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U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

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PILOT-SCALE PRODUCTION AND TESTING OF A RECOMBINANT STAPHYLOCOCCAL ENTEROTOXIN (SEB) TRIPLE MUTANT

Alena Calm
RESEARCH AND TECHNOLOGY DIRECTORATE

E. Randal Hofmann
Gabrielle Boyd
EXCET, INC.
Springfield, VA 22150-2519

Chris Mangaya
Kelley Betts
LEIDOS, INC.
Reston, VA 21010-6848

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Calm, Alena (ECBC); Hofmann, E. Randal; Boyd, Gabrielle (Excet); Mangaya, Chris; and Betts, Kelley (Leidos)

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Director, ECBC, ATTN: RDCB-DRB-M, APG, MD 21010-5424
Excet, Inc., 6225 Brandon Avenue, Suite 360, Springfield, VA 22150-2519
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The use of chemically or thermally inactivated biothreat toxins in detection methodologies can prove problematic when the resulting toxoids become attenuated or altered to the point that they are no longer recognizable by the antibody-based test methods for which they were intended. Recombinant technology was used to assess whether a nontoxic surrogate could be developed that retains the relative epitopes for antibody-based detection platforms. Here, three small batches of staphylococcal enterotoxin B triple mutant (L45R, Y89A, and Y94A) were prepared for the Defense Biological Products Assurance Office (DBPAO) to demonstrate the reproducibility of recombinant antigen production. Yields of starting material and final purified product were determined. Post-production testing for these batches was performed to identify purity, homogeneity, and activity as determined by the DBPAO using a Meso Scale Discovery SEB SinglePlex PR2 electrochemiluminescence assay (Meso Scale Diagnostics; Rockville, MD). Test results revealed that this product demonstrates high levels of reproducibility and consistency in yield, purity, homogeneity, and antigenicity.

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PREFACE

The work described in this report was started in March 2010 and completed in December 2011.

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PILOT-SCALE PRODUCTION AND TESTING OF A RECOMBINANT STAPHYLOCOCCAL ENTEROTOXIN (SEB) TRIPLE MUTANT

1. INTRODUCTION

The use of chemically or thermally inactivated biothreat toxins in detection methodologies can prove problematic when the resulting toxoids become attenuated or altered to the point that they are no longer recognized by the antibody-based test methods for which they were intended. To address this problem, recombinant technology has been applied to assess whether a nontoxic surrogate can be developed that retains the relative epitopes for antibody-based detection platforms.

Staphylococcal enterotoxins are members of a family of more than 20 different, functionally related staphylococcal and streptococcal exotoxins that share sequence homology.[1–3] These bacterial proteins are known to be pyrogenic and are connected to significant human diseases such as food poisoning and toxic shock syndrome. For the most part, these toxins are produced by *Staphylococcus aureus,* although other species are also enterotoxigenic.[1–3] Staphylococcal enterotoxins are broadly classified as superantigens, which have the ability to stimulate large populations of T-cells (~20–30%), leading to the production of a cytokine bolus.[1–3] At least 20 serologically distinct staphylococcal superantigens have been described that include staphylococcal enterotoxin A through V (SEA, SEB, etc.) and toxic shock syndrome toxin-1 (TSST-1). SEA, SED, and SEE share 70–90% sequence homology, whereas they share only 40–60% sequence homology with SEB, SEC, and TSST-1.[1–3] Staphylococcal enterotoxins have mature lengths of ~220–240 amino acids, depending on the toxin, and their molecular sizes are ~25 kDa, on average. Staphylococcal enterotoxins have significant sequence variability, but when they are folded, they have similar three-dimensional structures.

SEB is soluble in water and is quite stable to heat, proteolytic digestion, and a wide pH range. These characteristics make SEB easy to produce and distribute.[4] The effective dose of SEB for 50% of the population (ED_{50}) is 0.0004 µg/kg, and the lethal dose for 50% of the population (LD_{50}) is only 0.02 µg/kg;[2] therefore, inhalation of large quantities of SEB can lead to death by septic shock. Although SEB is responsible for only ~10% of food poisoning cases, in aerosol form, SEB is extremely toxic: the lethal dose for a 70 kg adult is only 1.4 µg.[1] Because of the low quantities needed to debilitate soldiers on the battlefield, SEB is considered a major biological threat. The relative ease of SEB transmission through food and water, as well as the toxin's ability to be aerosolized, make the need for detection assays extremely important. Several techniques are currently available for SEB detection.[1,4,5]

SEB toxicity is mediated through its interaction with the major histocompatibility complex (MHC) class II on target cells, which results in widespread leukocyte proliferation and cytokine release. Single mutations of key residues in the polar pocket (Y89A and Y115A) or the hydrophobic binding loop (L45R) eliminate binding to the MHC class II molecule, human leukocyte antigen–antigen D related (HLA-DRl). Previous studies of a recombinant SEB triple mutant (L45R, Y89A, and Y94A) produced in *Escherichia coli* cells demonstrated a lack of super-antigen activity in rhesus monkey leukocyte cultures from animals immunized with the

attenuated recombinant vaccine.[6] Furthermore, the SEB triple-mutant vaccine (SEBv) showed protection in both mice and Rhesus monkeys when challenged with wild-type SEB.[6]

To investigate whether recombinant SEB triple-mutant protein antigen could be reproducibly manufactured, three small batches of recombinant SEBv that contained the three mutations (L45R, Y89A, and Y94A) were prepared by the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) using expression constructs provided by Ellen Goldman and George Anderson at the Naval Research Laboratory (NRL; Washington, DC).[7] Following standard affinity purification protocols, we characterized the lots of recombinant protein for biophysical and immunological properties. Our test results demonstrated high reproducibility and consistency in yield, purity, homogeneity, and antigenicity.

2. METHODS

2.1 Expression of SEBv in *E. coli*

Eleven nanograms of pET15b plasmid DNA containing the coding region for SEBv (provided by NRL) was transformed into BL21 (DE3) pLysS electrocompetent *E. coli*. For each batch, 10 mL of Luria broth with 100 µg/mL of carbenicillin was inoculated from a single colony. The cultures grew overnight at 37 °C and were scaled up to six 500 mL cultures for a total of 3 L per lot in Novagen Overnight Express instant terrific broth media (EMD Millipore; Billerica, MA) containing 100 µg/mL of carbenicillin. The *E. coli* batches were cultured for 20 h. The cell mass was collected via centrifugation to create a cell paste and frozen at –80 °C until purification.

2.2 Affinity Purification of SEBv

To purify recombinant SEBv, the cell paste was thawed and resuspended in wash buffer (20 mM sodium phosphate, 20 mM imidazole, and 500 mM sodium chloride, pH 8.0) in a 1:5 weight–volume ratio. Cells were lysed using an M-110P microfluidizer (Microfluidics; Westwood, MA) at 20,000 psi. The lysate was clarified via centrifugation at 40,000 ×*g* for 2 h, and the supernatant was frozen at –80 °C. For the sake of reproducibility, the other lots were treated in the same manner. Supernatants were thawed and applied to a 1 mL nickel affinity column (GE Healthcare; Piscataway, NJ) at 0.4 mL/min on an ÄKTAxpress system (GE Healthcare). The column was washed with 20 column volumes of wash buffer to remove any loosely bound material, and SEBv was eluted with 500 mM imidazole. Peak fractions were detected by the ÄKTAxpress system, sent to an in-line desalting column (GE Healthcare), and eluted with phosphate-buffered saline (PBS; Fisher Scientific; Pittsburgh, PA).

2.3 Concentration Analysis of SEBv

The SEBv concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific; Waltham, MA). The absorbance at 280 nm was recorded, and the concentration was calculated using a 1.1 extinction coefficient (as calculated with the ProtParam tool on the ExPASy server [Expasy.org]) and the sequence for SEBv.

2.4 Assessment of Purity

Molecular weight and purity data were collected using an Experion automated electrophoresis system (Bio-Rad; Hercules, CA). For Experion analysis, each of the SEBv samples was processed using the validated procedure specified in the *Bio-Rad Experion Pro260 Analysis Kit* manual.[8] Briefly, a Pro260 microfluidic chip was prepared by adding 12 µL of Pro260 gel and gel stain to the designated wells. The chip was then placed on the priming station and primed for 1 min at the medium (B) pressure setting. The priming filled the fluidic channels with gel, which was used by the instrument to form a barrier between samples during the run. The sample was reduced with 2-mercaptoethanol (Bio-Rad), denatured in the kit-provided sample buffer at 95 °C, and then applied to the primed chip. The chip was placed in the instrument, and the lid was closed, lowering the sample needles into the wells. The instrument was operated using the Experion software, and each chip required 30 min to complete. All samples were run in triplicate, and analysis was performed using the Experion software.

2.5 Determination of Polydispersity and Hydrodynamic Radius Using Dynamic Light Scattering (DLS)

For DLS analysis, five 20 µL aliquots of the SEBv samples were placed into a quartz 384 well plate (Wyatt Technology Corporation; Santa Barbara, CA) and centrifuged for 2 min at 1000 ×*g* to remove trapped air bubbles. Mineral oil (Sigma-Aldrich; St. Louis, MO) was applied to the top of each sample to prevent sample evaporation, and the plate was then placed into a DynaPro temperature-controlled plate reader (Wyatt Technology). Each well was scanned 10 times for 5 s each at 25 °C. Wyatt Technology Dynamics software was used to obtain triplicate results, which were averaged to provide measurements of polydispersity, hydrodynamic radius, percent mass, and molecular weight for each sample.

2.6 Determination of Antigenicity

The reactivity to SEB-specific antibodies in an immunoassay was measured using the Meso Scale Discovery (MSD) PR2 model 1800 electrochemiluminescence (ECL) detection system (Meso Scale Diagnostics; Rockville, MD). Defense Biological Products Assurance Office (DBPAO) provided the MSD singleplex anti-SEB plates. SEBv samples were diluted in PBS containing 0.1% Triton X-100 solution (PBS-T). Serial dilutions were generated for assay linearity determinations, estimated limits of detection (LODs), signal-to-noise ratios (S/Ns), and assay variability. Assays were performed in accordance with the MSD protocol and using the detection antibody solution, diluent, and read buffer that were provided with the assay. Background signal was determined from the average of four blank (buffer only) samples, and test sample values greater than 3 standard deviations above background were considered significant.

3. RESULTS

3.1 Expression and Purification of SEBv in *E. coli*

SEBv with a C-terminal histidine tag was grown as three separate batches on separate days and purified using nickel affinity purification. The wet mass of the bacterial paste averaged 34 ± 3 g of starting material. The final yield for each 3 L batch was 11.2 ± 0.3 mg, for an average of 3.8 ± 0.1 mg/L of culture (Table 1). The protein concentration as estimated by the ÄKTAxpress software was confirmed using a NanoDrop spectrophotometer. These concentrations were 1.05, 1.25, and 1.23 mg/mL for lots 10052015-01, 10062015-01, and 10072015-01, respectively.

Table 1. SEBv Production Data

Lot Number	Pellet Weight (g)	Volume* (mL)		SEBv* Concentration (mg/mL)	Yield	
		Load	Elution*		Total SEBv* (mg)	SEBv/L Culture (mg/L)
10052015-01	33.6	210	10.0	1.1	11.0	3.7
10062015-01	31.3	185	10.1	1.1	11.1	3.7
10072015-01	37.2	210	10.5	1.1	11.6	3.9

*Data generated from ÄKTAxpress software.

3.2 Purity Analysis of SEBv

Each lot of SEBv was analyzed for purity using the Experion lab-on-a-chip Pro260 system. SEBv is a 31 kDa protein and should be visible as a single band on the electronic gel image. Figure 1A shows that all three lots were greater than 95% pure, and the molecular weight was between 32 and 33 kDa when stored at –4 °C. Figure 1B demonstrates that all three production lots were greater than 95% pure, and the molecular weight was between 32 and 33 kDa when stored at –80 °C. The cumulative results demonstrate that the production, extraction, and purification methods yielded a SEBv protein that was consistently pure across different production runs.

Figure 1. Experion system-generated gel image of SEBv stored at (A) 4 °C and (B) –80 ºC, at time zero. Lanes 1–3 represent lots 10052015-1, 10062015-1, and 10072015-1, respectively. The ladder (far left) is the molecular weight standard.

3.3 Homogeneity and Dispersity

DLS was used to interrogate each lot of SEBv to determine the physical properties of SEBv in solution. DLS identifies protein–protein interactions and the state of aggregation in solution. All data analyses were performed using DynaPro software (Wyatt Technology). Based on the globular protein prediction algorithm in the DynaPro software, SEBv was estimated to have a hydrodynamic radius of 2.6 nm. The correlation graphs for all samples exhibited a quick decay and a smooth sigmoidal curve, which are indicative of small, uniform particle size (data not shown). Table 2 summarizes the data gathered from the DLS analyses. In general, there were no significant differences in the physical properties of the different production lots stored at different temperatures. The average radius for all three lots was 2.8 nm, and the standard deviations were 1.4 and 1.6 nm for 4 and –80 °C, respectively. The average peak percentages of polydispersity were 17.6 ± 7.5 and 15.2 ± 4.5% at 4 and –80 °C, respectively. This data represented >99.9% of the mass measured and fell within the expected range based on the molecular weight of SEBv. The corresponding graphs are provided in the appendix.

Table 2. SEBv Production Data from DLS Analysis

Lot Number	Temperature (ºC)	Radius (nm)	Peak Polydispersity (%)	Peak Mol Wt (kDa)	Peak Mass (%)
10052015	4	2.8002	12.385	37.546	99.971
10062015	4	2.7377	14.114	36.102	99.985
10072015	4	2.7438	13.401	35.742	99.966
10052015	–80	2.6253	11.87	32.262	99.947
10062015	–80	2.7438	13.401	35.742	99.966
10072015	–80	2.9431	20.244	42.159	99.973

3.4 Activity of SEBv

Each lot of SEBv was tested for activity using the MSD ECL assay for staphylococcus enterotoxin B. The plates were read on the PR2 instrument, and results were analyzed using GraphPad Prism software (GraphPad Software; San Diego, CA). ECL assay indicated that the 4 and –80 °C samples exhibited the same activity, and there was no significant difference in activity between the three lots (Figure 2).

All three lots of SEBv stored at –80 ºC were detected at concentrations as low as 0.5 pg/mL (LOD) with an average S/N of 1.8. Two of the lots stored at 4 °C had an LOD of 0.5 pg/mL, and lot 10072015-01 was detected as low as 0.1 pg/mL. The average S/N for these three lots was 1.6. These LODs were in line with the confirmed LOD of a pilot batch that was used to characterize the MSD singleplex plates on the PR2 model 1800 ECL system for DBPAO.

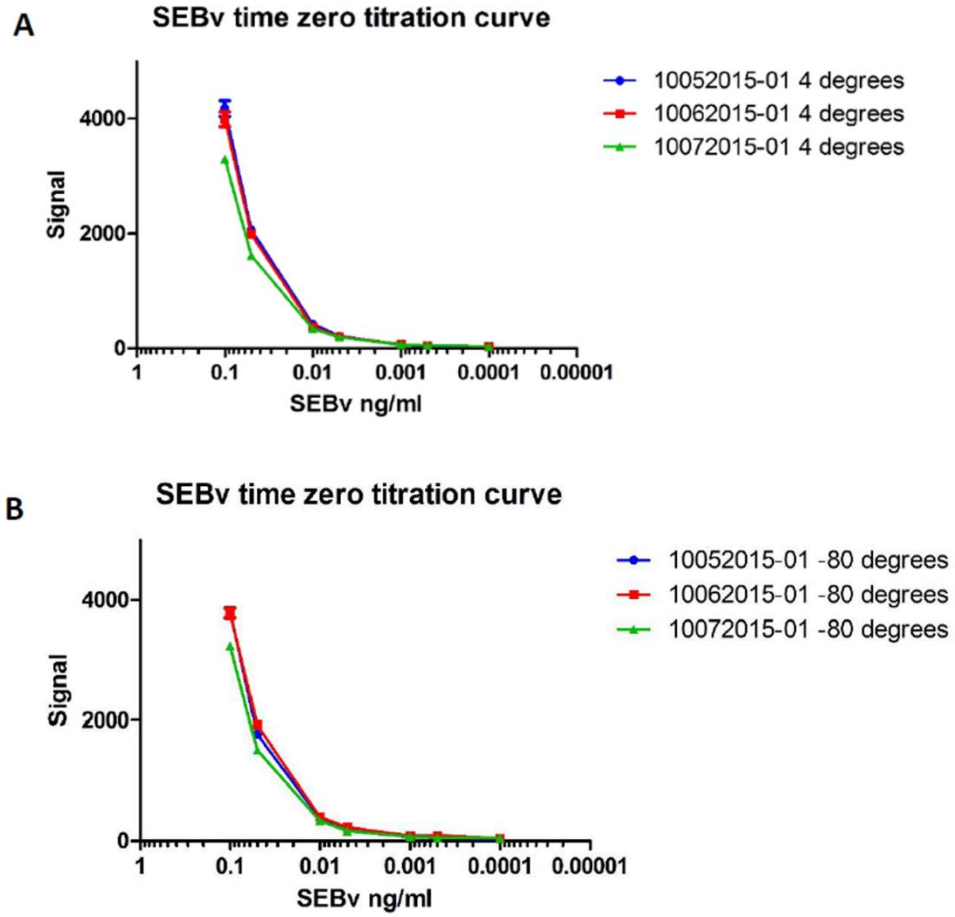

Figure 2. SEBv 4 and –80 °C ECL data plotted using GraphPad software. (A) SEBv 4 °C ECL data. The *x* axis shows a titration of the SEBv antigen, and the signal is shown on the *y* axis. (B) SEBv –80 °C ECL data. The *x* axis shows a titration of the SEBv antigen, and the signal is shown on the *y* axis. Error bars represent four replicates at each concentration.

4. DISCUSSION

To demonstrate the reproducibility of recombinant antigen production technology, three small batches of SEBv were prepared for the DBPAO. Post-production testing included DLS, Experion analysis, concentration analysis, and an activity assay. We found that when we followed a particular standard operating procedure for culturing the host bacterial strain, an affinity purification yielded a consistent and highly reproducible product. Our recombinant protein samples were consistently produced at high concentrations (>1 mg/mL) at purities exceeding 95%. The product demonstrated good solubility and homogeneity with no aggregation. Furthermore, the antigenicity was comparable to active wild-type toxin (data not shown). These lots are currently undergoing shelf-life studies that utilize the same conformance testing at storage conditions of 4 and –80 °C. A separate report will be provided for those studies.

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ACRONYMS AND ABBREVIATIONS

DBPAO	Defense Biological Products Assurance Office
DLS	dynamic light scattering
ECL	electrochemiluminescence
ED_{50}	effective dose for 50% of the population
LD_{50}	lethal dose for 50% of the population
LOD	limit of detection
MHC	major histocompatibility complex
MSD	Meso Scale Discovery
NRL	Naval Research Laboratory
PBS	phosphate-buffered saline
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
SEBv	staphylococcal enterotoxin triple-mutant variant
SEC	staphylococcal enterotoxin C
SED	staphylococcal enterotoxin D
SEE	staphylococcal enterotoxin E
S/N	signal-to-noise ratio
TSST-1	toxic shock syndrome toxin-1

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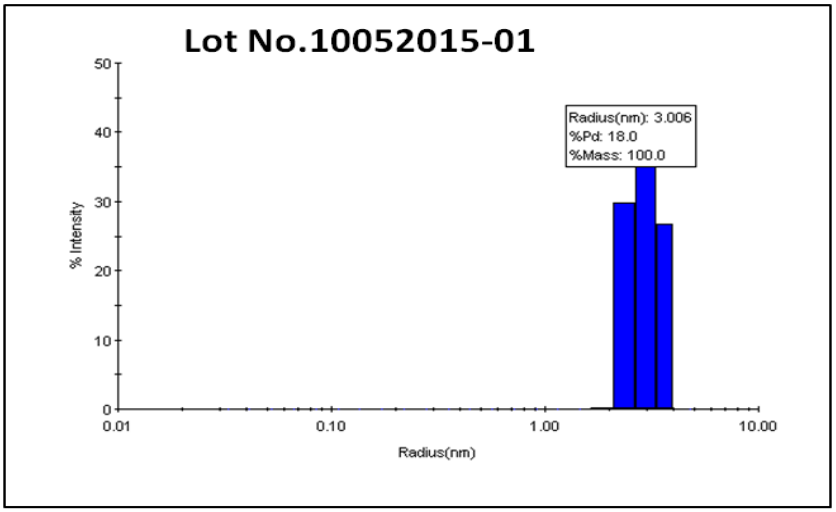
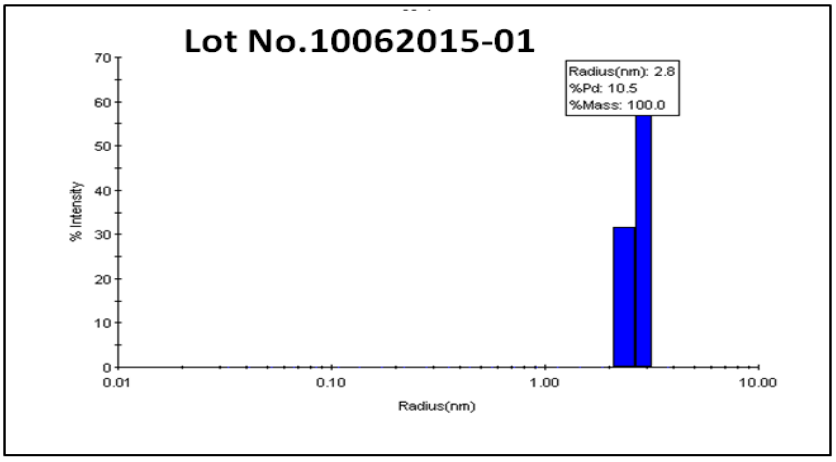
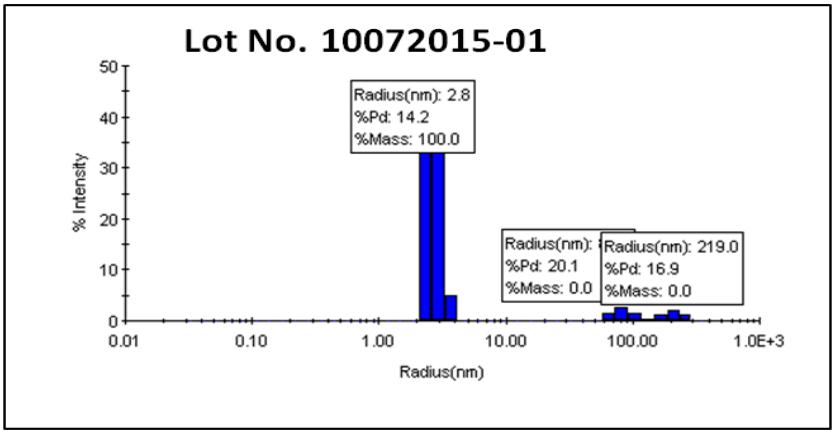
APPENDIX

RADIUS HISTOGRAMS







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