Selective Assay for LPS with Polylsine-Im mobilized Cellulose Beads and Limulus Amebocyte Lysate

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We developed an endotoxin (lipopolysaccharide; LPS) assay using poly(ε-lysine)-immobilized cellulose beads and Limulus amebocyte lysate (LAL). This method is composed of two steps. First, endotoxins are selectively adsorbed on the beads in protein solutions, and then the adsorbed endotoxins are separated from the compound such as protein by centrifugation. Second, the endotoxins adsorbed on the beads are directly reacted with the LAL reagent by turbidimetric time assay using Toxinometer ET-2000 (Wako). This assay method could be used widely as a means of assaying LPS in solutions containing LAL-inhibiting or enhancing substances, such as protein.

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I. INTRODUCTION

Endotoxin (lipopolysaccharide; LPS) [1] is a constituent of the cell walls of Gram-negative bacteria. It is a potential contaminant of protein solutions originating from biologic products. Products for intravenous administration must be tested for LPS contamination because of its potent biologic activities, which can cause pyrogenic and shock reactions. There are two methods for determining LPS. One is the pyrogen test using rabbits, and other is LAL test using Limulus amebocyte lysate (LAL).

Gel-clot [2], turbidimetric [3], chromogenic [4], and fluorometric methods are used in the LAL test. Gel-clot (1981), the turbidimetric (1985), and chromogenic techniques (1985) were previously approved by the USA Pharmacopoeia. The advantages of the LAL test over the rabbit pyrogen test are higher sensitivity, lower cost, and higher reproducibility. The LAL test is inhibited or enhanced by antibiotics, hormones, heavy metals, amino acids, alkaloids, plasma proteins, enzymes, and electrolytes in sample solution [5]. Dilution, heating and dialysis have been used to overcome these problems, but they have been unsatisfactory. In the dilution method, LPS sensitivity decreases because the LPS concentration is also diluted. Minobe et al. attempted to develop a new specific assay method for LPS using histidine-immobilized sepharose as an adsorbent [6, 7]. Adsorbed LPS by the adsorbent is separated from the compound (e.g., amino acids, enzymes) by centrifugation, and the LPS adsorbed on the adsorbent can react directly with the LAL. The great disadvantage of this type of adsorbent is the low adsorption activity of LPS in physiologic solution (neutral pH and ionic strengths (µ) = 0.15-0.2). We found that poly(ε-lysine)-immobilized cellulose beads (PεL-Celluline) can selectively adsorb LPS in bioproduts even under the physiologic conditions [8].

In this study, we describe the assay of LPS by a new adsorption method using the PεL-Celluline beads. We also compare this adsorption method with a standard solution method for LPS assay.

II. EXPERIMENTAL

When PεL-Celluline with amino-group content of 0.4-1.0 meq/g and matrix pore size of 2×10⁶ to 3×10⁶ as molecular mass exclusion (Mlim) of polysaccharides was used as adsorbent by a batchwise method, it selectively adsorbed LPS from a LPS solution containing protein at μ = 0.2-0.4 and neutral pH values [8]. PεL-Celluline (amino-group content: 0.8 meq/g; Mlim: 3×10⁶, Chisso Co. Ltd., Tokyo, Japan) was used as specific LPS adsorbent in this assay to separate LPS from the compound under physiologic conditions.

Assay of LPS using the PεL-Celluline beads was carried out as follows: (1) Removal of contaminating LPS from starting sample. Enzyme, ethanol and sodium chloride were used as compound. Contaminating LPS (natural LPS) in the compound solution was removed using PεL-Celluline by a column method. Purified LPS (Escherichia coli UKT-B, Wako Pure Chemical, Osaka, Japan) was added to compound solution. (2) Adsorption of purified LPS. The adsorption method and a standard method for LPS assay are shown in Fig. 1. Into a sterile test tube, 0.1 wet-g (0.015 dry-g) of PεL-Celluline and 2 mL of the LPS solution (containing compound or compound-free) were mixed, and the suspension was stirred at 25°C for 60 min. After being stirred, the tube was centrifuged (3000 rpm, 5 min) and the filtrate was discarded. To separate it from the compound, the PεL-Celluline (on which LPS was adsorbed) was washed with LPS-free water by centrifugation, and then suspended into 2 mL of water. (3) LAL test. One hundred microliters of LAL (Limulus II single test Wako) solution and 100 mL of the suspension of LPS adsorbed on PεL-Celluline were mixed in a LAL test tube. The solution was incubated at 37°C for 10-60 min. This is a test utilizing a coagulation (gelation) process in which LAL reacts with LPS to form a gel during incubation at 37°C. LPS concentration was determined by turbidimetric time assay [9] using Toxinometer ET-2000 (Wako).

The principle of the LAL test for the determination of LPS is based on LPS-induced coagulation reaction. The
turbidimetric time assay determines LPS concentration on the basis of turbidity (at 430 nm), which is induced by coagulation. LAL provides the transmitted light intensity curve by the reaction with LPS (Fig. 2). The toxinometer reads the coagulation (gelation) time \( T_g \) taken to reach a constant value (a value when \( R_t \) is 95%) of the ratio of decreasing transmitted light intensity accompanied by the proceeding gelation reaction to the initial transmitted light intensity [9].

### III. RESULTS AND DISCUSSIONS

The relationship between LPS concentration in water and LAL gelation time was investigated. The purified LPS (\( E. coli \) UKT-B, 1 endotoxin unit (EU) = 250 pg) was used as sample at a concentration of 0.056-560 EU/mL. The calibration curve of the adsorption method using P\( \varepsilon \)L-Cellufine was compared with that of the standard solution method without adsorbent (Fig. 3). Each showed a similar curve at a wide range of concentration (0.056-560 EU/mL). This adsorption method showed good recovery of LPS, which was added in LPS-free water.

LPS in various compounds was assayed by the adsorption method. Table I shows a comparison of the adsorption method with the standard method. In the latter, LPS concentration in the sample solution was directly investigated without dilution. Gelation times for LPS were determined as the average of five measurements. As a result of the LAL test by the standard method, apparent recovery of LPS from these compounds of 0.2, 0.4 M sodium chloride, methionine and phenylalanine was 40-60%. This suggests that these compounds inhibit the LAL procedure. For ethanol, a gelation reaction of the sample was not observed in the standard method. By contrast, the adsorption method showed good LPS recovery (88-120%) in all cases, without being inhibited by the compounds.

When LPS contamination was high, the dilution method was effective for decreasing LAL-inhibiting or LAL-enhancing substances in the sample solution.
TABLE I: Recovery of LPS from several compounds by the adsorption method and the standard solution method. To LPS-free solutions containing various compounds, 0.2–1.0 EU/mL of E. coli UKT-B was added. LPS concentration in the sample solutions was assayed by the adsorption method or standard solution method. Apparent recovery of LPS was estimated from the standard curve (LPS in water) in Fig. 3.

| Sample | Compound | Concentration of LPS added EU/mL | Adsorption method % | Standard method % |
|--------|----------|---------------------------------|---------------------|-------------------|
| Name   | Condition |                                  |                     |                   |
| NaCl   | 0.05 M in H2O (µ=0.05) | 0.5 | 100 | 98 |
| NaCl   | 0.2 M in H2O (µ=0.2) | 0.5 | 100 | 55 |
| NaCl   | 0.4 M in H2O (µ=0.4) | 0.5 | 105 | 40 |
| Methionine | 5 wt% in H2O | 0.5 | 105 | 60 |
| Phenylalanine | 5 wt% in 0.02 M-PBS (pH 7, µ=0.05) | 1.0 | 120 | 44 |
| Ethanol | 20 vol% in H2O (µ=0.4) | 1.0 | 90 | No gelation |
| Vitamin K | 1 mg/mL of 95 vol% ethanol aq. | 0.2 | 88 | No gelation |

Ionic strength (µ) of the phosphate buffered saline (PBS) was adjusted using NaCl.

sensitivity of the LAL used is 0.015 to 0.03 EU/mL. When LPS contamination is at very low (≤0.2 EU/mL), in the dilution method, the sensitivity decreased because of the dilution. In the adsorption method, LPS concentration could be accurately determined even at a low concentration of LPS (0.2–0.5 EU/mL), without sample dilution.

Minobe et al. reported similar data by a specific LPS assay using histidine-immobilized sepharose as adsorbent [6, 7], but the recovery was low at ionic strength (µ) ≥0.1. This was due to low adsorption of LPS on the adsorbent because of high ionic strength of the solution containing LPS [10]. Minobe also reported [6] that LPS adsorbed on polymyxin-immobilized sepharose [11] (commercially available LPS adsorbent) could not activate the LAL procedure. When P2L-Cellulose was used as an adsorbent in this adsorption-LAL assay, the recovery of LPS was high at a wide range of µ (0.05–0.4) (Table I). We reported [8] that P2L-Cellulose could selectively adsorb LPS in protein solution without adsorption of protein (recovery: 95–99%) at physiological pH and ionic strength. This high LPS selectivity of P2L-Cellulose may be due to the simultaneous effects of the cationic properties of P2L and its suitable hydrophobic properties.

We used LPS originating from E. coli UKT-B in this assay. Many kinds of LPS could be determined by this assay because P2L-Cellulose adsorbs LPS from E. coli O111:B4 and other natural LPSs (from various Gram-negative bacteria) in protein solution [8].

In summary, these data suggest that the adsorption method using P2L-Cellulose can be used widely to assay LPS in solutions containing LAL-inhibiting or LAL-enhancing compounds. This method showed good recovery and accuracy at a wide range of concentration (0.056–560 EU/mL). The operation time was long because of introduction of adsorption technique. Further studies on the adsorption method using P2L-Cellulose to find a simpler and more rapid method than this operation are in progress.

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