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Improved method for preparation of lipopolysaccharide-binding protein from human serum by electrophoretic and chromatographic separation techniques

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Abstract

Recent work has established the importance of serum proteins which interact with endotoxin (lipopolysaccharide, LPS) from Gram-negative bacteria. Thus human monocytes are activated after binding LPS complexed with a serum protein. LPS-binding protein (LBP) is a protein present in both normal and acute phase sera which binds LPS with high affinity. We describe the purification of LBP from human acute phase serum. The purification procedures combine preparative isoelectric focusing (IEF) and either preparative polyacrylamide gel electrophoresis (PAGE) or alternatively an anion-exchange chromatographic step using a Mono Q HR 5/5 column. This allows the isolation of biologically active LBP. LBP was characterized by N-terminal sequence analysis and by measuring the biological activity using flow cytometry (fluorescence-activated cell sorter, FACS) and a luminol enhanced chemiluminescence (LECL) assay.

1. Introduction

Endotoxins have clinical relevance as inducers of septic shock. Monocytes in the peripheral blood seem to play a key role in shock development. Activated by binding endotoxin (lipopolysaccharide) via the surface glycoprotein CD14 [1] they release large amounts of inflammatory mediators including tumor necrosis factor (TNF), interleukin-1, interleukin-6 (IL-1, IL-6), platelet activating factor (PAF), and reactive oxygen species [2]. The binding of LPS to CD14 is mediated by LPS binding proteins which are present in acute phase sera up to 50 times the concentration found in normal serum. Two proteins are known which support LPS binding to CD14: lipopolysaccharide-binding protein (LBP) [3,4] and, more recently described, septin [5]. LBP is a 64 kDa glycoprotein which enhances the binding of LPS to the cellular receptor CD14. LBP binds with high affinity ($K_d = 10^{-9}$ M) to LPS through its lipid A group [6]. By binding, LBP seems to change the physical state of LPS. This enables LPS to bind to CD14 via the lipid A structure. Other LPS binding proteins also present in sera, *e.g.* soluble CD14 [7,8] and bactericidal/permeability-increasing protein (BPI) [9,10], seem to have endotoxin neutraliz-

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ing effects. Although LBP and BPI have counterregulatory functions they possess a high structural homology [4]. The relationship between these interfering LPS binding serum factors is unknown. Generally available methods for the quantitation of LBP, septin or BPI are lacking. We were interested in the purification of the human LPS-binding protein (hLBP) in order to develop an immunological test system for the quantitation of hLBP in biological fluids and to perform functional studies [11] aimed at developing a sepsis intervention strategy.

The isolation of LBP has been first reported from rabbit serum by Tobias *et al.* [12]. Recently, Wright *et al.* [5] described the purification of hLBP from human ascites fluid by a modification of the method of Tobias *et al.* Both purification procedures combine chromatographic methods using cation- and anion-exchange supports.

Acute phase human serum has been shown to contain levels of LBP in the range of 120 to 320 μg/ml which is high compared with the LBP levels found in normal human serum which lie in the range of 3.2 to 11.2 μg/ml [13]. Because of the limited availability of this LBP source we have developed an improved method for the preparation of hLBP from acute phase serum using electrophoretic and chromatographic separation techniques.

2. Experimental

2.1. Chemicals and materials

All fine chemicals were from Merck (Darmstadt, Germany) or from Sigma (Deisenhofen, Germany). LPS (*Escherichia coli* 0111:B4, phenol extracted), fluoresceine isothiocyanate (FITC)-labeled LPS (*Escherichia coli* 055:B5, TCA extracted), luminol and poly-L-lysine were obtained from Sigma. RPMI 1640 medium was from Biochrom (Berlin, Germany). Human serum came from healthy volunteers or was obtained from Sigma.

Peripheral blood mononuclear cells (MNC) were prepared from heparinized venous blood of healthy volunteers by density gradient centrifugation. Blood was added to an equal volume of 50 m*M* phosphate buffer, 150 m*M* NaCl, pH 7.4 (PBS), and centrifuged on a Ficoll (Serva, Heidelberg, Germany)/Visotrast (Fahlberg-List, Magdeburg, Germany) gradient (density 1.077) at 280 *g* for 30 min. MNCs were harvested and washed two times with PBS.

2.2. Purification of the LPS-binding protein

Sample preparation

A 20-ml volume of human acute phase serum (hAPS) from patients showing an acute phase reaction defined by C-reactive protein (CRP) levels >100 μg/ml was mixed with 20 ml of saturated ammonium sulphate solution followed by gentle agitation for 30 min at 4°C. After centrifugation (1000 *g*, 30 min, 4°C), the precipitate was redissolved in 10 ml of deionized water containing 2 *M* urea and 1% glycine.

Preparative isoelectric focusing (IEF)

IEF was performed using a Rotofor cell (Bio-Rad, Munich, Germany).

The concentrated sample obtained was desalted using an Econo-Pac 10 DG column (Bio-Rad) equilibrated with water containing 2 *M* urea and 1% glycine. Carrier ampholytes (Bio-Lyte, pH range 3–10, 40%, w/v; Bio-Rad; final concentration 2%) and 10% glycerol (v/v) were added to the desalted material. The volume of the solution was adjusted to 50 ml with deionized water. The sample was then loaded into the Rotofor cell for focusing. To achieve the running temperature (4°C), the cell was rotated for 10 min before applying the electric current. Focusing required 4 h at constant power (12 W). Twenty fractions were collected, their pH values measured and neutralized with PBS. Biologically active fractions were pooled. Urea, glycine and glycerol were added to achieve the same final concentrations as in the first run and carrier ampholytes (Bio-Lyte, pH 4–6, 40%, w/v; Bio-Rad) were added to a final concentration of 2%. After dilution to a volume of 50 ml with deionized water the sample was reloaded and refractionated for 4 h at 12 W constant power at 4°C. After fraction collection, pH measurements and

neutralization with PBS, fractions containing biological activity were pooled and dialyzed against PBS.

Preparative sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

For preparative SDS-PAGE, the discontinuous buffer system of Laemmli [14] was used. Analytical SDS-PAGE was carried out to determine the acrylamide concentration (%T = weight percentage of total monomer (g/100 ml)) that would best separate the LBP from its nearest contaminants in the Model 491 Prep Cell (Bio-Rad, Munich, Germany). A 10-cm high 7.5% acrylamide (acrylamide–bisacrylamide, 30:0.8, w/w) separating gel was polymerized in the 37-mm diameter tube of the preparative gel apparatus. After formation of the separating gel, a 2-cm high 4% acrylamide stacking gel was polymerized on top. The sample contained approximately 1.5 mg of total protein dissolved in 2 ml of sample buffer (0.25 *M* Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 0.01% bromophenol blue) per run. After sample loading the gel was run for 4 h at 40 mA constant current by which time the bromophenol blue marker dye was *ca.* 5 mm from the bottom of the separating gel. Running buffer (25 m*M* Tris, 191 m*M* glycine, pH 8.3, 0.1% SDS) was pumped through the elution chamber at a flow-rate of 1.0 ml/min. The elution chamber outlet was connected to a fraction collector and eighty 2.5-ml fractions were collected. Fraction number one was the first fraction containing visible amounts of the bromophenol blue marker dye. In order to locate the fractions containing LBP, every fifth fraction was analyzed by analytical SDS-PAGE. Once the elution position of LBP was determined, adjacent fractions were analyzed by SDS-PAGE.

Anion-exchange chromatography

An FPLC system (Pharmacia, Freiburg, Germany) consisting of a LCC-Controller/500 Plus (gradient programmer), two P-500 pumps, an UV-M II monitor, a REC 2 recorder, a MV-7 multi position motor valve and a RediFrac fraction collector was used.

The anion-exchange chromatography was performed with a Mono Q HR 5/5 column 50 × 5 mm I.D. (Pharmacia). All buffers were degassed and ultrafiltrated (0.22-μm filter, Sartorius, Göttingen, Germany). The column was equilibrated with 20 m*M* triethanolamine-HCl, pH 7.5 (buffer A). The product from preparative IEF (pH 4–6) was transferred into buffer A using an Econo-Pac 10 DG gel-permeation column (Bio-Rad). After buffer exchange, the samples were injected in a 2-ml loop and loaded onto the Mono Q column at a flow-rate of 1.0 ml/min. Then the column was washed with 20 ml of buffer A to remove unbound proteins. Elution was performed using two buffers: buffer A and buffer B, which consists of buffer A supplemented with NaCl to a final concentration of 1 *M*. The bound proteins were eluted at a flow-rate of 1.0 ml/min, with a 30-ml linear gradient from 0 to 20% buffer B, followed by a 20-ml linear gradient from 20 to 100% buffer B. Protein peaks were detected using a Pharmacia UV-M-II (OD 280), collected in 0.5-ml fractions, and assayed by FACS analysis as described above.

Micro-affinity adsorption technique

Polystyrene microplates (Maxisorp; NUNC, Roskilde, Denmark) were coated with a solution of poly-L-lysine (10 μg/ml, 200 μl/well) at 25°C for 16 h. After washing three times with PBS, 0.05% Tween 20, LPS dissolved in PBS (10 μg/ml) was applied (200 μl/well). The plate was incubated at 37°C for 1 h and then washed three times with PBS, 0.05% Tween 20. Aliquots (100 μl) of the biologically active fractions from the preparative isoelectric focusing (pH 4–6; diluted 1/5 in 20 m*M* phosphate buffer, pH 7.4) or anion-exchange chromatography fractions (diluted 1/2 in 20 m*M* phosphate buffer, pH 7.4) were applied to each well and incubated at 4°C for 24 h. After washing three times with PBS, 0.05% Tween 20, the contents of the wells were eluted with SDS-PAGE sample buffer containing 50 m*M* Tris, pH 6.8, 1% SDS, 10% glycerol, and 0.01% bromophenol blue (diluted 1/10 in deionized water, 100 μl/well). The eluates were

collected, concentrated and characterized by analytical SDS-PAGE.

2.3. Analytical methods

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE on 9% or 12% acrylamide gels was performed with non-reduced samples by the method of Laemmli [14] using the Bio-Rad Minigel device (gel thickness: 1 mm). Proteins in the gel were Coomassie Blue or silver stained (Silver Stain Kit AG-5, Sigma). The apparent molecular masses of the proteins were determined using molecular mass standards (Bio-Rad).

Protein determination

Protein concentrations were determined according to the method described by Bradford [15] using bovine serum albumin as standard.

Analytical size-exclusion chromatography (SEC)

SEC was performed on a Superose 6 HR 10/30 column 300 × 10 mm I.D. (Pharmacia) equilibrated with 0.1 *M* degassed and ultrafiltrated (0.22-μm filter, Sartorius, Göttingen, Germany) PBS, pH 7.4 using the FPLC-system described above. A volume of 50 μl of protein standard solutions, or of anion-exchange chromatography fraction 51, which is highly enriched in LBP, was applied and chromatography was performed at a flow-rate of 0.2 ml/min with PBS. Molecular mass standards (Sigma) for calibration of the gel filtration column included the following: cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa).

N-Terminal sequence analysis

The samples of partially purified LBP were separated by SDS-PAGE. After electrophoresis the proteins were electroblotted onto a siliconized glass fiber sheet (Glassybond, Biometra, Göttingen, Germany), detected by Coomassie Blue staining, excised and applied to a pulsed liquid-phase sequencer (477a, Applied Biosystems, Weiterstadt, Germany) according to the method of Eckerskorn *et al.* [16].

Flow cytometry (FACS) analysis

LBP activity was determined by its ability to promote binding of fluoresceine isothiocyanate labeled LPS (FITC-LPS) (*E. coli*, 055:B5, TCA extracted) to human mononuclear cells. Human mononuclear cells (MNC) were washed and resuspended in PBS containing 0.1% NaN_3, and adjusted to $4 \cdot 10^6$ cells/ml; $2 \cdot 10^5$ MNCs were incubated with FITC-LPS (2.5 μg/ml) in the presence or absence of 20% human blood serum or purified LBP for 30 min at 4°C. After washing twice cells were analyzed by flow cytometry using a FACScan (Becton-Dickinson, Heidelberg, Germany). Fluorescence intensity of monocytes gated due to light scattering properties were calculated as average of median channel numbers, a measurement that reflects the amount of FITC-LPS bound per cell.

One unit of biological activity is defined as the amount of LBP required to achieve half the maximal rise in fluorescence intensity.

Luminol enhanced chemiluminescence (LECL)

This assay was used to measure the LPS inducible release of reactive oxygen species from human monocytes mediated by LBP. LPS was dissolved in deionized water to a final concentration of 1 mg/ml. A 10-μl sample of this solution was added to 100 μl serum or LBP solutions, incubated for 30 min at 20°C, and diluted 1:10 with PBS. MNCs were adjusted to $5 \cdot 10^5$/ml in serum-free, phenol-red-free RPMI 1640, luminol was added to a concentration of 10^{-4} *M* and the mixture was incubated for 30 min at 37°C. A 20-μl aliquot of the LPS/serum or LPS/LBP solution was added to 200 μl of a MNCs suspension. LECL was detected using an LB 953 luminometer (Berthold, Munich, Germany).

3. Results and discussion

A FACS assay which measures the biological activity of LBP was established. It was then used

for the detection of this protein in the starting material and throughout various fractionation steps.

As a first enrichment step, the acute phase serum proteins were fractionated by ammonium sulphate precipitation. After this, the recovery of total protein and biological activity was 31% and 62%, respectively. Buffer exchange of the redissolved precipitate did not result in additional losses.

Fractionation by isoelectric focusing for further enrichment of LBP in a preparative manner was possible using the Rotofor system. After a 4-h run at 12 W constant power, 20 fractions were collected. The problem of considerable precipitation during the run was substantially

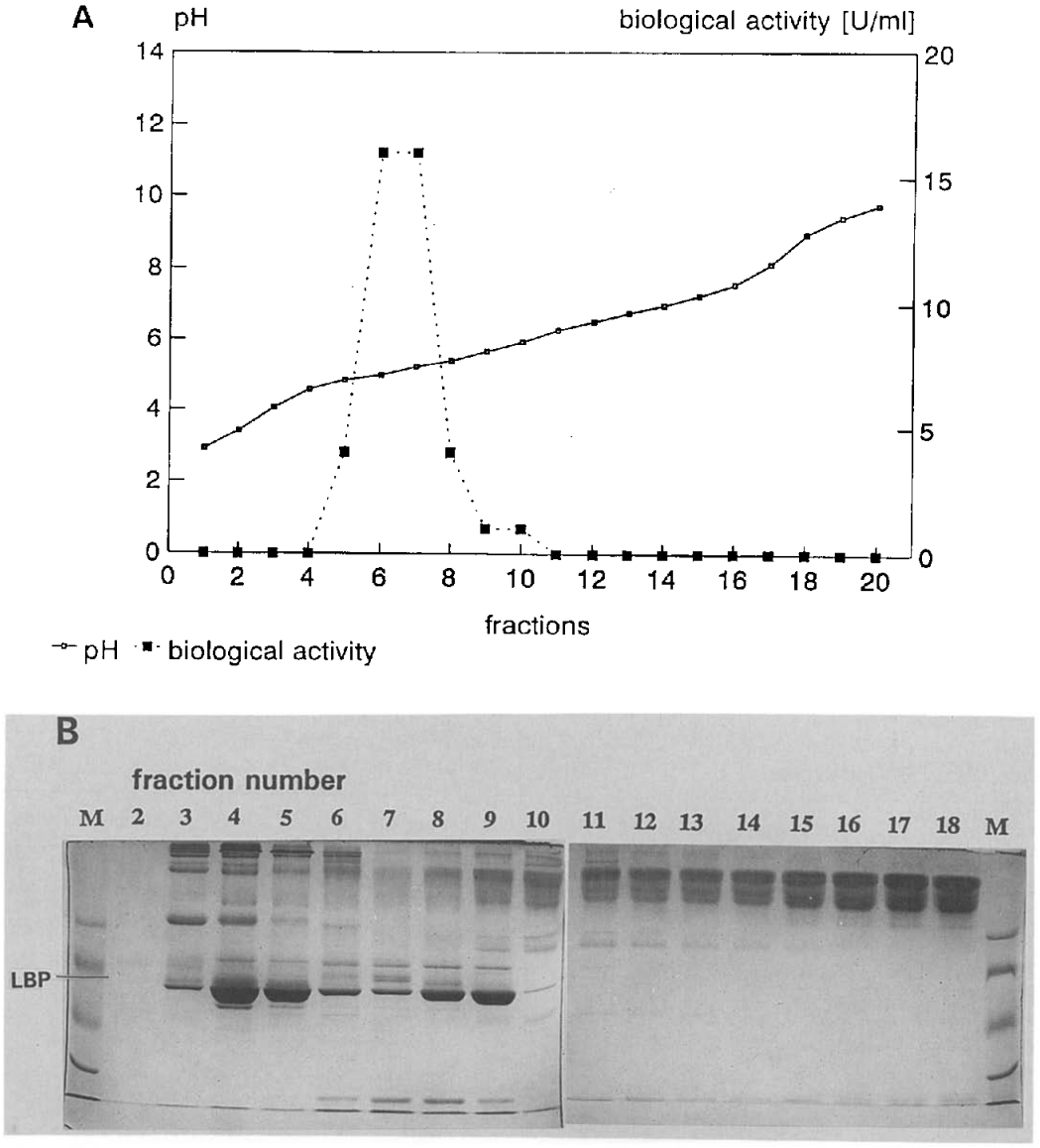


Fig. 1. Analysis of preparative isoelectric focusing (pH 3–10) run using the Rotofor system. (A) Plot of pH (□) and relative biological activity (■) (FACS analysis) for Rotofor fraction numbers 1–20. (B) SDS-PAGE analysis of Rotofor fractions 2–18. 9% Polyacrylamide gels were Coomassie Blue stained. M = molecular mass markers: 97.4, 66.2, 45 and 31 kDa.

alleviated by adding urea and glycerol to the sample solution. A single Rotofor run concentrated the LPS binding activity into 5–6 fractions, which contained different amounts of contaminating proteins. The pH gradient, biological activity profiles and PAGE analysis of selected fractions are shown in Fig. 1. The LPS binding activity focused within a pH range of 4.9 to 5.3 (fractions 5–10). The Rotofor cell yielded 1.6% of the protein and 42% of the total biological activity found in the starting material with a 27-fold purification of the LPS-binding protein (Table 1). Further purification was achieved by refractionation in a narrow pH gradient (pH 4–6). The protein of interest was localized in fractions 10–17, corresponding to a pH range of 5.0–5.5 with fractions 11–14 containing the majority of the biological activity. At this stage the yield of activity with respect to acute phase serum was 38% and the pool contained 0.4% of the original protein. Selected fractions were analyzed by SDS-PAGE (Fig. 2). Possibly two populations of LBP were separated by this purification step, fractions 8–13 and fractions 13–18, respectively. The presence of two populations of LBP was described previously for rabbit LBP [12]. The structural basis of this heterogeneity is unknown. However, Wright *et al.* [5] reported a single population for human LBP. The LBP containing fractions clearly required further purification.

For isolation of preparative quantities of LBP in a denaturated form, we utilized the Bio-Rad Model 491 Prep Cell for preparative SDS gel electrophoresis. With the help of analytical slab gel electrophoresis we defined conditions which were optimal for the preparative run. Fig. 3 shows the fractions from a preparative electrophoresis analyzed by analytical SDS-PAGE. The 64 kDa protein was collected in fractions 33–37. Of the 6.5 mg total protein from IEF (pH 4–6) fractions 11–16 fractionated on the Prep Cell, 200 μg of purified 64 kDa protein, or 0.012% of the total, was recovered. The recovered material was suitable for N-terminal sequencing. Microsequence analysis revealed the following N-terminal sequence: Ala-Asn-Pro-Gly-Leu-Val-Ala-Arg-Ile-Thr-Asp-Lys-Gly-Leu-Gln-, which is identical to that reported for LBP [4].

The material from the second IEF was fractionated on a Mono Q column, to yield highly enriched LBP. The biologically active protein was eluted at *ca.* 135–145 m*M* NaCl (Fig. 4). Analysis by SDS-PAGE (Fig. 5) revealed that the LBP was still contaminated with the human serum protein hemopexin. This was confirmed by N-terminal microsequence analysis and immunoblotting studies (data not shown). The final recovery of LBP measured as protein was approximately 150 μg or 0.009% at the end of the anion-exchange chromatography. The results of the purification of LBP from human acute phase

Table 1
Purification of the LPS-binding protein

Purification step	Protein (mg)	Biological activity of LBP		Purification factor	
		U	U/mg	Per step	Total
Starting material (hAPS)	1600	400	0.25	–	–
Salt precipitation	500	250	0.5	2	2
Preparative IEF (pH 3–10) (fractions 5–10)	25	168	6.7	13.4	26.8
Preparative IEF (pH 4–6) (fractions 11–16)	6.5	153	23.3	3.5	93.2
Anion-exchange chromatography (fractions 50 and 51)	0.24	35	145	6.2	580
Preparative SDS-PAGE	0.2	–	–	–	–

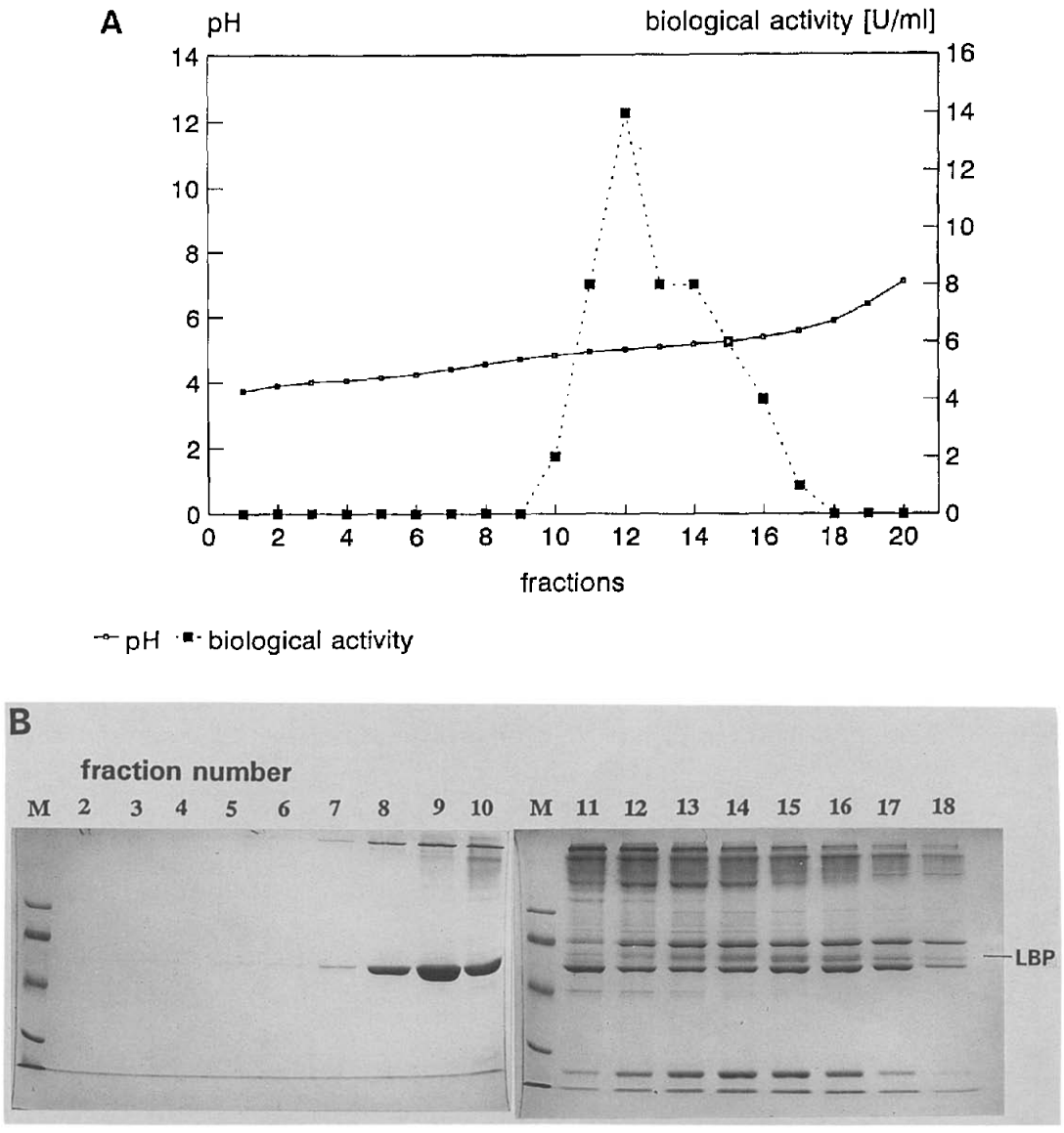


Fig. 2. Analysis of preparative isoelectric focusing (pH 4–6) run using the Rotofor system. (A) Plot of pH (□) and relative biological activity (■) (FACS analysis) for Rotofor fraction numbers 1–20. (B) SDS-PAGE analysis of Rotofor fractions 2–18. 9% Polyacrylamide gels were Coomassie Blue stained. M = molecular mass markers: 97.4, 66.2, 45 and 31 kDa.

serum are summarized in Table 1 and Fig. 6. The high-molecular-mass band visible in lane 4 of this gel is generated during the work-up of the protein recovered from the gel shown in Fig. 3. This was carried out in the absence of a reducing agent.

LBP was further purified to homogeneity on a micropreparative scale using a micro-affinity adsorption technique. LBP from biologically active fractions of isoelectric focusing or anion-exchange chromatography was captured by LPS coated on polystyrene microplates. LBP was then eluted with SDS-PAGE sample buffer, fractionated on analytical SDS-PAGE and visual-

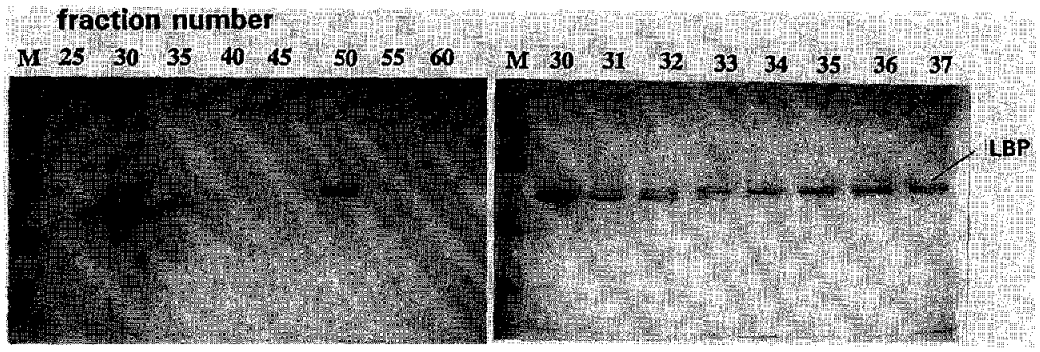


Fig. 3. Analysis of protein fractions eluted from the Prep Cell by SDS-PAGE. Aliquots from every fifth Prep Cell fraction were analyzed on Coomassie Blue stained SDS-PAGE mini gels. Every Prep Cell fraction near the peak of eluted LBP was then analyzed. M = molecular mass markers: 97.4, 66.2, 45 and 31 kDa.

ized by silver staining of the gel (not shown). Surprisingly, an additional LPS binding protein with an apparent molecular mass of 40 kDa was found in a fraction of isoelectric focusing (pH 4.5) used as negative control.

The molecular size of LBP was independent of whether the SDS-PAGE analysis was performed in the presence of a reducing agent (beta-mercaptoethanol) or not, and was determined as 64 kDa. A slightly different molecular mass of 62 kDa was found by analytical size-exclusion chromatography.

The biological activity was determined using a flow cytometric (FACS) assay and a luminol

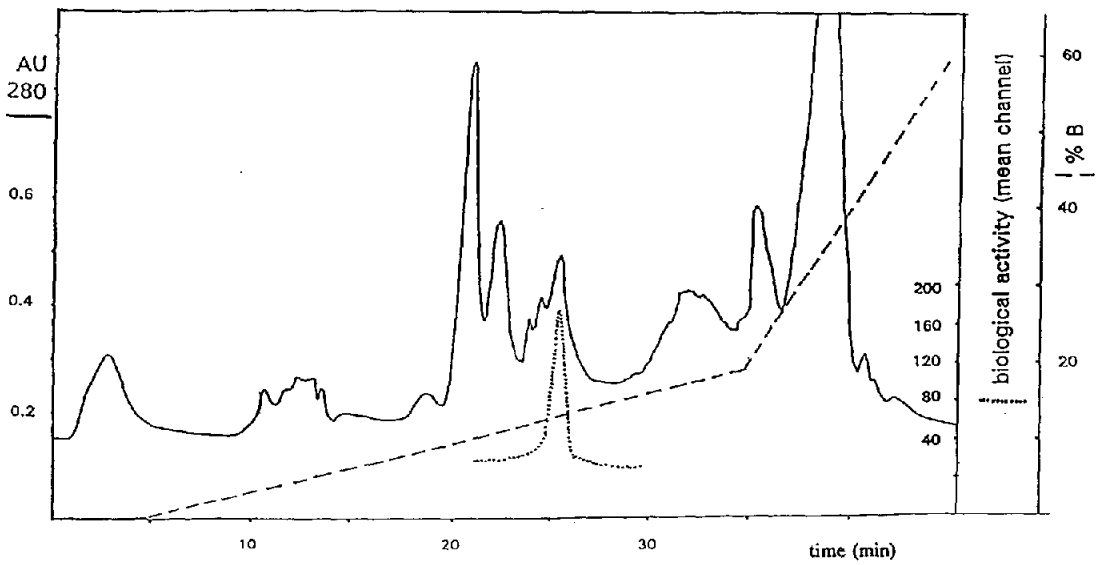


Fig. 4. Anion-exchange chromatography of LBP on a Mono Q HR 5/5 column. About 4 ml of LBP containing fractions refractionated in a narrow pH gradient (pH 4–6) using the Rotofor system were loaded on a Mono Q HR 5/5 column. LBP activity was eluted with a salt gradient from 0 to 200 m*M* NaCl in 30 ml of triethanolamine-HCl, pH 7.5. The flow-rate was 1.0 ml/min, pressure 2 MPa and fraction size 0.5 ml.

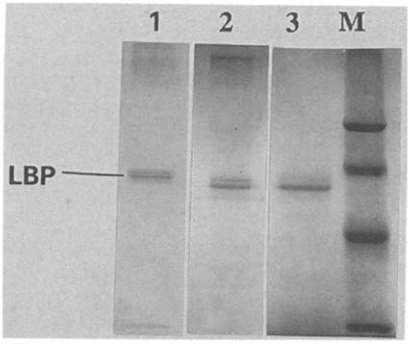


Fig. 5. SDS-PAGE of selected fractions from anion-exchange chromatography of LBP. Lane 1, fraction 51 containing LBP highly enriched; lane 2, fraction 49 containing LBP and hemopexin; lane 3, fraction 48 containing hemopexin highly enriched; lane M, molecular mass markers: 97.4, 66.2, 45, and 31 kDa. 9% Polyacrylamide gel was Coomassie Blue stained.

enhanced chemiluminescence (LECL) assay. The first measures the ability of LBP to promote binding of LPS to human monocytes. The resulting activation of these cells could be detected by LECL. The isoelectric point of LBP was determined by the pH of the IEF fractions which exhibited this activity. The p*I* is in the range of pH 5.0–5.4. As shown in Fig. 7, the biological activity of LBP was preserved during the purification procedure using isoelectric focusing and anion-exchange chromatography.

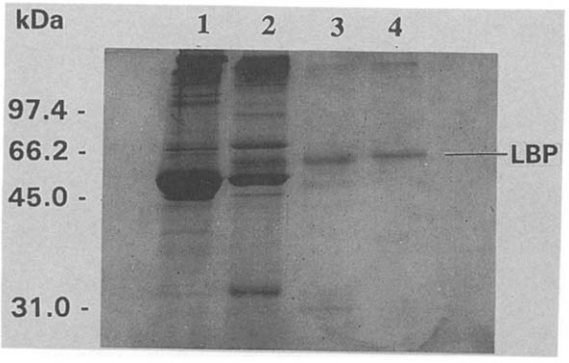
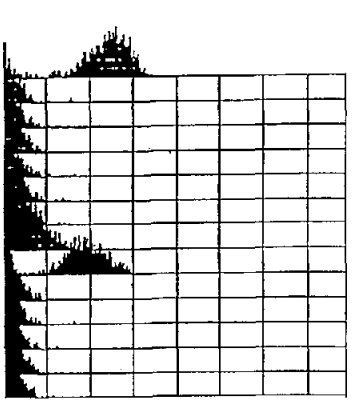


Fig. 6. SDS-PAGE analysis of the preparations of LBP. Lane 1, sample after IEF (pH 3–10); lane 2, sample after IEF (pH 4–6); lane 3, sample after anion-exchange chromatography; lane 4, sample after preparative SDS-PAGE. 12% Polyacrylamide gel was Coomassie Blue stained.



Fraction number	Mean channel
NCS	353
serum free	66
I	40
II	54
III	32
IV	25
V	29
VI	168
VII	263
VIII	46
IX	22
X	31
XI	29
XII	27

Fig. 7. Detection of the biological activity of LBP by FACS analysis. FITC-LPS binding to human monocytes is mediated by neonatal calf serum (NCS) and LBP purified by anion-exchange chromatography. The maximal biological activity of LBP is found in sample VII. Fluorescence histograms (*y*-axis, number of monocytes; *x*-axis, intensity of fluorescence) and fluorescence intensities (mean channels) detected are shown.

4. Conclusions

The LPS-binding protein (LBP) is a key component in macrophage recognition of Gram-negative LPS. The original purification procedure of LBP from rabbit acute phase serum reported by Tobias *et al.* [12], which was modified by Wright *et al.* [5] for the isolation of hLBP, is time-consuming and requires large amounts of starting material. Because of the limited availability of human acute phase serum, it was necessary to develop an improved method for the preparation of hLBP.

LBP was effectively isolated from human serum by preparative electrophoretic techniques. Primary fractionation by isoelectric focusing in the Rotofor cell resulted in a purification of the protein of interest of nearly 27-fold. After refractionation of the relevant fractions by IEF using a shallower gradient, LBP was isolated by preparative SDS-PAGE. The purification procedure typically yields 100–300 μg of the purified protein. In order to obtain LBP in a biologically active form, the material from the second IEF step was fractionated by anion-exchange chromatography using a Mono Q HR 5/5 column.

Using a micro-affinity adsorption technique it

is possible to separate LBP on a micropreparative scale. In addition to LBP a new LPS binding protein with an apparent molecular weight of 40 kDa was found by use of this technique. This affinity adsorption technique may therefore represent a useful method for the purification of LPS binding proteins.

The LBP purified by anion-exchange chromatography was used to raise monoclonal antibodies in order to develop an immunoassay which can measure a potential prognostic marker in pathological situations like ongoing septic shock. In addition, the availability of the LPS-binding protein in purified form is essential for functional studies as part of a project aiming at the development of a sepsis intervention strategy [11].

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