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Tandem repeats of Sushi3 peptide with enhanced LPS-binding and -neutralizing activities

Changgui Li[1], Miang Lon Patricia Ng[1], Yong Zhu[1], Bow Ho[2] and Jeak Ling Ding[1,3]

Departments of [1]Biological Sciences and [2]Microbiology,
14 Science Drive 4, National University of Singapore, 117543 Singapore

[3]To whom correspondence should be addressed.
E-mail: dbsdjl@nus.edu.sg

Endotoxin, also known as lipopolysaccharide (LPS), is the major mediator of septic shock due to Gram-negative bacterial infection. Chemically synthesized S3 peptide, derived from Sushi3 domain of Factor C, which is the endotoxin-sensitive serine protease of the limulus coagulation cascade, was previously shown to bind and neutralize LPS activity. For large-scale production of this peptide and to mimick other pathogen-recognizing molecules, tandem multimers of the S3 gene were constructed and expressed in *Escherichia coli*. The recombinant tetramer of S3 (rS3-4mer) was purified by anion exchange and digested into monomers (rS3-1mer). Both the rS3-4mer and rS3-1mer were functionally analyzed for their ability to bind LPS by an ELISA-based method and surface plasmon resonance. The LAL inhibition and TNFα-release test showed that rS3-1 mer can neutralize the LPS activity as effectively as the synthetic S3 peptide, while rS3-4mer displays an enhanced inhibitory effect on LPS-induced activities. Both recombinant peptides exhibited low cytotoxicity and no haemolytic activity on human cells. This evidence suggests that the recombinant sushi peptides have potential use for the detection, removal of endotoxin and/or anti-endotoxin strategies.

Keywords: endotoxin binding and neutralization/Factor C/limulus/S3 tandem repeats/Sushi3 peptide

Introduction

Sepsis remains a leading cause of death in critical care units, and is also frequently associated with serious consequences such as multiple organ failure. Gram-negative bacterial endotoxin, also known as lipopolysaccharide (LPS), has been suggested to play a pivotal role in such septic complications (Houdijk *et al*., 1997). The acute phase plasma protein, LPS binding protein (LBP), binds circulating LPS to extract it from micelles, and transfers it to either soluble or membrane-bound CD14 receptor in monocytes and macrophages. The interaction of this complex with Toll-like receptors is thought to initiate intracellular signaling reactions, via transcription factor NF-κB (Ulevitch and Tobias, 1999). Activation of protein kinases mediates the production of inflammatory cytokines, which contribute to septic shock. It has also been shown that in the absence of plasma LBP, the LPS is able to directly interact with CD14, yielding similar effects (Wyckoff *et al*., 1998). Thus, treatment of endotoxaemia and sepsis would be greatly aided by blocking the activity of endotoxins and/or removing them from the body fluids of patients, as cationic peptides and synthetic analogues do (de Haas *et al*., 1998; Scott *et al*., 2000).

LPS from Gram-negative bacteria induces the amoebocytes of limulus to aggregate and degranulate. This response underlies the important defense mechanism of limulus against invasion of Gram-negative bacteria (Ding *et al*., 1995). As a molecular biosensor, Factor C can be autocatalytically activated by femtograms of LPS to trigger the coagulation cascade (Ho, 1983), suggesting that it contains high affinity LPS-binding domains. Recently, two regions of Factor C that exhibit exceptionally high LPS binding affinity were defined as the Sushi1 and Sushi3 domains (Tan *et al*., 2000a). Two 34-mer synthetic peptides, S1 and S3, that span the 171–204 and 268–301 amino acid residues of Factor C (DDBJ/EMBL/GenBank accession No. S77063) are derived from Sushi1 and Sushi3 domains, respectively. Both peptides inhibit the LPS-induced limulus amoebocyte lysate (LAL) reaction and LPS-induced hTNF-α secretion (Tan *et al*., 2000b). Thus, the S1 and S3 peptides are promising endotoxin antagonists. The application value of these two peptides would be boosted if they could be obtained by cost-effective and large-scale methods such as recombinant expression in prokaryotic systems. However, expression of small peptides tends to encounter technical difficulties (Le and Trotta, 1991; Latham, 1999). It is reported that some of these problems were resolved by multimerization of the small peptide followed by *in vitro* digestion to restore their activity (Mauro and Pazirandeh, 2000). Besides Factor C, the tachylectin family members identified in circulating hemocytes and hemolymph plasma also contribute to the recognition of invading pathogens. Five types of lectins, named tachylectin-1 to -5, have different specificities for carbohydrates exposed on pathogens. Interestingly, all these lectins contain a different number of tandem repeats in their structure (Iwanaga, 2002). Thus, studying the tandem repeats of S3 may provide explanations as to why these proteins adopt repetitive structures, and how they contribute strategically towards pathogen recognition. In this work, tandem repeats of S3 gene were cloned into a modified vector, which was subsequently transferred to an expression vector, pET22b. Induced expression of the most robust tetramer clone was scaled-up. Recombinant S3 tetramer (rS3-4mer) was purified and digested into monomers (rS3-1mer) by acid treatment, and both the recombinant peptides were tested for their endotoxin-binding and -neutralizing activities.

Materials and methods

Materials

LPS from *Escherichia coli* 055:B5 was purchased from Sigma (St. Louis, MO). LAL kinetic-QCL kit was supplied by BioWhittaker (Walkersvile, MD). Human TNF-α kit (OptEIA ELISA) was from Pharmingen (San Diego, CA). CellTiter 96 Aqueous One Solution Reagent for cytotoxicity assay was purchased from Promega (Madison, WI). Enzymes for DNA

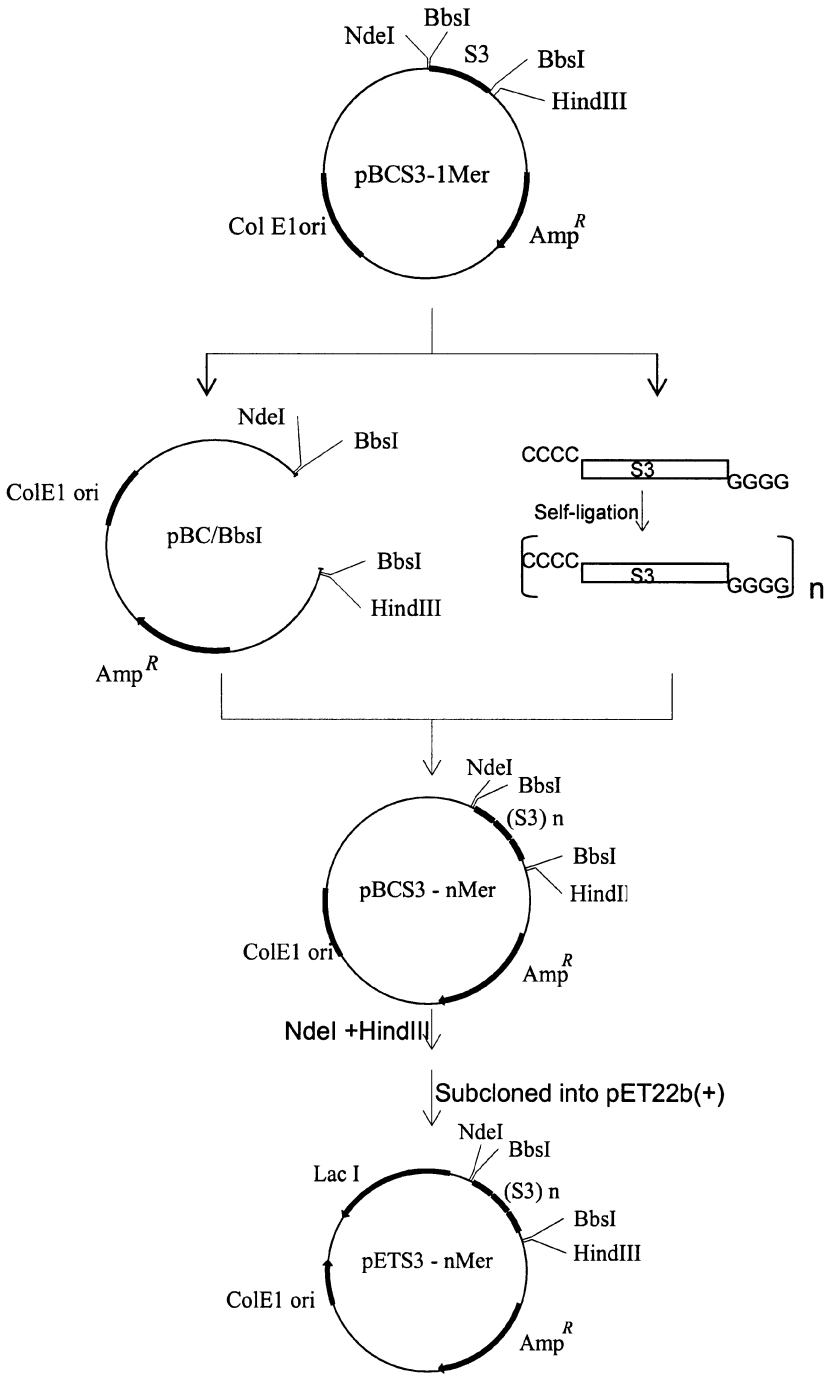

Fig. 1. Schematic representation of the multimerization of S3 gene using the gene amplification vector, pBC. The *Bbs*I site was introduced into the S3 primers, and the amplified gene was cloned into pBC vector. After the *Bbs*I digestion, the S3 gene with overhang terminals was self-ligated at 16°C for 2 h, and inserted into pBC which was previously linearized with *Bbs*I. The CCCC head motif on the sense strand and the GGGG tail motif on the anti-sense strand allowed the fragments to self-ligate directionally, giving rise to multimers of pBCS3-nMer constructs. These multimeric inserts were subsequently released and recloned into expression vector pET22b.

manipulation and polymerase reactions were purchased from NEB (Beverly, MA). DNA purification and extraction kits were from Qiagen (Chatsworth, CA). Pyrogen-free water for making buffers was from Baxter (Morton Grove, IL).

Construction of multimers of S3 gene

Using a cloned Factor C Sushi3 domain, pAC5.1Sushi3EGFP (Tan *et al*., 2000b), the LPS-binding motif, S3, was amplified by PCR. A cloning strategy, which allows for directional multimerization and cloning is shown in Figure 1. Briefly, the amplification vector pBBSI (Lee *et al*., 1998) was modified to include an *Nde*I site containing the start codon adjacent to *Bbs*I site. This modified vector was named pBC. Forward primer 5′-TCGAAGAC*GGCCCC*AG**GATCCC**CATGCTGAACACA-AGG-3′ was designed with *Bbs*I restriction site (GAAGAC) followed by *GGCCCC* in addition to the S3 flanking sequence. On the reverse primer, 5′-TAGAAGAC*CCGGGG*GTCCA-TCAAAGAAAGTAGTTA-3′, a similar motif, was also intro-

duced. Digestion of the PCR product by *Bbs*I yielded fragments with a complementary overhang of *CCCC* on the sense strand and *GGGG* on the anti-sense strand, which can be used for directional multimerization and cloning. In addition, the **GATCCC** sequence, which codes for aspartate (D) and proline (P), was introduced into the forward primer. The peptide bond between D and P can be cleaved under acidic conditions (Szoka *et al*., 1986), thus releasing single S3 units from the recombinant multimers. In this case, the PCR products of S3 were cloned into pBC vector, and the S3 gene was released by *Bbs*I digestion and allowed to self-ligate first, before cloning into the pBC vector, which was previously linearized with *Bbs*I. The multimers of S3 gene were selected and identified by enzyme digestion and sequencing.

Expression of the multimers of S3 gene in E.coli

To construct expression vectors bearing tandem S3 genes under the control of T7 promoter, the fragments flanked by *Nde*I and *Hin*dIII (containing the multimeric S3 genes) were cloned into the vector pET22b, previously linearized with *Nde*I and *Hin*dIII. The constructs were transformed into the *E.coli* host, BL21 (DE3), for expression. The colonies were cultured overnight in LB medium with 100 μg/ml ampicillin at 37°C, then diluted 1:100 into fresh LB medium with 100 μg/ml ampicillin and grown to an OD_{600nm} of 0.6 before induction with 0.5 mM IPTG (Promega). The cells were harvested every hour up to 12 h, and the expressed products were monitored by SDS–PAGE.

Solubilization of inclusion bodies and purification of rS3-4mer

One litre cultures were pelleted at 5000 *g* for 10 min at 4°C and resuspended in 60 ml of lysis buffer containing 20 mM Tris–Cl, pH 8.0 and 0.5 mM DTT. The bacterial cells in the suspension were passed through a French Press (Basic Z 0.75KW Benchtop Cell Disruptor, UK) operated at 15 kpsi for four rounds in order to generate >90% cell disruption. The inclusion bodies were recovered by centrifugation at 12 000 *g* for 20 min at 4°C and washed with 20 mM Tris–Cl buffer containing 1 M urea and 0.5% Triton X-100. The inclusion bodies were denatured and solubilized in 20 mM Tris–Cl with 8 M urea at room temperature for 2 h. Insoluble materials were removed by centrifugation at 16 000 *g* for 20 min, and the supernatant was filtered and purified by anion exchange using ÄKTA explorer (Pharmacia). Briefly, 30 ml of solubilized proteins were applied to a Q-Sepharose column (26×300 mm) equilibrated with buffer A (4 M urea, 20 mM Tris–Cl pH 6.7). After washing with four column volumes of buffer A, the proteins were eluted with a linear gradient of 0–30% buffer B (4 M urea, 20 mM Tris–Cl pH 6.7, 1 M NaCl) and the fractions were collected for SDS–PAGE analysis. The collected fractions were pooled and dialyzed in 10 kDa molecular weight cut-off (MWCO) pore size dialysis tubing (Snakeskin; Pierce, IL), against refolding buffer A containing 50 mM glycine, pH 9.5, 10% sucrose, 1 mM EDTA and 2 M urea at 4°C for 16 h, followed by buffer B containing 20 mM diethanolamine pH 9.5, 10% (w/v) sucrose and 1 mM EDTA at 4°C for another 8 h.

Monomerization of rS3-4mer into rS3-1mer by acid digestion

Two adjacent amino acids, aspartate and proline were added between the S3 units, so as to act as cleavable DP linkers. The renatured rS3-4mer was precipitated with nine volumes of ethanol, frozen at –80°C for 1 h or at –20°C overnight. The mixture was centrifuged at 16 000 *g* for 10 min and the pellet was washed with 90 % ethanol, dried, dissolved in digestion buffer (70% formic acid, 6 M guanidine–Cl) and digested at 42°C for 72 h. The final products were subjected to ethanol precipitation and dissolved in 20 mM Tris–Cl pH 7.3. The cleaved rS3 peptides were then dialyzed overnight against the same buffer using dialysis tubing of 1.5 kDa MWCO pore size (Sigma), thus removing the small linkers and residual salt. The endotoxin contaminant in rS3-4mer and rS3-1mer was removed by Triton X-114 phase separation (Liu *et al*., 1997) followed by polymyxin B affinity chromatography (Detoxi-Gel™; Pierce).

Tricine SDS–PAGE and western blot analysis

The recombinant proteins were resolved on tricine SDS–PAGE, using 5% stacking gel and 15% separating gel, and detected by Coomassie blue staining (Schagger and von Jagow, 1987). Western blot analysis was performed according to the manufacturer's instruction, using an ECL western analysis system (Pierce). The blot was probed with polyclonal rabbit anti-S3 antibody followed by goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; Dako, CA). The blots were visualized using Supersignal West Pico Chemiluminescent Substrate and exposed to X-ray film.

ELISA-based LPS-binding assay

The polysorp 96-well plate (MaxiSorp™; Nunc) was first coated with 100 μl per well of 4 μg/ml (~1 μM) of LPS diluted in pyrogen-free phosphate-buffered saline (PBS). The plate was sealed and incubated overnight at room temperature. The wells were aspirated and washed four times with 300 μl wash solution (PBS containing 0.05 % Tween-20). The wells were blocked with wash solution containing 2% BSA for 1 h at room temperature. After washing twice, varying concentrations of peptides were allowed to interact with bound LPS at room temperature for 3 h. Bound peptides were detected by incubation with rabbit anti-S3 antibody and 1:2000 of goat anti-rabbit antibody conjugated with HRP. Each antibody was incubated for 2 h at 37°C. In the final step, 100 μl of substrate, ABTS (Boehringer Mannheim), was added. The absorbance was measured at 405 nm with reference wavelength at 490 nm.

Endotoxin neutralization assay based on anti-LAL test

The LAL Kinetic–QCL kit utilizes the initial part of the LPS-triggered cascade in LAL to achieve an enzymatic reaction, which catalyzes the release of *p*-nitroaniline from a synthetic substrate, producing a yellow color, which is quantifiable by absorbance at 405 nm. The ENC_{50} (endotoxin neutralization concentration) refers to the peptide concentration required to neutralize 50% of a predetermined quantity of endotoxin. A low ENC_{50} indicates high potency of the peptide for endotoxin neutralization.

In this assay, peptides of different concentrations were incubated for 1 h at 37°C with or without an equal volume of LPS in disposable, endotoxin-free borosilicate tubes. Fifty microliters of each mixture was then dispensed into wells of a sterile microtiter plate (Nunclon™ Δsurface; Nunc). Fifty microliters of freshly reconstituted LAL reagent was dispensed into each well. The absorbance at 405 nm of each well was monitored after 45 min, and the concentration of peptides corresponding to 50% inhibition of LAL activity was designated ENC_{50}.

Suppression of LPS-induced hTNF-α secretion in human THP-1 cells

THP-1 cells were cultured at 37°C in a humidified environment in the presence of 5% CO_2. RPMI 1640 medium was supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were maintained at a density of $2.5\times10^{5-6}$ cells/ml. THP-1 monocytes were transformed into macrophages by addition of phorbol myristic acid (PMA; Sigma) at a stock of 0.3 mg/ml in dimethyl sulfoxide to give a final concentration of 30 ng/ml and 0.01% dimethyl sulfoxide. PMA-treated cell suspensions were immediately plated into 96-well microtiter plate at a density of 4×10^5 cells/ml and allowed to differentiate for 48 h at 37°C. The culture medium was removed and the cells were washed twice with serum-free RPMI 1640. Thereafter, the macrophages were stimulated with 50 EU/ml LPS (a specific activity of LPS that has been standardized by LAL test against FDA-approved LPS standards), peptides alone or LPS (pre-incubated with various concentrations of peptides) and incubated at 37°C. After 6 h, the culture medium was collected and hTNF-α concentration in the supernatants was assayed using ELISA.

Real time interaction analysis between peptides and LPS

Surface plasmon resonance (SPR) analysis of the real time interaction between peptides and LPS was performed with BIAcore 2000 (Pharmacia) using HPA chip (Tan *et al*., 2000b). The affinity constant was calculated using BIAevaluation software 3.0. The mean values were obtained from three independent experiments.

Cytotoxicity of peptides in eukaryotic cells

THP-1 monocytes in 50 μl of 2×10^4 cells/ml in RPMI 1640 were mixed in a microtiter plate with 50 μl of two-fold serial dilutions of peptides ranging in concentration, and incubated for 60 min at 37°C. To determine the cytotoxicity induced by the peptides, 20 μl of CellTiter 96 Aqueous One Solution Reagent was added into each well for 90 min at 37°C. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] is bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium. For detection, the absorbance was measured at 490 nm. To determine the ratio of cell lysis induced by the peptides, two controls were included by incubating cells in PBS containing 0.2% Tween-20 instead of medium only. This absorbance value corresponds to the background, as those cells could not metabolize MTS.

Results

Recombinant expression of S3 tandem repeats, purification and cleavage to monomers

A 143 bp S3 gene fragment was obtained by PCR using pAC5.1Sushi3EGFP as the template. The S3 gene was cloned into pBC vector by digestion with *Bbs*I. After multimerization, the clones containing one, two, four and eight copies of S3 were selected (Figure 2a) and named pBCS3-1, -2, -4 and -8mer, respectively. The *Nde*I and *Hin*dIII-flanking fragments of these clones were inserted into pET22b for expression of the multimeric S3 gene, and the expression levels were examined by SDS–PAGE. Of all the expression cassettes, the tetramer yielded the highest expression level, giving the expected recombinant S3 tetramer (rS3-4mer) of 18.4 kDa, which represented 25% of the total cell proteins (Figure 2b). The monomer construct was not expression-competent, while the octamer construct expressed poorly.

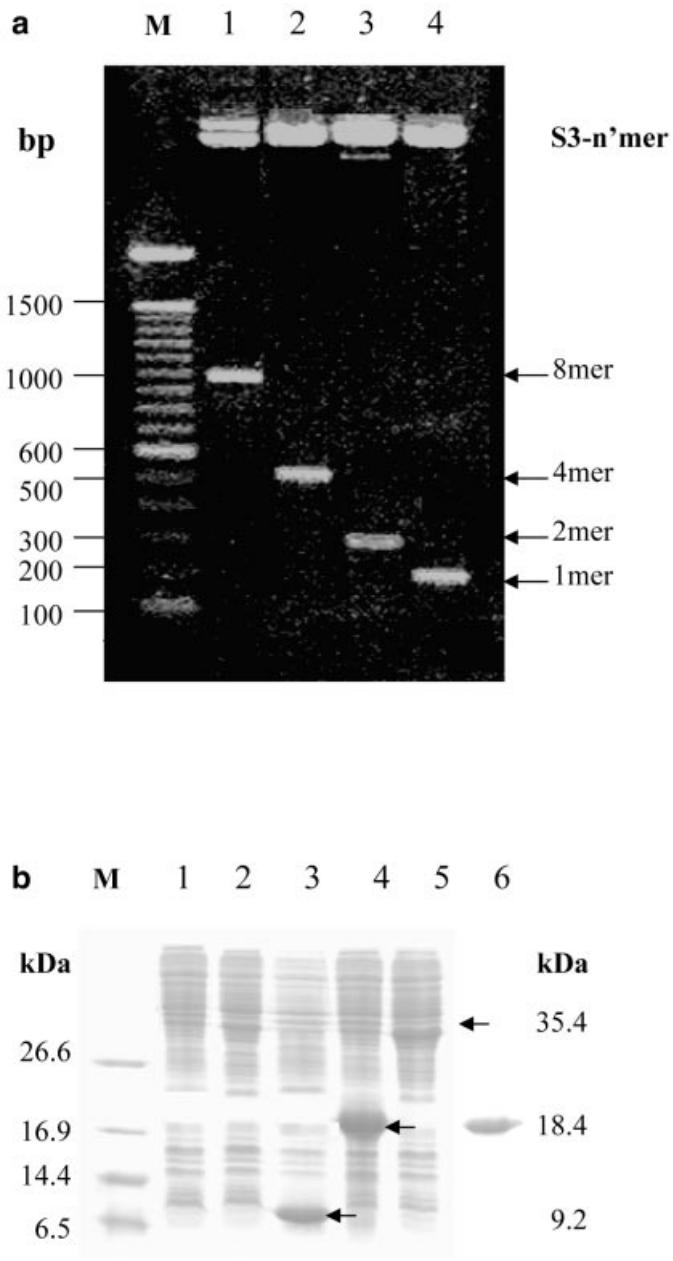

Fig. 2. Identification of multimers of S3 gene and expression in *E.coli*. (**a**) Electrophoretic analysis of the multimeric S3 genes. The number of S3 inserts cloned in the pBC was determined by digestion with *Nde*I and *Hin*dIII, which flank the multimers. The digests were resolved on 2% agarose gel. Lane M, 100 bp DNA ladder; lanes 1–4, *Nde*I and *Hin*dIII digested pBCS3-1, -2, -4 and -8mer, which contain 1, 2, 4 and 8 copies of S3 gene, respectively. (**b**) Expression of multimers of S3 gene in *E.coli* BL21 (DE3). The recombinant peptides were resolved on SDS–PAGE constituting 5% stacking gel and 18% resolving gel. Lane M, peptide markers; lane 1, BL21 containing pET22b; lanes 2–5, BL21 containing S3-1, -2, -4 and -8mer, respectively; lane 6, purified rS3-4mer. The arrows indicate the recombinant proteins.

The rS3-4mer was expressed as inclusion bodies in *E.coli*. The solubilization in 8 M urea and purification through Q-Sepharose anion exchange chromatography produced more than 95% purity of rS3-4mer (Figure 2b), yielding 42 mg rS3-4mer per litre of culture. The purified protein was dialyzed and

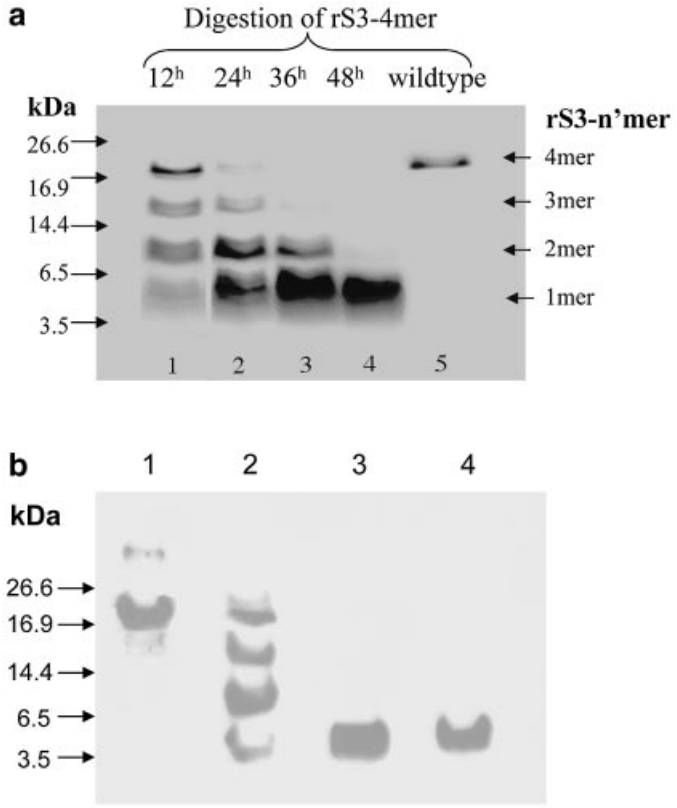

Fig. 3. Time course of formic acid cleavage of rS3-4mer into monomers and western blot analysis of recombinant peptides. (**a**) Digestion of rS3-4mer into monomers. The rS3-4mer was dissolved in cleavage buffer and incubated at 42°C with constant and gentle shaking. At 12, 24, 36 and 48 h, aliquots of 100 µl of samples were sampled and added to 900 µl of ethanol, chilled at –20°C for 30 min, centrifuged at 15 000 *g* for 10 min, and dissolved in loading buffer for electrophoretic resolution on tricine SDS–PAGE with 5% stacking gel and 15% resolving gel. Lanes 1–4, samples digested for 12, 24, 36 and 48 h, respectively; lane 5, intact rS3-4mer. (**b**) Western blot analysis of recombinant peptides. Lane 1, total expressed cell proteins. The expressed 18.4 kDa rS3-4mer strongly reacts with anti-S3 antibody; lane 2, partially digested peptide mixtures containing rs3-4mer, rS3-3mer, rS3-2mer and rS3-1mer; lane 3, rS3-1mer derived from the rS3-4mer; lane 4, synthetic S3 peptide. All peptides derived from rS3-4mer reacted with the antibody.

urea was removed gradually to allow the samples to refold. Dialysis also removed unspecific small molecular weight bacteria proteins, hence further improving the purity of the rS3-4mer. SDS–PAGE under non-reducing conditions showed a majority of one band with the expected size (data not shown). A minor form of a larger aggregate was removed by size exclusion using a Superose® 12 column (Pharmacia). The refolded protein was precipitated with 90% ethanol and redissolved in acid digestion buffer to obtain the monomers (rS3-1mer). The process of acid digestion is time dependent. A 1 day treatment yielded polymeric mixtures of four kinds of rS3 peptides: rs3-4mer, rS3-3mer, rS3-2mer and rS3-1mer (Figure 3a). Within 2 days, >90% of the multimers were cleaved to the monomers.

Recombinant Sushi3 peptides show stronger binding potency to LPS

Samples from the total cell protein, purified rS3-4mer, partially digested rS3 polymers, rS3-1mer and synthetic S3 peptide were resolved on tricine SDS–PAGE and subjected to western blot analysis against anti-S3 antibody. The rS3-1mer and its

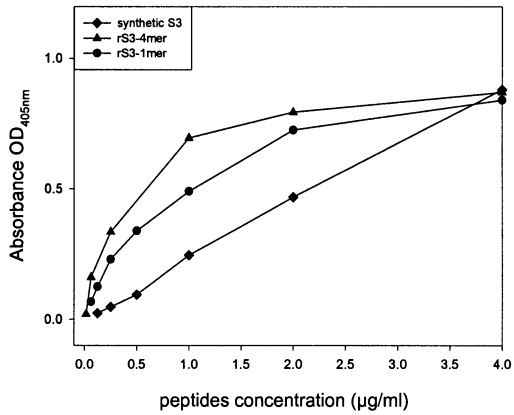

Fig. 4. ELISA-based LPS binding assay. LPS was coated overnight on 96-well plates. Varying concentrations of peptides were allowed to interact with the immobilized LPS. The amount of bound peptides was determined by rabbit anti-S3 IgG and quantitated by ABTS substrate. The average OD_{405nm} values of the triplicate samples were calculated and plotted with the corresponding concentration.

partially digested polymeric repeats (2, 3 and 4mers) were immunoreactive to the polyclonal rabbit anti-S3 antibody (Figure 3b). Thus, the antibody can be employed for the ELISA-based LPS-binding assay.

ELISA-based LPS-binding assay revealed different binding capabilities with rS3-1mer, rS3-4mer and synthetic S3. At 4 µg/ml, both recombinant peptides reached saturation of binding to LPS (Figure 4), while the synthetic peptide continued linearly and required 20 µg/ml to reach saturation of binding with LPS (data not shown). The EBC_{50} of the peptide, which achieves 50% of maximum binding to LPS on the ELISA plate, reflects the binding activity of peptide to LPS, with the lower EBC_{50} indicating higher potency. The rS3-4mer, rS3-1mer and synthetic S3 peptides displayed EBC_{50} at 0.41, 1.02 and 9.74 µg/ml, respectively. The kinetics of binding of peptides to LPS in 20 mM Tris–Cl pH 7.3, was also measured by SPR analysis with BIAcore 2000 using HPA chip, which was immobilized with LPS. The K_d values of synthetic S3, rS3-1mer and rS3-4mer are $(7.80 \pm 2.18)\times10^{-7}$ M, $(4.74 \pm 2.34)\times10^{-8}$ M, $(1.71 \pm 1.86)\times10^{-8}$ M, respectively. Thus, both the results from ELISA and SPR suggest that rS3-4mer is most efficient at binding LPS.

The recombinant S3 peptides inhibit endotoxin-induced LAL reaction and hTNF-α release from THP-1 cells

The ENC_{50} value of the peptides against 5 EU/ml of LPS was determined to be 5.4 µg/ml for rS3-4mer, 9.2 µg/ml for rS3-1mer and 11.2 µg/ml for synthetic S3 (Figure 5a). A lower ENC_{50} indicates higher potency of endotoxin neutralization. The binding isotherm of the two monomeric peptides, whether it is recombinant or synthetic, is similar, but rS3-4mer shows a 2-fold stronger LPS neutralization efficacy.

Similar results were also obtained by measuring the ability of rS3-4 and rS3-1mer to inhibit LPS-induced hTNF-α production by THP-1 cells, which were incubated with 50 EU/ml of LPS containing various concentrations of peptides. As shown in Figure 5b, rS3-1mer required 83.2 µg/ml, whereas rS3-4mer required 40.4 µg/ml to achieve 50% inhibition.

The peptides show minimal cytotoxicity to eukaryotic cells

Both recombinant peptides had minimal effect on cell permeabilization (data not shown). At the highest concentra-

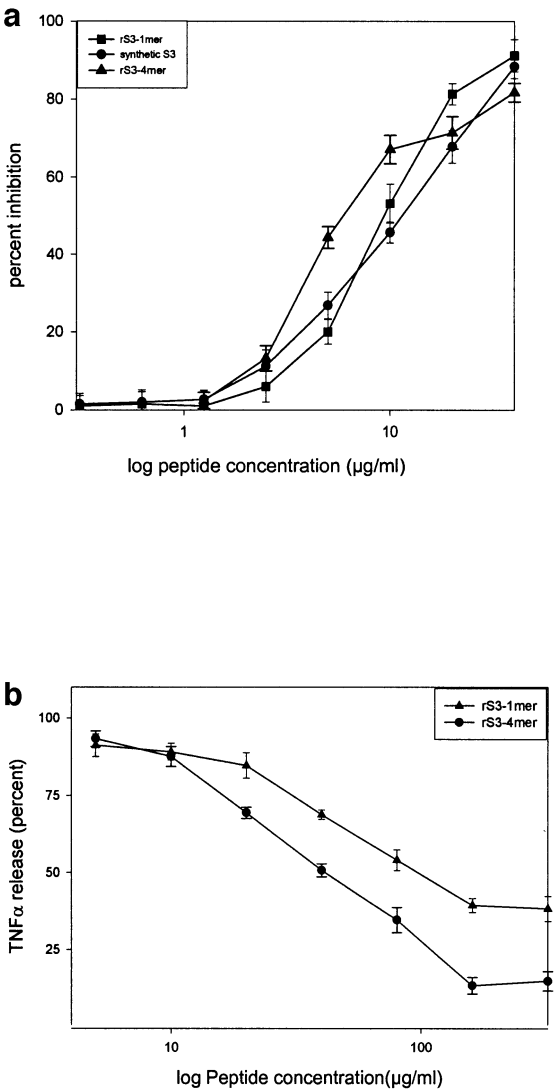

Fig. 5. Comparison of rS3-4mer and rS3-1mer with synthetic S3 in inhibition of the LPS-induced LAL assay and hTNF-α secretion in human THP-1 cells. (**a**) Inhibition of the LPS-induced LAL assay. Binding of the peptides to LPS would competitively inhibit the chromogenic reaction in the kinetic-QCL LAL test. The ENC_{50} values of rS3-4, rS3-1mer and synthetic S3 peptide were determined to be 5.4, 9.2 and 11.2 μg/ml, respectively. (**b**) Suppression of LPS-induced hTNF-α secretion in human THP-1 cells. The rS3-4mer and rS3-1mer were tested for their ability to suppress LPS-induced hTNF-α secretion from THP-1 cells. Both peptides inhibit hTNF-α production in a dose-dependent manner, albeit with different efficiency. rS3-4mer required only 40.4 μg/ml to achieve ENC_{50}, compared to 83.2 μg/ml needed for rS3-1mer. The decrease in TNF-α secretion was expressed as a percentage of control (LPS only).

tion of 50 μM, rS3-4mer caused only 2–3% of cell lysis, indicating that the recombinant multimers of S3 would have negligible contraindications, although the LPS-binding activity is amplified significantly.

Discussion

S3 has been shown to be one of the LPS-binding sites of Factor C, and is able to suppress the LPS-induced cytokine production in macrophages (Tan *et al.*, 2000b). The immobilized S3 peptide analogue can remove LPS from culture medium with high efficiency (Ding *et al.*, 2001). Thus, this promising reagent can be applied to prevent sepsis due to circulating LPS, which is released by viable or injured Gram-negative bacteria. Chemical synthesis is an uneconomical approach to obtain a large quantity of this peptide, whereas expression in *E.coli* may be more cost-effective (Latham, 1999). However, the yield from *E.coli* may be low and unstable (Le and Trotta, 1991). Thus, expression of the multimers of peptides would circumvent the above mentioned problems (Kajino *et al.*, 2000). A more important attribute for recombinant multimers of S3 is the expected enhancement in ligand-binding affinity and LPS-neutralization activity achieved through synergistic effects of multiple LPS-binding units in one molecule (Mauro and Pazirandeh, 2000).

Many methods can be applied to construct the tandem repeats of a peptide (Dolby *et al.*, 1999; Lee *et al.*, 2000; Mauro and Pazirandeh, 2000). We chose the amplification vector that readily allows us to obtain various multimers of S3 gene. Furthermore, we designed the DP linker between the repetitive units, to afford convenient cleavage under mildly acidic buffer to release the monomers. The multimeric constructs exhibit different expression levels. No expression was observed with the pETS3-1mer. As the copy number increases, the expression level improved dramatically, especially with the S3 tetramer, where the expression level reached 25% of the total cell proteins. However, further doubling to 8mer reduced the expression level, suggesting that the copy number is not always proportional to the expression level for this peptide. The ELISA-based LPS-binding test and SPR results show differential binding efficiencies of rS3-4mer, rS3-1mer and the synthetic S3 for LPS, with highest binding achieved by rS3-4mer. Both the LAL inhibition test and suppression of TNF-α release in THP-1 cells showed that rS3-1mer works equally well as the synthetic S3 peptide to neutralize LPS, while rS3-4mer displayed a 2-fold higher anti-LPS activity. However, the rS3-1mer and synthetic S3 showed inconsistent results in ELISA and SPR tests.

Two major forces mediate the interaction between LPS and LPS-binding peptides. The positive charge on the peptides forms an electrostatic attraction with the negatively charged phosphate head groups of the LPS. The other is the hydrophobic interaction between them (Farley *et al.*, 1988; Goh *et al.*, 2002). In fact, mutation of amino acid residues of S3 aimed at introducing positive charges, only achieved a slight increase in LPS-neutralizing activity (Tan *et al.*, 2000b). Besides charge modification, little effort has been taken to enhance the LPS-binding ability of such peptides. Herein, by creating tandem repeats of the LPS-binding units, instead of increasing the number of positive charges, we demonstrate a two-fold improvement in the activity of the tetramer compared to the original monomeric unit, thus providing an alternative strategy to improve the LPS-binding activity of similar peptides. The result of secondary structure analysis by the DNAMAN program (Version 4.15, Lynnon Biosoft) shows that both S1 and S3 have a distinctive structure of four regular β-sheets alternately spaced by turns and coils. We presume that this structure may be important to the interaction with LPS, and in addition, the multiple β-sheets in rS3-4mer, may form the β-barrel structure to provide better shielding of hydrophobic acyl chains of LPS (Ferguson *et al.*, 1998). Further structure analysis by CD or NMR will help to

explain the enhanced activity of the recombinant S3 tetramer.

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