Published in final edited form as:

Biotechnol J. 2011 July ; 6(7): 807–811. doi:10.1002/biot.201100024.

Rapid Communication Fast and easy protocol for the purification of recombinant S-layer protein for synthetic biology applications

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Abstract

A goal of synthetic biology is to make biological systems easier to engineer. One of the aims is to design – with nanometer-scale precision – biomaterials with well-defined properties. The surface layer protein SbpA forms two-dimensional (2D) arrays naturally on the cell surface of *Lysinibacillus sphaericus* but also as purified protein in solution upon addition of divalent cations. Its high propensity to form crystalline arrays, the simple way by which its crystallization can be controlled by divalent cations and the possibility to genetically alter the protein make SbpA an attractive molecule for synthetic biology. To be a useful tool, however, it is important that a simple protocol can be used to produce recombinant wild-type as well as modified SbpA in large quantities and in a biologically active form. The present study addresses this requirement by introducing a mild and non-denaturing purification protocol to produce milligram quantities of recombinant, active SbpA.

Keywords

S-layer protein; SbpA; non-denaturing purification; synthetic biology; electron microscopy

Introduction

A major goal in synthetic biology is to simplify the design and construction of biological systems [1]. SbpA [2–4], the surface layer (S-layer) protein of *Lysinibacillus sphaericus* and some other S-layer proteins are of particular interest to the synthetic biology community due

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The authors have declared no conflict of interest.

to their modular structure and their characteristic to form two-dimensional (2D) arrays in a divalent cation-dependent manner [5]. Although a non-denaturing purification protocol exists for the S-layer protein RsaA from *Caulobacter crescentus* and RsaA fusion proteins [6], purification of both native SbpA from *Lysinibacillus sphaericus* and recombinant SbpA (expressed in *E. coli*) involves the use of denaturing conditions [2, 4–10], often followed by lyophilizing and refolding the denatured protein by dialysis.

Here we present a simple method, a one-step, non-denaturing nickel affinity purification procedure, that makes it possible to produce milligram amounts of a recombinant, His-tagged version of SbpA (SbpA-His₇; residues 31-1068 [9]) as well as other N- and C-terminally truncated variants in *E. coli*. Previously, SbpA has been expressed at 37°C (see Supporting information). Here, expression in *E. coli* at low temperature (see Supporting information) allowed SbpA to fold properly and made it possible for the first time to purify recombinant SbpA under non-denaturing conditions. The recombinant SbpA variants retain their ability to form crystalline arrays in solution, which in the examples we examined correlates with the appearance of a white precipitate.

Materials and Methods

Preparation of recombinant SbpA protein under non-denaturing conditions

The gene encoding SbpA (residues 31-1268) was amplified by PCR from genomic DNA of *Lysinibacillus sphaericus* (ATCC number 4245) and ligated into the NdeI and BamHI restriction sites of the pET28a vector (EMD Biosciences) with a C-terminal 7xHis tag. From this construct, the three constructs used in this study were generated: SbpA-His₇ encoding residues 31-1068, a construct commonly used in engineering applications [9, 11–13], C-terminal truncation mutant SbpA_{C-trunc}-His₇ encoding residues 31-918 [14] and N-terminal truncation mutant SbpA_{N-trunc}-His₇ encoding residues 203-1068 [14].

Each construct was expressed in *E. coli* HMS174 (DE3) (EMD Biosciences). The culture was grown at 37°C in LB medium containing 50 µg/ml kanamycin and 50 µg/ml rifampicin until the OD₆₀₀ reached 0.6. Upon induction by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), the incubation temperature was lowered to 18°C. Eighteen hours after induction, cells were pelleted by centrifugation at 16,000g for 15 minutes at 4°C. The cell pellet was resuspended in lysis buffer [15] containing 20 mM Tris, pH 8.0, 300 mM NaCl, 10 mM imidazole and 1 mg/ml lysozyme (EMD Biosciences) and sonicated on ice. Insoluble debris was removed by centrifugation at 20,000g for 30 minutes at 4°C. The cleared lysate was incubated with Ni-NTA agarose beads (Qiagen, Valencia, CA) for 1 hour at 4°C or centrifuged through a Ni-NTA spin column (Qiagen, Valencia, CA). Following washing with buffer containing 100 mM imidazole in 20 mM Tris, pH 8.0, 300 mM NaCl, SbpA was eluted in buffer containing 250 mM imidazole as described [15]. The eluted protein was finally dialyzed at 4°C against 20 mM Tris, pH 8.0.

Purification of native SbpA

Native SbpA was prepared as described previously [8, 16].

SDS-PAGE, Western blotting, Quantitation

SDS-PAGE—Samples were run on 10% Bis-Tris gels (Invitrogen) and stained with Coomassie blue stain.

Western blots—His-tagged proteins were detected with anti-His antibody (GE Healthcare) and blots were developed with the alkaline phosphatase method using the Sigma Fast system (Sigma-Aldrich) [17].

Quantitation—The ratios of SbpA-His₇ in inclusion bodies and the soluble fraction were analyzed using quantitatively stained SDS-PAGE gels [18].

Specimen preparation, electron microscopy and image processing

Negative staining—Purified SbpA and 2D crystals of SbpA formed in solution or on lipid monolayers were stained with uranyl formate using the conventional negative staining technique described in [19].

Electron microscopy—Samples were imaged with an FEI Tecnai 12 electron microscope (FEI, Hillsboro, OR) equipped with an LaB₆ filament and operated at an acceleration voltage of 120 kV. Images were recorded on imaging plates under low-dose conditions at a nominal magnification of 67,000x and a defocus value of about −1.5 μm. Imaging plates were scanned with a Ditabis scanner (Pforzheim, Germany) using a step size of 15 μm, a gain setting of 20,000 and a laser power setting of 30% [20]. The images were then binned over 2×2 pixels for a final sampling of 4.5 Å/pixel at the specimen level.

Image processing—Fourier transforms were calculated using the *2dx* software package [21].

Results and Discussion

To produce SbpA under non-denaturing conditions, we amplified the SbpA gene from the genomic DNA of *Lysinibacillus sphaericus* (ATCC number 4525) [7, 9], cloned it into the pET28a vector following an established protocol [9] and expressed the protein in *E. coli* strain HMS174(DE3) (EMD Biosciences). Once induced, cells were cultured for 18 hours at 18°C, and then harvested and lysed as described before [15].

The cell lysate containing the recombinant SbpA-His₇ protein (Fig. 1A and B, lane 1) was incubated with nickel-nitrilotriacetic acid (Ni-NTA) affinity resin for 1 hr at 4°C. After washing with buffer containing 100 mM imidazole (Fig. 1A and B, lane 2), the protein was eluted with the same buffer containing 250 mM imidazole (Fig. 1A and B, lane 3). SDS-PAGE analysis showed that the one-step purification procedure yielded recombinant SbpA, running at an apparent molecular weight of 127 kDa (Fig. 1A, lane 3, black arrow), that is over 95% pure. A faint band at 53 kDa seen on the gel (Fig. 1A, lane 3, gray arrow) is also detected by the anti-His antibody (Fig. 1B, lane 3) and therefore must represent a C-terminal fragment of SbpA-His₇. For a comparison of the yield of different SbpA variants and a description of how SbpA-His₇ partitioned between the soluble fraction and inclusion bodies at different expression temperatures, see Supporting information and Fig. S1 and S2.

To characterize recombinant SbpA-His₇, we visualized the purified protein by negative stain electron microscopy (EM) (see Supporting information, Methods for details). Although the protein stains poorly and is thus difficult to see, the images reveal a heterogeneous particle population with particles adopting either a compact or a flexible, extended conformation (Fig. 1C). To determine whether the observed heterogeneity is an inherent characteristic of SbpA or specific to the recombinant protein, we also purified native SbpA from the cell walls of *Lysinibacillus sphaericus* (ATCC 4525) using a denaturing procedure [8]. Images of negatively stained samples of native SbpA showed a similarly heterogeneous particle population (Fig. 1D).

To assess the activity of both native and recombinant SbpA, we tested the two proteins for their ability to form 2D crystals in solution (see Supporting information, Methods for details). Addition of CaCl₂ to SbpA induces the protein to crystallize in solution, promoting the formation of self-assembly products [9, 14, 22]. When we added 50 mM CaCl₂ to native SbpA at concentrations of 1–2 mg/ml, we observed the formation of a white precipitate that settled over time at the bottom of the sample tube (Fig. 2A, top panel). For both native and recombinant SbpA at 7.6 nmol (i.e., 1 mg/ml for native SbpA), the white precipitate appeared after 18 hours, and no differences could be detected in the rate at which the precipitates formed and in the final amounts of the precipitates. Since precipitates never occurred in the absence of CaCl₂ (Fig. 2A, bottom panel), we speculated that the white precipitate is caused by the formation of SbpA crystals. This notion was verified by negative stain EM, which only showed 2D crystals in SbpA samples that contained the white precipitate. The same white precipitate also formed when 50 mM CaCl₂ was added to solutions containing different recombinant His-tagged SbpA versions, namely a long construct often used in bioengineering, SbpA-His₇ (residues 31-1068), an N-terminal truncation mutant, SbpA_{N-trunc}-His₇ (residues 203-1068), and a C-terminal truncation mutant, SbpA_{C-trunc}-His₇ (residues 31-918), which were all expressed in *E. coli* and prepared using our non-denaturing purification protocol.

To assess whether the recombinant proteins purified under non-denaturing conditions were functional, we used negative stain EM (see Supporting information, Methods) to compare the quality of 2D arrays formed by the recombinant proteins in solution with that of arrays formed by native SbpA (purified using a denaturing protocol). We prepared three samples each for native SbpA and the recombinant proteins (crystallized at a 7.6 nmol concentration) and found that 2D crystals were prevalent on all the grids (typically 10–14 crystals were present per grid square). The appearances of crystals formed by SbpA-His₇ (Fig. 2B), SbpA_{N-trunc}-His₇ (Fig. 2D), SbpA_{C-trunc}-His₇ (Fig. 2C) and native SbpA (Fig. 2E) were also indistinguishable. All arrays had side lengths that ranged from 0.5 to 2 μm, but the arrays formed by SbpA_{N-trunc}-His₇ were on average 30% smaller than those formed by the other SbpA variants. Furthermore, in lipid monolayer crystallization experiments, N-terminally truncated SbpA_{N-trunc}-His₇ consistently failed to form crystals on lipid monolayers (see Supporting information and Fig. S3). These results suggest that the N terminus of SbpA is predominantly required for surface binding but may also play a minor role in promoting array formation.

Fourier transforms calculated for five images of each sample of 2D crystals formed in solution indicated that all crystals had a similar degree of order with diffraction spots visible to a resolution of at least 25 Å (insets in Fig. 2B-E), close to the resolution limit imposed by the negative stain. The lattice constants for arrays formed by the various SbpA proteins were $a = b = 131 \pm 0.4$ Å for SbpA-His₇, $a = b = 131 \pm 3$ Å for SbpA_{N-trunc}-His₇ and $a = b = 129 \pm 4$ Å for SbpA_{C-trunc}-His₇ compared to $a = b = 131 \pm 0.9$ Å for the native protein.

Examination of purified native SbpA and recombinant SbpA-His₇ by negative stain EM revealed that the particle population in both preparations was heterogeneous. Although more of the SbpA-His₇ particles appeared to be in a compact conformation (Fig. 1C) and more of the native SbpA particles appeared to be in an extended, flexible conformation (Fig. 1D), both protein preparations were equally effective in forming 2D arrays in solution (Fig. 2). Thus, the different appearance of the two protein preparations does not appear to represent proteins with different properties but may simply indicate that SbpA is in a dynamic equilibrium that can shift between a compact and extended, flexible conformation.

All SbpA proteins we tested formed crystals in solution, and the presence of SbpA crystals in solution correlated in every case with the formation of a white precipitate in the sample solution. Previously, however, an expressed C-terminal truncation mutant of SbpA was purified using a denaturing purification procedure and formed crystals that displayed *p*1 rather than *p*4 symmetry [14]. By contrast, crystals formed by our identical SbpA_{C-trunc}-His₇ construct (except for an additional histidine residue in the affinity tag) that was isolated using our non-denaturing purification procedure still formed crystals with the native *p*4 symmetry (Fig. 2E). This result suggests that the loss of four-fold symmetry observed in the previous study does not result from the C-terminal truncation but from the harsh purification conditions that were used to purify the truncation mutant.

Concluding Remarks

Our non-denaturing purification procedure to prepare recombinant active SbpA variants in milligram amounts is likely to aid in the engineering and production of new biomaterials. In the field of synthetic biology, most chemical biosynthesis reactions are currently performed using enzymes produced within cells, which can be problematic if toxic products are produced [23], if the ratio of different enzymes needs to be tuned genetically [24], or if enzymes have to be brought together to obtain high yields [25]. Our protocol may be particularly advantageous to produce SbpA with C-terminally fused enzymes, which are likely more challenging to refold. Such fusion proteins would make it possible to bring different enzymes together by assembling them onto beads [26] or in solution and to vary their ratios simply by altering the concentration of each SbpA fusion protein before assembly. The ability to purify SbpA under non-denaturing conditions thus makes SbpA a powerful tool for *in vitro* synthetic biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a KAUST Scholar Graduate Research Fellowship (JEN), the SynBERC NSF ERC (www.synberc.org) (JEN and TFK), National Institutes of Health grant GM52580 (to S. C. Harrison) and the U.S. Army Research Office through both the Institute for Soldier Nanotechnologies and the Institute for Collaborative Biotechnologies (AMB). T.W. is an investigator in the Howard Hughes Medical Institute.

Abbreviations

ATCC	American Type Culture Collection
EM	electron microscopy
<i>E. coli</i>	<i>Escherichia coli</i>
His	histidine
Ni-NTA	nitrilotriacetic acid
SbpA-His₇	7 histidine-tagged SbpA containing residues 31-1068
SbpA_{C-trunc}-His₇	C-terminally truncated SbpA containing residues 31-918
SbpA_{N-trunc}-His₇	N-terminally truncated SbpA containing residues 203-1068
S-layer	surface layer

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Figure 1. Expression and purification of recombinant SbpA-His₇ containing residues 31 to 1068
 The non-denaturing purification process was followed by (A) SDS-PAGE and (B) western blot analysis with anti-His antibody. Lane 1, cell lysate; Lane 2, 100 mM imidazole wash; Lane 3, 250 mM imidazole elution. (C) Negative stain EM image of SbpA-His₇ purified using the non-denaturing protocol. (D) Negative stain EM image of full-length native SbpA purified using a standard, denaturing protocol. The black arrows in C and D point to protein in the extended conformation, which stains poorly and is difficult to see. Scale bar shows 50 nm. Experiments were reproducible within ten different preparations.

Figure 2. Native and recombinant SbpA are active and form 2D crystals in solution

(A) Addition of 50 mM CaCl_2 to full-length native SbpA results in a white precipitate (top panel) that does not occur in the absence of CaCl_2 (bottom panel). (B-E) Images of crystalline arrays formed by SbpA variants in solution; (B) SbpA-His₇ (residues 31-1068), (C) SbpA_{C-trunc}-His₇ (residues 31-918), (D) SbpA_{N-trunc}-His₇ (residues 203-1068) and (E) native SbpA. Scale bar shows 100 nm. SbpA-His₇ and native SbpA experiments were reproducible within ten different preparations. SbpA_{C-trunc}-His₇ and SbpA_{N-trunc}-His₇ were reproducible within three different preparations.