

# The specificity of Sushi peptides for endotoxin and anionic phospholipids: potential application of POPG as an adjuvant for anti-LPS strategies

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## Abstract

Sushi peptides [S1 (Sushi 1 peptide) and S3] are derived from the LPS (lipopolysaccharide; also known as endotoxin)-binding domains of an LPS-sensitive serine protease, Factor C, from the horseshoe crab (*Carcinoscorpius rotundicauda*). S1 and S3 interact at high affinity with LPS. The intermolecular disulphide bonding in the S3 dimer is indispensable for its LPS binding, disruption and consequent neutralization. Simultaneously, the specific interaction between the Sushi peptides and bacterial membrane phospholipids further explains the selective propensity of these peptides for the Gram-negative bacteria. Our findings yield insights into a complex molecular paradigm in which the juxtaposition of LPS molecules and the anionic phospholipid POPG (1-palmitoyl-2-oleoyl phosphatidylglycerol) on the bacterial outer membrane confers such interfacial properties which create the optimal environment for the interaction between the peptides and bacterial membrane lipids.

## Introduction

Gram-negative bacteria (GNB) represent a major group of pathogens responsible for a wide variety of infectious diseases. During GNB infection, LPS (lipopolysaccharide; also known as endotoxin) causes excessive release of inflammatory cytokines, leading to multiple organ failure and death [1]. The indomitable feature of LPS has been a major challenge to the pharmaceutical and medical industries [2]. Thus the development of a drug which is effective in neutralizing LPS is urgently required. Intensive research efforts are currently under way worldwide to innovate peptides which would represent a new generation of antibiotics with increased potency and specificity for endotoxin [3–7]. Currently, our group has developed Sushi peptides from the LPS-sensitive protein, Factor C, from a ‘living fossil’, the horseshoe crab (*Carcinoscorpius rotundicauda*).

## Derivation of Sushi peptides from a horseshoe crab serine protease

The evolutionary success of horseshoe crabs over 500 million years attests to their strong immune defence mechanisms. The

horseshoe crab haemolymph contains mainly one type of cell called amoebocytes, which are extremely sensitive to GNB infection. The amoebocytes release granular components into the plasma to participate in self-defence via blood coagulation to incapacitate the invading GNB. Studies suggest that coagulation factors, such as Factor C, are localized in the amoebocytes [8]. Factor C is a serine protease zymogen that functions as a biosensor of LPS at the initial step of the coagulation cascade [9]. Therefore it is conceivable that it harbours LPS-binding motif(s) that exhibits exceptionally high affinity for LPS [10,11]. Thus it was logical to derive LPS-binding peptides from Factor C.

We have characterized the LPS-binding region of *Carcinoscorpius rotundicauda* Factor C (CrFC), CrFCES, a 132 kDa glycoprotein, which consists of a heavy chain (80 kDa) and a light chain (52 kDa). Near the N-terminus of the heavy chain, there are several repeating units of Sushi domains of approx. 60 amino acids each. Expression and analysis of the N- and C-terminal fragments of recombinant Factor C protein revealed the major LPS-binding site to be located in the N-terminal fragment [12]. The N-terminal fragment (CrFCES) was first cloned and expressed and was proved to bind LPS with high affinity [10]. Subsequently, small fragments in CrFCES were subcloned and expressed, showing that the Sushi 1 and 3 domains are the major LPS-binding regions [11,13]. Based on our understanding of the amino acid sequence in the Sushi domains and comparison of LPS-binding motifs of several other LPS-binding proteins, we showed a predominance of lysine and arginine residues in alternation with hydrophobic residues [6]. Thus, based on the sequences of the Sushi 1 and

**Key words:** bacterial lipid, endotoxin, Factor C, lipopolysaccharide-binding peptide, phospholipid, Sushi peptide.

**Abbreviations used:** CrFCES, lipopolysaccharide-binding region of *Carcinoscorpius rotundicauda* Factor C; GNB, Gram-negative bacteria; LPS, lipopolysaccharide; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoyl phosphatidylglycerol; S1, Sushi 1 peptide.

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3 domains, the corresponding S1 (Sushi 1 peptide) and S3 peptides of 34 amino acids each were synthesized.

## Sushi peptides bind LPS with high affinity to neutralize the endotoxicity

Because Sushi peptides (S1 and S3) were derived from the LPS-binding domains of Factor C protein, we first tested the interaction between the peptides and LPS. S1 and S3 have been found to bind LPS with high affinity [11]. We observed that in solution, S3 exists in two probable forms, a monomeric peptide free of disulphide bonds and a dimeric peptide linked by an intermolecular disulphide bond. In order to gain a global perspective on the importance of the disulphide bond in S3, a mutant peptide named S3-C27S was synthesized (where Cys<sup>27</sup> was mutated to serine). The S3-C27S mutant remained as an inactive monomer. The ability of the S3 dimer to neutralize LPS was found to be superior over that of the S3-C27S, suggesting that disulphide bond is important in S3 activity. On the other hand, S1 exhibited full anti-LPS activity as a monomeric peptide (Figure 1A). Congruent to the anti-LPS activity, fluorescence COSY assay showed the critical role of the disulphide bond in the dimeric S3 peptide, which confers detergent-like properties to bind avidly to LPS, equipping the S3 dimer to preferentially disrupt the LPS micelles [7].

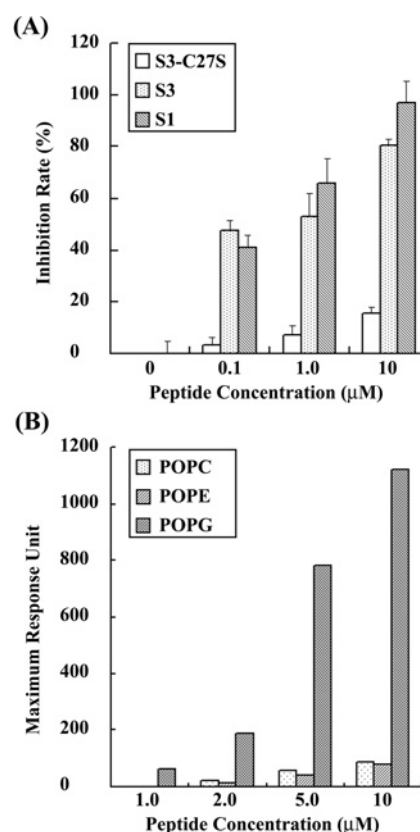
## Sushi peptides bind specifically to bacterial membrane lipids rather than mammalian cell membrane lipids

Besides the anionic LPS molecules, the GNB outer membrane also contains substantial amounts of unique anionic phospholipids such as phosphatidylglycerol [14,15]. In contrast, the outer layer of the mammalian cell membrane comprises mainly phosphatidylcholine and phosphatidylethanolamine and small amounts of other phospholipids, most of which are neutral at physiological pH [15]. We envisage that this subtle compositional difference could be responsible for the different mechanisms of action of antimicrobial peptides, which explains the specificity of the Sushi peptides for the anionic bacterial membrane. This hypothesis is consistent with our observation that the S1 peptide specifically binds POPG (1-palmitoyl-2-oleoyl phosphatidylglycerol), rather than POPC (1-palmitoyl-2-oleoyl phosphatidylcholine), or POPE (1-palmitoyl-2-oleoyl phosphatidylethanolamine) on-chip (Figure 1B).

Sequence analysis showed that there are several positively charged amino acids such as lysine and arginine at the N-termini of S1 and S3. These positively charged amino acids probably contribute to electrostatic interaction with the negatively charged diphosphoryl head groups of the LPS. This is in agreement with our previous observation that mutation of the N-terminus to introduce two extra lysine residues into the peptide resulted in an increase in LPS-neutralizing activity [11]. The positively charged N-termini of Sushi

## Figure 1 | Sushi peptides interact specifically with anionic bacterial lipids

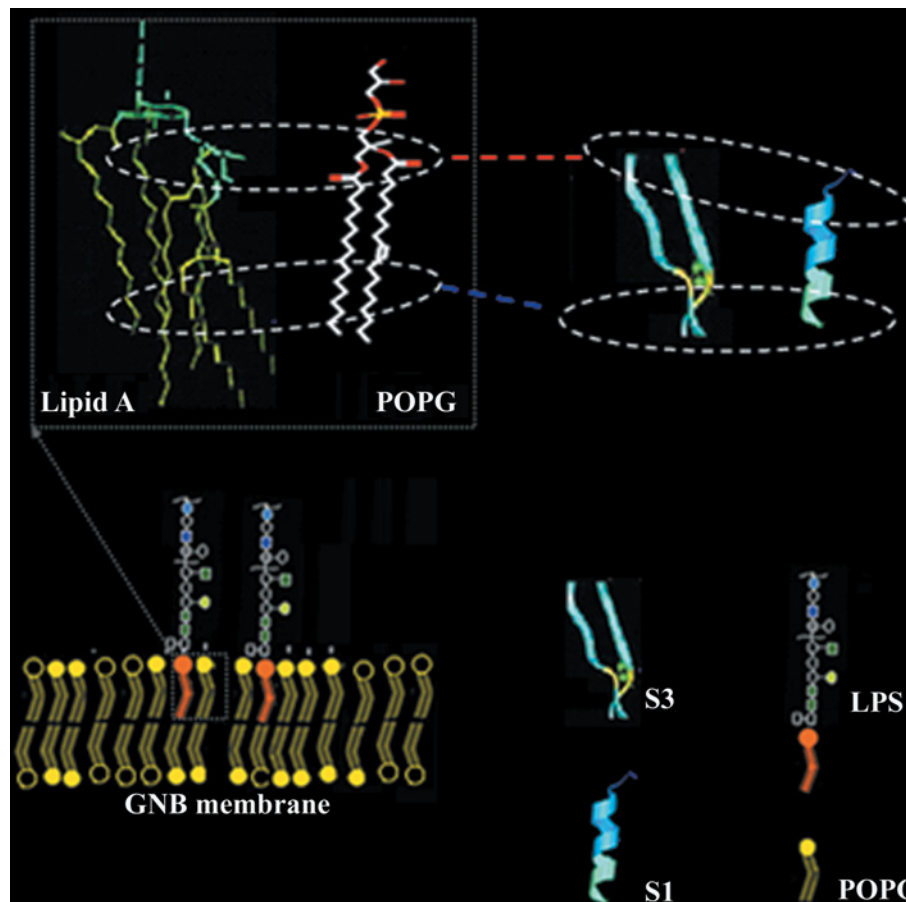
(A) The anti-LPS activity of Sushi peptides assayed using the recombinant Factor C PyroGene assay. Binding of the peptides to LPS inhibits the LPS-induced recombinant Factor C activity. Compared with the mutant S3-C27S, S3 dimer shows superior ability to inhibit LPS activity. In contrast, S1 peptide is active as a monomer. (B) The real-time biointeraction between the Sushi peptides and the immobilized phospholipids (POPG, POPE and POPC). The maximum binding capacity (in response units) of the peptides to the three phospholipids indicates the preference of S1 and S3 for POPG, rather than POPC or POPE.



peptides probably interact with the negatively charged phosphate group of POPG. The C-termini of Sushi peptides are more hydrophobic, and probably interact with the hydrophobic acyl chains of the LPS or POPG, forming a stable molecular complex (Figure 2). Taken together, we have shown that electrostatic and hydrophobic interactions are both important for the Sushi peptides to bind specifically to LPS. Furthermore, preferential interaction with unsaturated phospholipid, POPG, may be interpreted as a means by which the peptides gain easier insertional access through a more fluid bacterial membrane environment, to enhance and stabilize the peptide-LPS complex. In view of the dual preference of the Sushi peptides for LPS and POPG on the GNB membrane, we envisage that POPG could be applied as a potential adjuvant to improve the anti-LPS activity of the Sushi peptides.

**Figure 2 | Sushi peptides interact with bacterial membrane lipids**

The positively charged amino acids at the N-termini of S1 and S3 contribute to electrostatic interactions with the diphosphoryl head groups of the LPS/POPG; the C-termini are more hydrophobic, and probably interact with the hydrophobic acyl chains of the LPS/POPG, forming a stable molecular complex. Taken together, both the electrostatic (red line) and hydrophobic (blue line) interactions are important for the Sushi peptides to bind to bacterial lipids, and contribute to the specific binding of Sushi peptides to GNB membrane lipids.

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