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Screening of LPS-specific peptides from a phage display library using epoxy beads

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Abstract

The selection of identical or highly homologous peptides from phage display combinatorial peptide libraries has been unsuccessful in biopanning experiments using microtiter plates. In the present study, by biopanning on LPS-conjugated epoxy beads, we repeatedly enriched clones encoding AWLPWAK and NLQEFLF. These peptides were found to interact with the polysaccharide moiety of LPS, which is highly variable among gram negative bacterial species. In addition, phages encoding these peptides preferentially bound to the LPS of *Salmonella* family. AWLPWAK-conjugated beads absorbed *Salmonella enteritidis* from solution and showed a preference for *S. enteritidis* over *Escherichia coli*. In summary, this study shows for the first time that a peptide screened from phage displays of combinatorial peptide libraries can be synthesized on beads and be used practically to concentrate bacterial cells from solution.

Keywords: Peptide library; Lipopolysaccharide; Phage display

Lipopolysaccharide (LPS; endotoxin) is the major surface-exposed structural component of the outer membrane of gram-negative bacteria [1,2]. Its structure can be divided into three regions: (1) a phospholipid (lipid A) that is responsible for most of its biological activities [3,4], (2) a core oligosaccharide, which can be further divided into an inner core containing a characteristic sugar acid, 2-keto-3-deoxy-octulonic acid (KDO), and an outer core, and (3) an O-specific chain, which is an antigenic polysaccharide composed of a chain of highly variable repeating oligosaccharide subunits (O antigenic side chain) [5].

The appearance of LPS in the human circulatory system as a consequence of systemic bacterial infections or due to the injection of LPS-contaminated pharmaceuticals frequently induces uncontrolled systemic activation of the immune system. If systemic levels of pro-inflammatory cytokines like tumor necrosis factor- and interleukin-1 remain elevated for long enough, the patient may experience "endotoxic shock" and/or "sepsis," which are characterized by hypotension, coagulation abnormalities, and multiple organ failure. Although the mortality of sepsis is 20–65%, treatment still remains

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anti-symptomatic and supportive [6]. Therapeutic strategies in cases of sepsis are aimed at eradicating bacteria by antibiotics, blocking the actions of cytokines, and most importantly at neutralizing LPS [7]. A few antibodies and peptides reactive to LPS are able to efficiently block its pro-inflammatory activity, and their potential uses as therapeutic agents are being tested [8–10]. These LPS-binding proteins and peptides are also being incorporated into the development of fast reliable methods for detecting LPS in agricultural and pharmaceutical products [11–15].

Phage display of combinatorial peptide libraries has become one of the standard technologies for selecting peptides that target specific molecules. But in the case of LPS, several attempts to select peptides with a sufficiently high sequence identity or homology have failed [16–18]. All of these previous biopannings were performed using LPS absorbed on a microtiter plate. Therefore in this study, we tested whether biopanning using beads, which provide a higher concentration of LPS in its various three-dimensional conformations, could enable the selection of peptides that share sequence identity or homology.

Materials and methods

Conjugation of LPS, lipid A, and polysaccharide to epoxy beads. Polyvinyl alcohol (1.2 g; OCI Chemicals, Seoul, Korea) dissolved in a mixture of 120 ml of degassed distilled water and 10 ml toluene (OCI Chemicals, Seoul, Korea), 2.4 ml glycedyl methacrylate (Aldrich, Milwaukee, WI), 2.4 g acrylamide (Aldrich, Milwaukee, WI), and 9.6 ml ethylene glycol dimethacrylate (Aldrich, Milwaukee, WI) monomers containing 72 mg dibenzoyl peroxide (Aldrich, Milwaukee, WI) were sequentially added to a reactor. After completing the synthesis at 65 °C with constant stirring at 300 rpm for 8 h under a nitrogen atmosphere, the synthesized beads ranging from 38 to 150 μm in diameter were collected by mesh sieving and sequentially washed several times with distilled water, methanol (Aldrich, Milwaukee, WI), and dichloromethane (Aldrich, Milwaukee, WI). Finally, the prepared beads were thoroughly washed with hot ethanol overnight in a Soxhlet apparatus and dried under vacuum.

LPS of *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella typhosa*, *Escherichia coli* K-235, *E. coli* O111:B4, or *Pseudomonas aeruginosa* were purchased from Sigma (St. Louis, MO). Beads were conjugated with LPS as follows. One hundred milligrams of beads was suspended in 800 μl of 0.5 M sodium hydroxide, mixed with 2 mg LPS in 200 μl of distilled water, and incubated with gentle rotation for 40 h at room temperature. After washing with distilled water, the beads were resuspended in 6 ml of 0.5 M NaOH and 10 μmol β-mercaptoethanol was added. The beads were incubated for 2 h to block remaining functional epoxide groups. Then the beads were washed with distilled water until a neutral pH was obtained and stored at 4 °C until required.

To prepare lipid A and the polysaccharide components from LPS, LPS was hydrolyzed by incubating it with 10 mM sodium acetate, pH 4.5, at 100 °C for 1 h. Under these conditions, the labile glycosidic linkage between KDO and lipid A is selectively hydrolyzed [19–22]. The hydrolyzed lipid A was extracted in the mixture of chloroform/methanol (2:1) from the remaining aqueous solution containing the polysaccharide moiety. Both samples were dried using a centrifugal vacuum concentrator and analyzed by mass spectrometry to confirm sample purity. The polysaccharide and the lipid A moiety were then individually conjugated to epoxy beads using the procedure described above for LPS conjugation to epoxy beads.

Biopanning. The Ph.D.-7 Phage Display Peptide Library with a complexity of 2.8×10^9 (New England Biolabs, Beverly, MA) was used for biopanning. The epoxy beads were incubated with a blocking buffer (1% bovine serum albumin (BSA), 0.02% NaN_3 in $NaHCO_3$, pH 8.6) for 1 h at 4 °C, washed five times with Tris-buffered saline–Tween 20 (TBST; 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20), and then used for biopanning. Phages were first incubated with LPS-unconjugated epoxy beads for 1 h and then the unbound fraction was transferred to LPS-conjugated beads. After incubation for 2 h at room temperature, the beads were washed with TBST once during the first round, five times during the second and third rounds, and 10 times during the fourth and the fifth rounds. Bound phages were eluted from the beads by incubating them in 50 μl elution buffer (0.2 M glycine–HCl, pH 2.2) for 10 min. After adding 8 μl of a neutralizing buffer (1 M Tris(hydroxymethyl)aminomethane–HCl, pH 9.1), the eluted phages were used to infect *E. coli* 2738 cells. After being left overnight to propagate, the bacterial cells were removed by centrifugation at 1000*g* for 15 min. The phages were then precipitated by adding PEG solution (20% (w/v) polyethylene glycol-8000, 2.5 M NaCl in distilled water) to the culture supernatant as previously described [23]. After centrifugation at 10,000*g* for 20 min, phage pellets were resuspended in 1% BSA in TBS and used for the next round of biopanning. For each round of panning, the number of phages applied to wells and those finally eluted were determined as described previously [23].

Bead-based enzyme immunoassay. The epoxy beads (100 mg) prepared as described above were added to the individual wells of a microtiter plate (high binding, Costar, NY), incubated with blocking buffer for 1 h, and washed six times with 150 μl PBST (phosphate-buffered saline containing 0.1% Tween 20). Individual clones were isolated from the titration plate for output phages of the last round and infected into *E. coli* ER2738 cells as described previously [23]. After overnight culture 100 μl of the growth medium was added to individual wells containing the beads and incubated for 2 h at 37 °C. The beads were then washed five times with PBST and incubated with horseradish peroxidase-conjugated anti-M13 antibody (1:5000 in 1 mg/ml BSA in PBS, Amersham-Pharmacia Biotech UK, Buckinghamshire, UK) for 1 h at room temperature. After washing five times with PBST, the beads were incubated for 30 min at 37 °C with ABTS substrate solution, which was prepared by dissolving 10 mg of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma, St. Louis, MO) in 10 ml of 50 mM sodium citrate, pH 4.0, and adding 30μl of 30% H_2O_2. After 30 min, the substrate solution in each well was separated from the beads by a brief centrifugation and transferred to another microtiter plate. Then the optical densities of these solutions were measured at 405 nm.

Surface plasmon resonance (SPR) analysis. Real-time interaction analysis was performed using a BIAcore 3000 (BIAcore AB, Uppsala, Sweden) and a HPA sensor chip (BIAcore) by following the procedures reported previously [16]. Before immobilization onto a HPA chip, the LPS of *S. enteritidis* was resuspended in 0.5 mg/ml distilled water and sonicated at 37 °C for 15 min. Chips were pre-cleaned and conditioned by washing with 40 mM *n*-octyl β-D-glucoside (Sigma, St. Louis, MO). LPS was then injected into the flow cell at 1 μl/min until the surface of the chip was saturated. To remove non-covalently bound LPS, 0.1 M NaOH solution was pulsed into the flow cell for 1 min. After the chip had been conditioned with TBS, phages dissolved in TBS were injected at various concentrations into the flow cell for 2 min followed by 2 min of dissociation at a flow rate of 10 μl/min. Before each injection, the chip was washed with 0.1 M NaOH until the sensogram became stable. Background signals were monitored using an LPS-unconjugated HPA chip treated with the same chemicals used to immobilize LPS. M13KO7 helper phages were used as a negative

control. The affinity constant of phage was calculated using BIAcore Evaluation 3.1 software.

Synthesis of peptide-conjugated beads. Core-shell type amino beads (Beadtec, Seoul, Korea) were modified in order to make peptide-conjugated beads. After coupling Boc-PEG1900-COOH to the beads, a photolabile linker was introduced by applying a mixture of 12.5 mg Fmoc-photolabile linker (RT1095. Advanced ChemTech, Louisville, KY), 10.6 mg benxotriazole-1-yloxy-tris-(dimethylamino) phosphonium hexafluorophosphate (BOP), 3.2 mg of 1-hydroxybenxotriazole (HOBt), and 8.4 μl diisopropylethylamine (DIEA) in 3 ml *N*-methyl-2-pyrrolidone (NMP) to the beads and incubating for 2 h at room temperature with constant shaking. After washing the beads three times with methylene chloride (MC), the Fmoc group was removed by incubating beads with 20% piperidine in NMP for 20 min at room temperature. Then Fmoc-6-aminocaproic acid was coupled to the beads as a spacer. After Fmoc group of the spacer was removed, lysine protected with Foc and Boc was conjugated. Then the Fmoc group of lysine was removed and then alanine, tryptophane, proline, leucine, tryptophan, and alanine were coupled to the beads as described previously [24]. Finally, the Boc group was removed from Lys side chains by treating with 50% trifluoroacetic acid and 5% triethylsilane in MC for 20 min. A fraction of the beads was then irradiated with UV (360 nm, 2.5 mW/cm^2) for 30 min and analyzed by mass spectrometry. The peptide released by the beads was detected as a sodium adduct peak at $m/z = 1005.60$.

Enrichment of S. enteritidis from solution on AWLPWAK-conjugated beads. Salmonella enteritidis (ATCC13076) and *E. coli* O157:H7:K$^-$ (ATCC 700927) were stained using a *Live Baclight* Bacterial Gram Stain Kit (L-7005, Molecular Probes, Eugene, OR) as described by the manufacturer's. Briefly bacterial cells were cultured in nutrient broth at 37 °C to the late log phase (10^8–10^9 bacteria/ml). After centrifugation for 5 min at 10,000*g*, the bacterial cell pellets were washed with PBS and incubated in the mixture of compound A containing green fluorescent SYTO 9 and compound B containing red fluorescent hexidum iodine (supplied in the kit) for 15 min at room temperature. The labeled bacterial cells were then resuspended in PBS at 2×10^7 or 2×10^6 cells/ml and mixed with AWLPWAK-conjugated beads. After washing four times with PBST, the beads were transferred onto a slide glass and scanned by confocal laser microscopy (LSM5 Pascal, Carl Zeiss, Germany).

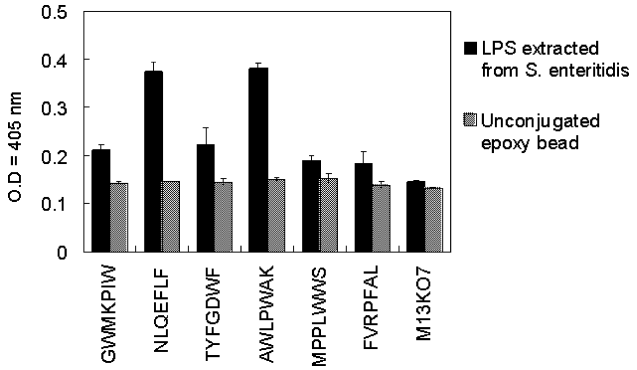

Fig. 1. Reactivity of the phage clones selected by biopanning using the LPS of *S. enteritidis*. Phage clones were allowed to react with beads conjugated with LPS extracted from *S. enteritidis*. The beads were exposed to horseradish peroxidase conjugated anti-M13 antibody and then ABTS substrate solution sequentially with an intermittent washing step. M13KO7 helper phage was used as a negative control. Unconjugated epoxy beads were employed to determine the background signal level. Data shown are mean values of triplicate experiments and their standard deviations.

Table 1
Peptide sequences encoded by phage clones selected by biopanning using the LPS of *Salmonella enteritidis*

Sequences	Number of clones with identical sequence
AWLPWAK	4
NLQEFLF	2
WMKPIW	1
TYFGDWF	1
MPPLWWS	1
FVRPFAL	1

Results

The phage display of the random heptameric peptide library was subjected to biopanning on LPS-conjugated to epoxy beads. After five rounds of biopanning, we selected phage clones from the titration plate for the output phages of the last round and tested their affinity to LPS by the bead-based enzyme immunoassay (Fig. 1) and determined the sequences of the 10 positive clones. AWLPWAK and NLQEFLF were encoded by four clones and two clones, respectively (Table 1). We then tested the reactivity of phages encoding these two peptides toward the LPS of six different bacterial species using a bead-based enzyme immunoassay. Phages encoding both AWLPWAK and NLQEFLF were found to be reactive to the LPS of all six bacterial species, with some preference toward the LPS of the *Salmonella* family (Fig. 2).

The binding characteristics of the phage displaying AWLPWAK for the LPS of *S. enteritidis* were examined by real time interaction analysis. The LPS of *S. enteritidis* was immobilized on a HPA chip successfully with a total binding of 800 RU per flow-cell. Because multiple copies of peptide are displayed per M13 phage and these peptides are likely to be cooperative in binding, the actual binding mechanism may be too complicated to model [16]. Therefore, we counted a phage, probably displaying five copies of the peptide, as one particle and analyzed the interaction accordingly. The calculated k_{on}, k_{off}, and K_D (K_D=k_{off}/k_{on}) values were 1.68×10^6 M^{-1}s^{-1}, 0.0457 s^{-1}, and 2.72×10^{-8} M.

As AWLPWAK and NLQEFLF bound to the LPS of *S. enteritidis* preferentially (Fig. 2), we hypothesized that these peptides would bind to the O-specific chain of LPS, because the O-specific chain is highly variable among bacterial species unlike the conserved lipid A moiety. To confirm this hypothesis, we first hydrolyzed LPS into its lipid A and polysaccharide constituents. The individual components were then conjugated separately to epoxy beads and subjected to bead enzyme immunoassay. As we expected, these peptides bound to the polysaccharide moiety (Fig. 3).

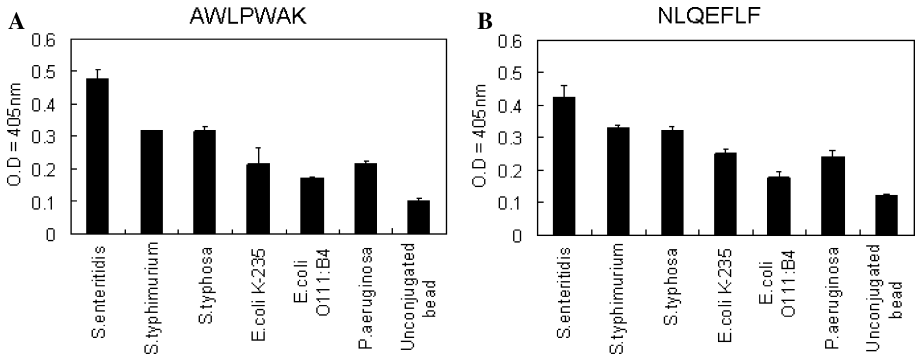

Fig. 2. Reactivity of the phage clones to LPS of several bacterial species. Epoxy beads were conjugated with LPS extracted from individual bacterial species and allowed to react with individual phage clones displaying AWLPWAK (A) or NLQEFLF (B). The amounts of bound phages were determined by exposing the beads to horseradish peroxidase, conjugated anti-M13 antibody, and then ABTS substrate solution sequentially with an intermittent washing step. The data shown are mean values of triplicate experiments and their standard deviations.

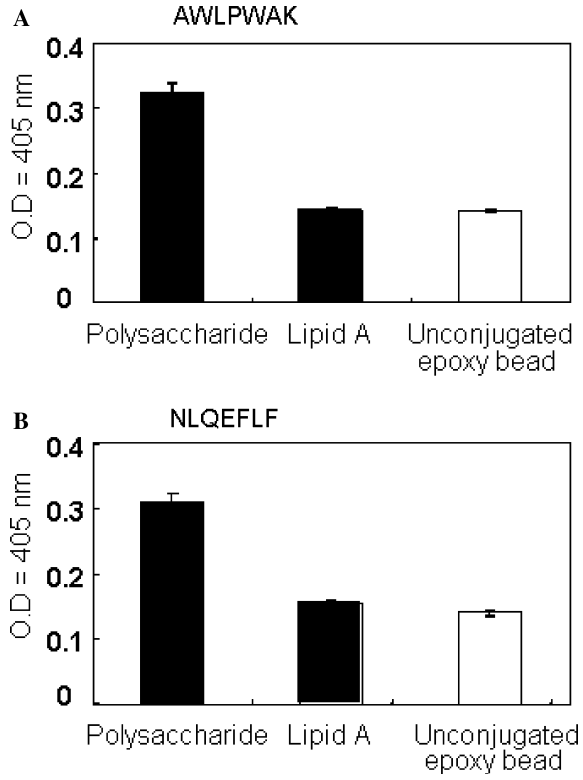

Fig. 3. Reactivity of phage clones to polysaccharide and to the lipid A moiety derived from the LPS of *S. enteritidis*. After hydrolysis of this LPS into its lipid A and polysaccharide components, these components were conjugated individually to epoxy beads. Phages displaying AWLPWAK (A) or NLQEFLF (B) were allowed to react with beads conjugated with each component. The beads were then exposed to horseradish peroxidase conjugated anti-M13 antibody and then ABTS substrate solution sequentially with an intermittent washing step. Unconjugated epoxy beads were used to determine the background signal level. The data shown are the mean values of triplicate experiments and their standard deviations.

The practicality of using AWLPWAK to enrich *S. enteritidis* from solution was tested using chemically synthesized peptide and fluorescence-labeled bacteria. AWLPWAK was chemically synthesized on core-shell type amino beads, and *S. enteritidis* and *E. coli O157* were fluorescence labeled and allowed to react with AWLPWAK-linked beads and with mock beads. The amount of bacteria bound to beads was determined by confocal microscopy as a fluorescent intensity. AWLPWAK-linked beads preferentially interacted with *S. enteritidis* in a dose-dependent manner (Fig. 4), whereas mock beads showed no signal.

Discussion

Several efforts have been made to select LPS-binding peptides from phage display of combinatorial peptide libraries [16–18]. However, these biopanning experiments used microtiter plates and failed to enrich phage clones with identical or highly homologous sequences, which is commonly observed in the biopanning experiments against protein targets [23,25]. Noda et al. reported the selection of clones encoding RVVKGSR, YSALDDG, and MMGVGTS in individual clones [18], and Thomas et al. [17] have reported the enrichment of clones with the sequences . . .h.H.hhhhhhb. . ., . . .h.H.Hhhhhhhb. . ., . . .hhH.Hhhhhhh+. . ., and .. hshH.HhhALhh+b.. (h, denotes hydrophobic; +, a positive; b, a big; and s, denotes a small amino acid). Zhu et al. [16] reported the enrichment of clones with the sequences b(p)hb(p)hb(p), bbbb, hhhh (b, denotes a basic; p, a polar; and h, a hydrophobic residue.)

In the present study, we observed the conventional enrichment of clones with identical sequences, which has not been achieved previously. The most distinctive feature of the present study compared with the previous studies is that epoxy beads were used for biopanning. This may have been advantageous for two reasons. First, contrary to the simple adsorption that occurs on the surface of a microtiter plate, the epoxy groups of beads can bind covalently with any spatially available hydroxy group, which renders a more diverse conformation of the bound

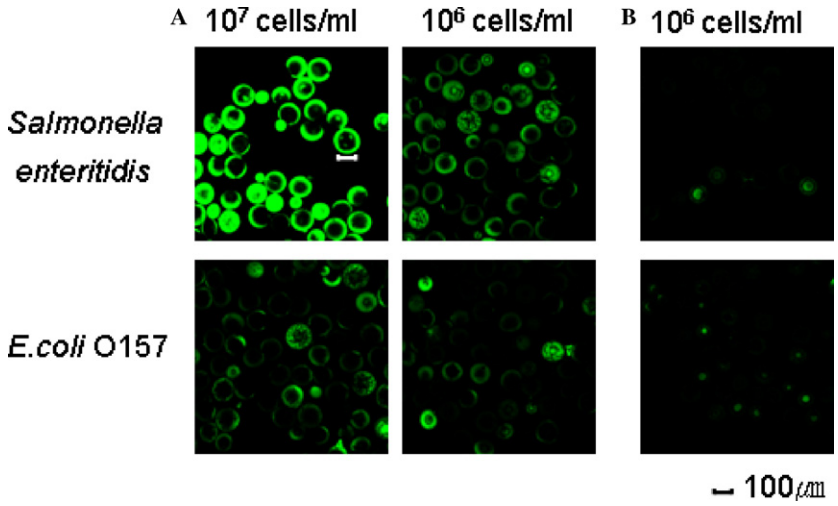

Fig. 4. Preferential binding of AWLPWAK to *S. enteritidis*. *S. enteritidis*, and *E. coli O157* were fluorescence labeled and allowed to react with AWLPWAK-linked-beads (A) or mock beads (B). The relative amounts of bacteria on beads were determined by analyzing fluorescence intensities measured by confocal microscopy.

LPS [26]. Second, the molar ratio of LPS molecules versus peptide molecules is substantially increased when beads are used due to their much greater surface area.

In addition, the enrichment of clones might be enhanced because whole LPS rather than an LPS component was used for biopanning. As lipid A is believed to be responsible for most of the biological activities of LPS [3,4], and is relatively homogeneous in nature, previous biopannings have tended to use lipid A [17,18]. Moreover, in the present study AWLPWAK and NLQEFLF appear to have been selected because of their affinity for the polysaccharide component of LPS not lipid A (Fig. 3). Therefore, it is possible that heterogeneous O-specific chains contain the structure that defines reactivity with a limited set of peptides. And this structure might exist only in a limited bacterial spectrum because this enrichment was not achieved by biopanning the whole LPS molecule of *E. coli* O55:B5 [16].

In this study, we also tested whether a selected peptide could be used practically. We synthesized AWLPWAK on beads and tested its ability to adsorb gram-negative bacterial species from solution. It was found that AWLPWAK-linked-beads successfully adsorbed *S. enteritidis* from solution, and they showed a preference for *S. enteritidis* over *E. coli* O157:H7:K$^-$ (Fig. 4). To the best of our knowledge, the present study is the first to demonstrate a working scheme for screening peptides reactive to LPS using a phage display combinatorial peptide library, chemically synthesizing the peptide identified, and then using this peptide for a practical purpose.

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