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Thermostable single domain antibody–maltose binding protein fusion for *Bacillus anthracis* spore protein BclA detection

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ABSTRACT

We constructed a genetic fusion of a single domain antibody (sdAb) with the thermal stable maltose binding protein from the thermophile *Pyrococcus furiosus* (*Pfu*MBP). Produced in the *Escherichia coli* cytoplasm with high yield, it proved to be a rugged and effective immunoreagent. The sdAb–A5 binds BclA, a *Bacillus anthracis* spore protein, with high affinity ($K_D \sim 50$ pM). MBPs, including the thermostable *Pfu*MBP, have been demonstrated to be excellent folding chaperones, improving production of many recombinant proteins. A three-step purification of *E. coli* shake flask cultures of *Pfu*MBP–sdAb gave a yield of approximately 100 mg/L highly purified product. The *Pfu*MBP remained stable up to 120 °C, whereas the sdAb–A5 portion unfolded at approximately 68 to 70 °C but could refold to regain activity. This fusion construct was stable to heating at 1 mg/ml for 1 h at 70 °C, retaining nearly 100% of its binding activity; nearly one-quarter (24%) activity remained after 1 h at 90 °C. The *Pfu*MBP–sdAb construct also provides a stable and effective method to coat gold nanoparticles. Most important, the construct was found to provide enhanced detection of *B. anthracis* Sterne strain (34F2) spores relative to the sdAb–A5 both as a capture reagent and as a detection reagent.

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Introduction

Bacillus anthracis (BA)[2] is the etiological agent that causes lethal anthrax infections unless prompt medical intervention is implemented. Much of BA's threat arises due to its spore form, which persists in harsh environments [1]. It is this spore form that is the principal threat agent and the target for detection in environmental samples. Although it is possible to detect proteins derived from the vegetative cell such as EA1, an S-layer protein that is commonly found in BA spore preparations unless washed extensively [2,3], spore coat proteins, such as BclA (*B. anthracis* collagen-like protein), are often favored because they are intrinsic to the target [4]. BclA is an immunodominant glycoprotein and the major component of the hair-like projections that cover the exosporium of BA spores [5–8].

A number of immunoreagents have been described for the detection of BclA [9–15]; however, the development of immunoreagents is not without difficulties. In many cases, particularly where a recombinant antigen is used, the resulting antibody may target epitopes not accessible in the intact organism [16–18]. In addition, traditional immunoreagents lack the thermostability necessary to eliminate the need for cold storage prior to use, a difficulty when fielding detection equipment. Recently, our group developed a number of single domain antibodies (sdAbs) toward BA spore-specific proteins [19]. The sdAbs derived from camelid heavy-chain-only antibodies are remarkable for their small size, high stability, solubility, and ability to bind epitopes not accessible to

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[2] *Abbreviations used:* BA, *Bacillus anthracis*; BclA, *B. anthracis* collagen-like protein; sdAb, single domain antibody; MBP, maltose binding protein; *Pfu*, *Pyrococcus furiosus*; ECBC, Edgewood Chemical Biological Center; TB, Terrific Broth medium; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; IMAC, immobilized metal affinity chromatography; UV, ultraviolet; OD, optical density; FPLC, fast protein liquid chromatography; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; PBST, PBS containing Tween 20; Bt, biotinylated tracer; SPR, surface plasmon resonance; DSC, differential scanning calorimetry; CD, circular dichroism; T_m, melting temperature; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AuNP, gold nanoparticle; ELISA, enzyme-linked immunosorbent assay.

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conventional antibodies; these features make them especially suitable for many therapeutic and diagnostic/biodetection applications [20–22]. Of the sdAbs developed toward the BA spore proteins, those isolated toward BclA appeared to be the most useful, likely due to its abundance on the spore surface [23]. Although we have successfully used unfused sdAbs for assay applications [24,25], we have also expressed sdAb fusion constructs to augment their utility [26,27].

To date, most of the sdAbs prepared by our group used shake flask *Escherichia coli* fermentation with overexpression of our heterologous protein into the periplasm; this method typically provides protein yields ranging from 1 to 20 mg/L. To increase production without the cost of simply scaling up volume, a number of alternatives have been employed, including production in yeast, filamentous fungi, or insect cells [28]. Another common approach is to move to cytoplasmic expression in *E. coli*, an approach that, while having the advantages of increased yields and familiarity, is not without complications. Overexpression of recombinant proteins in the cytoplasm often results in inclusion body formation, and the reducing environment of the cytoplasm can also prove to be problematic for proteins such as sdAbs that include disulfide bonds [29].

The use of chaperones, either coproduced or as a fusion, to improve production has been a fruitful approach [30]. For our application, we desired a chaperone that would improve cytoplasmic production without compromising the thermostability of our sdAbs. Maltose binding protein (MBP) has proven to be one of the most effective chaperones to date, enabling cytoplasmic production of high levels of active antibody fragments [31]. The MBP variant derived from the thermophile *Pyrococcus furiosus* (*Pfu*) has been shown both to be an effective chaperone and to help thermostabilize the resulting fusion construct [32,33]. Here, we investigated the utility of *Pfu*MBP to improve cytoplasmic production of an sdAb and its impact on affinity, thermostability, and utility for the detection of BclA and BA spores.

Materials and methods

Materials

BclA (locus_tag: BA_1222) with N-terminal histidine tag, recombinantly produced in *E. coli* (cat. no. NR-9577), was obtained from BEI Resources Repository, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). A C-terminal fragment of *B. anthracis* collagen-like protein, iBclA, was more soluble and was a kind gift from Michael Weiner (AxioMx); iBclA included both a His tag and a biotinylation tag (see Fig. S1 of online supplementary material). Spores from *B. anthracis* Sterne strain (34F2) were produced using protocols described previously [2]. Depending on the assay, spores were used as either intact or broken samples. Broken samples were obtained via mechanical shearing with glass beads and a BioSpec Mini BeadBeater [19].

The affinity-purified polyclonal goat anti-*B. anthracis* antibody was a kind gift from Jill Czarnecki (Naval Medical Research Center). The polyclonal goat anti-*B. anthracis* antibody was a kind gift from Thomas O'Brien (Tetracore, Rockville, MD, USA). The anti-BclA Fab BA21 was a kind gift from James Carney (Edgewood Chemical Biological Center, ECBC).

Unless otherwise specified, chemical reagents were acquired from Sigma–Aldrich, Fisher Scientific, or VWR International. Restriction endonucleases and ligation reagents were obtained from New England Biolabs. DNA amplification was accomplished with the Roche Expand High Fidelity DNA polymerase kit. Specific kits and assays are defined where applicable.

Cloning and production of sdAbs and PfuMBP–sdAb fusion

The sdAb–A5 and sdAb–B7 were selected from an sdAb phage display library and periplasmically produced as described previously [19]. The gene for *Pfu*MBP [33] was synthesized by Genescript with *Nco*I and *Xho*I sites for integration into the pET22 vector for periplasmic expression or the pET28 vector for cytoplasmic expression. In addition to the sequence of *Pfu*MBP, the construct included a poly-asparagine flexible linker, factor Xa site, and directional *Sfi*I restriction endonuclease sites for cloning the sdAbs. The sdAb–A5 was cloned into this vector, and the fusion of the sdAb–A5 with the *Pfu*MBP was termed *Pfu*MBP–A5. See Fig. S2 of the supplementary material for predicted amino acid sequence.

To prepare soluble *Pfu*MBP–A5, the expression vector was first transformed into *E. coli* Rosetta (Novagen, Madison, WI, USA). Single colonies were grown overnight in Terrific Broth medium (TB) with 2% glucose. The next day, 0.5 L of TB was inoculated with the overnight culture and grown at 37 °C for 3 h and then moved to a 30 °C incubator, and protein production was induced with 1 mM isopropyl β-D-1-thiogalactoside. After 3 h, the cells were pelleted by centrifugation and frozen (−20 °C) overnight prior to purification. For protein purification, the cell pellet from one-third of a liter (~5 g) was resuspended in 20 ml of phosphate-buffered saline (PBS, pH 7.4) and 1 mM ethylenediaminetetraacetic acid (EDTA). Then, 1 mg of lysozyme, 0.2 ml of 100 mM phenylmethylsulfonyl fluoride (PMSF), and 2 ml of glycerol were added to the bacterial suspension. The solution was transferred to a 50-ml conical tube and sonicated two times for 1 min using 50% power output and 50% duty cycle on a Branson sonifier. Tween 20 was added to a final concentration of 0.5% (v/v), and the cells were sonicated another two times as before. The solution was then centrifuged for 15 min at a speed of 15,000 rpm in a Dupont SS-34 rotor to pellet insoluble cell debris. The supernatant was moved to a 50-ml conical tube, diluted to 45 ml with water, and 0.1 ml of 0.5 M $MgCl_2$, Ni^{2+} Sepharose High Performance (~5 ml, GE Healthcare), and 5 ml of 10× immobilized metal affinity chromatography (IMAC) buffer (200 mM phosphate buffer [pH 7.4], 4 M NaCl, and 0.2 M imidazole) were added. Tubes were rotated for 1 to 2 h at 4 °C using a Dynal Rotisserie. The resin was pelleted via low-speed centrifugation and then washed with 1× IMAC buffer (~20 ml). The resin was washed a minimum of two times. After washing, the resin was loaded into a 25 × 200-mm column attached to a Bio-Rad Econo pump system with an in-line ultraviolet–visible (UV–Vis) detector. The resin was washed with 1× IMAC buffer to establish a baseline of approximately 5 bed volumes. Protein was eluted using an acidic buffer (50 mM citrate [pH 4.0], 0.5 M NaCl, and 0.1 mM PMSF). To remove aggregates of the *Pfu*MBP–A5 from the column, the Ni^{2+} resin was washed with 10 ml of 0.25 M imidazole; the resin was then regenerated prior to reuse. The acid-eluted *Pfu*MBP–A5 was further purified using an amylose resin column (New England Biolabs) having double the capacity of the measured quantity of *Pfu*MBP–A5 loaded. The column was washed with PBS until baseline; the *Pfu*MBP–A5 was eluted using PBS containing 200 mM D-(+)-maltose monohydrate (type II, ⩾95%, ⩽1.5% glucose, ⩽5% maltotriose) from Sigma–Aldrich. The most concentrated fractions (>5 mg/ml) were further purified by gel filtration using a GE Superdex High-Load G200 column on a Bio-Rad Duo-Flow System fitted with a 5-ml sample loop. Protease inhibitor cocktail (Sigma–Aldrich) was added to the final product at a 1:1000 dilution. Proteins were quantified by optical density (OD) at 280 nm on a Nanodrop 1000 instrument. Aliquots were either stored at 4 °C or frozen on dry ice for storage at −0 °C prior to analysis. Dilute eluent from the amylose column was concentrated using Ni^{2+} Sepharose resin prior to gel filtration. Cytoplasmically produced sdAb–A5 was produced as above with the following exceptions. The IMAC