

Development of a Separation and Detection System for *Bacillus anthracis* Spores Based on Peptide Conjugates Identified from Peptide Library

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Abstract

A simple, specific and rapid identification system against *Bacillus anthracis* spores was developed using specific capture peptides conjugated with a bead-based biosensor, which was tightly and specifically bound onto the spore surface of *B. anthracis*. We successfully detected and separated *B. anthracis* spores from the spores of *B. thuringiensis* and *B. cereus* using this peptide-magnetic bead conjugates by fluorescence and anthrax-specific analyses. For more convenient mediation of high-throughput detection against *B. anthracis* spores, streptavidin-biotin interactions of spore-peptide and spore-peptide-magnetic bead conjugates were performed, and we demonstrated the separation and detection of *B. anthracis* spores using this method. In the presence of mixed condition of several types of *Bacillus* spores, the *B. anthracis* spores were easily separated using the oligopeptide-conjugated magnetic beads, thus allowing the clear detection of *B. anthracis* spores from *B. cereus*, *B. subtilis*, and *B. thuringiensis* spores. This oligopeptide-based strategy was rapidly and unambiguously identified as little as one viable *B. anthracis* spore in less than 1 h with a simple binding assay format. When assessed for its effectiveness for specifically and selectively detection of environmental spores phylogenetically similar to *B. anthracis* spores, such as *B. thuringiensis* and *B. cereus* spores, the system was free of false-positive signals.

Keywords: *Bacillus anthracis*; Anthrax detection; Magnetic separation; Capture peptide; Oligopeptide-conjugated ligand

Abbreviations: PCR: Polymerase Chain Reaction; PS-SPCLs: Positional Scanning-Synthetic Peptide Combinatorial Libraries; FITC: Fluorescein Isothiocyanate; HPLC: High-Performance Liquid Chromatography; SULFO-NHS: N-HydroxySulfosuccinimide

Introduction

With recent threats of biological terrorism and outbreaks of microbial pathogens, a great need for biosensors that can quickly and accurately identify infectious agents has arisen. *Bacillus anthracis* is a gram-positive, spore-forming bacterium that causes anthrax, a lethal disease for both humans and animals [1-5]. Because of its potential use for bioterrorism, *B. anthracis* has become a critical threats to many countries [6,7]. Once exposed to internal tissues, *B. anthracis* spores germinate and enter a vegetative state, often resulting in the death of the host within several days. Furthermore, great similarity between *B. anthracis* and other members of the *Bacillus* genus such as *B. cereus*, *B. thuringiensis*, and *B. mycoides*, exists. Therefore, the rapid and specific detection of environmental *B. anthracis* spores prior to infection is of extreme importance for the preservation of human safety and national security.

Various biological and chemical techniques have been developed. Nanoparticle-based colorimetric or fluorometric assay are most common detection methods. Recently, direct and specific detection studies with the help of hydrogel-based materials and physics-based methods were developed to detect virus and bacteria [8,9]. Furthermore, label-free methods implemented for the specific detection of DNA strands were developed [10,11]. Among the most important biological methods for the detection of anthrax, Polymerase Chain Reaction (PCR) [5-7] and immunoassay [12-14] have been used as representatives. PCR, a primer-mediated enzymatic DNA amplification method, requires expensive reagents and takes 3-4 h

or longer several hours to obtain the result. Moreover, PCR needs considerable effort in sample preparation processing prior to analysis. The detection limit of PCR, which is based on the detection of bacterial *pagA* and *lef* genes encoding the protective antigen toxic protein, has been $\sim 10^3$ spores. Immunoassays, which rely on the interaction between *B. anthracis* spore surface antigens and their specific antibodies, can detect 10^5 spores in approximately 12 h. However, in the case of immunoassays, specific antibodies and additional molecular fluorophores and/or enzymatic reactions must be employed for the desired agents to be detected, and mobile-phase conditions must also be adjusted depending on their capture, elution, and separation properties. In addition, although this direct spore detection system is relatively fast, the lack of accuracy and limited sensitivity of current antibody-based detection methods result in unacceptably high levels of both false-positive and false-negative responses [12-14]. Therefore, the development of a more robust detection system is required.

Many biological actions such as ligand-receptor interactions are based on the specificity of proteins conferred by the primary sequence of amino acids as well as the secondary and tertiary structures dictated by the primary sequence. Of particular importance is the formation of local structures, such as the active site and motif, which play a key role

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in protein function. Among the random sequences of short peptides, there may be sequences that can ideally act on such local environments, and also serve as the focal point for the development of new and more effective drugs. Recently various methods have been developed for rapid and easily identification of the sequences of interest from vast mixtures of random peptide sequences or polymers (template) with various side chain groups (chemical diversity libraries) [15-19]. Successful screening of these libraries has been described not only for epitopes recognized by their antibodies [20-22], but also for the identification of the biologically active peptides, such as antibacterial and antifungal peptides [16], human immunodeficiency virus protease inhibitors [23], substrate-analog trypsin inhibitors [24], and interleukin-8-specific antagonists [25].

In this study, we developed a high-throughput detection system for *B. anthracis* spores with oligopeptides using fluorescence, magnetic beads, and positional scanning-synthetic peptide combinatorial libraries (PS-SPCLs). Specific capture oligopeptides were developed to specifically bind against the surface of *B. anthracis* spores. Several peptide ligands were conjugated with Fluorescein Isothiocyanate (FITC) to detect fluorescent signals, and magnetic beads were used to facilitate rapid isolation by magnetic polarity conjugated with streptavidin to bind with biotin-conjugated oligopeptides. The resulting system was capable of the detection of specific spores with high sensitivity in less than 1 h. We also optimized the system to maximize its binding affinity to spores of two *B. anthracis* strains, namely, *B. anthracis* ΔSterne (pXO1⁻, pXO2⁻) and *B. anthracis* Sterne 34F2 (pXO1⁺, pXO2⁻), by using fluorescence assay and magnetic separation. Based on genome sequence comparisons, two species of *Bacillus*, *B. cereus* and *B. thuringiensis*, which are most similar to *B. anthracis*, were used in this study as model strains for comparison before the actual diagnosis of *B. anthracis*. Finally, to further improve the performance of the capture peptides, we newly designed their amino acid sequences using PS-SPCLs that can bind *B. anthracis* spores with greater specificity. The detection system based on these specific capture peptides is a high-throughput fluorescence-based assay that employs quantum dots. These specific binding systems can be employed to rapidly, specifically and selectively detect and separate *B. anthracis* spores in mixed conditions.

Materials and Methods

Materials

Streptavidin-conjugated magnetic beads (~ 1-2 μm in diameter) and a portable handheld magnetic separator were purchased from PureBiotech (San Diego, CA). The capture peptides used in this study (Table 1) were synthesized by Pepton (Korea). For rapid separation of *B. anthracis* spores from the spore mixture, the magnetic microbeads was functionalized with the capture peptides and used for detection of spores.

Bacterial strains and spores

The *Bacillus* strains used in this study are listed in Table 2.

Capture peptide	Sequence (N-terminus to C-terminus)	Reference
BA-1	biotin-ATYPLPIRGGGC	5
B-Negative	biotin-SLLPGLPGGGC	5
Negative-amine	SLLPGLPGGGC-NH ₂	5
BaS-01	EWWWNW-NH ₂	This study
BaΔS-01	DWDHDW-NH ₂	This study
BaΔS-02	DWDSEW-NH ₂	This study
BaΔS-03	DWDKDW-NH ₂	This study

Table 1: Capture peptides used in this study.

Strains	Relevant characteristics	Reference or source
<i>B. cereus</i> 1092	KCTC 1092	KCTC ^a
<i>B. subtilis</i> DB104	<i>His nprR2 nprE18 ΔaprA3</i> (BGSC 1E75)	BGSC ^b
<i>B. thuringiensis</i> 4Q7	Plasmidless mutant of <i>B. thuringiensis</i> var. <i>israelensis</i>	BGSC ^b
<i>B. anthracis</i> ΔSterne	(pXO1 ⁻ , pXO2 ⁻)	Prof. Chai ^c
<i>B. anthracis</i> Sterne 34F2	(pXO1 ⁺ , pXO2 ⁻)	Prof. Chai ^c

^aKorean Collection for Type Cultures, Daejeon, Korea

^b*Bacillus* Genetic Stock Center, Columbus, OH

^cProf. Y.G. Chai's laboratory, Hanyang University, Ansan, Korea

Table 2: Bacterial strains and plasmids used in this study.

Experiments with *B. anthracis* ΔSterne (pXO1⁻, pXO2⁻) and *B. anthracis* Sterne 34F2 (pXO1⁺, pXO2⁻) were carried out at Professor Y.G. Chai's laboratory at Hanyang University to avoid transportation and handling problems.

Synthesis and conjugation of individual capture peptides

The capture peptides were chemically synthesized and purified by High-Performance Liquid Chromatography (HPLC) according to the manufacturer's protocol (Pepton, Daejeon, Korea). Biotin was attached for conjugation at the N-terminus of capture peptides. The coupling efficiency was determined by analyzing the peptide solution, before and after coupling, by prominence HPLC (Shimadzu, Japan) and then reading the absorbance at a wavelength of 220 nm. The peptides were characterized by chromatography on a C18 column (Shiseido Capcell Pak, 22×300 mm). The peptides were eluted with a 0-30% gradient of water and acetonitrile in 0.1% (v/v) trifluoroacetic acid. The peptide composition was confirmed by amino acid analysis [26], and the peptides were sequenced using an Applied Biosystems' 473A protein/peptide microsequencer (Life Technologies, Grand Island, NY).

Preparation of positional scanning-synthetic peptide combinatorial libraries

Libraries were synthesized according to the protocol of Houghten et al. [16] and Pinilla et al. [19]. Briefly, PS-SPCLs, consisting of six-residue peptide sequences having free N termini and amidated C-termini, were synthesized. A single position in each peptide mixture was individually and specifically defined with 19 of the 20 natural L-amino acids (cysteine excluded), while the five remaining positions consisted of mixtures of the same 19 amino acids. Defined positions with known amino acids are represented by O and the positions with mixed amino acids are represented by X. The six sets of PS-SPCLs (a total of 114 pools) are represented by the following formulae: O₁XXXXX-NH₂, XO₂XXXX-NH₂, XXO₃XXX-NH₂, XXXO₄XX-NH₂, XXXXO₅X-NH₂, and XXXXXO₆-NH₂. Libraries of peptides were constructed on Rapidamide resin beads as described elsewhere called fluorenylmethyloxycarbonyl (Fmoc) chemistry [27]. The resin beads were distributed into different reaction vessels for each amino acid at each coupling step, then pooled, washed, and thoroughly mixed for randomization. Beads were then deprotected and redistributed into the various vessels again for the next coupling step and so on. The amount of each amino acid used to yield approximately equimolar coupling was determined empirically. The completeness of each reaction was checked with ninhydrin [26]. Side chains were deprotected with a mixture of trifluoroacetic acid (ethanedithiol:water:thioanisole; 90:5:4:1, vol/vol). The 114 peptide mixtures were individually extracted with water, lyophilized, and dissolved in water at a final concentration

of 27 nM for a peptide sequence in each pool.

Cultivation of cells and purification of spores

Cells were cultivated in CDSM media at 37°C or 30°C and 250 rpm for 48–60 h [28]. Spores mixed with vegetative cells were harvested from 50 mL of the culture by centrifugation (5,000 rpm) and then resuspended in 0.2 mL of 20% (w/v) urografin (Sigma, St. Louis, MO). This suspension was gently layered over 1 mL of 50% (w/v) urografin in a 1.5 mL microcentrifuge tube, and then centrifuged for 10 min at 4°C and 13,000 rpm. The collected pellet contained only free spores.

Polymerase chain reaction

PCR experiments were performed with a PCR Thermal Cycler (Bio-rad, Hercules, CA) using the High Fidelity PCR System. DNA fragments encoding lethal factor 2 were obtained by PCR using the genomic DNA of *B. cereus*, *B. anthracis* Sterne and Δ Sterne as templates, and the universal primers as follows: Forward primer: 5'-AAGCTTTGAGCAAGTTCATTCAAAGC-3'; Reverse primer: 5'-ATTGAAAGTTTCGGAGCA-3'.

Fluorescence analysis

Fluorescence assays were performed using a spectrofluorometer (Model VICTOR², PerkinElmer, Shelton, CT). Green fluorescence samples were excited by a 488/543 nm HeNe laser and were filtered by a long pass 505/575 nm filter.

Results and Discussion

Peptide labeling with biotin

Each of the capture peptides created from the PS-SPCL peptide library have free N-termini and amidated C-termini. These peptide termini can be reacted with *N*-hydroxysulfosuccinimide (sulfo-NHS)-biotin reagents to enable simple and efficient biotin labeling of peptides, proteins and any other primary amine-containing molecules (Figure 1). Differing only in their spacer arm lengths, this reagent offers the possibility of optimizing labeling and detection experiments where steric hindrance of biotin binding is an important factor. Sulfo-NHS esters of biotin are the most popular type of biotinylation reagent. NHS-activated biotins react efficiently with primary amine groups ($-NH_2$) in pH 7–9 buffers to form stable amide bonds. Several different NHS esters of biotin are available, with varying properties and spacer arm lengths. The sulfo-NHS ester reagents are water soluble, enabling reactions to take place in the absence of organic solvents such as dimethyl sulfoxide or dimethylformamide.

Proteins, including antibodies, generally have several primary

amines in the side chain of lysine (K) residues and the N-termini of each polypeptide that are available as targets for labeling with NHS-activated biotin reagents. However, because these molecules dissolve readily in polar solutions and are charged by the sulfonate groups, they cannot penetrate the cell and spore. As long as the spore remains intact, only primary amines of peptides bound on the anthrax spore surface will be biotinylated with sulfo-NHS-biotin reagents. Biotinylated-peptide or protein can be detected by streptavidin-conjugated fluorescent dye.

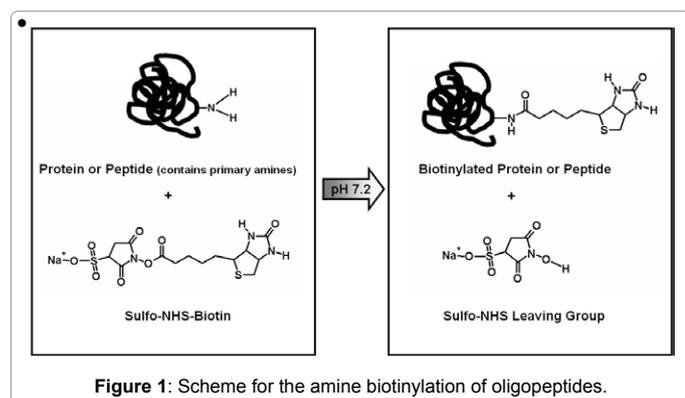
We have a library of 400 peptides (Peptide Library Support Facility of Pohang University of Science and Technology, Korea). These pools were individually dissolved in PBS solution at a final concentration of 27 nM per peptide having free N-termini and amidated C-termini. The peptide pools first react with anthrax spores in each 96-well, and are then washed three times to remove unbound peptides. These peptide-bound spores react with sulfo-NHS-biotin reagents, and streptavidin-FITC, and from these samples, we can detect specific bound peptides against anthrax spores. Next, the peptide sequences can be designed.

Screening and identification of specific binding peptides

We screened peptides bound to the *B. anthracis* Sterne spores, which were generated from PS-SPCLs, to examine their corresponding peptide-spore reactions. A total of 114 peptide pools of PS-SPCLs were subjected to testing, and the most effective amino acid sequence at each of the six positions in a hexapeptide was determined. The results of the initial screening of the peptide library are shown in Figure 2. The peptide mixture, EXXXNX-NH₂, was found to strongly bind to the *B. anthracis* Sterne spores. The amino acids with slightly less activity than glutamate (E) at the first position were aspartate (D) and valine (V). The active amino acids at the second, third and fourth positions were all tryptophan (W), with slightly more activity than others. The most active amino acid at the fifth position was asparagine (N). At the sixth position, tryptophan (W) was significantly more active than other amino acids. The amino acids chosen for reiterative synthesis of the peptide were as follows: 1st, E; 2nd, W; 3rd, W; 4th, W; 5th, N; and sixth, W. The final selected amino acid sequence was EWWW-NH₂, named BaS-01.

Likewise, we applied the same binding and screening procedure to the *B. anthracis* Δ Sterne spores, and the test elucidated the most active amino acid from 114 peptide pools at each amino acid position in the hexapeptide. The results for the initial screening of the peptide library are presented in Figure 3. The peptide mixture, DXXXXX-NH₂, was found to strongly bind to *B. anthracis* Δ Sterne spores. The most active amino acid at the first position was aspartate (D). The amino acids with slightly less activity than tryptophan (W) at the second position were alanine (A) and aspartate (D). The most active amino acid at the third position was aspartate (D). Histidine (H), lysine (K), and serine (S) appeared to be more active than other amino acids at the fourth position. The active amino acids at the fifth position were aspartate (D) and glutamate (E), with aspartate being slightly more active than the others. Tryptophan (W) was more active than other amino acids at the sixth position. The amino acids chosen for reiterative synthesis of the peptide were as follows: 1st, D; 2nd, A, D, and W; 3rd, D; 4th, E, H, K, and S; 5th, D and E; and sixth, W. The selected amino acid sequences used were DWDHDW-NH₂, DWDSEW-NH₂, and DWDKDW-NH₂ hexamers, named Ba Δ S-01, Ba Δ S-02, and Ba Δ S-03, respectively.

Each active peptide pool was synthesized at Pepton and confirmed by using HPLC on a C18 column. We subsequently investigated if the newly designed peptides had specific binding effects on *B. anthracis* spores with a binding assay incubating the active peptide



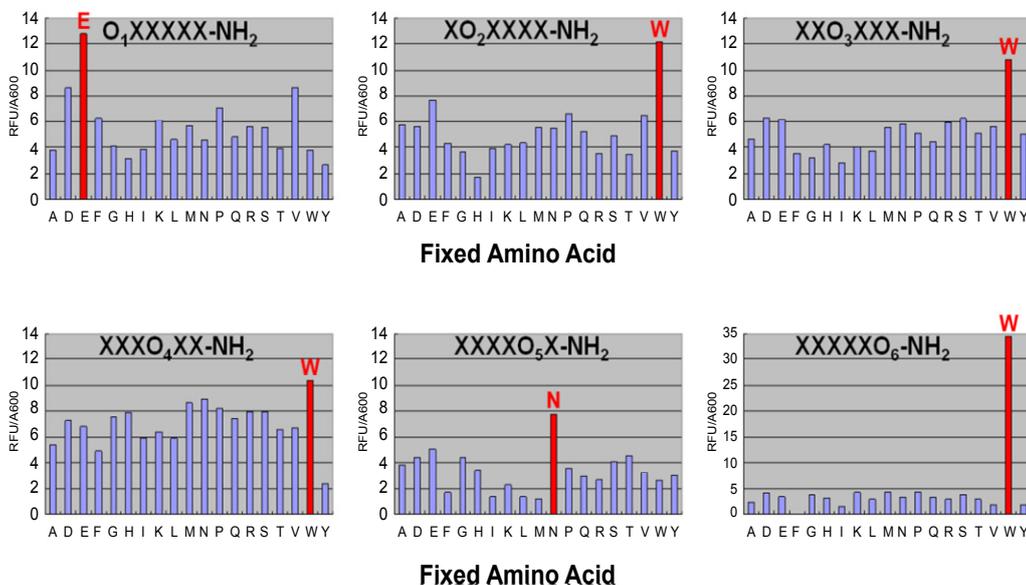


Figure 2: Screening of the PS-SPCLs to select peptides that bind to *B. anthracis* Sterne spores. Panels represent the results obtained with the peptide pools having a known amino acid at each of the six positions in the hexapeptide. One of the six positions (O_1 , O_2 . . . and O_6) is assigned with each of the 19 L-amino acids. The remaining five positions consist of mixtures (X) of 19 L-amino acids (cysteine excluded). The library consists of 114 peptide pools. The optimized binding peptide sequence was the EWWWNW hexamer, named BaS-01.

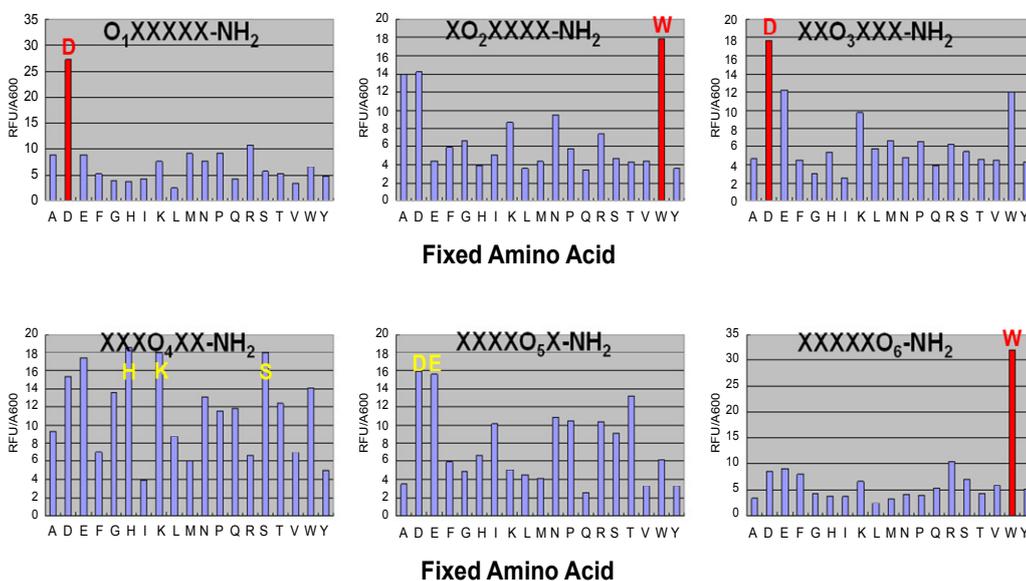


Figure 3: Screening of the PS-SPCLs to select peptides that bind to the *B. anthracis* Δ Sterne spores. Panels represent the result obtained with the peptide pools having a known amino acids at each of the six positions in hexapeptide. One of the six positions (O_1 , O_2 . . . and O_6) is assigned with each of the 19 L-amino acids. The remaining five positions consist of mixtures (X) of 19 L-amino acids (cysteine excluded). The library consists of 114 peptide pools. The optimized binding peptide sequences are DWDHDW, DWDSEW, and DWDKDW hexamers, named Ba Δ S-01, Ba Δ S-02, and Ba Δ S-03, respectively.

(EWWWNW-NH₂) with *B. anthracis* Sterne spores for 15 min at room temperature (Figure 4). The binding affinity of the other three active peptides (DWDHDW-NH₂, DWDSEW-NH₂, and DWDKDW-NH₂) was examined with *B. anthracis* Δ Sterne spores by 15-min incubation at room temperature (Figure 5). From these results, we developed the specific binding peptides with about 70-100% higher binding affinity against *B. anthracis* spores via peptide library screening, when compared with the BA1 peptide reported by Williams et al. [5].

Specificity and selectivity for *B. anthracis* spores of specific binding peptides

In order to verify their specificity corresponding peptide-spore reactions, the capture peptides bound onto the *B. anthracis* Sterne and Δ Sterne spores (1.0×10^8 CFU/mL) were checked with same amount of spores of other *Bacillus* strains, *B. cereus*, *B. subtilis*, and *B. thuringiensis*, respectively (Figure 6a). The newly designed peptides

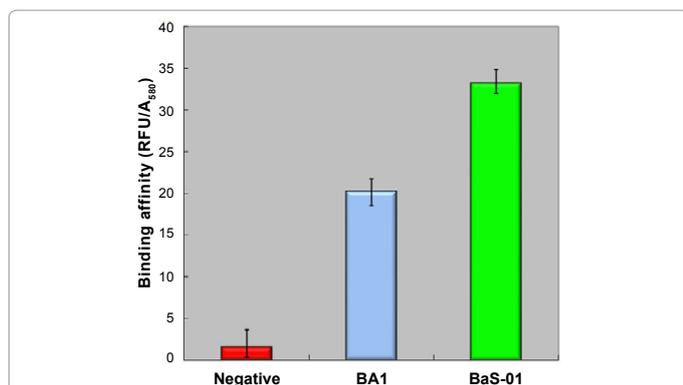


Figure 4: Binding affinity of the synthesized hexapeptide from the selected amino acids to *B. anthracis* Sterne spores. Negative, SLLPGLPGGGC as a negative control; BA1, ATYPLPIRGGGC; BaS-01, EWWWNW.

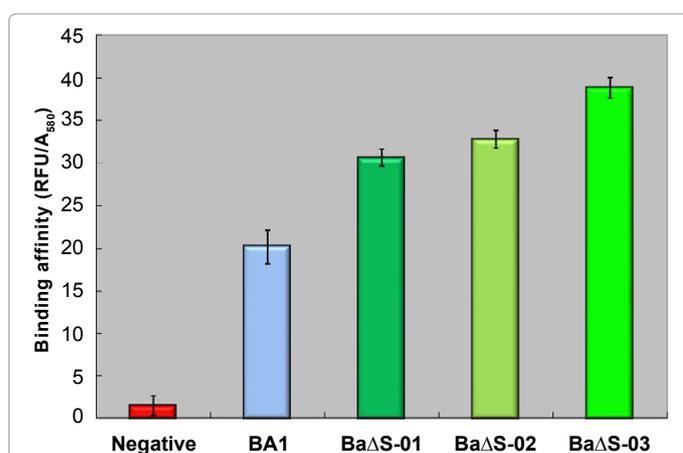


Figure 5: Binding affinity of the synthesized hexapeptides from the selected amino acids to *B. anthracis* ΔSterne spores. Negative, SLLPGLPGGGC as a negative control; BA1, ATYPLPIRGGGC; BaΔS-01, DWDHDW; BaΔS-02, DWDSEW; BaΔS-03, DWDKDW.

have been specific binding effects on *B. anthracis* spores after incubating the active capture peptide (EWWWNW-NH₂) with *B. anthracis* Sterne spores and other three *Bacillus* spores for 15 min at room temperature, respectively (Figure 6a). The binding affinities of the other three active peptides (DWDHDW-NH₂, DWDSEW-NH₂, and DWDKDW-NH₂) were examined with *B. anthracis* ΔSterne spores and other three *Bacillus* spores by 15-min incubation at room temperature (Figure 6b-6d). From these results, we confirmed the specific and selective binding peptides with higher binding affinity against *B. anthracis* spores, when compared with other several bacilli spores. Finally, the detection limit of the method was calculated one more time by 3-sigma rule. As a result, the limit was 1.8×10^2 CFU/mL for *B. anthracis* Sterne spores, upto 1.7×10^2 CFU/mL for *B. anthracis* ΔSterne spores, respectively. The screened capture peptides could be specifically and sensitively bound onto the *B. anthracis* spore surface.

Rapid separation of *B. anthracis* spores using oligopeptide-conjugated magnetic microbeads

We next examined the possibility of detecting *B. anthracis* spores when mixed with other microorganisms, as is the case in natural environments. As shown in the schematic diagram of Figure 7, rapid separation of *B. anthracis* spores from the spore mixture was performed using magnetic microbeads. The BaS-01 peptides were incubated with the streptavidin-conjugated magnetic microbeads for 15 min at room temperature and washed with PBS buffer to remove unbound peptides. The peptide-bound magnetic microbeads were then incubated with a 1:1 mixture of *B. cereus* and *B. anthracis* Sterne spores. The sample mixture became slightly turbid with the formation of brown precipitate after incubation. The precipitate could be moved to the side wall of the tube by using a handheld magnet separator. To confirm the separation of the two types of spores (*B. cereus* and *B. anthracis* Sterne), the supernatant (unbound spores) and precipitate (bound spores) fractions were separately spread on a Mueller Hinton agar plate for the detection of hemolytic activity. As shown in Figure 8a, a halo-zone caused by hemolytic activity was observed with the unbound spores, but not with the bound spores. Since *B. cereus*, not *B. anthracis*, is supposed to show hemolytic activity, these results indicate that this method allows for

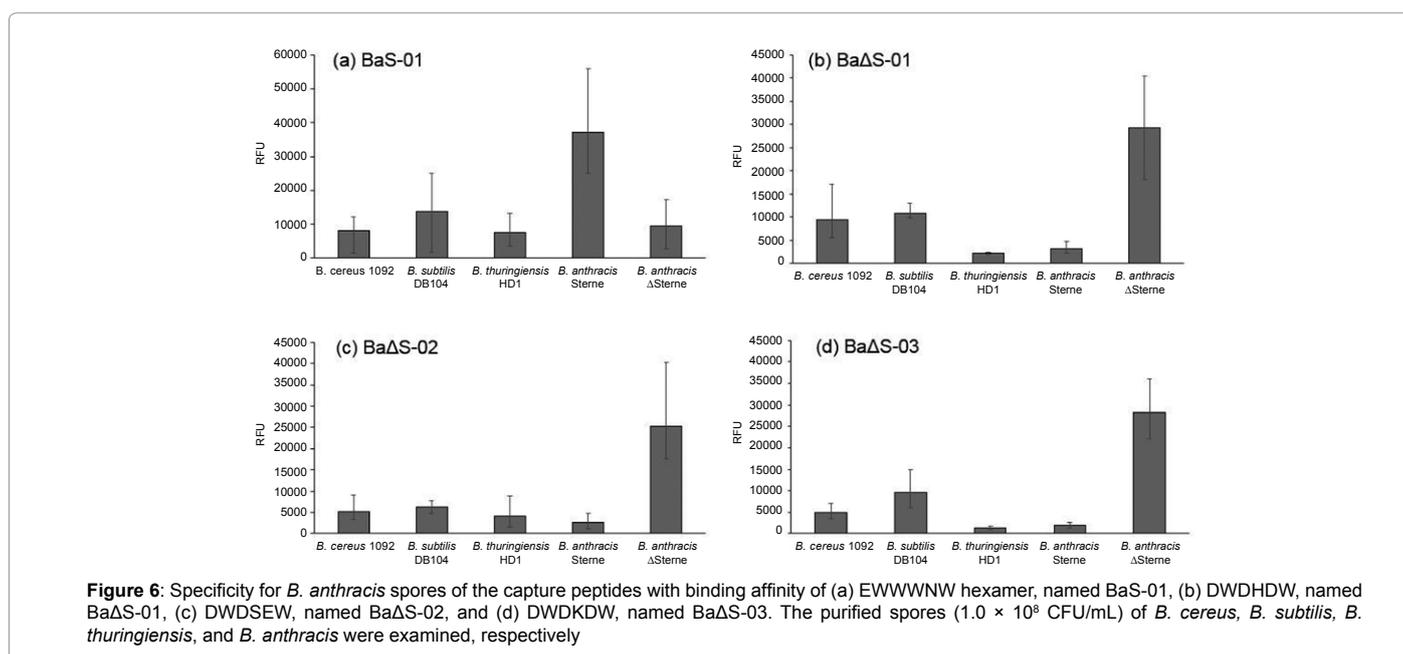
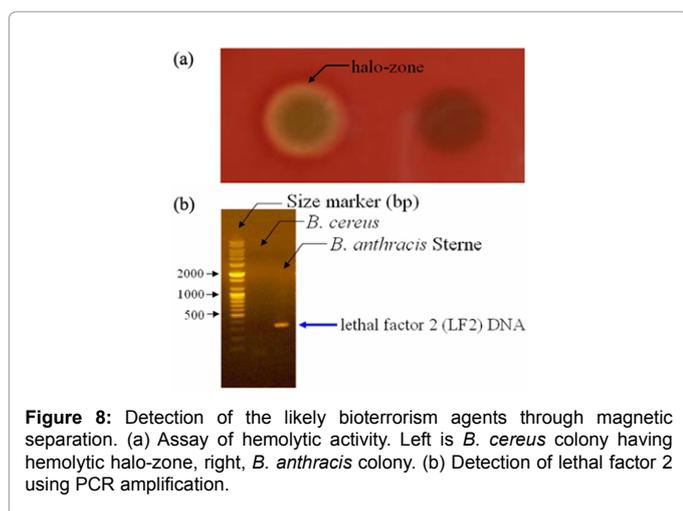
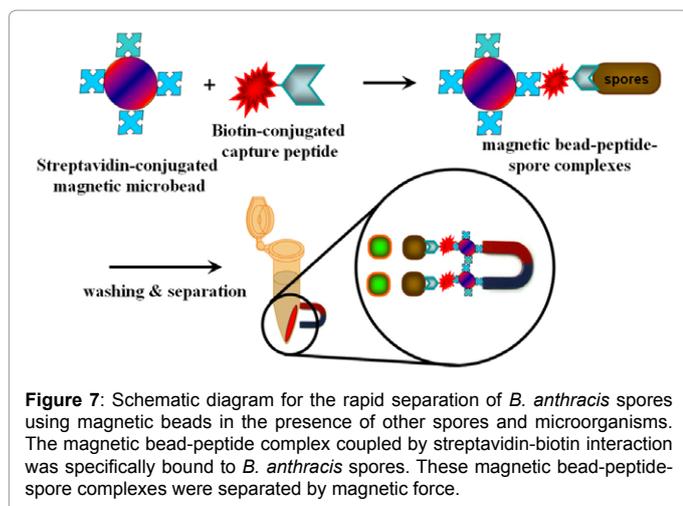


Figure 6: Specificity for *B. anthracis* spores of the capture peptides with binding affinity of (a) EWWWNW hexamer, named BaS-01, (b) DWDHDW, named BaΔS-01, (c) DWDSEW, named BaΔS-02, and (d) DWDKDW, named BaΔS-03. The purified spores (1.0×10^8 CFU/mL) of *B. cereus*, *B. subtilis*, *B. thuringiensis*, and *B. anthracis* were examined, respectively



simple and rapid isolation of *B. anthracis* spores from *B. cereus* spores. To further confirm the successful separation of the two types of spores, PCR was carried out with universal primers and genomic DNA of each fraction (*B. cereus* and *B. anthracis* after germination) as templates. We found that the PCR product encoding lethal factor 2 (ca. 400 bp) was only detected in *B. anthracis* Sterne (Figure 8a and 8b).

Even with the success of this method, the major disadvantages, including false-positive results and non-specific bindings, must be prevented for urgent use during times of a biowarfare threat. Therefore, additional assays involving PCR and/or other antibodies that target different spore surface antigens should be used to separate and detect all forms of *B. anthracis* spores in environmental mixed samples. From a practical standpoint, it will be necessary to develop a sample preparation method for the detection of dilute *B. anthracis* spores present in water, powder, or other environmental conditions. When a suitable sample preparation method is developed in aqueous condition, it will then be possible to employ this system for the early detection of a number of real-life situations encountering anthrax, a dangerous bacterium (e.g., for personal checks against anti-terrorism-targeted activities).

Conclusion

The capture peptide-nanobead-based method developed in this

study allows rapid, simple and accurate detection of *B. anthracis* spores. The high affinity to *B. anthracis* Sterne and Δ Sterne spores was obtained by generating high-affinity-peptides against *B. anthracis* Sterne and Δ Sterne spores via screening assays from the peptide library. Although our fluorescence-based methods require relatively expensive materials and sophisticated laboratory equipment for analysis, our assays described herein take less than 1 h to detect *B. anthracis* spores in the presence of other spores and microorganisms. We also developed a simple and rapid separation method for *B. anthracis* spores using magnetic microbeads; this method can specifically and sensitively detect *B. anthracis* spores with a detection limit of 1.7×10^2 CFU/mL when they are mixed with other microorganisms such as those characteristic of the natural environment.

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