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Screening and characterization of anti-SEB peptides using a bacterial display library and microfluidic magnetic sorting

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Bacterial peptide display libraries enable the rapid and efficient selection of peptides that have high affinity and selectivity toward their targets. Using a 15-mer random library on the outer surface of *Escherichia coli (E.coli)*, high-affinity peptides were selected against a staphylococcal enterotoxin B (SEB) protein after four rounds of biopanning. On-cell screening analysis of affinity and specificity were measured by flow cytometry and directly compared to the synthetic peptide, off-cell, using peptide-ELISA. DNA sequencing of the positive clones after four rounds of microfluidic magnetic sorting (MMS) revealed a common consensus sequence of (S/T)CH(Y/F)W for the SEB-binding peptides R338, R418, and R445. The consensus sequence in these bacterial display peptides has similar amino acid characteristics with SEB peptide sequences isolated from phage display. The K_d measured by peptide-ELISA off-cell was 2.4 nM for R418 and 3.0 nM for R445. The bacterial peptide display methodology using the semiautomated MMS resulted in the discovery of selective peptides with affinity for a food safety and defense threat. Published 2014. This article is a U.S. Government work and is in the public domain in the USA. *Journal of Molecular Recognition* published by John Wiley & Sons, Ltd.

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Keywords: SEB; peptide; bacterial display; microfluidic; peptide ELISA; peptide display; biothreat

INTRODUCTION

A bacterial peptide display library contains billions of unique bacteria that each express a single, randomized peptide sequence appended to an outer membrane protein (Bessette *et al.,* 2004). In the eCPX biterminal peptide display library, 3×10^{10} unique peptide sequences are displayed on the circularly permutated outer membrane protein, OmpX, of *E.coli* (Rice and Daugherty, 2008). The permutated OmpX displays a random 15-mer peptide at the N-terminus for biopanning against a target of interest and a fixed YPet-Mona peptide binding sequence, which enables quantitative affinity measurements relative to the overall peptide surface expression (Rice and Daugherty, 2008). The eCPX is an unconstrained 15 amino acid library (Daugherty, 2007) that was engineered to routinely isolate high-affinity peptides for arbitrary protein targets (Bessette *et al.,* 2004).

The use of a bacterial peptide display combinatorial library takes advantage of the fast growth rate of bacteria to enable biopanning of a large DNA-encoded library against a molecule of interest. Bacterial display libraries offer a more rapid reagent selection process than typical hybridoma technologies. New affinity reagents can be selected within 1 week using bacterial display libraries, compared to months for new antibodies, which is critical for isolating reagents for any new or emerging threats (Bessette *et al.,* 2004; Kogot *et al.,* 2011; Stratis-Cullum *et al.,* 2011; Stratis-Cullum *et al.*, 2012). Recently, peptide binders from a bacterial display library were isolated for protective antigen (PA) protein of *Bacillus anthracis* using a microfluidic magnetic sorter (MMS), which offers a semiautomated magnetic sorting method to improve reproducibility with a disposable microfluidic cartridge and limits the handling of noxious biological materials (Sooter *et al.*, 2009; Kogot *et al.,* 2011). The MMS library sorting has been shown to be successful at isolating high-affinity peptide binders against biothreats with a lesser focus on widespread specificity. Herein, we examine specificity and affinity of peptide binders for staphylococcal enterotoxin B.

Staphylococcal enterotoxin B (SEB) is one of the most studied and characterized enterotoxins of the seven total enterotoxins found in *Staphylococcus aureus.* SEB is a 29-kDa superantigen that causes extreme immune system effects, including toxic shock syndrome (Ulrich *et al.,* 1997), and SEB exposure in food (food poisoning) results in emesis and diarrhea (Iandolo and Tweten, 1988; Ulrich *et al.,* 1997). The SEB protein exhibits high thermal stability, retaining 50% biological activity after 5 min exposure at 100 °C, and is stable between pH 4–10 (Schantz *et al.,* 1965). SEB has relatively low effective quantity by inhalation ($ED_{50} = 0.4$ ng/kg, $LD_{50} = 20$ ng/kg), combined with broad thermal and pH stability that makes SEB a potential biothreat agent (Ulrich

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et al., 1997). The potential threat of SEB in food, a complex matrix, and use as biothreat agent with broad pH and thermal stability necessitates a highly specific and stable detection reagent, such as a peptide reagent. To limit the exposure to this biothreat molecule, the MMS method with disposable cartridge is an ideal technique for rapid, semiautomated isolation of new peptide display reagents to SEB.

To date, there have been reports of SEB-binding peptides from both phage display (Goldman *et al.,* 2000; Soykut *et al.,* 2008) and solid-phase combinatorial library (Wang *et al.,* 2004), but no reports of bacterial display peptides isolated for SEB binding. This is the first report of SEB binders isolated from a bacterial display library. A hexamer peptide isolated from a solid-phase combinatorial library, YYWLHH, exhibited high selectivity to SEB with very little cross-reactivity with staphylococcal enterotoxin A (31% homology) and staphylococcal enterotoxin C (67% homology) but greater binding to $TSST_1$ (16% sequence homology) in an affinity resin format (Wang *et al.,* 2004). The authors attribute the nonspecific binding to the least homologous $TSST_1$ because of tertiary and not primary sequence similarity to SEB. The SEB-selective hexamer was isolated as a peptide-capture reagent for isolating SEB from a complex mixture and no binding affinity was reported.

Selectivity or specificity of a target capture reagent is as important as binding affinity when detecting samples in a complex matrix. Further complicating the isolation of SEB-selective reagents using phage is the observed specificity difference between the phage-ELISA (on-cell) and the SPR analysis of the identical free peptide (off-cell), even for high-affinity phage clones (Soykut *et al.,* 2008; Dudak *et al.,* 2010). In this study of bacterial display libraries, we will compare the on-cell selectivity using a SEB concentration-dependent flow cytometry method with the selectivity of the synthetic peptide using a peptide-ELISA method (Kogot *et al.,* 2012). SEB-binding peptides selected from phage display with a consensus sequence of **WHK** have been used in whole-cell ELISA, with a SEB detection limit of 1.4 ng/well in a 96-well plate format without a direct comparison to the free peptide binding (Goldman *et al.,* 2000). In SEB-binding peptide studies from phage display using the free peptide, the affinity of the free peptides determined by SPR had binding association constants (K_a) of $4.2 \times 10^5\ M^{-1}$ ($K_d = 2.38\ \mu M$) (Soykut *et al.,* 2008) and (K_a) $2.3 \times 10^5\ M^{-1}$ ($K_d = 4.35\ \mu M$) (Dudak *et al.,* 2010); phage-ELISA on-cell was used as a screening method, and no binding constant was reported using phage-ELISA. In bacterial display, flow cytometry analysis provides a rapid screening tool, similar to the use of phage-ELISA in phage display.

In this study, SEB-binding peptides were selected from a bacterial display library after four rounds of biopanning (4 days) using a biotinylated SEB protein and streptavidin-coated magnetic bead in a MMS. After three rounds of sorting, a preliminary sample analysis of the positive clones was performed using a flow cytometer with fluorescently labeled SEB. After four rounds of sorting, additional screening of positive clones was completed and DNA sequencing was performed to determine the peptide sequence for each positive clone. The round four clones with the strongest affinity were analyzed further on-cell to generate a concentration-dependent binding curve (Boder and Wittrup, 1998; Getz *et al.,* 2012) along with a specificity measurement with a panel of potential cross-reacting proteins. Finally, the highest affinity and specificity clones from round four were synthesized and analyzed off-cell for affinity and specificity.

The results of this study present SEB-binding peptides from a bacterial display library that were more specific to SEB compared to other proteins tested and had single-nanomolar binding dissociation constants.

MATERIALS AND METHODS

Reagents

Luria Bertani Broth (LB) and chloramphenicol (Cm) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were used for all *E.coli* growth steps. SEB was purchased from Sigma-Aldrich and used for all bacterial sorting and analysis. Biotinylated SEB was made using EZ-Link Sulfo-NHS-Biotin (SEB-biotin) according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL, USA) and was coupled to Dynabeads® MyOne™ Streptavidin T1 superparamagnetic beads during MMS sorting. The Ypet-Mona (Nguyen and Daugherty, 2005) was provided by CytomX Therapeutics (San Francisco, CA, USA) for evaluating peptide display expression. The SEB and protective antigen (PA; List Biological Laboratories, Campbell, CA, USA) were fluorescently tagged using an amine reactive DyLight 488 (Thermo Fisher Scientific); the Streptavidin-R-phycoerythrin (SAPE) and Neutravidin-R-phycoerythrin (NAPE) were purchased from Invitrogen (Carlsbad, CA, USA); and FITC conjugated anti-Hemagluttinin (HA) Epitope Tag polyclonal antibody (US Biological; Salem, MA, USA) were used for on-cell specificity studies. For the peptide-ELISA, SEB, and PA were conjugated to horseradish peroxidase using EZ-Link Plus Activated Peroxidase (SEB-HRP and PA-HRP) according to the manufacturer's instructions (Thermo Fisher Scientific) and NeutrAvidin, Horseradish Peroxidase (HRP) Conjugated, and High Sensitivity Streptavidin-HRP were purchased from Thermo Fisher Scientific. The anti-Staphylococcal Enterotoxin B mouse monoclonal antibody was purchased from US Biological. Phosphate buffered saline (PBS), as BupH Modified Dulbecco's PBS Packs from Thermo Scientific, was also supplemented with 0.5% Albumin from bovine serum or 0.1% Tween-20 (PBST) purchased from Sigma-Aldrich. QuantaRed Enhanced Chemifluorescent HRP substrate (Thermo Fisher Scientific) was used as the substrate for ELISA detection.

MMS sorting

The MMS cell sorting procedure was similar to previously published procedures (Kogot *et al.,* 2011; Pennington *et al.,* 2012). Briefly, a bacterial display library (eCPX library; CytomX Therapeutics, San Francisco, CA, USA) with approximately 3×10^{10} unique peptide sequences was grown in 500 ml LB containing 25 μg/ml chloramphenicol (LB-Cm^{25}). The culture was grown to an OD_{600} of 0.6 and induced with 0.04% (w/v) L-arabinose (Rice and Daugherty, 2008). After 45 min of growth, 3×10^{11} cells (10-times oversampling of the initial library diversity) were pelleted by centrifugation at 3000 g for 20 min. The bacterial pellet was resuspended in 1.5 ml of PBS containing 1×10^9 streptavidin T1 beads and incubated at 4 °C for 45 min to deplete the library of any streptavidin-binding peptides using the MMS negative selection program. Using the MMS, bacterial cells bound to the streptavidin beads were isolated from the entire cell library, which was designated as the streptavidin-depleted library. The streptavidin depletion step is only performed prior to the first sort when streptavidin beads are used in the sorting method.

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In target sorting, the streptavidin-depleted library was resuspended in 1 ml PBS and was incubated with 600 nM SEB-biotin for 45 min. After incubation, the cells were centrifuged at 3000 g for 5 min and resuspended in 1 ml PBS containing 1×10^9 T1 streptavidin beads. After 45-min incubation with the magnetic beads, the cells were loaded onto the MMS and separated using the MMS positive selection program. The bacterial cells selected as SEB positive binders by the MMS were plated in serial dilutions to determine the resultant library diversity, and the remaining cells were grown overnight in LB-Cm^{25}. In the second, third, and fourth rounds of sorting, the SEB concentration was decreased from 600 nM in round one to 300 nM in round two, 150 nM in round three, and 75 nM in round four. The number of cells used in each round was dependent on the resultant library diversity after each round of sorting. Typically, a 5–10 times oversampling of the library was used in each round as determined by the cell counts on the overnight plates after each positive sorting round (Hall and Daugherty, 2009; Kogot *et al.,* 2011).

On-cell affinity and specificity

Positive SEB clones, and non-binder R441 (FTSSPSKHPQVEAGV), were measured for affinity and specificity from an overnight culture of a single clone in LB-Cm^{25}. After overnight growth, each clone was diluted 1:200 in fresh LB-Cm^{25}, grown to $OD_{600} = 0.6$, and induced for 45 min using 0.04% (w/v) L-arabinose. For the on-cell affinity measurements, 5 μl of each clone was added to 25 μl of varying concentrations of SEB-488 (150, 75, 50, 25, 5, and 0 nM) and incubated for 45 min. The overall peptide display expression level was measured separately using 75 nM Ypet-Mona. The specificity measurements were performed using 5 μl of each clone added to 25 μl of 1000 nM of PA-488, αHA-488, NAPE, or SAPE, and incubated for 45 min. After incubation, both the affinity samples and specificity samples were measured by flow cytometry using a BD FACSCanto II (BD Biosciences, San Jose, CA, USA). The on-cell, apparent dissociation constant K_d^{APP} was determined by plotting the fraction of cells bound at varying SEB-488 concentrations and fitting to a sigmoidal dose–response function with variable slope using Prism (GraphPad, LaJolla, CA, USA). The on-cell specificity was determined by comparing the median fluorescence by flow cytometry of each specificity protein tested at 1000 nM to the SEB-488 binding at 150 nM.

Off-Cell affinity and specificity

The off-cell affinity and specificity were measured using a PS-tag (polystyrene tag) peptide fusion in a direct peptide-ELISA that was developed in our lab (Kogot *et al.,* 2012). The peptide-ELISA was performed by first coating the PS-tag-modified R418 and R445 peptides containing a four Gly spacer (PS-tag; RAFIA-SRRIRRP) (Kumada *et al.,* 2006; Kumada *et al.,* 2010) to a Maxisorp (Nalge Nunc) 96-well plate at 3 μg/ml in PBS for 2 h. A 3 μg/ml peptide sample, 300 ng of peptide, is expected to result in approximately 100 ng of PS-tag peptide bound per well on a Maxisorp surface (Kumada *et al.,* 2010). An anti-SEB monoclonal antibody (αSEB-mAb) was run as a positive control at 1 μg/ml in each experiment. The wells were blocked against nonspecific binding using PBST for 1 h. After blocking, a 45 min binding step was performed with each protein dissolved in PBST and serially diluted across each well in the 96-well plate: SEB-HRP initial concentration 14 nM, Strep-HRP initial concentration 250 nM, HRP initial concentration 200 nM, and PA-HRP initial concentration 100 nM. The plate was washed three times using PBS prior to addition of the QuantaRed ELISA substrate. After 10 min, the substrate was quenched using QuantaRed stop solution according to the manufacturer's instructions and measured using a Synergy HT Microplate reader (Biotek, Winooski, VT, USA) with a 530/25 nm excitation filter and 590/35 nm emission filter. For the peptide affinity measurements, the peptide samples and a buffer negative control were measured in five replicates. The off-cell K_d for both affinity and specificity was determined by plotting the relative fluorescence versus protein concentration and fitting to a one site-specific binding function ($Y = B_{max} * [ligand]/(K_d * [ligand])$ using Prism (GraphPad, LaJolla, CA, USA).

Peptide competition with monoclonal antibody

A peptide competition assay was performed by coating a 96-well Maxisorp plate with a 1 μg/ml sample of αSEB-mAb in PBS for 2 hours. The wells were blocked with PBST for 1 h. After blocking, a 7 μM sample of SEB-HRP was concurrently mixed with 1:2 serial dilutions of the R338, R418, and R445 peptides in PBS using an initial peptide concentration of 850 μM. The plate was washed three times using PBS prior to addition of the QuantaRed ELISA substrate. After 10 min, the substrate was quenched using QuantaRed stop solution according to the manufacturer's instructions and measured using a Synergy HT Microplate reader (Biotek, Winooski, VT, USA) with a 530/25 nm excitation filter and 590/35 nm emission filter. The samples were run in triplicate.

RESULTS

After three rounds of sorting by MMS, 40 clones were selected at random and analyzed for SEB binding at 150 nM SEB-488, for streptavidin binding with 150 nM SAPE, and for peptide display expression using 75 nM Ypet-Mona. Only two clones were identified as SEB binders, R330 and R338. After four rounds of sorting, a total of 80 additional bacterial colonies were screened using flow cytometry to identify SEB-binding peptides from the display library. In Table 1, clones that either bound to SEB or had more than one sequence incidence are presented. For the round four samples, the percent SEB binding was measured using 75 nM SEB-488 (or 150 nM SEB-488 for R338), and the overall streptavidin percent binding was determined using 75 nM SAPE (or 150 nM SAPE for R338). After four rounds, 11 of the 39 clones shown in Table 1 were identified as SEB binders. All 80 of the clones were sequenced (Genewiz; Germantown, MD, USA), and the frequency of each sequence is given in Table 1 along with the ratio of SEB-488 mean fluorescence to SAPE mean fluorescence for each sequence. Three of the five sequences with the highest SEB : SAPE binding ratio, R338, R445, and R418, contain a consensus sequence of **(S/T)CH(Y/F)W,** whereas R403 and R401 did not have this consensus (Table 1). In addition, most of the sequences with the lowest SEB : SAPE ratio each contained an **HPQ** consensus sequence (Table 1). The round three clone R330 did not contain either consensus sequence and was not studied further.

In addition to measuring the overall surface expression and SEB binding, the three clones with the highest SEB : SAPE binding ratio were analyzed by flow cytometry for percent binding to (percentage of cells outside of the gated, unlabeled control population for each clone) and median fluorescence intensity of (Chan *et al*., 2013) four proteins for specificity analysis: NAPE,

Table 1. Compilation of isolated clones, sequences, sequence occurrence frequency, and SEB : SAPE mean fluorescence ratio above negative control as determined by flow cytometry. The R338, R445, and R418 SEB binding sequences shared a similar consensus, shown in bold and indicated with a box around the consensus. The remaining sequences were designated as streptavidin-binding clones, with many containing a HPQ consensus (underlined) that had been previously reported as a streptavidin binding sequence

Sequence Frequency	Clone	Peptide sequence														SEB : SAPE Ratio
1	R338	S	W	T	C	L	V	N	I	V	K	**S**	**C**	**H**	**F**	**W**	429:1
4	R445	C	L	L	R	L	R	D	T	**T**	**C**	**H**	**Y**	**W**	T	Q	574:1
1	R418	S	Y	**S**	**C**	**H**	**Y**	**W**	L	S	S	A	V	P	Y	M	19:1
1	R408	W	R	S	S	A	V	H	P	Q	V	S	G	L	I	R	7:1
1	R409	T	S	A	L	H	P	M	G	G	Q	V	V	P	Q	I	1:78
1	R403	S	Q	R	V	F	N	D	D	G	L	Y	H	P	Q	G	294:1
1	R422	V	K	H	D	P	H	P	Q	H	W	I	L	P	F	P	0.5:1
7	R402	S	L	Y	Q	Y	H	P	Q	V	A	G	G	Q	P	L	3:1
4	R423	R	C	V	H	Y	C	Q	Q	D	E	R	L	G	R	P	1:25
3	R424	T	I	A	R	Q	P	H	P	H	P	Q	F	A	P	M	1:125
3	R401	R	S	A	I	P	D	R	L	S	H	P	Q	F	H	L	36:1
3	R428	G	P	H	P	Q	N	T	P	L	R	Q	G	L	L	N	1:1
2	R427	L	A	Q	E	S	L	R	H	P	Q	S	G	V	I	V	3:1
2	R435	A	P	T	L	S	R	L	A	S	H	P	Q	F	L	G	1:20
2	R430	F	V	V	H	S	H	P	Q	T	G	I	W	T	S	H	1:10

SAPE, PA-488, and αHA-FITC. There was minimal binding observed for the three clones at concentrations less than 1000 nM; therefore, the on-cell specificity analysis was conducted using 1000 nM of each protein, a concentration almost 7-fold greater than the 150 nM used for SEB (Figure 1; Supplemental Figures 4, 5). The median fluorescence values for R418 were 19 for PA, 24 for SAPE, 22 for αHA, and 27 for NAPE at 1000 nM, as compared to 493 for SEB at 150 nM. The corresponding median fluorescence values for R445 were 10 for PA, 12 for SAPE, 13 for αHA, and 7 for NAPE at 1000 nM, as compared to 601 for SEB at 150 nM. For R338, the median fluorescence values were 21 for PA, 16 for SAPE, 21 for αHA, and 25 for NAPE at 1000 nM, as compared to 467 for SEB at 150 nM. A representative nonbinding peptide from the round four sort, R441, is shown as a negative control for SEB median fluorescence (Figure 1). Binding to SEB and SAPE for the R441 sample, as measured by median fluorescence, is comparable to the unlabeled controls for R338, R418, and R445. The result is similar to using percentage of cells outside of a gated, buffer-only control, which is the method previously utilized in peptide bacterial display studies (Kogot *et al*., 2011; Stratis-Cullum *et al.,* 2011). The R418 clone was bound at 11.6% to PA, 4.5% to SAPE, and approximately 11% to αHA, and 10.2% to NAPE. The R445 clone was bound at 3.2% by NAPE, 4.0% by SAPE, and at 2.0% or less by αHA and PA. The percent binding by R338 to NAPE was 10.5%, 8.7% by SAPE, 6% by PA, and 2% by αHA. For comparison, the R338, R445, and R418 approach 100% bound between 50–150 nM SEB, at a concentration nearly 10-fold lower than the concentration used for the specificity studies (Supplemental Figure 1, Supplemental Figures 4, 5). The apparent on-cell equilibrium dissociation constant, K_d^{APP}, for the three sequences (R338, R418, and R445) was also measured using flow cytometry (Supplemental Figure 1). The SEB fraction bound is the percent cell population bound as a function of concentration of SEB-488 measured at 530/30 nm by flow cytometry compared to unlabeled cells (negative control). The R445 and R418 round four clones had higher SEB affinity than the R338 clone. The R418 had an on-cell K_d^{APP} of 19 nM and the R445 on-cell K_d^{APP} was 16 nM, while the R338 clone had an on-cell K_d^{APP} of 57 nM. The two round four peptides, R418 and R445, were also analyzed using direct ELISA. The synthetic peptide K_d, off-cell, was determined using peptide-ELISA as previously described (Kogot *et al.,* 2012). The K_d for the R445 and R418 were 3.0 and 2.4 nM, respectively (Figure 2).

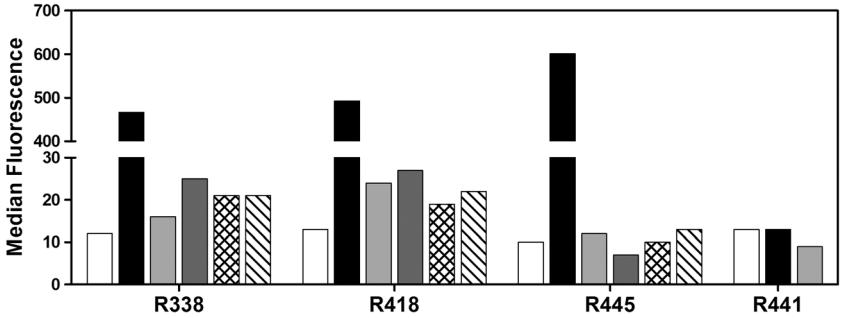

Figure 1. On-cell specificity analysis of staphylococcal enterotoxin B (SEB) binding clones measured against PBS buffer alone (white), 150 nM SEB (black), or 1000 nM each of streptavidin-R-phycoerythrin (light gray), neutravidin-R-phycoerythrin (dark gray), protective antigen (cross hatched), and anti-hemagluttinin (striped). The R441 SEB non-binder was tested against PBS, SEB, and SAPE only. Note the disjointed scale on the *y*-axis.

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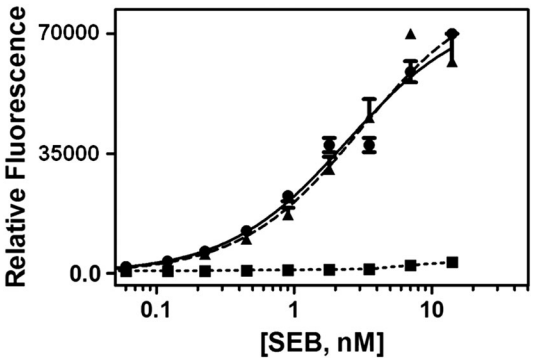

Figure 2. Plot of the relative fluorescence of staphylococcal enterotoxin B (SEB) by ELISA as a function of varying SEB concentration using a constant 3 μg/ml peptide per well: PS-tag R418 peptide (circle), PS-tag R445 peptide (triangle), and PS-tag alone (square). The K_d was determined by fitting the binding isotherm with the R445 peptide $K_d = 3.0$ nM and R418 $K_d = 2.4$ nM.

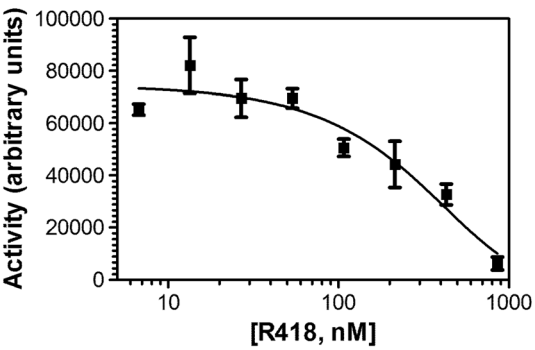

Figure 3. Plot of the competition ELISA for the displacement of the staphylococcal enterotoxin B (SEB) mAb at varying concentrations of R418 peptide measured in triplicate. The decreased activity with increasing peptide was fit to an exponential decay function with $K_d = 296$ nM for the R418.

In peptide competition studies with the monoclonal antibody (mAb), only the R418 peptide (Figure 3) and not the R445 or R338 (Supplemental Figures 2, 3) peptides were able to compete with the mAb for SEB binding. The loss of activity from the SEB-HRP during the ELISA was plotted as a function of R418 concentration. The resultant competition ELISA was fit to an exponential decay function resulting in a calculated K_d of 296 nM for the R418 in competition with the mAb. Peptide competition ELISA's for SEB binding were also performed between the R445 and R418 PS-tag peptides and a soluble R445, R418, and R338 peptide without the PS-tag. Only the R418 competition with the PS-tag for SEB binding resulted in a 50% decrease in activity (Supplemental Figures 6, 7).

In addition to off-cell affinity, the two round four synthetic peptides were measured for off-cell specificity using peptide-ELISA. The R418 and R445 synthetic peptides were both more specific to SEB (Figure 2) than to the other proteins tested: PA, streptavidin, and HRP. The R418 was bound to PA-HRP with a K_d of approximately 32 nM, to Strep-HRP at approximately 10 nM, and to HRP at approximately 30 nM, (Figure 4); the SEB K_d for R418 was at least 5-fold greater (2.4 nM) than the cross-reactive protein panel (Figure 2). The R445 was bound to PA with a K_d of approximately 18 nM, to Strep-HRP at approximately 15.4 nM, and at approximately 25 nM, to HRP alone (Figure 5). The R445 K_d to SEB was 3.0 nM, which is more than 5-fold higher than the K_d of the next strongest binding affinity, PA-HRP (Figure 2).

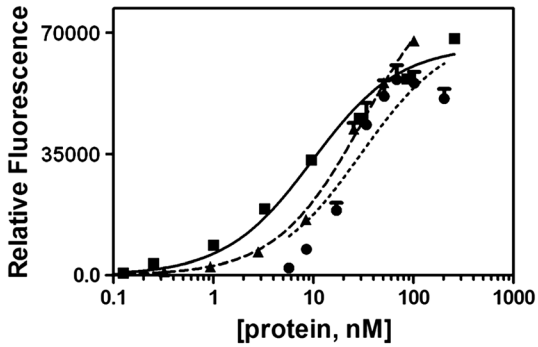

Figure 4. Plot of the relative fluorescence by ELISA for specificity analysis of R418 peptide with varying concentrations of proteins. The binding dissociation constant (K_d) for PS-tag R418 and Horseradish Peroxidase (HRP) alone (circle) is 30 nM, Strep-HRP (square) is 10 nM, and PA-HRP (triangle) is 32 nM.

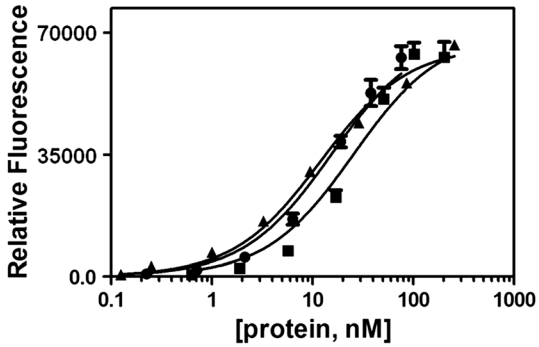

Figure 5. Plot of the relative fluorescence by ELISA for specificity analysis of R445 peptide with varying concentrations of target proteins. The binding dissociation constant (K_d) for PS-tag R445 and Horseradish Peroxidase (HRP) alone (square) is 25.4 nM, Strep-HRP (triangle) is 15 nM, and PA-HRP (circle) is 18 nM.

DISCUSSION

From a total of 120 clones analyzed in round three and round four during MMS sorting, three isolated clones were identified as strong SEB binders; each clone that was analyzed by flow cytometry had approximately 100% SEB binding using a 75 nM SEB-488 sample. A multiple sequence alignment using Clustal Omega (Goujon *et al.,* 2010; Sievers *et al.,* 2011) was performed to determine a SEB binding consensus sequence of **(S/T)CH(Y/F)W** for clones R338, R445, and R418, while a second consensus sequence of **HPQ** emerged in clones R401, R402, R403, R408, R422, R424, R427, R428, R430, R432, and R435. The **HPQ** consensus has been isolated as a streptavidin binding consensus sequence in other peptide libraries (Devlin *et al.,* 1990; Lam *et al.,* 1991; Giebel *et al.,* 1995). The high incidence of streptavidin binding sequences is surprising because a streptavidin-depleted library was used in this study. Further studies are needed to evaluate the SEB binding portion of these sequences beyond the **HPQ** streptavidin binding sequence.

The R418 and R445 clones shared the **CHYW** consensus sequences, and the R338 had a Tyr➔Phe substitution. Given that the R418 and R445 on-cell binding affinities were 3-fold greater than the R338 affinity, the Tyr➔Phe substitution is hypothesized to impact the SEB protein binding by the peptide. This difference

is likely because the overall sequence similarity of R418 and R445 is low outside of the consensus sequence. The two sequences have a similarity score of 25.0, which is comprised of the four consensus residues and the similarity of Thr and Ser (polar, uncharged amino acids) preceding the consensus in both sequences. The aligned consensus sequence of **CHYW** from this bacterial display library is very similar to the **WHK** and **FYW** consensus from peptides isolated during phage library sorting (Goldman *et al.,* 2000; Soykut *et al.,* 2008) and the solid phase library sequence of YYWLHH (Wang *et al.,* 2004). These sequences all contain a hydrophobic Trp residue in proximity to a His residue and/or Tyr residue.

The binding of SEB to the off-cell peptide in the peptide-ELISA was approximately 10-times greater than the apparent on-cell K_d. The on-cell versus off-cell binding affinity differences has also been observed for phage peptides with SEB; high-affinity samples were identified in phage-ELISA but did not bind during peptide-ITC experiments (Dudak *et al.,* 2010). The specificity difference between on-cell and off-cell reagent (Figures 1, 4, 5) was less disparate than the affinity results (Supplemental Figure 1, Figure 2). Specificity measurements by flow cytometry on-cell indicated specificity to SEB, with approximately 100% binding at 75 nM and less than 12% binding at 1000 nM for all of the cross-reactivity proteins tested: αHA, streptavidin, neutravidin, and protective antigen. The 10-fold greater on-cell specificity from the bacterial display peptides against SEB was greater than the observed 2-fold specificity improvement for the phage clones cross-specificity to SEB measured against potential cross-reactive proteins (Soykut *et al.,* 2008). The nonspecific protein binding in the phage clones was attributed to the hydrophobic portions of the phage coat proteins (Soykut *et al.,* 2008), which is not apparent in the bacterial display library clones tested against αHA, streptavidin, neutravidin, and PA presented herein. By median fluorescence intensity (MFI), the specificity of these peptides to SEB is even more pronounced when comparing MFI of SEB to MFI of the highest binder from the selectivity panel; approximately an 18-fold greater binding to 150 nM SEB than to 1000 nM NAPE for R418 and R338, and approximately a 46-fold greater binding to 150 nM SEB than to 1000 nM αHA for R445 is observed. The R445 and R418 peptides had similar off-cell specificity as well. Both peptides had at least 5-fold greater specificity to SEB than to the other proteins tested. The R418 was measured by peptide-ELISA to have a binding affinity of 10 nM to streptavidin, 30 nM to HRP, and 32 nM to PA (Figure 4). The R445 had a higher affinity to both HRP (25 nM) and PA (18 nM) (Figure 5). The R445 affinity was lower for streptavidin, 15.4 nM, compared to the R418 affinity at 10 nM. Therefore, screening of on-cell cross-reactivity by flow cytometry can provide an initial qualitative estimate of the bacterial display peptide specificity prior to more complete peptide-ELISA analysis.

The R445 had stronger affinity to the cross-reactive proteins tested off-cell compared to the R418, although R418 had an overall higher cross-reactivity on-cell (Figures 1, 4, 5). The difference could be due to the presentation of the peptide on-cell and off-cell or the remaining residues outside of the consensus region. In the R418 clone, the consensus sequence is at the N-terminus of the polypeptide and would be more accessible in solution to bind only the intended SEB target. The consensus sequence for the R445 clone is at the C-terminus in close proximity to the outer membrane; therefore, the residues outside of the consensus region would be located at the N-terminus and may bind to other targets nonspecifically. The amino acids outside of the consensus region may also play a role in the specificity and affinity. The most apparent difference between the R445 and R418 is that the R445 contains two Arg and one Asp residue that can bind through electrostatic interactions to other proteins. The R418 does not contain either polar amino acid; therefore, all nonspecific interactions would likely be attributed to hydrophobic interactions and hydrogen bonding. In the peptide-ELISA, the polystyrene tag is positioned at the terminus opposite of the consensus sequence to present a more accessible binding sequence to SEB (the PS-tag on the R418 was positioned at the C-terminus, while the R445 was positioned at the N-terminus).

The importance of the contribution of amino acids outside of the consensus region is further evidenced in binding competition studies. Although all three peptides contain a similar consensus sequence **(S/T)CH(Y/F)W,** only the R418 was able to compete with the monoclonal antibody for SEB binding (Figure 3) and neither the R445 (Supplemental Figure 2) nor the R338 (Supplemental Figure 3) was able to out-compete the mAb for SEB binding. Furthermore, only the R418 showed evidence of competing with the R445 peptide for SEB (Supplemental Figures 6 and 7), although both peptides shared the same consensus sequence. Future studies may include an affinity-matured daughter library built from degenerate primers for the regions outside of the consensus sequence region (Kenrick and Daugherty, 2010) creating new "daughter" libraries to improve affinity and specificity to SEB by randomizing the primary sequence outside of the strong consensus region.

In this work, we show selection of the first SEB binding peptides from a bacterial peptide display library using a rapid, operator-safe, and semiautomated MMS sorting method. Additionally, these SEB binding peptides share a similar consensus sequence with other peptides isolated using other peptide library methods. The similarity between the on-cell specificity by flow cytometry and the off-cell peptide-ELISA for the clones tested suggests that the on-cell specificity is a practical, rapid screening method for initial specificity analysis. In the current PS-tag peptide ELISA, both the R418 and R445 would be able to detect SEB at or below the inhalation LD_{50} of 20 ng/kg (Ulrich *et al.,* 1997) because each well in the ELISA was tested using approximately 20 ng (60 pM) of SEB per well at 2.5 times the signal above the buffer background. In conclusion, this study contributes a better understanding to the problem of correlating affinity and specificity in a peptide ligand (Hall and Daugherty, 2009) by presenting a method to obtain a high-affinity binder while retaining specificity for the target off-cell. For the specificity panel examined herein, an approximate five to 10-fold preference for the SEB target was observed. The SEB binding peptides isolated from a bacterial peptide display library using MMS sorting show the potential for developing specific recognition elements from a short peptide (15-mer). However, for practical detection applications, further optimization of binding specificity will be needed.

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