Affinity Purification of Plasmid DNA by Temperature-Triggered Precipitation

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Received 15 May 2003; accepted 29 September 2003

Published online 10 December 2003 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.10890

Abstract: This report describes a new plasmid DNA purification method, which takes advantage of the DNA-binding affinity and specificity of the bacterial metalloregulatory protein MerR, and of the temperature responsiveness of elastin-like proteins (ELPs). Upon increasing the temperature, ELP undergoes a reversible phase transition from water-soluble forms into aggregates, and this property was exploited for the precipitation of plasmid DNA containing the MerR recognition sequence by a simple temperature trigger. In one purification step, plasmid DNA was purified from *E. coli* cell lysates to a better purity than that prepared by a standard alkaline purification method, with no contaminating chromosomal DNA and cellular proteins. This protein-based approach, in combination with the reversible phase transition feature of ELP, makes the outlined method a promising candidate for large-scale purification of plasmid DNA for sensitive applications such as nonviral gene therapy or DNA vaccines. © 2004 Wiley Periodicals, Inc.

Keywords: DNA cloning; Plasmid binding; plasmid purification; affinity precipitation; Elastin-like proteins (ELPs)

INTRODUCTION

Medical applications of DNA-based therapy, such as gene therapy and vaccine, are gaining popularity (Duguid et al., 1998) and the first products are expected to soon reach the market. Two general strategies are available to deliver nucleic acids, both viral and nonviral (Ferreira et al., 2000). For higher efficiency, the use of viral vectors is more popular at this time, but serious safety and regulatory issues arise due to their toxicity and immunogenicity. Therefore, interest is shifting toward the safer nonviral alternative, based on plasmid delivery. Unfortunately, these methods are less efficient, requiring the use of higher doses of the therapeutic plasmid DNA (between 0.1 mg and 1 g), which in turn demands the manufacturing of large amounts of plasmid DNA at the industrial scale.

Classical methods, such as alkaline lysis (Birnboim and Doly, 1979) and CsCl-dye buoyant density-gradient ultracentrifugation (Sambrook and Russell, 2001), are not suitable for large-scale plasmid purification because they are difficult to scale up and require the use of toxic reagents. Chromatographic methods, including size-exclusion (Ferreira et al., 1997), ion-exchange (Yamakawa et al., 1996), and hydrophobic interactions (Diogo et al., 2000), have been suggested for large-scale purification of plasmids. In most cases, the presence of impurities, such as endotoxins, RNA, and genomic DNA, has limited their use in large-scale applications.

A number of affinity-based techniques have recently emerged (Ferreira et al., 2000). One common method is based on the formation of triple helices between single-stranded oligonucleotides and target plasmids (Wils et al., 1997). So far, only small-scale demonstrations of triplex-affinity chromatography have been reported, perhaps due to the relative slow helix formation and the need for high ionic strength and low pH (Ferreira et al., 2000). Affinity purification based on the specificity of DNA-binding proteins is an effective alternative. Target plasmids containing a specific recognition sequence have been selectively removed by either lac*I*–protein A fusions immobilized on IgG sepharose (Lundeberg et al., 1990) or by immobilized zinc-finger DNA-binding protein–glutathione *S*-transferase fusions (Woodgate et al., 2002). However, the use of chromatography resulted in relatively low yield and final separation of the protein–plasmid complex was not demonstrated.

Matteo et al., (2002) recently reported an improved method for plasmid DNA purification based on triplex-helix affinity precipitation using a thermoresponsive NIPAAM polymer as the carrier. Although the yield reported in this case was considerably higher than previously achieved, this method remains tedious because it requires complicated organic synthesis as well as multiple buffer changes by dialysis.

Elastin-like proteins (ELPs) are artificial proteins consisting of a repeating pentapeptide, VPGVG. These biopolymers undergo a reversible phase transition from water-soluble forms into aggregates similar to NIPAAM polymer within a wide range of conditions that are controlled by the chain length and composition (Urry, 1997). Unlike the statistical

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Contract grant sponsors: National Science Foundation; U.S. Environmental Protection Agency

Contract grant numbers: BES0086698; R8296060

nature of polymerization reactions, ELP biopolymers are specifically preprogrammed within a synthetic gene template that can be precisely controlled over chain length, composition, and sequence. ELPs have been fused to other proteins while retaining their temperature responsiveness, as well as the functionality of the fusion partner (Kostal et al., 2001; Meyer and Chilkoti, 1999; Shimazu et al., 2003).

In this study, we introduce a new concept of affinity purification of plasmid DNA based on temperature-triggered precipitation of ELP biopolymers (Fig. 1). The underlying principle is based on the interaction of a DNA-binding protein to its cognate DNA sequence present on the target plasmid. An elastin biopolymer bearing a suitable DNA-binding protein is used to capture a target plasmid, which is removed from the cell extracts by temperature-triggered precipitation. Purified plasmids are recovered by dissociation from the ELP biopolymers, which are removed again by thermal precipitation. We have successfully demonstrated this principle for plasmid purification by exploiting the affinity of a bacterial metalloregulatory protein, MerR, toward its corresponding promoter sequence (O'Halloran, 1993). The use of DNA-binding proteins offers great flexibility as DNA-binding proteins can potentially be engineered to bind any DNA sequence of choice (Beerli and Barbas, 2002).

MATERIALS AND METHODS

DNA Cloning

DNA manipulations were performed according to standard methods (Sambrook and Russell, 2001). All cloning steps were carried out in *E. coli* JM109. Plasmid pET153 coding for ELP153H6 was obtained by insertion of a multimeric VPGVG-encoding sequence (Kostal et al., manuscript in preparation) into the expression vector pET38b+ (Novagen, Madison, WI) as an *Nde*I–*Pst*I fragment. The design and cloning of protein ELP153MR was described earlier (Kostal et al., submitted for publication). For high-yield protein expression, *E. coli* strain BLR (DE3; Novagen) containing the expression vector was grown in Terrific Broth (Sambrook and Russell, 2001) containing 30 μg/mL kanamycin at 30°C for 2 days without addition of IPTG, allowing for yields of up to 800 mg of purified protein per liter of bacterial culture (Guda et al., 1995). Both proteins were purified by temperature-cycling procedures, as described previously (Kostal et al., 2001), and stored at 4°C. To protect from oxidation, the ELP153MR protein was stored in the presence of 50 m*M* 2-mercaptoethanol.

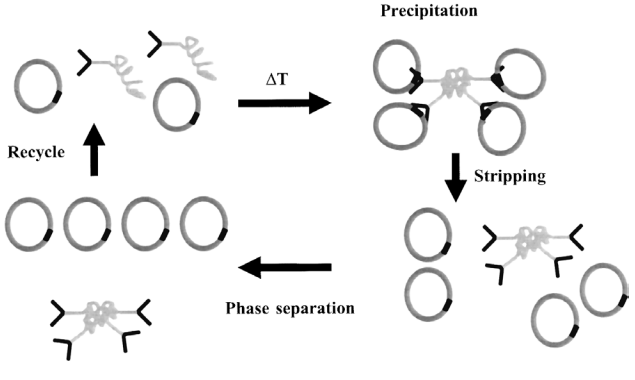

Figure 1. Principle of the affinity purification of plasmid DNA based on temperature-triggered precipitation of ELP biopolymers.

The Pt promoter region (−40 to +4) of the mer operon from the Tn501 transposon (O'Halloran and Walsh, 1987), containing the MerR-binding region, was assembled from two 70-bp oligonucleotides Pt-o1 (5′-*tcgactgcagccttgg*cgcttgactccgtacatgagtacggaagtaaggtta-cgctatcc*ttggctgcag*-3′) and Pt-o2 (5′-*tcgactgcagccaa*ggatagcgtaaccttacttccgtactcatgtacggagt-caagcg*ccaaggctgcag*-3′). Italicized letters indicate the restriction sites *Sal*I, *Pst*I, and *Sty*I, inserted to facilitate subsequent cloning. The Pt region was then inserted into the unique *Sal*I site of pBLUESCRIPT SK+ (Stratagene, La Jolla, CA) and the resulting plasmid was designated pBLU-Pt. Cells harboring pBLU-Pt or pBLUESCRIPT were grown in LB broth containing 100 μg/mL ampicillin. For the initial experiments, plasmids were prepurified by a standard alkaline midiprep method (Sambrook and Russell, 2001).

Plasmid Binding

Binding reactions were carried out in a binding buffer containing 50 m*M* Tris and 150 m*M* NaCl, pH 7.4 (TB74S). The binding mixture further contained, unless specified otherwise, 250×10^{-9} *M* DNA-binding protein ELP153MR, 15.5×10^{-6} *M* (i.e., 1 mg/mL) carrier protein ELP153H6, and 1.5×10^{-9} *M* (i.e., 3 μg/mL) of pBLU-Pt. Binding was allowed to occur for approximately 5 min at room temperature, unless specified otherwise. The mixture was then heated to 37°C for 2 min, and the precipitated biopolymer–DNA complex was recovered by gentle centrifugation at 37°C for 5 min at 5000 rpm in a benchtop microcentrifuge. For the selectivity experiments, similar conditions were employed, except either 3 μg/mL of pBLUESCRIPT was used instead of pBLU-Pt or no ELP153MR was added.

Plasmid Elution and Determination of DNA Content

The precipitated protein–DNA complex was first redissolved in fresh TB74S buffer on ice. Plasmid elution was performed by heating at 60°C in the presence of 0.15 *M* NaCl. The remaining biopolymers were removed by 2-min centrifugation at 37°C.

The amount of eluted DNA was analyzed by horizontal agarose-gel electrophoresis in the presence of 0.5 μg/mL ethidium bromide, followed by quantification by a gel-documentation system (GelDoc 2000, Bio-Rad, Hercules, CA).

Plasmid Purification and Characterization From Cell Lysates

E. coli strain JM109 harboring pBLU-Pt was grown overnight in LB broth (Sambrook and Russell, 2001) containing

100 μg/mL ampicillin. Cell-free extract was prepared from 1 mL of culture according to the boiling method (Sambrook and Russell, 2001). The cell pellet was resuspended in 300 μL STET (Sambrook and Russell, 2001) with 20 μL of lysozyme solution, (10 mg/mL of 10 m*M* Tris, pH 8) boiled for 40 s, and centrifuged for 10 min. The lysates were then subjected to the same binding and elution conditions as just described. For comparison, the plasmid was also purified by the standard alkaline lysis method. Purified plasmid was digested with *Sac*I and *Not*I, according to manufacturer's protocol (New England BioLabs).

Contaminating chromosomal DNA was detected by standard polymerase chain reaction (PCR) (Sambrook and Russell, 2001) (30 cycles, 95°C to 60°C to 72°C) with *Taq* polymerase (Promega, Madison, WI) using primers arsfor, 5′-gcaccggtggcatgtcatttctgttaccc-3′, and arsback 5′-cctctgcagttaactgcaaatgttctt-3′, specific for the amplification of the *E. coli* chromosomal gene arsR (Xu and Rosen, 1997). Contaminating proteins were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gel (Laemmli, 1970), followed by Coomassie blue staining.

RESULTS

Plasmid Purification by Affinity Precipitation

A biopolymer (ELP153MR) consisting of a bacterial metalloregulatory protein MerR (O'Halloran and Walsh, 1987) fused to the C-terminus of elastin-like domain $(VPGVG)_{153}$ was generated to demonstrate the utility of the affinity precipitation method. Purified ELP153MR biopolymers were produced as described previously (Kostal et al., 2003). Due to the temperature-responsive properties of the ELP domain, biopolymers were easily purified by inverse temperature cycling. Up to 800 mg/L of purified biopolymers were obtained after 48-h incubation.

As the mercury-binding functionality of MerR was retained when fused to the ELP (Kostal et al., 2003), we sought to establish whether the DNA-binding property of MerR to its promoter sequence could be similarly preserved for the purpose of plasmid purification. To generate a plasmid containing the recognition sequence for MerR, the P_t promoter region (−40 to +4) of the *mer* operon was inserted into the plasmid pBLUESCRIPT, resulting in plasmid pBLU-Pt (Fig. 2).

The ability of ELP153MR to bind to the P_t promoter region of pBLU-Pt was investigated by temperature-induced precipitation at different biopolymer concentrations. At a concentration of >12.5 μ*M*, nonselective binding to pBLUESCRIPT was observed. Because the transition temperature of the ELP biopolymer is concentration dependent, an inert carrier protein, ELP153H6 (Table I), was added to the binding mixture to achieve 100% precipitation efficiency below 37°C and to minimize nonselective binding.

Using the aforementioned binding conditions, plasmid pBLU-Pt was successfully coprecipitated with ELP153MR

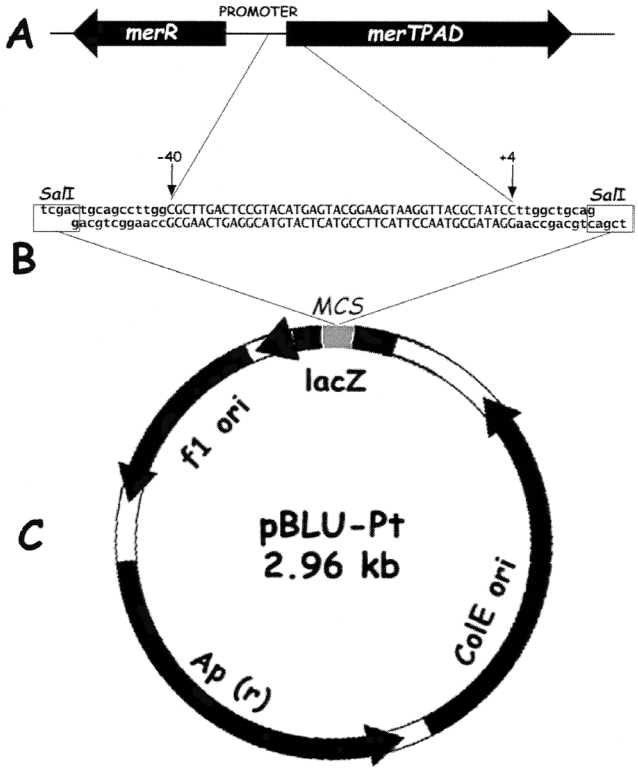

Figure 2. Construction of the plasmid pBLU-Pt. (A) The promoter region of the merTPAD operon (O'Halloran and Walsh, 1987). (B) The binding region of MerR from position −40 to +4 was reconstructed as a 70-bp DNA fragment with flanking *Sal*I sites. (C) Plasmid pBLU-Pt was constructed by inserting the 70-bp fragment into the *Sal*I site of pBLUESCRIPT.

(Fig. 3A, lane 2). To confirm that plasmid removal was a result of the interaction between MerR and the P_t promoter sequence, similar experiments were performed with either a control plasmid pBLUESCRIPT (Fig. 3A, lane 3) or with only the inert carrier ELP153H6 (Fig. 3A, lane 4). In both cases, no plasmid removal was observed, demonstrating that nonspecific interactions were minimal.

The kinetics of binding was investigated by measuring the percentage plasmid removal as a function of incubation time. As shown in Figure 3B, binding occurred rapidly with maximum recovery obtained within 5 min. More than 70% of the initial plasmid was removed by temperature-triggered precipitation.

Elution of Bound Plasmids From Biopolymers

To release the bound plasmids from the MerR moiety, different combinations of NaCl concentrations (0 to 0.5 *M*) and elution temperatures (37° to 80°C) were tested. The elution efficiency increased with increasing NaCl concentration as well as with temperature up to 60°C. The optimum condition for elution (91% recovery efficiency) was found to be heating at 60°C for 2 min in the presence of 0.15 *M* NaCl. Higher temperatures or longer heating times decreased plasmid recovery, whereas higher sodium chloride

Table I. ELP proteins used in this study.

Symbol	Protein sequence[a]	Amino acids	Da
ELP153MR	$MGP(GVGVP)_{153}$ –GVGPGT–linker–MerR	933	80,111
ELP153H6	$MGP(GVGVP)_{153}$ –GVGPGT–GM–H_6	782	64,434

[a]Linker = GGGSGAGGAGSGGG.

concentrations (tested up to 0.5 *M*) did not have a detectable effect (data not shown).

Plasmid Purification From Cell Lysates

Using the optimized conditions for plasmid binding and elution, we tested whether the ELP153MR biopolymer could be used for direct plasmid purification from crude cell lysates. Crude cell extracts (Fig. 4A, lane 1) were first prepared by a standard boiling method (Sambrook and Russell, 2001). The ELP153MR biopolymers were added directly to the cell lysates and the biopolymer–plasmid complex was precipitated by increasing the temperature to 37°C. The bound plasmid was eluted as described earlier and the biopolymers were removed by temperature-induced precipitation and centrifugation. Figure 4A shows the result from a typical purification process. As can be seen in Figure 4A, plasmid pBLU-Pt was successfully recovered from the cell lysates in one simple precipitation step and no contaminating RNA was visible, again highlighting the specific nature of the affinity precipitation method. Although some relaxed forms of the plasmid were visible, this was likely attributable to physical shearing damage and could be avoided by devising a more sensible handling procedure such as filtration instead of centrifugation. For comparison, purification was performed using a commonly employed alkaline lysis method with a slightly higher yield (Fig. 4A, lane AM).

To ensure that the purified plasmids were devoid of contaminating chromosomal DNA and proteins, the recovered

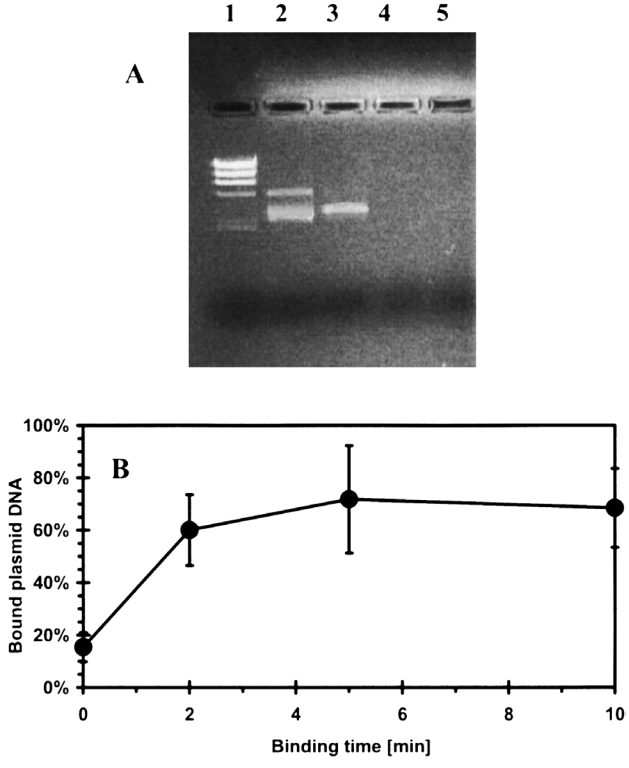

Figure 3. (A) Selectivity of plasmid binding by the ELP153MR protein. Binding was carried out as described in the text and eluted by heating at 60°C. Equal amounts of the eluted plasmid were analyzed by agarose-gel electrophoresis. Line 1: λ-*Hind*III molecular weight marker (Promega, Madison, WI); line 2: original amount of plasmid pBLU-Pt before binding; line 3: plasmid pBLU-Pt recovered; line 4: same as line 3, but ELP153MR was omitted from the binding mixture; line 5: same as line 3, but a control plasmid pBLUESCRIPT was used. (B) Kinetics of pBLU-Pt binding to ELP153MR. Binding was allowed to proceed for various time periods at room temperature before precipitation and the amounts of plasmid recovered are shown. Error bars represent standard deviation of three independent experiments.

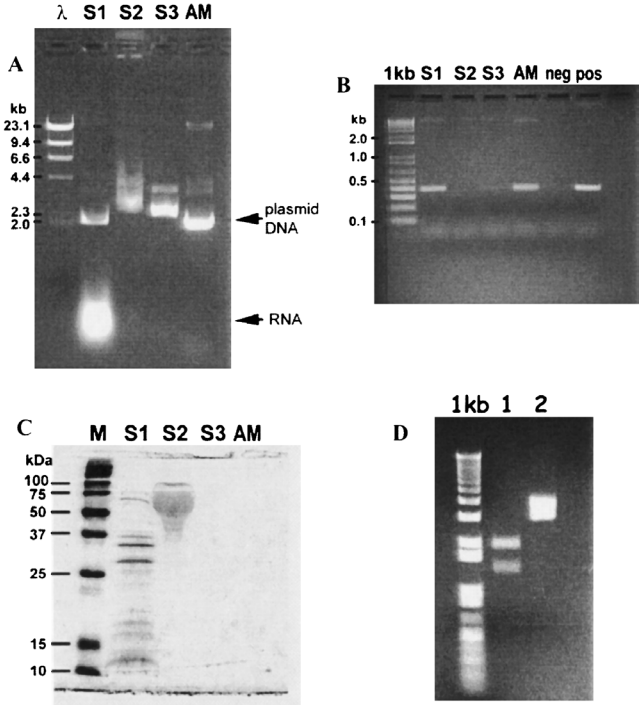

Figure 4. Plasmid purification from crude cell lysates. (A) Agarose-gel electrophoresis of purified plasmid samples. (B) Detection of contaminating chromosomal DNA by agarose-gel electrophoresis of PCR-amplified arsR. (C) Detection of contaminating protein by SDS-PAGE. (D) Restriction digest of the purified plasmid. Lane description: λ, *Hind*III-digested λ-DNA molecular weight marker (Promega, Madison, WI); 1kb, 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA); M, precision protein standards broad range (Bio-Rad, Hercules, CA); S1, cell lysates prepared from a standard boiling method (Sambrook and Russell, 2001); S2, biopolymer–plasmid DNA complex dissolved in TB74S buffer; S3, eluted plasmid DNA from the biopolymer–DNA complex; AM, plasmid DNA obtained from a standard alkaline lysis method; neg, negative control in the PCR reaction with the addition of water only; pos, positive control in the PCR reaction containing 20 ng of plasmid pETR harboring the ArsR gene (Xu and Rosen, 1997).

samples were subjected to further analysis. To probe the presence of chromosomal DNA, a sensitive PCR amplification method was used with a chromosomally encoded *arsR* gene as the target (Fig. 4B). As little as 20 ng of plasmid DNA containing the *asrR* gene was detected with this method. Again, no amplified fragment was observed with the affinity method, whereas traces of chromosomal DNA were detected with the alkaline lysis method. The presence of proteins in the purified samples was investigated by SDS-PAGE (Fig. 4C). In both cases, no contaminating protein was detected. More importantly, all biopolymers were removed from the purified plasmids after elution by a simple precipitation and centrifugation step. The recovered plasmids were easily digested with *Sac*I and *Not*I, indicating that they were intact and free of inhibiting materials (Fig. 4D).

DISCUSSION

In this report we have presented a new method for plasmid purification based on the interaction of a DNA-binding protein with its cognate promoter sequence. Rather than employing immobilized binding proteins as previously described (Lundenberg et al., 1990; Woodgate et al., 2002), plasmid was precipitated by a simple temperature trigger using the ELP biopolymer. This results in excellent plasmid yield, while removing contaminating chromosomal DNA, RNA, and proteins, all of which are essential factors for gene therapy or DNA vaccine applications. It remains to be demonstrated, however, whether this method is equally efficient for endotoxin removal. Plasmids used for gene therapy experiments tend to be larger than plasmids used in this study and it would of interest to elucidate how plasmid size influences the purification results. Although the MerR protein was used as a model in this investigation, the number of other DNA-binding sequences that could be used is virtually unlimited and may result in improved efficiency. One promising approach to generalize the utility of this method to a broader class of target plasmids is to exploit the interaction between the replication origin and its binding protein. A single ELP biopolymer containing this binding protein may be used for a wide range of plasmids containing the same replication origin.

Unlike triplex-helix affinity precipitation using thermoresponsive NIPAAM polymers, our method is unique in that it is a protein-only approach and no chemical synthesis is required. Rather than relying on the statistical nature of polymerization reactions, ELP biopolymers are specifically preprogrammed within a synthetic gene template that can be precisely controlled over chain length, composition, and sequence to satisfy any given applications of interest.

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