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Sequence and structural diversity in endotoxin-binding dodecapeptides

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Abstract

For the study of sequence or structure requirement of short peptides for endotoxin binding, and to search for potential endotoxin antagonists, biopanning was carried out on a phage-displayed random dodecapeptide library against immobilized lipopolysaccharide (LPS) or lipid A (LA), the core toxic portion of LPS. Specific binding of selected phage-displayed peptides to LPS/LA was confirmed by surface plasmon resonance (SPR) analysis. These peptides are rich in basic and hydrophobic amino acids, especially histidine, proline and tryptophan, highlighting apparent amphiphilicity and bacterial membrane activity. These dodecapeptide sequences have no predictable secondary structure in solution, indicating the importance of a random structure before their interaction with LPS/LA. Sequence alignment reveals various potential secondary structures with these selected peptides, which contain specific signature motifs such as ***b(p)hb(p)hb(p)***, ***bbbb***, ***hhhh*** (***b***—basic, ***p***—polar, ***h***—hydrophobic residue), capable of binding LPS/LA. However, none of these peptides exhibit a significant calculated structural amphiphilicity while assuming a secondary structure. This study suggests that for these short dodecapeptides to bind LPS/LA, the potential for their structural adaptation is more important than an amphipathic structure.

Keywords: Phage-displayed peptide; Endotoxin antagonist; Endotoxin-binding motif; Amphiphilicity; Surface plasmon resonance (SPR)

1. Introduction

To fight against bacterial infection, nature has developed in almost all forms of life, an effective innate immune system, of which short antimicrobial cationic peptides play the key role [1,2]. Endotoxin, also known as lipopolysaccharide (LPS), is the major component of the outer membrane of Gram-negative bacteria. It causes lethal septic shock to infected hosts [3]. LPS from most species is composed of three distinct regions: the O-antigen region, a core oligosaccharide and lipid A (LA). The latter is a highly conserved hydrophobic structure and is considered to be the toxic moiety of the LPS molecule [4–7]. Because of high morbidity and mortality of septic shock, agents that can bind LPS and neutralize its toxic effects with low toxicity toward host cells, are of clinical importance. While increased resistance of various bacteria toward available traditional antibiotics becomes a very serious challenge, antimicrobial peptides are thought to be a promising new generation of antibiotics. This is attributable to their unique structure and nature of interaction with bacteria, which makes it almost impossible for the bacteria to develop resistance by genetic recombination and mutation [1,2,8]. Although it is generally believed that antibacterial peptides kill bacteria through disruption of the membrane structure, such as the proposed 'carpet mechanism' [9] and 'pore formation' [10,11], the exact mechanism(s) of action remains unknown [2]. Further information on the peptide–LPS interaction would shed light on the precise mechanisms of antimicrobial action and help in the rational design and development of new peptides based on structure–activity relationship (SAR).

Two common and functionally important features that have emerged for most antibacterial peptides are a net cationicity and the ability to assume an amphipathic struc-

Abbreviations: IPTG, isopropyl β-D-thiogalactoside; LA, lipid A; LPS, lipopolysaccharide; RU, response unit; SAR, structure–activity relationship; SPR, surface plasmon resonance; TBS, Tris-buffered saline; TBS-T, TBS with Tween-20; Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside

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ture (α-helix or β-sheet) upon interaction with negatively charged vesicle mimicking bacterial membranes, with no stringent primary structural organization [12,13]. These features account for the selective preference for negatively charged bacterial membrane over zwitterionic mammalian plasma membranes. The efficacy of these peptides results from their ability to disrupt prokaryotic membranes at concentrations that are not harmful to host membranes [14]. For a better understanding of the selective interaction of antibacterial peptides with the Gram-negative bacterial outer membrane, many SAR studies have been carried out in relating the structural parameters such as peptide charge, helicity, hydrophobicity and hydrophobic moment, to the activity and selectivity (for recent reviews, see Refs. [15,16]). However, most studies have not considered or solved the problem that sequence modifications usually result in complex changes of more than one structural parameter, making it very difficult to trace the activity differences to a specific structural motif [16].

By molecular modeling of peptide–LA interaction, our earlier study [17] predicted that the minimum LA binding motif, ***bhphb***, has the best affinity for LA amongst various derived or predicted LPS/LA-binding motifs, such as ***bhhhbhhhb***, ***bhb(p)hb***, ***bbbbb***, and ***hhhhh***. In this investigation, we use biopanning of LPS/LA binding peptides from a random dodecapeptide library to further test our computational prediction. The analysis of peptide sequences screened from this phage-displayed random library is expected to help to elucidate the physicochemical nature of binding of LPS/LA, via possible specific binding motifs. Furthermore, in combination with surface plasmon resonance (SPR) technology, this approach would allow a rapid search for potential new anti-endotoxin peptides with high affinity for LPS/LA and provide guidance for the rational design and development of new peptides.

2. Materials and methods

2.1. Materials

Unless otherwise specified, reagents of analytical grade were obtained from Fisher (Tustin, CA), or Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO). Pyrogen-free water used for all buffer preparations was from Baxter Healthcare, Australia. BigDye™ Terminator Cycle Sequencing kit was from Perkin Elmer Applied Biosystems. LPS (from *Escherichia coli* 055:B5) and LA in 1,4′ -diphosphoryl form (from *E. coli* F-583) were from Sigma. HPA biosensor chip, which is developed for the immobilization of a lipid layer on the hydrophobic chip surface, was purchased from BIAcore (Uppsala, Sweden). Ph.D.-12™ Phage Display Peptide Library kit was purchased from New England BioLabs (Beverly, MA). The library consists of about 3.7×10^9 distinct sequences.

2.2. Biopanning of the phage-displayed peptide library against immobilized LA or LPS

Aliquots of 1.5 ml of LPS or LA at 100 μg/ml in 0.1 mM $NaHCO_3$, pH 8.6, were each added to polystyrene Petri dishes (60 × 15 mm) and incubated overnight at 4 °C with gentle agitation in a humidified container. The coated plates were blocked for 1 h at 4 °C with blocking buffer (5 mg/ml BSA and 0.02% NaN_3 in 0.1 M $NaHCO_3$, pH 8.6) and washed 10 times with Tris-buffered saline (TBS) with Tween-20 (TBS-T; 50 mM Tris–HCl, pH 7.5, 150 mM NaCl with 0.1% Tween-20). The peptide library was panned for three rounds using the LPS- or LA-coated plates, while the stringency increased from 0.1% Tween-20 in the first round to 0.5% in the following two rounds. In the panning procedure, 1-ml aliquots of TBS-T containing 2×10^{11} phages were loaded onto the coated plates and incubated for 1 h at room temperature with gentle agitation. After removing the unbound fraction and washing with TBS-T, phages bound to LA- or LPS-coated plate were eluted with 1 ml of 0.2 M glycine–HCl, pH 2, containing 1 mg/ml BSA for 10 min with gentle rocking. The eluates were neutralized with 150 μl of 1 M Tris–HCl, pH 9.1. The eluted phages were amplified and titrated for the next round of panning. The eluted phages from the third round of panning (named as LPS/3rd and LA/3rd) were titrated, and well-separated blue plaques on the LB/isopropyl β-D-thiogalactoside (IPTG)/5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) plates from the titering experiment were used to obtain individual clones for sequencing and further characterization. The non-binding phages from the first round of panning (named as NLPS/1st and NLA/1st) were kept and amplified for use as negative controls in subsequent SPR measurements.

Phage amplification, purification and titration were carried out according to manufacturer's instruction, with minor modifications. The host strain used was *E. coli* 2738. The modification was made for the purification of amplified eluted phages in panning procedures. After centrifugation of the amplified culture for 10 min at 12,000 × *g*, the bacteria pellet was subjected to 'elution' by acid glycine buffer (0.2 M glycine–HCl, pH 2) to recover those bacteria-bound phages. The eluted phages were neutralized and combined with the first supernatant, and purified by repeated precipitation with 1/6 volume of polyethylene glycol and NaCl (20% PEG 8000, 2.5 M NaCl).

2.3. Phage DNA purification and sequencing

Phage DNAs from 1 ml amplification culture of individual clones were extracted and purified according to the manufacturer's instruction. For DNA sequencing, a gene specific-96 primer for peptide-gIII fusion protein was used in the automated sequencing using BigDye™ Terminator kit.

2.4. Real-time measurement of bio-interaction between phage-displayed peptides and LA

Realtime bio-interaction analysis of the selected phage-displayed dodecapeptides with LA was measured by surface plasmon resonance, SPR, using the BIAcore 2000 (BIAcore AB, Uppsala, Sweden). 1,4-Diphosphoryl LA (from *E. coli* F-583) at 0.5 mg/ml in water was sonicated at 37 °C for 15 min before immobilization onto HPA chip. Briefly, after the HPA chip was washed with 40 mM of *n*-octyl β-*D*-glucoside for 5 min at a flow rate of 5 μl/min, LA was injected into a flow cell at 1 μl/min until saturation level was achieved. After immobilization, 0.1 M NaOH was injected in 1 min pulses into the flow cell to remove excess LA so that only a monolayer of LA remained. Washing was considered sufficient when the basal SPR response unit (RU) in the sensorgram was stable and had returned to baseline. Typically, around 1000 RU per flow-cell surface coating of LA was obtained.

To determine the condition for specific binding of phage-displayed peptides with immobilized LA, buffers for running and resuspension of phage at different pH (NaOAc for acidic pH, Tris–HCl for neutral and alkaline pH) were tested, using non-binding phage NLPS/1st or NLA/1st as controls. To study the effect of ionic strength of buffer on the binding, LA/3rd (containing mixture of phage-displayed peptides) at 1.6 nM (1×10^{11} pfu/ml) in 10 mM Tris buffer with different salt concentrations were injected and the sensorgrams recorded. Identical buffer injections as blank were always recorded for background subtraction. For the measurement of apparent affinity constant of an individual phage-displayed peptide, samples in TBS (10 mM Tris–HCl, pH 7.0, 100 mM NaCl) at a series of appropriate concentrations were injected into the flow cell for 2 min followed with 3 min of dissociation at 20 μl/min. After each injection of phage-displayed peptide, the sensor chip surface was regenerated with 100 mM NaOH in 1 min pulses until the basal RU in the sensorgram was stable. All BIAcore experiments were conducted at 25 °C, but samples were kept in ice before injection. The apparent binding affinity of phage-displayed peptide for LA was calculated by fitting the sensorgrams of kinetic injections using the bivalent binding model with BIAcore evaluation software version 3.1.

2.5. Physicochemical properties and helix content prediction of the peptides

The mean hydrophobicity and hydrophobic moment, which are the average values of the whole sequence in either α-helical or β-sheet conformation, were calculated as described by Eisenberg et al. [18], using the Eisenberg–Weiss consensus scale of hydrophobicity [19]. Cationicity (p*I*) and amino acid composition were calculated online using ProtParam at ExPASy (http://tw.expasy.org/tools/). The helix content of peptides at pH 7, 278 K and ionic strength 0.1 M NaCl, was predicted by the Agadir algorithm [20–23], with reference to http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html.

3. Results

3.1. Combination of phage display and SPR to screen for endotoxin-binding peptides

After three rounds of panning and amplification procedures, 15 phage clones each from the panning against immobilized LPS and LA were randomly selected and named as LPS/1–15 and LA/1–15, respectively. Because one of the clones, LA/7, succumbed to contamination

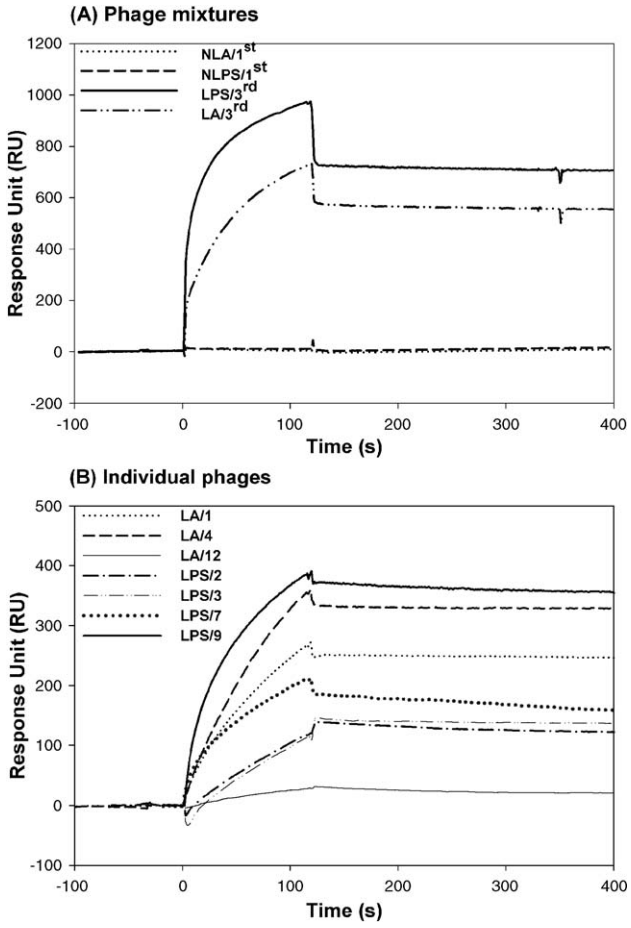

Fig. 1. Phage-displayed peptides selected by biopanning show specific binding to immobilized LA. Binding sensorgrams shown were for injection of (A) 8 nM (5×10^{11} pfu/ml) of mixtures LA/3rd and LPS/3rd, using NLA/1st and NLPS/1st (non-binding phages in the first round of panning) as control, and (B) 1.6 nM (1×10^{11} pfu/ml) of individual clones (e.g. LA/1, LA/4, LA/12, LPS/2, LPS/3, LPS/7, LPS/9). All phage-displayed peptides were purified by repeated PEG/NaCl precipitations, resuspended in 0.1 M NaCl at high stock concentration (1×10^{13} pfu/ml) and diluted to the indicated concentrations with TBS (10 mM Tris–HCl, pH 7.0, 0.1 M NaCl) before injection into LA-immobilized HPA chip. Degassed TBS was used as running buffer for BIAcore measurement. Injections were maintained for 2 min at a flow rate of 20 μl/min.

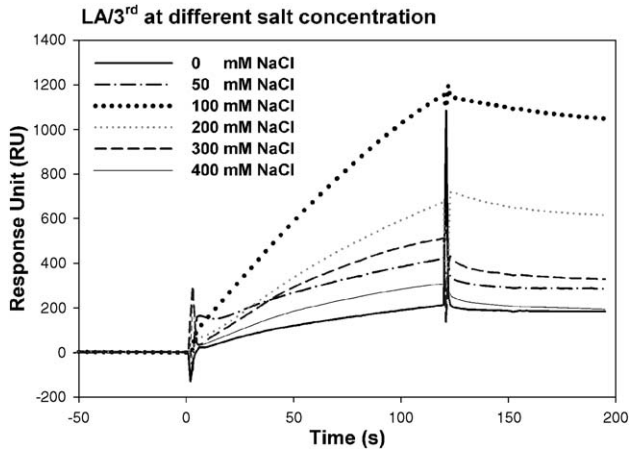

Fig. 2. Moderate ionic strength enhances binding of phage-displayed peptide mixture (LA/3rd) to immobilized LA. Purified LA/3rd (1×10^{13} pfu/ml in 0.1 M NaCl) was diluted to 1.6 nM (1×10^{11} pfu/ml) in 10 mM Tris–HCl, pH 7.0, with different concentrations of NaCl (0–400 mM) before injection into LA-immobilized HPA chip. Identical buffers for phage dilutions were injected for background subtraction. Degassed 10 mM Tris–HCl (pH 7.0) was used as running buffer. Injections were maintained for 2 min at the flow rate of 20 μl/min. The binding sensorgrams shown are normalized against identical background buffer injections.

during various rounds of passages, the remaining total of 29 LPS/ and LA/clones were further studied.

To confirm the LPS-binding affinity of phage-displayed peptides selected by biopanning, we used SPR technology to monitor the interaction of phage-displayed peptides with immobilized LA in a real-time mode. In a preliminary experiment (data not shown), we found non-specific binding of the M13 phage body to the LA-immobilized HPA chip at acidic pH (pH < 6), likely due to the positively charged phage body. Specific binding of phage-displayed peptides only occurs at pH≥7. But above pH 9, the binding signal is very weak, conceivably because the phage body becomes more negatively charged, causing repulsion of the phage from the negatively charged chip surface. Under optimised condition at neutral pH, mixtures of phage-displayed peptides represented in LA/3rd and LPS/3rd, and individual phage-displayed peptides gave significant specific binding signals (Fig. 1A and B).

In an effort to attain an optimum condition for monitoring specific interaction of phage-displayed peptides with immobilized LA, we investigated the effect of ionic strength on binding. Phage displayed peptides in 10 mM Tris–HCl, pH 7 with different concentrations of NaCl (0, 50, 100, 200, 300, 400 mM) were injected. Interestingly, specific binding is drastically enhanced at 100–200 mM NaCl, in comparison to low binding response at low ionic strength (Fig. 2). At 300–400 mM NaCl, the binding signal is reduced to levels comparable to that at 50 mM NaCl. This indicates that upon weakening of the electrostatic interaction by higher ionic strength, hydrophobic interaction contributes significantly to the specific binding, thus maintaining the high affinity of these peptides for LA. This evidence supports the idea that, hydrophobic interaction between peptide side chains and lipid acyl chains of LA may be the primary force that drives secondary structure formation [24,25], while electrostatic interactions, which drive the accumulation of the cationic peptide at the negatively charged membranes, have little influence on the peptide conformation [26].

The apparent variation in the association and dissociation rates of individual phage-displayed peptides on the LA chip (Fig. 1B) suggests the potential of strategically using SPR technology to rapidly rank the LPS/LA binding affinities of phage-displayed peptides with high throughput, to guide subsequent chemical synthesis of interested peptides. Establishment of optimized conditions of pH and salt for the specific binding would at least allow qualitative affinity comparison between different phage-displayed peptides,

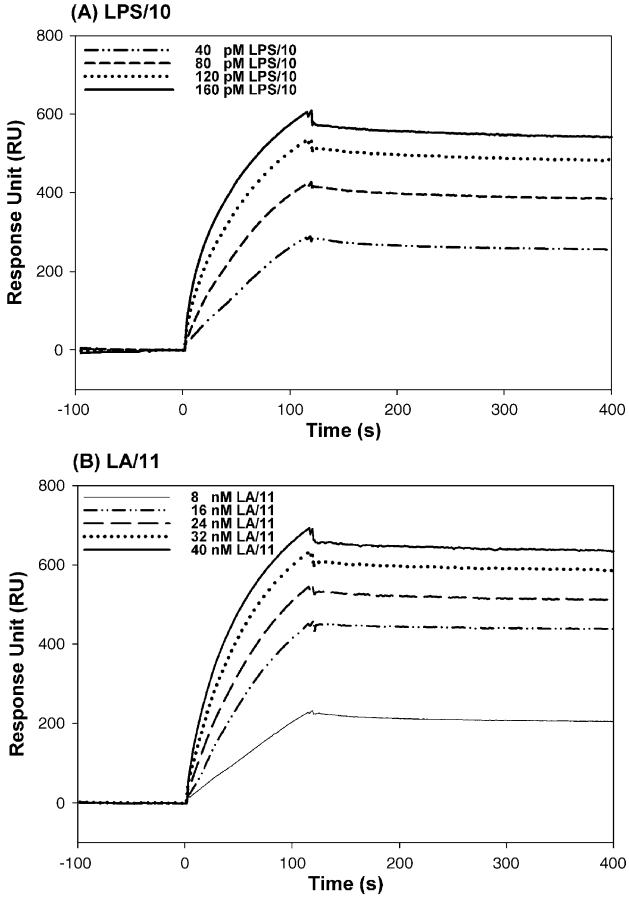

Fig. 3. Comparison of LPS/LA-binding affinity of two individual phage-displayed peptides (LPS/10 and LA/11). Binding sensorgrams shown are for kinetic injections of (A) LPS/10 and (B) LA/11, which are predicted to have different affinities for LA, at a series of concentrations indicated. Stock solutions of phage-displayed peptides in 0.1 M NaCl were diluted into different concentrations with TBS (10 mM Tris–HCl, pH 7.0, 0.1 M NaCl) before injection into LA-immobilized HPA chip. Injections were maintained for 2 min at the flow rate of 20 μl/min. Degassed TBS was used as running buffer.

with evaluation of apparent association rates and net binding at the same concentration, or the peptide concentrations required to obtain similar binding responses. Fig. 3A and B shows the comparison of binding sensorgrams of phage-displayed peptides, LPS/10 (40–160 pM) and LA/11 (8–40 nM) at a series of concentrations each. An overview shows that, to obtain similar binding rates, the concentrations required for the two peptides differ by about 200-fold. Because multiple copies of peptides (generally five) are displayed on one end of the phage M13 and the binding is very likely co-operative, the actual binding mechanism is too complicated to model and in fact, the calculation of absolute K_D is impossible. Nevertheless, efforts were made with different binding models available with BIAcore Evaluation 3.1. As a result, the satisfactory fitting is only obtained by bivalent binding model. The best fitting result was achieved at χ^2 around 10, which was acceptable for affinity ranking. The calculated apparent K_D is 5.5×10^{-12} M for LPS/10 and 3.7×10^{-7} M for LA/11. Of all 29 selected phage-displayed peptides, the measured apparent binding affinities for LA in K_D ranged from 5.2×10^{-7} M for LPS/4 to 5.5×10^{-12} M for LPS/10 (the full data set is not shown).

3.2. The selected dodecapeptides exhibit cationicity, amphiphilicity and membrane activity

The amino acid sequences of phage-displayed peptides LPS/1–15 and LA/1–15 were deduced from the determined DNA sequences (Fig. 4). Analysis of amino acid composi-

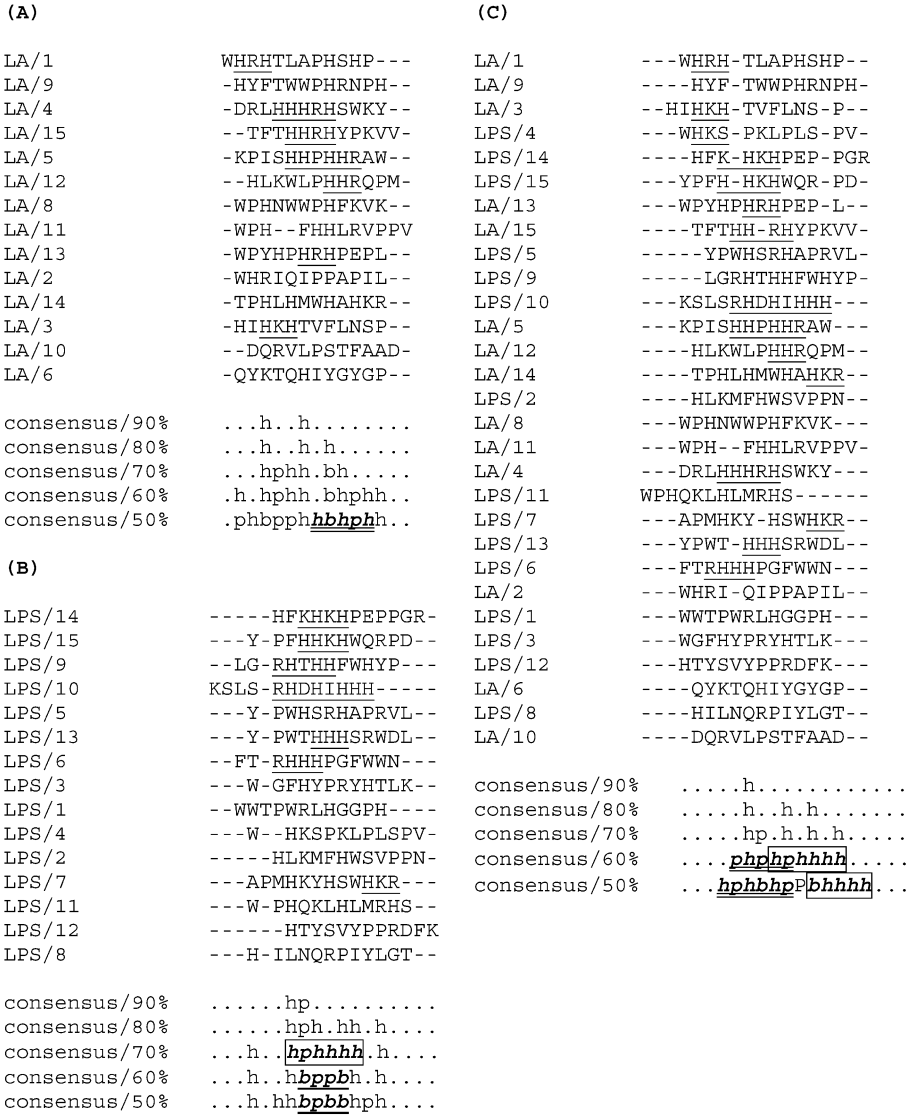

Fig. 4. Multiple sequence alignment and consensus patterns of LPS/LA-binding peptides. Program CLUSTAL W (ver. 1.8) [40] was used for the analysis. Results shown are for alignments of (A) LA/1–15, (B) LPS/1–15 and (C) all peptides. In the pattern scheme, the annotations are: ***h***, hydrophobic residues (G, A, V, L, I, M, P, F, W); ***b***, basic residues (K, R, H); ***p***, polar residues (S, T, N, Q, Y, C, D, E, K, R, H). The sequence of LA/7 was omitted because this clone was contaminated.

tion revealed two common features of these peptides: cationicity and amphiphilicity, which are consistent with those found in natural antimicrobial peptides. Most of these peptides were found to be rich in cationic residues (35% on average), and have an average p*I* of 11.42 (Table 1). These peptides contained about 45% lipophilic hydrophobic residues. Arginine and single cysteine in the random peptide sequence interfere with secretion of M13 pIII and phage infectivity, respectively. Consequently, clones of peptides containing arginine or cysteine are selected against [27]. This may explain the prevalence of histidine over arginine in the selected peptides. However, while no cysteine was found in all the selected peptides, arginine was still present at a high level and is the fourth richest residue (~ 7.5%, 0.9/12), as shown in Table 1.

Table 1
Amino acid composition of phage-displayed peptides with high affinity for LPS/LA[a]

Amino acid	LA/1–15[b]		LPS/1–15[b]		Total[b]	
	Number[c]	% Mol/mol	Number	% Mol/mol	Number	% Mol/mol
P (Pro)	1.79	14.88	1.53	12.78	1.65	13.79
W (Trp)	0.93	7.74	1.00	8.33	0.97	8.05
L (Leu)	0.71	5.95	0.87	7.22	0.79	6.61
A (Ala)	0.43	3.57	0.13	1.11	0.28	2.30
F (Phe)	0.43	3.57	0.53	4.44	0.48	4.02
G (Gly)	0.14	1.19	0.47	3.89	0.31	2.59
I (Ile)	0.43	3.57	0.20	1.67	0.31	2.59
M (Met)	0.14	1.19	0.20	1.67	0.17	1.44
V (Val)	0.50	4.17	0.27	2.22	0.38	3.16
Total (non-polar)	5.50	45.83	5.20	43.33	5.35	44.55
C (Cys)	0.00	0.00	0.00	0.00	0.00	0.00
N (Asn)	0.21	1.79	0.20	1.67	0.21	1.72
Q (Gln)	0.29	2.38	0.20	1.67	0.24	2.01
S (Ser)	0.36	2.98	0.67	5.56	0.52	4.31
T (Thr)	0.57	4.76	0.47	3.89	0.52	4.31
Y (Tyr)	0.50	4.17	0.67	5.56	0.59	4.89
Total (polar)	1.93	16.08	2.20	18.35	2.07	17.24
D (Asp)	0.21	1.79	0.27	2.22	0.24	2.01
E (Glu)	0.07	0.60	0.07	0.56	0.07	0.57
Total (negative)	0.29	2.39	0.33	2.78	0.31	2.58
H (His)	2.71	22.62	2.53	21.11	2.62	21.84
R (Arg)	0.86	7.14	0.93	7.78	0.90	7.47
K (Lys)	0.64	5.36	0.80	6.67	0.72	6.03
Total (positive)	4.21	35.12	4.27	35.56	4.24	35.34
Average p*I*	11.49		11.21		11.42	

[a] The amino acid composition and average p*I* were calculated online using ProtParam at ExPASy (http://tw.expasy.org/tools/).

[b] The composition and p*I* were calculated based on all relevant peptide sequences (all LPS-binding peptides, or LA-binding peptides, or all of them as the total).

[c] The average number of amino acid residues in the dodecapeptides.

The amino acid analysis also revealed the prevalence of histidine (average ~ 22%, 2.6/12), proline (~ 14%, 1.7/12) and tryptophan (~ 8%, 1.0/12). All these amino acids have been implicated in the assembly and structure of membrane proteins [28–31]. Proline might be involved in the packing of bulky aromatic side-chains, hence, lowering the high cost needed to partition them into the membrane interface, as in the case of indolicidin [32]. Tryptophan is of particular interest with regard to the partitioning of peptides into membranes because of its propensity to position itself near the membrane/water interface [33,34]. Examples of antimicrobial peptides, which are rich in tryptophan, include tritrpticin [35] and indolicidin [36,37]. Histidine, together with the other basic amino acids, renders cationicity to the peptides, which contribute to the binding affinity and selectivity for bacterial membrane. Examples of antimicrobial peptides that are histidine-rich include histatin [38]. This indicates that the identified LPS/LA binding peptides are likely to be membrane-active.

3.3. Sequence diversity and structural adaptation are characteristic hallmarks of endotoxin-binding dodecapeptides

It is a general belief that the binding affinity of antimicrobial peptides to negatively charged bacterial membrane is related to the higher content of basic residues and potential to adopt amphipathic structure. However, using the multiple sequence alignment analysis program CLUSTAL W [39], no specific consensus sequence was found in the alignment of the 29 selected peptides (Fig. 4A–C). This is not surprising. In fact, it is consistent with the observation of the sequence diversity of natural antimicrobial peptides. Furthermore, there was no statistically significant difference between peptide sequences selected by panning against immobilized LPS and those by panning against immobilized LA (see also Table 1).

The lack of consensus sequence in the LPS/LA binding peptides supports the idea that an effective LA-binding motif is neither sequence-specific nor structure-specific [40]. However, it may require specific distribution of cationic residues, which form a structural pattern that is complementary to the LA head-group. Different minimum structural binding motifs were predicted in our earlier investigation [17], including ***hhhhh***, ***bhhhb***, ***bhb(p)hb***, and ***bbbbb*** and other non-symmetric patterns, amongst which ***bhphb*** has the highest binding affinity for LA. Indeed, similar patterns were found in the analysis of the selected peptides in this study. First, the regular alternating pattern of basic (or polar)-hydrophobic amino acid residues (double underlined patterns in Fig. 4A: ***hbhph***; and Fig 4C: ***hphbhp*** and ***phphph***), which tends to form β-sheet structure upon binding with the negatively charged LA, was observed at 50–60% consensus scale. Second, the clustering of hydrophobic residues (Fig. 4B and C, framed patterns, and ***phhhh*** ***bhhhh***) or positively charged residues (Fig.

4B, single-underlined pattern ***bppb***, ***bpbb***), which tends to form complicated random structure [17], was also observed. The clustering of basic amino acids, from three to eight residues in length (e.g. LPS/10), was observed in almost half of the identified sequences (underlined in the sequences, Fig. 4). However, a distinct standard amphipathic α-helical motif ***bhhhb(p)hhhb*** or ***bhhhb(p)hhb*** was not obvious in the consensus analysis, although expected. This can be attributed to the observed rich structural potentials of LPS/LA binding peptides, and that the minimum consensus scale in the analysis is 50%.

In conclusion, the diversity of sequences and structural propensities of the LPS/LA binding peptides was prominent. Together with hydrophobic moment analysis and helix content prediction below, we propose that the potential of an amphipathic structure may not necessarily be the prerequisite of endotoxin-binding affinity of at least short peptides. Instead, flexibility and structural adaptation could be the crucial factor.

3.4. Structural variety but not amphiphilicity is the prominent feature in dodecapeptides

The calculated average hydrophobicity ($\langle H \rangle$) values of selected peptides (Table 2) range from −0.26 to 0.15 in the Eisenberg–Weiss hydrophobicity scale [19]. Almost all values are within the thresholds (< −0.51, hydrophilic; >0.10, hydrophobic), with only two exceptions (0.11 for LA/3 and 0.15 for LA/11). The calculation indicates the general amphiphilicity of these peptides. However, the potential of these peptides to fold into distinct amphipathic secondary structures, is not close to expectations, as shown by the calculation of hydrophobic moments of peptides in either helical or sheet conformation. The calculated hydrophobic moments range from 0.08 (LPS/3 as a β-sheet) to 0.37 (LPS/5 as an α-helix).

Antimicrobial peptides exert cell lysis effect by a two-step mechanism, that is, binding to the cell surface and membrane permeabilization [41]. Linear antimicrobial peptides are largely unstructured in solution and fold into an amphipathic helix upon binding to the target membrane [41]. In accordance to this general observation, all the peptides selected by biopanning are predicted to be unordered in solution, based on the predictions of helix content by Agadir algorithm [20–23]. The predicted helix content of the peptides ranged from 0.0 to 0.10 (Table 2), with the only exception in LPS/1 (0.26). This indicates that a flexible and unordered structure of peptides in solution, and the versatile structural potential are required for their binding and consequential perturbation of the bacterial membranes.

Only few sequences may have relatively high hydrophobic moment (bold in Table 2) upon folding to either α-helix or β-sheet. Therefore, it suggests that high hydrophobic moment may not necessarily be required for bacterial membrane binding or antibacterial activity. Thus, distinct amphipathic structures, marked by high hydrophobic moment, may have been overemphasized in the modulation of activities in the SAR-based synthetic design.

Table 2
Physicochemical properties and secondary structure propensity of the peptide sequences selected by biopanning

Peptide	Sequence	$\langle H \rangle$[a]	$\langle \mu_H \rangle/\alpha$[b]	$\langle \mu_H \rangle/\beta$[c]	[α][d]
LPS/1	WWTPWRLHGGPH	0.05	0.21	0.17	0.26
LPS/2	HLKMFHWSVPPN	0.08	0.14	0.13	0.02
LPS/3	WGFHYPRYHTLK	−0.05	0.06	0.08	0.01
LPS/4	WHKSPKLPLSPV	−0.05	0.21	0.11	0.00
LPS/5	YPWHSRHAPRVL	−0.12	**0.37**	0.27	0.02
LPS/6	FTRHHHPGFWWN	0.05	0.17	0.12	0.00
LPS/7	APMHKYHSWHKR	−0.19	**0.28**	0.07	0.10
LPS/8	HILNQRPIYLGT	−0.02	0.08	0.09	0.01
LPS/9	LGRHTHHFWHYP	0.10	0.16	0.21	0.05
LPS/10	KSLSRHDHIHHH	−0.07	0.21	0.23	0.02
LPS/11	WPHQKLHLMRHS	−0.09	0.07	0.16	0.16
LPS/12	HTYSVYPPRDFK	−0.22	0.19	0.10	0.01
LPS/13	YPWTHHHSRWDL	−0.05	0.11	0.23	0.02
LPS/14	HFKHKHPEPPGR	−0.24	0.15	**0.27**	0.02
LPS/15	YPFHHKHWQRPD	−0.19	0.26	0.25	0.05
LA/1	WHRHTLAPHSHP	0.03	0.16	0.20	0.01
LA/2	WHRIQIPPAPIL	0.09	0.17	**0.30**	0.00
LA/3	HIHKHTVFLNSP	0.11	0.16	0.20	0.02
LA/4	DRLHHHRHSWKY	−0.26	0.04	0.29	0.04
LA/5	KPISHHPHHRAW	−0.03	0.08	0.17	0.01
LA/6	QYKTQHIYGYGP	−0.10	0.08	0.17	0.06
LA/8	WPHNWWPHFKVK	0.01	0.05	**0.28**	0.01
LA/9	HYFTWWPHRNPH	−0.02	0.12	0.16	0.02
LA/10	DQRVLPSTFAAD	−0.19	0.08	0.18	0.02
LA/11	WPHFHHLRVPPV	0.15	0.10	0.21	0.01
LA/12	HLKWLPHHRQPM	−0.07	0.25	0.17	0.01
LA/13	WPYHPHRHPEPL	−0.05	0.24	0.22	0.00
LA/14	TPHLHMWHAHKR	−0.01	0.18	0.04	0.10
LA/15	TFTHHRHYPKVV	−0.04	**0.30**	0.22	0.05

[a] $\langle H \rangle$, mean hydrophobicity values, were calculated based on the Eisenberg consensus scale [18].

[b] $\langle \mu_H \rangle$, mean hydrophobic moment of peptide, calculated by the method of Eisenberg et al. [19] for the whole sequence, as an α-helix ($n = 12$, $\delta = 100°$).

[c] $\langle \mu_H \rangle$, mean hydrophobic moment of peptide, calculated by the method of Eisenberg et al. [19] for the whole sequence, as a β-sheet ($n = 12$, $\delta = 170°$).

[d] Helix content of peptides at pH 7, 278 K, ionic strength of 0.1 M NaCl was predicted by the Agadir algorithm [37–40] at http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html.

4. Discussion

While all identified natural linear short peptides adopt α-helical structure upon interaction with negatively charged membrane, different synthesized peptides containing 6–15 residues [42,43] that adopt a β-sheet structure in the presence of lipid were reported to exhibit variable antimicrobial potentials. Because the length of 12 residues is sufficient to assume a distinct secondary structure, we have used an experimental approach involving biopanning of a phage-displayed dodecapeptide library to select endotoxin (LPS/LA)-binding peptides and study the minimum LA/LPS-binding motif. This study was carried out with the

long-term aim of searching for potential pharmaceutical anti-endotoxin peptides. However, at this stage, it is infeasible to search for antimicrobial activity per se because the presence of the phage body may restrict the action of the displayed LPS/LA-binding peptides, either by steric hindrance and/or other interferences that cause the loss of bactericidal activity. Thus, the displayed peptides on the surface of phage M13 were assumed to be ineffective in killing the bacterial host. As such, they could still be selected and amplified in the *E. coli* host, although probably to a lesser extent. In amplification of the phage of interest, it was found that indeed under the same conditions, different phage clones were amplified to varying titer with over a 1000-fold difference (2.4×10^8 to 3.6×10^{11} pfu/ml), suggesting variable antibacterial potentials.

In our earlier study, a minimum LPS/LA-binding motif, ***bhphb***, was predicted to have the highest affinity amongst other motifs derived from sequence analysis of several LPS-binding proteins and antibacterial peptides from natural sources [17]. In addition to the alternating pattern with β-sheet preference, this study also predicted several other minimum binding motifs, including disordered ***hhhhh***, ***bbbbb*** and α-helical ***bhhhb(p)hhhb*** motifs [17]. Indeed, the pattern of alternate distribution of basic and hydrophobic residues, ***b(p)hb(p)hb(p)***, is also found in the present LPS/LA binding dodecapeptide sequences selected by biopanning. In addition, other patterns similar to ***hhhhh***, ***bbbbb*** were also observed. However, the present analyses, which combines empirical and theoretical approaches revealed a more remarkable diversity of sequence and structure propensities than expected, not limited to an amphipathic structure (α-helix or β-sheet). It is noteworthy that many of the sequences in this study contain clusters of histidine residues, like the natural histidine-rich peptide, histatin, which shows no homology to other known peptides and does not have an amphipathic character [38]. Furthermore, contrary to expectation, the structural amphipathicity, which is usually quantitatively measured by hydrophobic moment, is also generally far from being significantly high in the selected peptides. Many efforts have been undertaken to improve the activity of antimicrobial peptides with emphasis on the modulation of hydrophobic moment [16]. However, based on our findings, an amphipathic structure may be too simple to account for LPS/LA binding of peptides, and the importance of the hydrophobic moment might have been overemphasized.

The growing opinion of antimicrobial peptides is that their biological activity is a function of amphipathic structure and high cationic charge. This widely accepted dogma of SAR was recently challenged by Rao [44], who suggested that the maintenance of balance between peptide hydrophobicity and hydrophilicity, is a critical parameter in addition to structure. This property is also observed in the peptides selected in this study, and is deemed to be an important consideration in future design of peptides with pharmaceutical relevance. This idea is further corroborated by a study of short peptides of 9 or 10 residues with little or no α-helicity, where Bessalle et al. [45] argued that it might be an overinterpretation to correlate bioactivity strictly with structural amphiphilicity. Another supportive evidence comes from a study of synthetic diastereomeric peptides which also showed that derivatives that are totally devoid of α-helical structure but have a high ratio of hydrophilic to hydrophobic residues still retain the full antimicrobial activity of the parent peptide [46].

While the peptides in this study were selected by affinity, the antibacterial potential of the peptides may not be directly related to their affinity for LPS/LA. However, identical membrane affinity and permeabilization efficiency revealed in other studies [47] suggest that high affinity is sufficient to destabilize highly negatively charged lipid membranes despite distinct structural differences. With the consideration of the difficulty and limitations in SAR-based modulation of antibacterial activity and selectivity [16] due to the fact that replacement of residues often does not only affect a single structural parameter, we propose that screening the random peptide library in combination with BIAcore technology may be a very efficient initial approach toward the development of antiendotoxin peptides. Further investigations using chemically synthesized peptides based on the identified phage-displayed sequences in this study are in progress to determine the structure, absolute binding affinity to LPS/LA and antibacterial activity.

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