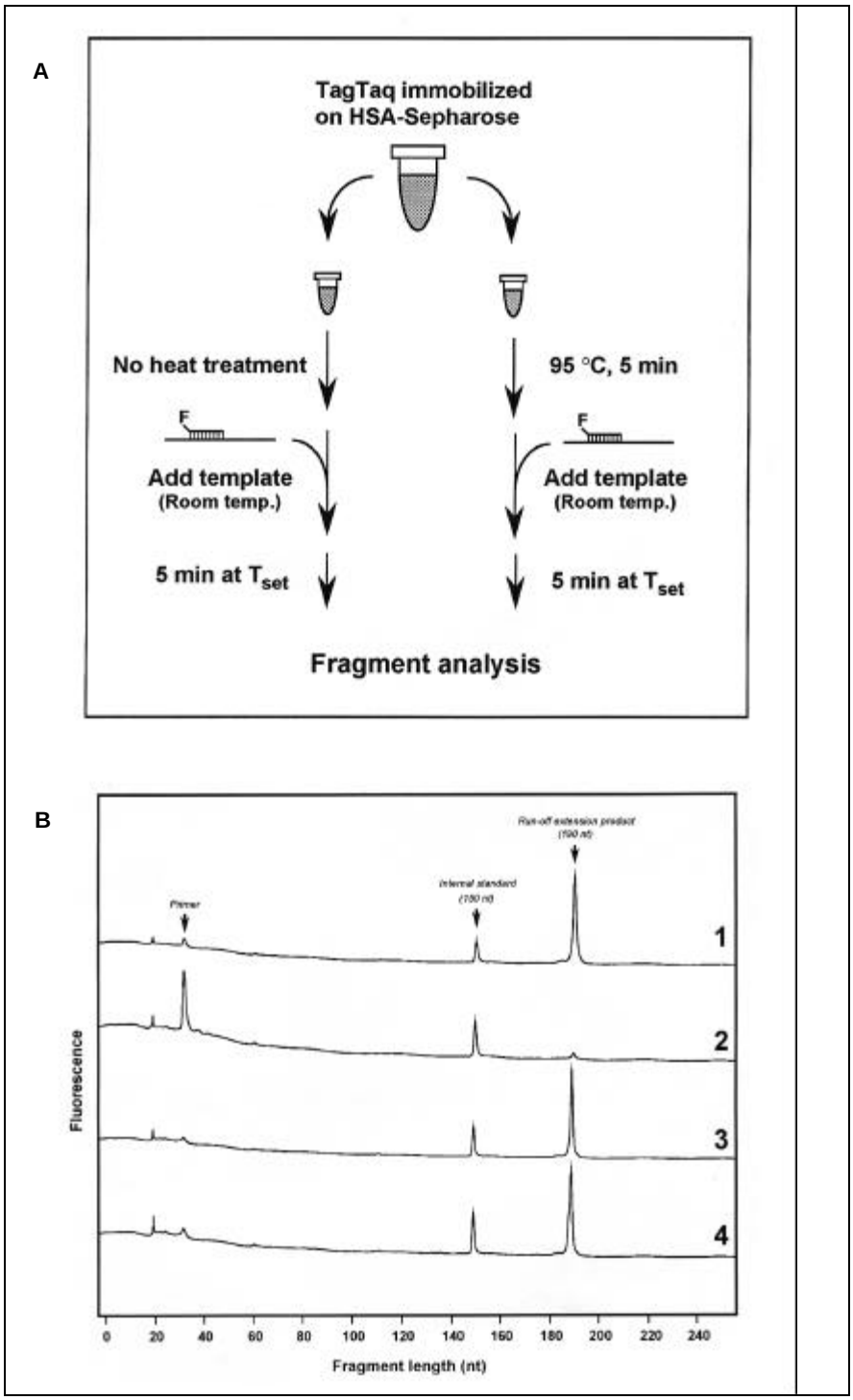

Figure 2. Analysis of the TagTaq polymerase activity. (A) The experimental outline with the two parallel alternative routes. (B) Fragment analysis: rows 1 and 2 correspond to pre-heat-released (right route) and immobilized (left route) samples, respectively, after extensions at 60°C (T_{set}). Rows 3 and 4 correspond to pre-heat-released (right route) and immobilized (left route) samples, respectively; before the extension at 70°C (T_{set}), both samples were incubated at 95°C for 5 min.

Samples analyzed in Figure 2B were prepared identically. Fragment Manager Version 1.1 Software (Pharmacia Biotech) was used for fragment size calculations and peak quantification. The peak area ratios shown in Figure 3 were calculated by the formula: product peak area × 1 or 4.5/internal standard peak area.

Hot-Start PCR

The TagTaq fusion protein was immobilized onto HSA-coated superparamagnetic Dynabeads M-280 by resuspending 10 mg of HSA-coated Dynabeads in 0.5 mL of storage buffer and adding 0.5 mL of HSA-affinity-purified TagTaq fusion protein (ca. 100 µg/mL). The suspension was incubated at room temperature for 60 min with gentle agitation. The beads were washed with 4× 1-mL storage buffer. After washing, the beads were resuspended to a concentration of 10 mg beads/mL storage buffer and stored at 4°C. In PCR experiments, Dynabeads with the affinity-immobilized TagTaq fusion protein were compared with pre-heat-released TagTaq fusion protein (achieved by incubation at 94°C for 5 min and subsequent transfer of supernatant to the PCR mixture) and AmpliTaq DNA polymerase. PCR mixtures contained 1× PCR buffer II (Perkin-Elmer), 2.5 mM $MgCl_2$, 200 µM dNTPs, 1 µM primers (K-*ras* forward 5′-TGA AAA TGA CTG AAT ATA AAC TT-3′ and K-*ras* reverse 5′-GAT CAT ATT CGT CCA CAA AAT GA-3′) and 25 ng of human genomic DNA as template in a total volume of 30 µL. The reaction mixtures were prepared at room temperature. The PCR amplifications were performed on a GeneAmp PCR System 9600 (Perkin-Elmer). The PCR program was: 94°C for 2 min followed by 34 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s. A final extension was performed at 72°C for 5 min. The PCR products were analyzed by 3% agarose (NuSieve® GTG; FMC BioProducts, Rockland, ME, USA) gel electrophoresis and stained with ethidium bromide.

RESULTS

Fusion Protein Design and Affinity Purification

A gene fusion approach was designed for the production of *Taq* DNA polymerase fused to an affinity tag capable of immobilizing the fusion protein by a heat-labile interaction. The gene encoding *Taq* DNA polymerase was amplified from genomic *T. aquaticus* DNA and subsequently inserted into an affinity fusion *E. coli* expression vector encoding an N-terminal multifunctional tag containing three different affinity domains: an in vivo biotinylated peptide, a hexahistidine tag and a serum ABP. These domains can be used separately or in combination for purification, immobilization and detection of fusion proteins (20). The resulting 110-kDa fusion protein, designated TagTaq, was produced in *E. coli* and purified by HSA-affinity chromatography from a clarified cell lysate. By using low pH elution (22), the expression level of the TagTaq fusion protein was calculated to be approximately 3 mg/L of culture. Analysis by SDS-PAGE showed that the majority (ca. 90%) of the material was full length (data not shown).

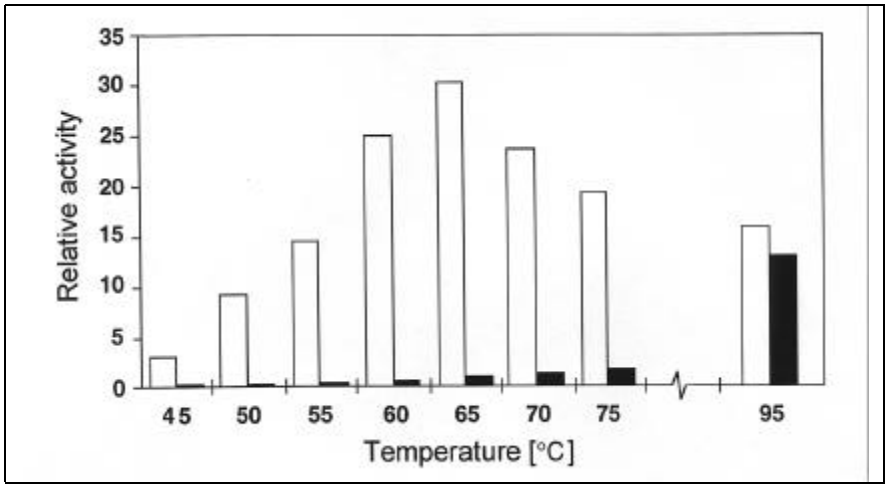

Figure 3. Summary of the TagTaq fusion protein polymerase activities at different temperatures. The open and filled bars represent the relative activities of pre-heat-released (right route) and immobilized (left route) TagTaq, respectively, corresponding to the scheme shown in Figure 2A. The bars at 95°C correspond to rows 3 and 4 described in the legend to Figure 2B.

Heat Elution of HSA-Affinity-Purified Fusion Protein

To evaluate the ABP-HSA interaction for use as a heat-susceptible bond at temperatures relevant for standard PCR protocols, we investigated the release of TagTaq fusion protein from HSA-Sepharose at different temperatures (Figure 1). Aliquots of HSA-affinity-immobilized fusion protein were incubated for 5 min at temperatures ranging from 45° to 95°C. An analysis by SDS-PAGE of collected supernatant samples showed that no significant release of the fusion protein was observed at temperatures below 70°C (Figure 1). However, at temperatures above 70°C, increasing amounts of TagTaq fusion protein (110 kDa) were observed in the supernatants, with the largest amounts in the sample corresponding to incubation at 95°C. Taken together, the data suggest that the HSA-ABP interaction is broken in the same temperature range routinely desired for initiation of polymerase activity in hot-start PCR protocols. It is noteworthy that little or no HSA ligand (67 kDa) is present in the supernatants even at 95°C, indicating that the covalent coupling to the gel matrix is unperturbed even under these conditions.

Polymerase Activity at Different Temperatures

The results from the heat-elution experiment suggested that elevated temperatures (above 70°C) are required to significantly release TagTaq fusion protein from the HSA-affinity resin. To determine if the DNA polymerase activity correlated with the heat-release temper-

ature profile, we investigated the polymerase activity of the affinity-immobilized TagTaq fusion protein at different temperatures by using an experimental setup presented in Figure 2A (left route). In parallel, we analyzed control samples containing equal amounts of affinity-immobilized TagTaq fusion protein, preincubated at 95°C to release the fusion protein (Figure 2A, right route). To assay eventual DNA polymerase activities in the samples, a substrate consisting of a fluorescein-labeled primer annealed to an ssDNA fragment was used. Full extension of the template strand would result in the production of fluorescein-labeled 190-nt runoff fragments, suitable for detection and quantification by a DNA sequencing electrophoretic system. Before analysis, each sample was spiked with an equal amount of a fluorescein-labeled 150-nt internal standard fragment, facilitating the comparison of peak areas between samples.

The results from the incubation of pre-heat-released TagTaq fusion protein (right route) at 60°C shows a major peak corresponding to a fragment size of 190 nt, resulting from successful extension of almost all available substrate templates, as indicated by the small peak at 30 nt corresponding to the remaining unextended primers (Figure 2B, lane 1). In contrast, the immobilized TagTaq fusion protein (left route), incubated with the substrate at the same temperature, resulted in only very small amounts of the 190-nt extension product. However, a major peak was observed at 30 nt, suggesting that almost all substrate templates remained unextended under these conditions (Figure 2B, lane 2). Note that no extension products of intermediate lengths can be observed, suggesting that extensions are either complete or not initiated. This is also supported by the approximately equal peak areas for unextended and extended samples.

The results from incubations of parallel samples at various temperatures in the range of 45°–75°C are summarized in Figure 3, which shows the relative activities as calculated from ratios between peak areas corresponding to the 190-nt extension product and the internal standard 150-nt fragment. The pre-heat-released TagTaq fusion protein (right route) shows a high relative activity at all temperatures tested, with an apparent maximum at 65°C. The immobilized TagTaq fusion protein generally shows a very low relative activity, with a small increase at 65°–75°C. To further rule out the possibility that the immobilized preparation of TagTaq fusion protein (left route) was inactive or that HSA-Sepharose acted as an inhibitor for the polymerase activity, both pre-heat-released (right route) and immobilized (left route) TagTaq fusion proteins containing the substrate template were subjected to an authentic first PCR cycle, with subsequent incubations at 95°C and then at 70°C (Figure 2B, lanes 3 and 4). Taken together, the results shown in Figure 2B and Figure 3 indicate indeed that TagTaq is

active in the presence of HSA-Sepharose, but only after incubation at a temperature high enough to break the ABP-HSA affinity interaction.

PCR Amplification Using HSA-Affinity-Immobilized TagTaq

To investigate the performance of the immobilized and pre-heat-released TagTaq fusion proteins in a full multiple-cycle PCR amplification, a region of the human K-*ras* gene (104 bp) was amplified from genomic DNA using a standard amplification protocol (Figure 4). For comparison, a commercially available *Taq* DNA polymerase was used both in a standard protocol and in a conventional hot-start procedure. Analyses of the PCR products by agarose gel electrophoresis show that, for both the immobilized (lanes 1–3) and pre-heat-released (lanes 4–6) TagTaq, the expected PCR product is achieved. However, for the amplification of this fragment, the use of either pre-heat-released TagTaq or a commercial *Taq* DNA polymerase results in the formation of artifactual primer-dimer products (lanes 4–6 and lane 7), which also reduce the product yield. In contrast, by using either a conventional hot-start procedure or immobilized TagTaq, high yields of essentially pure, full-length PCR products free from primer-dimers are obtained.

DISCUSSION

In this study we have characterized and applied a heat-mediated release of an affinity-immobilized recombinant *Taq* DNA polymerase (TagTaq) for use in hot-start PCR procedures. This *Taq* DNA polymerase fusion protein can be purified and immobilized by a one-step affinity capture for direct use in PCR. This approach circumvents additional procedures that usually follow the elution step in affinity chromatography to obtain the target protein in a suitable buffer for further use.

For the affinity immobilization, a recently described multifunctional Bio-His_6-ABP tag (20) was used as a fusion partner with *Taq* DNA polymerase. This single-fusion partner allows for the evaluation of three separate purification and immobilization strategies for a fused target protein: *(i)* biotin-avidin (streptavidin), *(ii)* hexahistidyl-immobilized metal ions and *(iii)* ABP-HSA. In this study, the TagTaq fusion protein was purified by HSA-affinity chromatography (20,22) using the ABP affinity tag, but any of the other two domains may also be used. However, for PCR applications, the chelating matrices used for the capture of polyhistidine fusion proteins may disturb PCR running conditions through metal ion binding, and the biotin-avidin (streptavidin) interaction is probably too heat-stable, described to withstand temperatures above 100°C (5). The ABP tag was an attractive alternative since preliminary data (P.-Å. Nygren, unpublished) indicated that its interaction with HSA ($K_{aff} \approx 10^9\ M^{-1}$) is disrupted at elevated temperatures. In addition, any leakage of the ligand (HSA) would probably not affect the PCR, since its homolog bovine serum albumin is frequently included in PCR buffers at relatively high concentrations to improve PCR performance.

We have shown that at temperatures below 70°C, TagTaq fusion protein, associated with HSA-Sepharose, shows negligible polymerase activity compared with TagTaq fusion protein released from the resin by heat before the analysis. The reason for this low activity needs further investigation; however, possible explanations may be that the immobilization prevents the translocation of the DNA polymerase along the DNA template (12,23) and/or that the immobilized polymerase is physically separated from the DNA template in solution. Furthermore, similar hot-start results have also been obtained for a truncated *Taq* DNA polymerase gene fusion (J. Lundeberg, unpublished), which may indicate a general behavior of immobilized DNA polymerases. The question remains whether this convenient heat-mediated activation can also be used for other enzymes such as reverse transcriptases and DNA ligases.

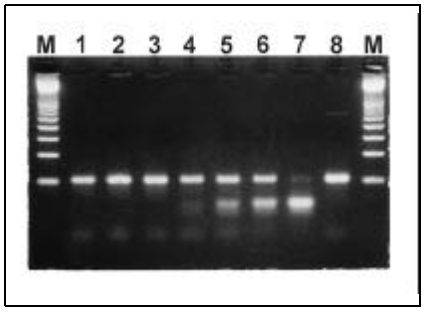

Figure 4. Results of the PCR experiments analyzed by agarose gel electrophoresis. Lanes 1–3: K-*ras* gene amplification using 50, 100 and 200 µg of HSA-Dynabeads with affinity-immobilized TagTaq fusion protein, respectively; lanes 4–6: K-*ras* gene amplification using TagTaq fusion protein pre-heat-released from 50, 100 and 200 µg of HSA-Dynabeads, respectively; lane 7: K-*ras* gene amplification using 2 U of AmpliTaq DNA polymerase; lane 8: same as lane 7, but after use of a conventional hot-start procedure involving the addition of 2 U of AmpliTaq DNA polymerase at 94°C. The expected size of the K-*ras* amplicon was 104 bp. Lane M: DNA size marker (100-bp ladder).

The slight decrease in relative activity observed for pre-heat-released TagTaq fusion protein (Figure 2A, right route) at temperatures above 65°C can probably be explained by a partial strand separation of the heteroduplex DNA used as extension substrate or a change in the characteristics of the fusion protein. But the PCR data presented in Figure 4 indicate that the former explanation is more likely, since the TagTaq fusion protein showed efficiencies similar to those of AmpliTaq DNA polymerase [maximum activity at ca. 75°C (15)]. In addition, further PCR experiments using standard PCR conditions (i.e., extension at 72°C) with immobilized TagTaq (amplification of targets up to 2.1 kbp) have been performed successfully (J. Nilsson, unpublished).

Interestingly, the ABP affinity fusion partner is still functional after 30 PCR cycles, allowing post-PCR re-immobilization of TagTaq fusion protein onto fresh HSA-Sepharose (J. Nilsson, unpublished). This would facilitate the removal of the DNA polymerase from a reaction mixture after PCR and thereby allow efficient endonuclease restriction without prior purification of the PCR product, which is usually performed to circumvent polymerase-dependent fill-in reactions of generated protrusions. The use of TagTaq fusion protein in one-step RT-PCR procedures can also be envisioned, in which the fusion protein is inactivated during the reverse transcription using mesophilic reverse transcriptases and subsequently activated during the first heating cycle of PCR.

Conventional hot-start PCR proce-

dures usually involve extra manipulations such as the addition of a critical PCR component to the reaction during the first heat cycle, the inclusion of a pre-PCR melting step for wax covers or the pre-PCR mixing of *Taq* DNA polymerase with an inhibiting anti-*Taq* DNA polymerase antibody. In contrast, using an immobilized *Taq* DNA polymerase to obtain hot-start PCR, these extra procedures can be avoided, thereby creating a robust integrated system. Our data show that affinity-immobilized TagTaq fusion protein can be used directly in PCR, enabling a hot-start PCR through the heat-labile interaction between ABP and HSA. Here we have used both Sepharose and paramagnetic beads as solid supports, the latter of which improves the overall handling.

In conclusion, we describe for the first time that heat-mediated elution can be used to regain the enzymatic activity of an affinity-immobilized enzyme fusion protein. This procedure allows the release of a recombinant *Taq* DNA polymerase fusion protein, immobilized through the specific affinity between a bacterial albumin-binding domain and HSA covalently coupled to a solid support, under conditions suitable for hot-start PCR.

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