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Single-stranded DNA-binding protein facilitates gel-free analysis of polymerase chain reaction products in capillary electrophoresis

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Abstract

It has been recently demonstrated that single-stranded DNA-binding protein (SSB) can facilitate quantitative analyses of DNA, RNA, and proteins in gel-free capillary electrophoresis (CE). Here, we report the application of SSB-mediated gel-free CE for analyses of polymerase chain reaction (PCR) products. The unique ability of SSB to bind ssDNA but not double-stranded DNA (dsDNA) allows efficient separation of three types of DNA molecules in the PCR reaction mixture: primers, products (amplified templates), and by-products, which originate from non-specific DNA hybridization. SSB-mediated gel-free CE analysis of PCR products combines simplicity, high sensitivity, and outstanding quantitative capabilities. The ability of the method to distinguish between products and by-products makes this method an indispensable tool in preparative PCR (e.g., in the development of nucleotide aptamers).

Keywords: DNA; Proteins

1. Introduction

Many proteins involved in DNA replication, DNA damage control, DNA repair, and gene expression are capable of binding DNA and RNA with different affinities and sequence specificities [1–3]. This unique ability of DNA- and RNA-binding proteins makes them potentially valuable means in different kinds of analyses. So far, only a few DNA-binding proteins were used as analytical tools. The majority of applications relate to polymerase chain reaction (PCR), an in vitro procedure for the amplification of DNA. DNA-replicating enzymes (thermostable polymerases) are necessary for the synthesis of complimentary oligonucleotide chains. Moreover, single-stranded DNA binding proteins (SSB), which bind single-stranded DNA (ssDNA) sequence non-specifically but do not bind double-stranded DNA (dsDNA), are often used in PCR to prevent non-productive binding of DNA polymerases to ssDNA and thus increase the processivity of polymerases [4–6].

We recently proposed another analytical application of DNA- and RNA-binding proteins gel-free capillary electrophoresis (CE) [7]. In the proof-of-the-principle work, we demonstrated that SSB-mediated CE could facilitate gel-free affinity analyses of DNA, RNA, and proteins. The affinity probes used were hybridization probes for analyses of DNA and RNA and aptamer probes for analyses of proteins—both types of probes were ssDNA. We proved that SSB, when present in the CE run buffer, bound differently to free probes and probe-target complexes and by doing so, mediated their efficient separation in the gel-free environment. Here we report the application of SSB-mediated CE for gel-free analysis of PCR products.

In PCR, DNA templates are replicated by enzymatic extension of primers, so that the number of copies of the template grows exponentially with the number of cycles of PCR until the primers are depleted. The after-PCR reaction mixture may contain different amounts of three types of DNA molecules: remaining primers, products (amplified templates), and by-products, which originate from non-specific amplification of primers and products (Fig. 1A). The primers are ssDNA, the products are dsDNA, and the by products may contain both single stranded and double stranded regions and are denoted here as ss–dsDNA. Gel-based CE has been proven to enhance sensitivity and allow for the quantitation of PCR products [8–10]. In this work, we demonstrate highly

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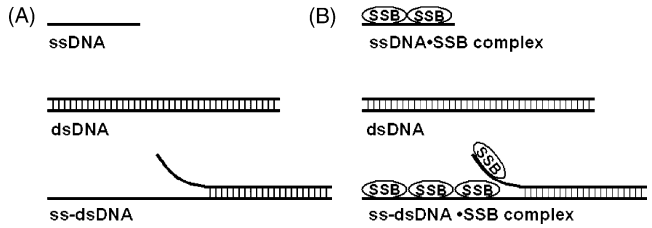

Fig. 1. (A) Schematic illustration of three types of DNA molecules, which may be present in the reaction mixture of PCR after its completion: ss-DNA remaining primers, dsDNA desirable products (amplified templates), and non-specific ss–dsDNA products of primer-primer or template-template hybridization. (B) Schematic illustration of binding of SSB to ssDNA and ss–dsDNA and the lack of such binding to dsDNA.

efficient gel-free analyses of PCR products in SSB-mediated CE. When present in the run buffer, SSB binds ssDNA and single-stranded regions of ss–dsDNA but does not bind dsDNA and double-stranded regions of ss–dsDNA (Fig. 1B). By doing so, SSB changes electrophoretic mobilities of the primers and by-products but does not change the mobilities of the products. These mobility shifts allow for the separation of products from the primers and by-products. Moreover, due to different SSB/DNA ratios in the SSB-primer and SSB-by-product complexes, the primers and by-products are also separated. The unique feature of the method is that it separates primers, products, and by-products, while masking the heterogeneity within the three groups of molecules. Therefore, even for highly heterogeneous samples, the three groups of molecules are perfectly separated and can be unambiguously identified and accurately quantitated. SSB-mediated gel-free CE analysis of PCR products offers simplicity, high sensitivity, and outstanding quantitative capabilities. The ability of the method to distinguish between products and by-products makes it a very valuable tool in preparative PCR, for example, in applications, such as the development of oligonucleotide aptamers [11,12].

2. Materials and methods

2.1. Chemicals and materials

Single-stranded DNA binding protein from *E. coli*, Taq DNA polymerase, fluorescein, and buffer components were obtained from Sigma-Aldrich (Oakville, Canada). DNA template and PCR primers were custom-synthesized by Integrated DNA Technologies (Coralville, IA, USA). The forward primer (3′→5′) was synthesized with a 6-carboxyfluorescein (6-FAM) functional group at the 5′-end and was 41 bases long; the reverse primer was labeled with biotin at the 5′-end and consisted of 45 bases. The DNA template was 122 bases long and had two constant regions (41 bases and 45 bases long) complementary to the primers at its ends and a 36-base long random region in the middle. All solutions were made, using the Milli-Q-quality deionised water and filtered through a 0.22 μm filter (Millipore, Nepean, Canada).

2.2. Polymerase chain reaction

The PCR reagent mixture consisted of 200 mM of each deoxyribonucleotide triphosphate (dNTP), 1 μM of each primer, and 0.05 units/μL of Taq enzyme in standard PCR buffer (Sigma-Aldrich) in the final volume 50 μL. DNA template was added in the amounts of 0, 10^3, 10^4, 10^5, 10^6, 10^8, 10^{10}, and 6×10^{11} molecules per reagent mixture. Thirty-two cycles of "touch-down" PCR were used for the amplification of template DNA. The first cycle included: melting for 30 s at 94 °C, annealing for 10 s at 80 °C, and extension for 10 s at 72 °C. Cycles 2–12 included: melting for 10 s at 94 °C, annealing for 10 s at (81-*N*) °C, where *N* is the cycle number, and extension for 10 s at 72 °C. Cycles 13–32 included: melting for 10 s at 94 °C, annealing for 10 s at 69 °C, and extension for 10 s at 72 °C. When 32 cycles were completed, a final extension was carried out for 30 s at 20 °C.

2.3. Slab-gel electrophoresis

Slab-gel electrophoresis of PCR products was performed with a Mini Gel Migration Tank GelXL (National Labnet, NJ, USA). DNA molecules were separated in a 1% agarose gel for 15 min with an electric field of 7 V/cm and stained with ethidium bromide.

2.4. Capillary electrophoresis

A P/ACE MDQ CE system with laser-induced fluorescence detection (Beckman Coulter, Fullerton, CA) was used for CE analyses. Fluorescence was excited with a 488 nm line of an argon-ion laser. Fused-silica capillaries (75 μm inner diameter and 365 μm outer diameter) were purchased from Polymicro (Phoenix, AZ, USA). Two capillary lengths were employed: 30.5 and 50 cm with distances to the detector of 20.3 and 39.8 cm, respectively. The electric fields across the capillaries were 655 and 400 V/cm, respectively. The running buffer was 25 mM sodium tetraborate at pH 9.4. For separation of dsDNA from ssDNA and ss–dsDNA, the running buffer was supplemented with SSB at the final concentration of 100 nM. Prior to every CE run, the capillary was flushed with 100 mM HCl, 100 mM NaOH, water, and CE run buffer for 2 min each. Samples were injected onto the capillary by a pressure pulse of 3.4 kPa for 5 s.

3. Results and discussion

3.1. Template/primers system

This work was inspired by the need to analyze the products of preparative PCR, which is used in selection of nucleotide aptamers. Therefore, as a DNA sample, we chose a

combinatorial library of DNA with an internal random sequence and two constant regions complementary to the primers at the ends. To facilitate fluorescence detection of primers and products in CE, we used a fluorescently labeled forward primer. To have the template/primers system similar to those employed in selection of aptamers, the reverse primer was labeled with biotin at its 5′-end—biotin facilitates separation of DNA strands on solid supports with immobilized streptavidin.

3.2. SSB-mediated separation of primers and products in gel-free CE

First, we used a moderate amount of the template (10^6 molecules), which did not cause a noticeable over-amplification under our PCR conditions; the after-PCR reaction mixture contained mainly primers and products and did not contain significant amounts of by-products. The after-PCR reaction mixture was analyzed by CE with and without SSB in the run buffer. Without SSB, electropherograms contained multiple unresolved peaks, which complicated both identification and quantitation of dsDNA. In the presence of SSB, ssDNA and dsDNA eluted as two well-resolved uniform peaks (Fig. 2); a resolution of 6.8 was achieved within the analysis time shorter than 10 min.

Second, we demonstrated that SSB-mediated CE facilitates sensitive and accurate quantitative analyses of PCR products. Our mode of PCR (“touch-down” amplification, primers lengths, number of cycles) was chosen to reduce unspecific reactions as much as possible while amplifying low-copy short templates. This approach is important in preparative PCR, which is used in the development of nucleotide aptamers [11,12]. The SSB-mediated CE analysis proposed here allows us to optimize preparative PCR conditions and estimate the quantity of dsDNA products and undesired by-products. In this work, we used the

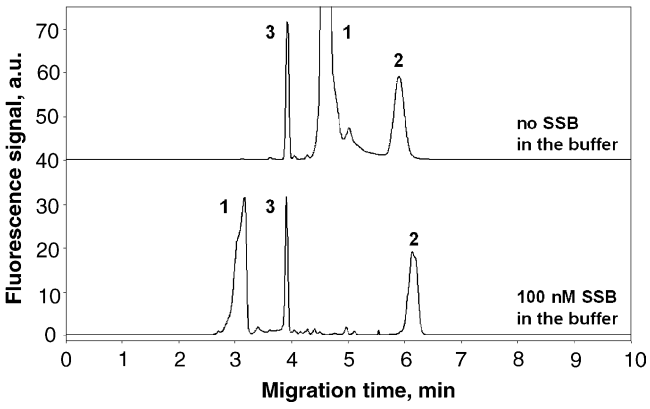

Fig. 2. SSB-mediated separation of ssDNA primers (peak 1) from dsDNA PCR products (peak 2) in gel-free CE with fluorescein (peak 3) as an internal standard. The top and the bottom electropherograms show the separation in the absence and in the presence of 100 nM SSB in the run buffer, respectively. PCR was started with 10^6 template copies. The separation electric field in CE was 655 V/cm.

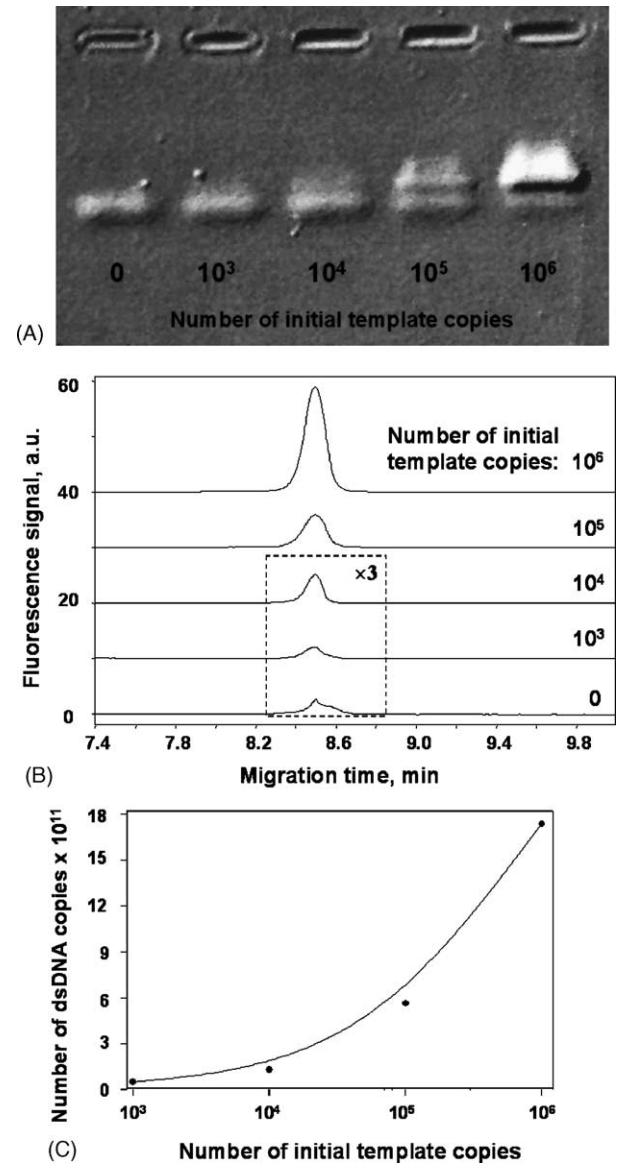

Fig. 3. Analysis of PCR products by slab-gel electrophoresis (A) and CE with 100 nM SSB in the run buffer (B). (C) Dependence of the amount of dsDNA formed in PCR on the initial number of template molecules in PCR. The separation electric field in CE was 400 V/cm.

SSB-mediated analysis to unambiguously identify dsDNA products of PCR and determine their absolute amounts using fluorescein as an internal standard (Fig. 3). The relative quantum yield of the fluorescein label in the primers was found to be approximately 0.5 which can be explained by fluorescence quenching upon binding to the oligonucleotide chain [13].

3.3. Detection of by-products

By-products are predominantly ss–dsDNA molecules which have both single-stranded and double-stranded regions (Fig. 1A). Although SSB forms complexes with both ssDNA primers and single-stranded regions of ss–dsDNA by-products, the SSB/DNA ratios in two types of complexes

are different (Fig. 1B). This leads to differences in electrophoretic mobilities of SSB·ssDNA and SSB·ss–dsDNA complexes—these mobilities also differ from that of dsDNA products, which do not form a complex with SSB. As a result, SSB-mediated CE allows for the separation of three types of molecules: ssDNA primers, dsDNA products and ss–dsDNA by-products. It is important to emphasize that ss–dsDNA by-products may have different lengths, but despite this heterogeneity, in the SSB-mediated electrophoresis, they elute as a single peak, which significantly simplifies the separation of products from by-products. Fig. 4 illustrates such an analysis for different initial numbers of template molecules in the sample. Slab-gel electrophoresis of PCR products led to the results that were hard to interpret (Fig. 4A). SSB-mediated CE, however, provided easily interpreted information on the product formation (Fig. 4B). When no template was introduced, only the peak of ssDNA primers was present (Fig. 4B, lower trace). When a moderate initial amount of the template was used, dsDNA products were formed, but by-products formation was insignificant (Fig. 4B, middle trace). For a large initial amount of the template, the formation of dsDNA products was negligible with respect to that of the ss–dsDNA by-products (Fig. 4C, upper trace). SSB-mediated CE analysis also allowed us to quantify the amounts of formed products and by-products for different initial numbers of template copies (Fig. 4C).

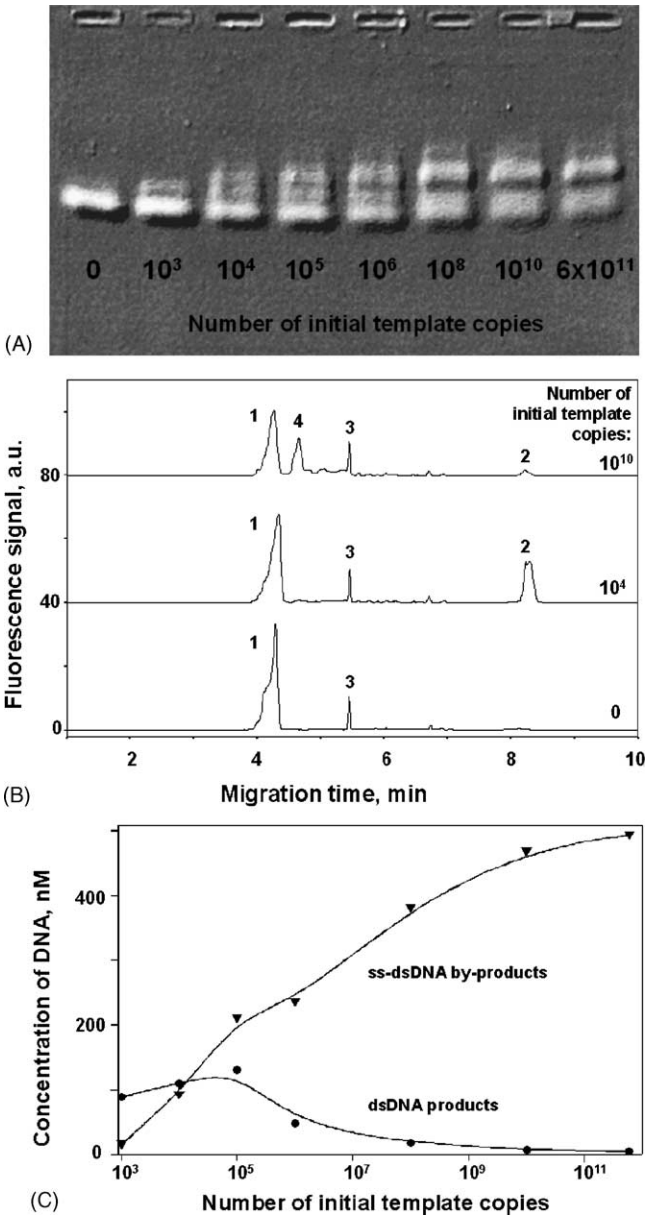

Fig. 4. Detection of various products in a post-PCR reaction mixture. Slab-gel electrophoresis (A) failed to elucidate dsDNA products while SSB-mediated CE analysis allowed separation (B) and quantitation (C) of ssDNA primers (peak 1), dsDNA products (peak 2), and ss–dsDNA by-products (peak 4). Thirty nanomolar fluorescein was used as an internal standard (peak 3). The separation electric field in CE was 400 V/cm.

Finally, although the developed method can separate dsDNA products from ssDNA primers and ss–dsDNA by-products it cannot distinguish dsDNA products of PCR amplification of specific template-primer hybridization from those of non-specific primer dimerization. The elimination of the primer–dimer amplification should be achieved in a classical way—through optimizing PCR conditions (mainly the number of cycles), so that there are no detectable dsDNA products in a "no-template control."

4. Conclusions

The proposed SSB-based CE analysis facilitates reliable and sensitive quantitation of products and by-products in PCR. In contrast to conventional slab gel electrophoresis and gel-filled CE, the SSB-based separation allows highly efficient separation of three groups of DNA molecules ssDNA primers, dsDNA desirable products, and ss–dsDNA undesirable by-products, while masking the heterogeneity within the groups. As a result, the new method is applicable to heterogeneous samples, such as combinatorial libraries of DNA used for aptamer selection. Since SSB binds only single-stranded regions of DNA, it will not interfere with DNA intercalating dyes, which bind only double stranded regions, thus allowing for versatile detection schemes [10]. In addition, the method can be automated and multiplexed for high-throughput analyses if multi-capillary instrumentation is used [14].

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References

[1] B. Ren, F. Robert, J.J. Wyrick, O. Aparicio, E.G. Jennings, I. Simon, J. Zeitlinger, J. Schreiber, N. Hannett, E. Kanin, T.L. Volkert, C.J. Wilson, S.P. Bell, R.A. Young, Science 290 (2000) 2306.

[2] F. Leng, R. McMacken, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 9139.
[3] D.B. Oh, Y.G. Kim, A. Rich, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 16 666.
[4] R.R. Meyer, P.S. Laine, Microbiol. Rev. 54 (1990) 342.
[5] R. Rapley, Mol. Biotechnol. 2 (1994) 295.
[6] E.K. Davydova, L.B. Rothman-Denes, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 9250.
[7] M. Berezovski, S.N. Krylov, J. Am. Chem. Soc. 125 (2003) 13 451.
[8] C.M. Roth, Curr. Issues Mol. Biol. 4 (2002) 93.
[9] Y. Zhang, Y. He, E.S. Yeung, Electrophoresis 22 (2001) 2296.
[10] J. Skeidsvoll, P.M. Ueland, Anal. Biochem. 231 (1995) 359.
[11] A.D. Ellington, J.W. Szostak, Nature 346 (1990) 818.
[12] C. Tuerk, L. Gold, Science 249 (1990) 505.
[13] R. Sjöback, J. Nygren, M. Kubista, Biopolymers 46 (1998) 445.
[14] M.S. Liu, V.D. Amirkhanian, Electrophoresis 24 (2003) 93.