Title
Limulus amebocyte Lysate assay for endotoxins by an adsorption Method with Polycation-immobilized Ce...

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Introduction

The pharmaceutical products for parenteral administration must be tested for contamination of bacterial endotoxins (LPS) because of their potent biologic activities, which can cause pyrogenic and shock reactions. LPS, a constituent of the cell walls of Gram-negative bacteria, is a potential contaminant of protein solutions originating from biologic products. There are two methods for determining LPS. One is the rabbit pyrogen test; the other is the limulus test using Limulus amebocyte lysate (LAL).

The gel-clot, turbidimetric, and chromogenic testing methods, methods previously approved by the USA Pharmacopoeia in 1981, 1985, and 1985, respectively, are typically employed in the LAL test. 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 the sample solution. Dilution, heating and dialysis have been used to overcome these problems, but the results have been unsatisfactory. Minobe et al. attempted to develop a new specific assay method for LPSs using histidine-immobilized Sepharose as an adsorbent. LPSs adsorbed by the adsorbent were separated from the compounds (e.g., amino acids, enzymes) in solution by centrifugation, and the adsorbed LPSs can react directly with the LAL. The great disadvantage of this type of adsorbent is the low adsorption activity of the LPSs in physiologic solution (neutral pH and ionic strength (μ) = 0.1 – 0.2).

In this study, we developed a novel assay of LPSs with a new adsorption method using a turbidimetric time assay system. We previously found that poly(ε-lysine)-immobilized cellulose beads (PL-Cellufine) can selectively adsorb LPSs in bioproducts over a wide range of ionic strengths (μ) from 0.05 – 0.84 at neutral pH. On the other hand, a simple turbidimetric time assay system has been developed for the quantitative assaying of LPS; an automated apparatus is available commercially. In this paper, we describe the LPS-adsorbing activities of PL-Cellufine. Then, using the turbidimetric time assay, we describe the optimum conditions for the LPS assay using the PL-Cellufine as adsorbent. We also compare the apparent quantity of LPS provided by the adsorption method with the apparent quantity of LPS provided by a normal method.

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Experimental

Reagents and materials

Purified LPS (Escherichia coli UKT-B and O111:B4) and Limulus ES-II test Wako (Limulus amebocyte lysate) were purchased from Wako Pure Chemicals (Osaka, Japan). Poly(ε-lysine)-immobilized cellulose beads (PL-Cellufine) was obtained from Chisso Co. Ltd. (Tokyo, Japan). PL (Mw, 4500; pK, 7.6; Chisso) was produced by Streptomyces albulus. DEAE-Sepharose CL-6B was purchased from GE Healthcare Bio-Sciences Corp. (USA). Ovalbumin (from egg), bovine serum albumin (BSA), γ-globulin (from human serum), lysozyme (from horse heart), and vitamin K, were purchased from Wako. Myoglobin (from horse muscle), methionine, phenylalanine, and cysteine were purchased from Nacala Tesque (Kyoto, Japan). Distilled water (Otsuka Pharmaceuticals Co., Tokyo, Japan) was used as LPS-free water. All other chemicals were of analytical reagent grade.
Selective adsorption of LPS on PL-Cellufine

LPSs and proteins were used as samples. The samples were dissolved in one of the following buffers: 0.02 M sodium acetate (pH 4, 5), 0.02 M phosphate (pH 6, 7, 8), and 0.02 M Tris (pH 9), and the pH and ionic strength were adjusted. The ionic strength of the buffer was adjusted by changing the sodium chloride concentration. PL-Cellufine adsorbent (0.1 wet-g) was suspended in 2 mL of the sample solution. The 0.1 wet-g amount of adsorbent is equivalent to about 0.03 of dry-g adsorbent. The suspension was shaken for 1 h at 25°C and then filtered through a 0.8-μm cellulose acetate filter (Advantec Toyo Kaisha, Ltd., Tokyo, Japan) to separate the adsorbent from the suspension. The residual concentration of LPSs and the recovered protein in the filtrate were investigated.

Preparation of LPS-free sample solution

Various sample solutions such as protein, enzyme, buffer and sodium chloride, were naturally contaminated with LPSs. The contaminating LPSs (natural LPSs) in the sample solution were removed by a batchwise method using the PL-Cellufine adsorbent as follows. In 10 mL of a sample solution, 0.5 wet-g of wet PL-Cellufine adsorbent was suspended. The suspension was shaken for 1 h at 25°C and then filtered through the cellulose acetate filter (0.8 μm) to separate the adsorbent from the suspension. The filtrate was used as an LPS-free sample solution.

Assay of LPS by adsorption method

Adsorption of purified LPS onto PL-Cellufine. The adsorption method and a standard method (not using adsorbent) for the LPS assays are shown in Fig. 1. The adsorption method was investigated as follows. Purified LPS (E. coli UKT-B) was added to the LPS-free sample solution. Into a sterile test tube, 0.05 wet-g (0.015 dry-g) of PL-Cellufine and 1 mL of the LPS solution (containing compound or compound-free) were mixed, and the suspension was shaken at 25°C for 10 - 120 min. After being shaken, the tube was centrifuged (3000 rpm, 5 min) and the supernatant was discarded. The PL-Cellufine adsorbent, on which the LPSs were adsorbed, was then washed with LPS-free water by centrifugation, and finally suspended into 1 mL of LPS-free water. The suspension was then assayed by the LAL test.

LAL test

A 100-μL of LAL (Limulus II single test, Wako) solution and 100 μL of a suspension of PL-Cellufine, on which LPS was adsorbed, were mixed in a test tube. The principle of the LAL test for the determination of LPS is based on the LPS-induced coagulation reaction. The LPS concentration in the tube was determined by a turbidimetric time assay using an ET-2000 toxinometer (Wako). The toxinometer measures the turbidity change at 430 nm during coagulation (gelation) of the sample in the tube and records the gelation time (Tg) of the sample automatically. As shown in Fig. 2, LAL provides a transmitted light intensity curve due to its reaction with LPS. The gelation time is defined as the reaction time required to obtain a 5% decrease of R(t) (the ratio of amount of transmitted light to that of incident light at any given time). The toxinometer, which consists of an analysis module and a control module, monitors the ratio R(t) of sequential to initial transmittance of up to 16 samples simultaneously. The sample suspension in the tube was incubated statically at 37°C for 60 min.

Assay of LPS by standard method

A 100-μL portion of the LAL solution and 100 μL of a LPS or LPS/compound solution were mixed in a test tube. The determination of LPS in the tube was investigated by a method similar to the LAL test in the adsorption method.

Assay of protein

The concentration of protein in the sample solution was measured at 280 nm using a V-650 spectrophotometer (JASCO).
Selective adsorption of LPS onto PL-Cellufine

PL-Cellufine (amino-group content, 0.8 meq g⁻¹; $M_{lim}$, $3 \times 10^5$) was used as a specific LPS adsorbent in this assay under physiologic conditions. The adsorption capacity of PL-Cellufine is 0.48 mg of LPS (E. coli O111:B4) per 1 mL of wet-type PL-Cellufine at pH 7.0 and an ionic strength of $\mu = 0.05$. The dissociation constant of PL-Cellufine for LPS is $1.1 \times 10^{-11}$ M when the molecular weight of the LPS is taken as $1 \times 10^6.8,9$

For an assay of LPS using PL-Cellufine, it is necessary that the LPSs are adsorbed onto PL-Cellufine quantitatively. The most suitable processing time for LPS adsorption was investigated by a batchwise method. As shown in Fig. 3, the residual concentration of LPS after treatment with PL-Cellufine decreased with increasing shaking time. As for the initial concentration of 1 EU mL⁻¹, almost all the LPSs were adsorbed after 20, 40, 60, and 80 min of shaking; the residual concentrations of LPS were below 0.005 EU mL⁻¹. As for the initial concentration of 10 EU mL⁻¹, almost all the LPSs were adsorbed after 60 and 80 min of shaking.

For assays of LPSs in various solutions of bioproduct, it is necessary to check the LPS-adsorbing activity of PL-Cellufine in the bioproduct solution under physiological condition (neutral pH, ionic strength ($\mu$) 0.15 – 0.2). The effects of the buffer’s ionic strength and pH on the LPS-adsorbing activity of PL-Cellufine were compared with those of DEAE-Sepharose (commercial ion-exchanger); the results are shown in Figs. 4a and 4b. The LPS adsorption was determined by a batchwise method with shaking for 1 h. As shown in Fig. 4a, the higher the ionic strength of the buffer, the lower the LPS-adsorbing activity of the adsorbent. PL-Cellufine showed a greater LPS-adsorbing activity (>99%) over a wide range of ionic strength ($\mu$) from 0.05 – 0.8. By contrast, the adsorbing activity of DEAE-Sepharose decreased markedly when the ionic strength was increased to 0.2 or higher. Figure 4b shows the effect of pH on the adsorption of LPS by the two adsorbents. Over a wide pH range of 6.0 – 9.0 and at $\mu = 0.05$, PL-Cellufine showed a high activity (>97%), although it decreased from 99 to 80% as the pH decreased from 6.0 to 4.0. DEAE-Sepharose showed high adsorbing activity only at pH 7.0 and $\mu = 0.05$.

The results indicate that PL-Cellufine can adsorb enough LPS (100 ng mL⁻¹ (480 EU mL⁻¹)) under physiological condition, but that the adsorbing activity of DEAE-Sepharose is insufficient under the same condition.

It is also desirable that LPS is selectively adsorbed onto PL-Cellufine without adsorption of other compounds, because these compounds may have an influence on the LAL test. For selective adsorption of LPS from a protein solution, it is also necessary to adjust buffer conditions such as ionic strength and pH. The effect of ionic strength on the selective adsorption of LPS by PL-Cellufine was compared with that of DEAE-Sepharose. The results are shown in Figs. 5a and 5b. A BSA solution (1 mg mL⁻¹) to which purified LPS (100 EU mL⁻¹) was added was used, at pH 7.0 and various ionic strengths, as the sample solution. After batchwise treatment, the adsorbent was removed by filtration. The filtrate was diluted 10 times with LPS-free water, and then the residual concentration of LPS was assayed by a common LAL test. As shown in Figs. 5a and 5b, the higher the ionic strength of the buffer, the lower the BSA-adsorbing activity of each adsorbent. PL-Cellufine selectively adsorbed LPS from the BSA solution at $\mu = 0.4$ to 0.8, without adsorption of BSA; the adsorption of LPS was >99% and that of BSA was <2% (Fig. 5a). By contrast, DEAE-Sepharose showed strong adsorbing activities for both proteins.

Results and Discussion

Selective adsorption of LPS onto PL-Cellufine

![Fig. 3 Time course of adsorption of LPS by PL-Cellufine. Adsorption of LPS (E. coli UKT-B, 1 endotoxin unit (EU) corresponds to 250 pg LPS) was determined by the batchwise method with 50 wet-mg of PL-Cellufine and 1 mL of a LPS solution (○, 10 EU mL⁻¹; ●, 1 EU mL⁻¹).](image)

![Fig. 4 Effect of buffer’s ionic strength (a) and its pH (b) on the adsorption of LPS by PL-Cellufine (○) and DEAE-Sepharose (●). The adsorption of LPS was determined by a batch method with 0.1 mL of wet adsorbent and 2 mL of a LPS solution (LPS from E. coli O111:B4, 100 EU mL⁻¹) for 1 h.](image)
LPS and BSA at a low ionic strength ($\mu = 0.05$), and adsorbing activities for both LPS and BSA decreased with an increase in ionic strength from 0.05 to 0.8 (Fig. 5b). This adsorbent could not selectively adsorb LPS from a BSA solution containing LPS at any ionic strength.

Table 1 shows the effects of a buffer's ionic strength on the selective adsorption of LPS from various proteins by PL-Cellufine. Various protein solutions, which were naturally contaminated with LPS at concentrations of 8 - 160 EU mL$^{-1}$, were used as sample solutions. PL-Cellufine typically shows a high LPS-adsorbing activity; each residual concentration of LPS after treatment was <0.1 EU mL$^{-1}$ (Table 1). In the recovery of proteins under the condition of ionic strength ($\mu$) 0.2 and pH 7.0, ovalbumin and BSA both adsorbed (recovery 55 and 74%, respectively) to PL-Cellufine. Interestingly, other proteins showed higher recovery (>98%). When the ionic strength was increased to $\mu = 0.4$, all of the proteins tested showed higher recovery (>98%).

We previously reported$^8,9$ that the adsorption activity of PL-Cellufine for bio-products is due to the simultaneous effects of their cationic and hydrophobic or other properties. PL-Cellufine and DEAE-Sepharose, being cationic in nature, can adsorb LPS and acidic proteins mainly by ionic interaction, because the charge of LPS ($pK_\text{a} = 1.3$)$^{13}$ and acidic proteins (ovalbumin ($pI = 4.6$), BSA ($pI = 4.9$)) are anionic at neutral pH. As shown in Figs. 5a, 5b, and Table 1, the adsorption of acidic protein was dependent on the ionic strength. This finding suggests that the acidic protein ($pI = 4.6 - 4.9$) was also adsorbed mainly by ionic interaction. On the other hand, LPS adsorption was independent of ionic strength at $\mu = 0.05$ - 0.8 (Fig. 5a). These findings suggest the participation of hydrophobic binding between PL-Cellufine and LPS. Hou et al.$^{14}$ also reported that a hydrophobic bond was formed between LPS and the polymeric affinity matrix. Furthermore, as shown in Table 1, PL-Cellufine binds more strongly with LPS than with proteins. This is because the $pK_\text{a}$ of the phosphate residues of LPS is lower than the $pI$ of the proteins ($pI = 4.6 - 11.0$) tested, and probably because the LPS is adsorbed by the adsorbent through its multipoint attachment onto the polycation chain of the adsorbent surface. By contrast, the adsorption activity of DEAE-Sepharose for
both LPS and BSA was dependent strongly on the ionic strength (Fig. 5b). The finding suggests that DEAE-Sepharose, which has monomeric ligands, adsorbs both mainly by ionic interaction. It is also considered that an anion-exchanger, such as DEAE-Sepharose, is less able to selectively adsorb LPS from acidic protein solution under physiological condition. As a result, it was found that PL-Cellufine can selectively adsorb sufficient natural LPS (8–160 EU mL⁻¹) under physiological condition (neutral pH and μ = 0.2–0.4). For the selective adsorption of LPS from a bio-product, we also found that it was necessary not only to select the adsorbent, but also to adjust the buffer’s conditions (pH, ionic strength).

Assay of LPS using PL-Cellufine

The relationship between LPS concentration and LAL gelation time was investigated by an adsorption method using PL-Cellufine. As shown in Fig. 6, each calibration (n = 5) curve obtained between 0.0056 and 5.6 EU mL⁻¹ was well correlated.

LPS in various compounds were also assayed by the adsorption method. Table 2 shows a comparison of the adsorption method with the common standard method. In the latter, the LPS concentration in the sample solution was directly investigated without dilution. Gelation times for LPS were determined as the average of five measurements. When the standard method was used, 0.2–0.4 M sodium chloride, serum proteins, methionine and phenylalanine showed false negative results. As a result of the LAL test by the standard method, the apparent recovery of LPS from these compounds was 40–95%. This suggests that these compounds inhibit the LAL procedure. By contrast, the adsorption method showed good LPS recovery (88–120%) in all cases, without being inhibited or enhanced by the compounds. Vitamin K does not dissolve in water, and so it is difficult to measure the amount of LPS in vitamin K by the standard solution method. For a 95 vol% aqueous ethanol solution of vitamin K, the gelation reaction was not observed in the LAL standard method because of the inhibition by ethanol. As for the adsorption method, the LPS recovery (88%) from the vitamin K sample was close to the LPS added.

When the LPS contamination was high, the dilution method was effective for decreasing the LAL-inhibiting or LAL-enhancing substances in the sample solution. However, in the dilution method, the LPS sensitivity decreases because the LPS concentration is also diluted. It is essential to assay the concentration of LPS in fluids used for injections to less than 0.1 EU mL⁻¹. Because even if present in only 0.1 EU quantities, the potent biological activities associated with LPS can cause pyrogenic reactions in mammals. When we try to measure the LPS contamination of a very low (≤0.1 EU mL⁻¹) concentration by a standard LAL assay using a 10 times-dilution method, it is very difficult to measure a fixed quantity precisely because of the LAL sensitivity of 0.0056 EU mL⁻¹ (Fig. 6). In the adsorption method, the LPS concentration in various compounds could be accurately determined even at a low concentration of LPS (0.1–0.5 EU mL⁻¹), without sample dilution.

Minobe et al. reported similar data for a specific LPS assay using histidine-immobilized sepharose as adsorbent, but the recovery was low at ionic strength (μ) ≥0.1. This was due to the low adsorption of LPS on the adsorbent because of the high ionic strength of the solution containing LPS. Minobe also reported that LPS adsorbed on polymyxin-immobilized sepharose (commercially available LPS adsorbent) could not activate the LAL procedure. As shown in Table 1, the PL-Cellufine could selectively adsorb LPS in protein solution without adsorption of the protein (recovery ≥98%) at a physiological condition (pH 7.0, μ = 0.4). When the PL-Cellufine was used in this adsorption-LAL assay, the recovery of LPS was high over a wide range of μ (0.05–0.4) near neutral pH (Table 2). This high LPS selectivity of PL-Cellufine may be due to the simultaneous effects of the cationic properties of PL and its suitable hydrophobic properties. As a standard purified LPS in this assay, we used only two kinds of LPS (E. coli UKT-B and O111:B4). Many kinds of LPS could be determined by this assay because PL-Cellufine satisfactorily adsorbs other natural LPSs in protein solution (Table 1). As further applications, this adsorption assay is currently being tested for natural LPS in serum samples, such as Anti-thrombin-III. The application data will be described elsewhere.

Conclusions

The present results indicate that the adsorption method using PL-Cellufine can be used widely to assay LPS in solutions containing LAL-inhibiting or LAL-enhancing compounds.
This method showed good recovery and accuracy over a wide range of LPS concentration (0.0056 – 5.6 EU mL⁻¹). This high recovery of LPS is due to the high LPS selectivity of PL-Cellufine, which has cationic properties originating from PL and suitable hydrophobic properties originating from the matrix. However, the operation time is long in comparison with the common method because of the introduction of the adsorption technique. Further studies on the adsorption method using PL-Cellufine to find a simpler and more rapid method than this operation are in progress.

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