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Interaction of peptide-bound beads with lipopolysaccharide and lipoproteins

Masatsugu M. Suzuki [a], Megumi Matsumoto [a], Hiroyuki Omi [a], Tomomi Kobayashi [a], Akio Nakamura [b], Hiroko Kishi [c], Sei Kobayashi [c], Takashi Takagi [a,*]

[a] Peptide Door Co. Ltd., Fukushima-machi 794-21, Takasaki, Gunma 370-3523, Japan
[b] Graduate School of Medicine, Gunma University, Showa-machi 3-39-15, Maebashi, Gunma 371-8511, Japan
[c] Department of Molecular Physiology and Medical Bioregulation, Graduate School of Medicine, Yamaguchi University, Minami-Kogushi 1-1-1, Ube, Yamaguchi 755-8505, Japan

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ABSTRACT

We previously reported the generation of lipopolysaccharide (LPS)-binding peptides by phage display and chemical modification. Among them, a dodecapeptide designated Li5-025 (K′YSSSISSIRAC′; K′ and C′ denote D-lysine and D-cysteine, respectively) showed a high binding affinity for LPS and was resistant to protease digestion (Suzuki et al., 2010). In the current study, Li5-025-bound silica beads, hereafter referred to as P-beads, were generated and found to be devoid of LPS-neutralizing activity. Thus, LPS bound to the P-beads could be directly used in the Limulus amebocyte lysate (LAL) assay. P-beads bound LPS dissolved in solutions of ethanol, pH 4, pH 10, and 0.5 M NaCl and LPS bound to the P-beads was quantitatively assayed. The sensitivity of this assay was observed to be approximately 0.1 pg/mL LPS. P-beads bound LPS dissolved in antithrombin III (AT III) solution which is a strong inhibitor of activated factors C and B as well as the clotting enzyme in the LAL assay; the inhibitory effect of AT III was completely reversed upon washing the P-beads with 25% acetonitrile. This was employed as the first step for the detection of free LPS in plasma using the LAL assay. LPS added to human plasma at 0 °C followed by application to the P-beads and subsequent washing with 25% acetonitrile resulted in low LPS activity as detected by the LAL assay. However, further washing of the P-beads with 0.1% Triton X100 in 25% acetonitrile resulted in high LPS activity. This is the first instance of quantitative detection of free LPS in plasma using the LAL assay, and the sensitivity of this method was observed to be 1 pg/mL of LPS. The proteins eluted in the 0.1% Triton X-100 wash were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Two protein bands of 28 kDa and 18 kDa were predominantly observed. Mass spectrometry analysis revealed that the 28 kDa and 18 kDa bands corresponded to apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II), respectively. ApoA-I and apoA-II are components of high density lipoprotein (HDL). Thus, it is likely that the P-beads-bound LPS was sequestered by HDL, resulting in neutralization of its toxicity.
This study showed that by using P-beads, free LPS in plasma can be quantitatively measured by the LAL assay at a concentration of 1 pg/mL.

1. Introduction

Lipopolysaccharides (LPS) are major components of the outer membranes of gram-negative bacteria and evoke inflammatory responses by activating monocytes or endothelial cells. The activation of circulating monocytes by LPS is mediated by LPS-binding protein (LBP) and membrane CD 14 that are present on the surface of monocytes. LBP catalyzes the movement of LPS either to high density lipoprotein (HDL), which results in the neutralization of LPS, or to soluble membrane-bound CD 14, which results in cellular activation (Cohen, 2002; Van Amersfoort et al., 2003).

A large amount of LPS evokes an excessive cellular response and causes deleterious septic shock. Therefore, the removal of contaminated LPS in medicines dissolved in various solvents is necessary. Although removal of contaminated LPS is essential in the case of vaccines, or DNA used for gene therapy, the recovery of proteins or DNA should also be considered. A medical examination for sepsis, detection, and quantitation of LPS in plasma is important for controlling septic shock due to excess LPS. Limulus amebocyte lysate (LAL) assay is extremely sensitive to LPS detection; however, plasma contains inhibitors of the LAL assay, such as antithrombin III (AT III; Nakamura et al., 1986a,b). Elimination of such inhibitory effects is the first step toward the detection and quantitation of free LPS in plasma.

* Corresponding author. Tel.: +81 27 381 5131; fax: +81 27 381 5132.
E-mail address: pep-takagi@peptide-door.com (T. Takagi).

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We have previously reported the generation of LPS-binding peptides using phage-display and chemical modification (Matsumoto et al., 2010; Suzuki et al., 2010). One such peptide, a dodecapeptide termed Li5-025 (K′YSSSISSIRAC′; K′ and C′ denote D-lysine and D-cysteine, respectively) showed high affinity for LPS and resistance to protease digestion (Suzuki et al., 2010). In the present study, we observed that Li5-025 bound silica beads, hereafter referred to as P-beads, could bind to LPS dissolved in several solvents and that the nonspecifically bound materials could be easily washed out. The peptide bound beads did not show any LPS-neutralizing activity. This means that, LPS still retains its toxic activity; thus, the Li5-025-bound beads could be directly used in the LAL assay subsequent to LPS-binding. Using the P-beads, free LPS at a concentration of 1 pg/mL in plasma was quantitatively detected by the LAL assay.

2. Materials and methods

2.1. Materials

LPS [*Escherichia coli* O113:H10, 20000 Endotoxin Units (EU), Japanese Pharmacopoeia Reference Standard] and sterile barrier tips (Neptune Science) were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). LAL assay reagent (Endospecy) and diazo-coupling reagents (Toxicolor) were obtained from Seikagaku Biobusiness Co. Ltd. (Tokyo, Japan). Alkyl-chloride silica beads (Si-acid chloride) and human immunoglobulin G (IgG) were purchased from Sigma-Aldrich Chemical Co. (Tokyo, Japan). Iodoethanol was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Peptide Li5-025 was synthesized by Sigma-Aldrich Chemical Co. (Suzuki et al., 2010). Bovine serum albumin (BSA), salmon sperm DNA, and Coomassie Brilliant Blue R-250 were purchased from Wako Pure Chemical Ind. Ltd. (Tokyo, Japan). Human AT III (Neuart) was a kind gift from Dr. Horiuchi (National Institute of Infectious Diseases, Tokyo, Japan). Pooled samples of human citrate plasma were purchased from Cosmobio Co. Ltd. (Tokyo, Japan).

2.2. Generation of P-beads

Alkyl-chloride silica beads were treated with iodoethanol in 10% pyridine to replace the acidic chloride functional group with ethyl iodide. Li5-025 was added to the iodate silica to allow coupling (Matsumoto et al., 2010). Uncoupled ethyl iodide group was blocked by the addition of 2-mercaptoethanol. Peptide (Li5-025)-bound beads, P-beads, were washed with 0.2 M NaOH in 95% ethanol to remove any contaminating endotoxins (Niwa et al., 1969). Endotoxin-free P-beads were suspended in sterile water and maintained at 0 °C until further use. In the control experiment, the ethyl iodide groups of beads were blocked using 2-mercaptoethanol.

2.3. LPS binding and LPS activity

A total 15 μL of P-beads was pipetted into a sterile barrier tip and washed with sterile water. LPS-containing solution was added and first washed with washing solution and then with sterile water. The residual water was dispensed by pipetting. The barrier of the tip was removed by cutting and 50 μL of sterile water was added on top of the beads. The P-beads were dispensed into the well of a microtiter plate by pipetting and the LAL assay was performed by adding an LAL reagent to the well. The reaction was assayed at 37 °C for 30 min with gentle agitation. The absorbance of the supernatant was measured at 405 nm. In some experiments, diazo-coupling reagents were added for magenta color development after the LAL assay, which was measured by monitoring the absorbance at 537 nm. Standard curves were generated using different amounts of LPS standard.

2.4. Protein and DNA recovery

LPS (10 EU/mL) in protein or DNA solution (1 mg/mL) was applied to the barrier tip containing P-beads as described above. The tip was washed and the washing solution was collected. The concentration of protein or DNA in the washing solution was determined by measuring absorbance at 280 nm and 260 nm, respectively.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method by Laemmli (1970) and visualized by staining the gel with 0.25% Coomassie Brilliant Blue R250 in 50% methanol/10% acetic acid followed by multiple rounds of destaining in 5% methanol/10% acetic acid.

2.6. In-gel digestion

Purified proteins were detected using SDS-PAGE using Mini-PROTEAN TGX gels (Bio-Rad, Hemel Hempstead, UK). After electrophoresis, the gel was stained with Quick CBB Plus (Wako Pure Chemicals, Japan). Visible bands were excised and repeatedly destained in 100 μL of 50% acetonitrile/25% NH_4HCO_3 for 10 min with vortexing. After destaining, 100 μL of 100% acetonitrile was added and vortexed to remove the residual water. To reduce cysteine residues, the gel pieces were incubated with 10 mM dithiothreitol (DTT) prepared in 25 mM NH_4HCO_3 for 60 min at 56 °C. The gel-slices were then incubated for 45 min in the dark with 100 μL of 55 mM iodoacetamide that was prepared in 25 mM NH_4HCO_3. The iodoacetamide solution was subsequently removed, and the gel slices were washed with 200 μL of 25 mM NH_4HCO_3 for 10 min. This washing step was repeated twice, following which the solution was changed to 50% acetonitrile/25% NH_4HCO_3. The gel slices were vacuum-dried for 15 min and rehydrated with 100 μL of sequencing-grade modified trypsin (10 μg/mL in 50 mM NH_4HCO_3; Promega, USA). After incubation for 30 min on ice, the excess solution was removed and the gel was incubated overnight at 37 °C for digestion. Following digestion, the tryptic peptides were extracted using 100 μL of 50% acetonitrile acidified with 5% trifluoroacetic acid (TFA) at 25 °C for 30 min. The supernatant was collected and dried by vacuum centrifugation. The tryptic peptide solution was desalted using C18 ZipTip (Millipore, Bedford, USA) according to the manufacturer's instructions, and the trypsin-digested peptides were subjected to mass spectrometry analysis.

2.7. MS

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed using a Voyager DE-STR instrument (AB SCIEX, Framingham, USA). For sample preparation, tryptic peptides were dissolved in 10 μL of 70% acetonitrile/0.1% TFA and 1 μL of peptides was mixed with10 mg/mL α-cyano 4-hydroxycinnamic acid (α-CHCA; Sigma-Aldrich, St. Louis, USA). A mixture of the peptides and matrix solution was spotted on a sample plate, allowed to dry at room temperature, and subjected to MALDI-TOF MS analysis. The calibration mixture of the peptide mass standard kit (AB SCIEX, Framingham, USA) dissolved in the matrix solution was deposited near the samples for external calibration. Mass spectra were acquired by a reflector mode with 180 ns extraction delay time, positive, 76% grid voltage, and 20 kV accelerating voltage. Laser shots at 300 per spectrum were used to acquire the spectra with mass range from 700 to 4000 Da. The acquired mass spectra were calibrated, and subjected to protein identification using peptide mass fingerprinting (PMF) performed by the Mascot search engine (http://www.matrixscience.com) against the SwissProt protein database.