

Purification and Characterization of an Endotoxin-binding Protein with Protease Inhibitory Activity from *Limulus* Amebocytes*

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Using a lipopolysaccharide affinity column and ion exchange chromatography, a 12-kDa protein has been purified from *Limulus* amebocytes. In solid phase binding assays, the radiolabeled protein binds specifically to lipopolysaccharide (LPS) with a K_d value on the order of 10^{-7} M. A cDNA coding for this protein has been isolated and sequenced. The amino acid sequence deduced from the cDNA indicates that this protein shares no sequence homology with LPS-binding proteins isolated from different species of vertebrates (Schumann, R. R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S., and Ulevitch, R. J. (1990) *Science* 249, 1429-1431) and invertebrates (Aketagawa, J., Miyata, T., Ohtsubo, S., Nakamura, T., Morita, T., Hayashida, H., Miyata, T., Iwanaga, S., Takao, T., and Shimonishi, Y. (1986) *J. Biol. Chem.* 261, 7357-7365). The binding to LPS can be displaced by the unlabeled 12-kDa protein, polymyxin B, lipid A, and to a lesser extent by D-glucosamine. In whole cell binding assays, the 12-kDa protein has also been shown to bind to *Escherichia coli*. Using both [^{14} C]casein and a synthetic substrate, the protein has been shown to inhibit the proteolytic activity of trypsin, with an IC_{50} of approximately 10^{-7} M. In the presence of LPS, the antitryptic activity of the *Limulus* endotoxin-binding protein-protease inhibitor remains unaffected. The protein is a major component of the cytoplasmic proteins (1%). Immunocytochemical analysis reveals that this protein exists in the secretory granules of the amebocytes where enzymes and substrates for the clotting cascade reside. Based on the unusual dual functional properties, the newly isolated protein was named a "*Limulus* endotoxin-binding protein-protease inhibitor" (LEBP-PI).

Limulus amebocytes are normally discoid and highly granulated and constitute the only circulating cell type in the

hemolymph of the horseshoe crab. In isolated amebocytes, the presence of minute amounts of endotoxin (lipopolysaccharide, LPS)¹ from Gram-negative bacteria causes cellular degranulation followed by the formation of an extracellular gel (Levin and Bang, 1964). All of the factors which contribute to clot formation are sequestered within the secretory granules whose contents are released into the surrounding medium when cells degranulate (Shirodkar *et al.*, 1960). LPS has been shown to mediate the degranulation of amebocytes *in vivo* with subsequent activation of a zymogen in the early events of the clotting cascade (Levin and Bang, 1964; Tai and Liu, 1977). Several proteins and peptides have been isolated from *Limulus* amebocytes that interact with LPS. A protein with calmodulin-like activity has been isolated from amebocyte membrane and shown to enhance the activities of LPS (Liang *et al.*, 1980 and 1981). A protease precursor, factor C, was isolated from the soluble fraction of the amebocyte lysate and shown to be converted into an active form upon binding to LPS (Nakamura *et al.*, 1988a). Proteins and peptides which inhibit the activities of LPS have also been reported. A 15-kDa protein, named anti-LPS factor (Aketagawa *et al.*, 1986), and an octadecapeptide, tachyplesin (Nakamura *et al.*, 1988b), both isolated from *Tachyplesus tridentatus* amebocytes, have been shown to block LPS from triggering initiation of the *Limulus* clotting cascade.

Protease inhibitors have often been found to co-exist with proteases in biological systems (Laskowski and Kato, 1990). There is significant evidence to suggest that protease inhibitors play a key role in preventing unwanted proteolysis as seen in mammalian blood plasma, where proteolytic processes are tightly regulated in part by the presence of a number of inhibitors. Inhibitory activities to proteases have been observed in both the plasma and amebocytes of *Limulus*. In this regard, an α_2 -macroglobulin-like inhibitor was identified in plasma (Armstrong *et al.*, 1984), and a panel of protease inhibitory activities have been described (Armstrong and Quigley, 1985) in amebocytes. Most recently, a 16-kDa serine protease inhibitor has been purified from *Limulus* amebocytes (Donovan and Laue, 1991). In this paper, we describe the isolation and characterization of a novel protein from *Limulus* amebocytes that binds to LPS and inhibits trypsin activity. The possible physiological roles of this unique protein are discussed.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M65017.

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¹ The abbreviations used are: LPS, lipopolysaccharide; LEBP-PI, *Limulus* endotoxin-binding protein-protease inhibitor; LAL, *Limulus* amebocyte lysate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; IC_{50} , concentration required for 50% inhibition; bp, base pair(s).

EXPERIMENTAL PROCEDURES

Materials—LPS (*Escherichia coli* 055:B5) was from Difco, and lipid A (*Salmonella minnesota* Re 595) was from Calbiochem. Sephacryl S-100 and Mono S cation exchange columns were from Pharmacia LKB Biotechnology Inc. Pyrotell *Limulus* amebocyte lysate was from Associates of Cape Cod, Inc., Woods Hole, MA, and LAL chromogenic was from Whittaker Bioproducts. Affi-Gel Hz was from Bio-Rad. *E. coli* (LE392) was from Bethesda Research Laboratories. Trypsin and chymotrypsin were from Sigma, human Factor Xa was from Enzyme Research Laboratories, human thrombin was from Boehringer Mannheim, [¹⁴C]casein was from Du Pont-New England Nuclear, Boc-Leu-Gly-Arg-p-nitroanilide was from Sigma, and Chromozym TH (tosyl-Gly-Pro-Arg-4-nitroanilide acetate) was from Boehringer. Polyvinyl microtiter plates were from Beckton Dickinson. All other reagents were of the purest grades commercially available.

Isolation of *Limulus* Amebocytes—*Limuli* obtained from Cape Cod, MA were bled by cardiac puncture as described by Jorgensen and Smith (1973). The blood collected in ice-cold containers was centrifuged at $100 \times g$ for 15 min at 4 °C. The serum was removed, and the pellet containing the amebocytes was washed twice with sterile 3% NaCl, frozen, and stored at -70 °C.

Purification of LEBP-PI from *Limulus*—Two procedures were used for the isolation of endotoxin-binding protein (LEBP-PI) from *Limulus*. LPS affinity chromatography or gel filtration followed by cation exchange chromatography. Frozen amebocytes (5 ml) were thawed and homogenized in 4 volumes of 30% acetic acid with a Polytron Tissuemizer. The mixture was centrifuged at $6000 \times g$ for 15 min to remove debris. The supernatant was diluted with an equal volume of water and lyophilized. An endotoxin affinity column was prepared by coupling LPS to Affi-Gel Hz (Bio-Rad) according to the manufacturer's procedure. The lyophilized material was resuspended in 0.05 M NaHCO₃, pH 8.0, containing 1 mM CaCl₂ and applied to the LPS-Affi-Gel column (1 × 10 cm) equilibrated in the same buffer. The column was washed until the A₂₈₀ of the effluent was negligible. The proteins bound to the column were eluted with 0.05 M NaHCO₃ containing either 1 mg/ml LPS or 0.5 M trisodium citrate, pH 8.5. Fractions containing the bound proteins were desalted with a Sephadex G-25 column in 0.05 M ammonium acetate, pH 5.5, and lyophilized. The LEBP-PI was further purified on a C-4 reverse-phase column in 0.1% TFA and eluted with a 0–80% CH₃CN gradient (Fig. 1).

Alternatively, the supernatant of the acetic acid extract was filtered through a Millipore filter (0.45 μm) and applied to a Sephacryl S-100 gel filtration column (16 mm × 100 cm, Pharmacia) equilibrated with 0.05 M ammonium acetate at pH 5.5. Fractions containing the LEBP-PI were pooled and applied to a Mono S cation exchange column (5 × 50 mm) (Pharmacia). The proteins were eluted with a 0–1 M NaCl linear gradient in 0.05 M ammonium acetate buffer at pH 5.5. The LEBP-PI-rich fractions were further purified with a C-4 reverse-phase column as described above.

Amino Acid Analysis—The protein was hydrolyzed in 4 M methanesulfonic acid in a hermetically sealed microcapillary tube (Liu and Boykins, 1989), and the amino acid composition was determined in a Beckman 6300 Amino Acid Analyzer (Spackman, 1967).

Carbohydrate Determination—Samples of LEBP-PI were treated with 4 N TFA for 1 h at 125 °C. Following removal of TFA, the samples were reconstituted in water and analyzed with an AS-6 column (Dionex BioLC System). Monosaccharides were eluted isocratically with 0.25 M NaOH and detected with a pulsed amperometric detector.

Carboxymethylation of Protein—The presence of free SH groups was determined by exposing the native protein (1 nmol) to 1 μCi of [¹⁴C]iodoacetate and recrystallized iodoacetate (50 nmol) in 0.1 ml of 0.1 M Tris, pH 8.5, 6 M guanidine HCl, 1 mM EDTA, 25 °C for 1 h under nitrogen. The protein was subsequently separated from the reagents by reverse-phase HPLC on a C-4 column under conditions identical with those described in Fig. 1.

Amino-terminal and Internal Protein Sequence—The native protein, a CNBr fragment of the native protein (CNBr-I) (Fig. 3) and a peptide derived from endoproteinase Lys-C (Promega) digestion of CNBr fragment II were subjected to automated Edman degradation in a 477A gas-phase Microsequencer (Applied Biosystems) connected to an on-line model 120A PTH analyzer. The native protein (5 nmol) was treated with a 100-fold molar excess of CNBr in 1 ml of 88% HCOOH at 32 °C for 20 h (Gross and Witkop, 1962). Separation of the CNBr fragments by reverse-phase HPLC on a C-4 column gave rise to two fractions (Fig. 3). Fraction I was subjected to automated

Edman degradation and yielded the results shown in Table II. Fragment II was further digested with endoproteinase Lys-C (50-μg CNBr fragment, two additions of 1-μg aliquots of enzyme, at 6-h intervals) for 20 h at 25 °C in 0.6 M guanidine HCl, 0.05 M Tris at pH 7.6. The digestion mixture was fractionated on a reverse-phase HPLC column (Protein C-4, 2.1 × 250 mm, Vydac). Individual fractions were collected and subjected to automated Edman degradation. The result for CNBr II endoproteinase Lys-C-6 is shown in Table III.

Preparation of Rabbit Anti-LEBP-PI: Purification and Biotinylation—Rabbits were immunized subcutaneously with purified LEBP-PI solution (200 μg/ml in pyrogen-free water) and Freund's adjuvant. Blood samples were collected at 2-week intervals, and two booster injections of the protein solution mixed with incomplete Freund's adjuvant were performed. Enzyme-linked immunosorbent assay from samples of the final bleed showed a titer of approximately 1:1000. For immunocytochemistry studies, the antibody was purified on a DEAE-Affi-Blue column (Bio-Rad) to remove albumin and hemoglobin, and the clarified serum was passed through a Protein A-Sepharose CL-4B (Pharmacia) column equilibrated with PBS. The bound antibody was eluted with 4 M potassium thiocyanate and dialyzed against 0.1 M NaHCO₃ at pH 8. For enzyme-linked immunosorbent assay, purified antibody was biotinylated with biotin-N-hydroxysuccinimide ester (Pierce) according to the method of Bayer et al. (1979).

SDS-PAGE and Western Blot Analysis—SDS-PAGE was performed on 8–25% gradient gels according to Laemmli (1970) with a Phastgel System (Pharmacia) and stained with Coomassie Blue. For Western blot analysis, samples of proteins were transferred to nitrocellulose papers and reacted with rabbit anti-LEBP-PI. The protein bands were visualized through use of biotinylated anti-rabbit IgG and streptavidin-horseradish peroxidase conjugate (Bethesda Research Laboratories) followed by hydrogen peroxide and 4-chloro-1-naphthol (Sigma) for color development.

Use of PCR to Synthesize cDNA Coding for LEBP-PI—Based on the amino-terminal amino acid sequence, mixed oligonucleotides, GGNA(A/G)GA(T/C)GA(T/C)TGGCC, corresponding to the coding sequence of amino acids 16–20 were synthesized with an Applied Biosystems DNA Synthesizer. First strand cDNA was synthesized from the total amebocyte mRNA by reverse transcriptase with oligo(dT) as a primer and subsequently used as a template for PCR. The mixed oligonucleotides and oligo(dT) were used as primers for the Taq polymerase extension and amplification. A band of 550 bp was visible when the PCR mixture was analyzed on an 0.8% agarose gel stained with ethidium bromide. The band was excised from the gel and subcloned into the pUC13 vector. DNA sequence analysis was performed using the dideoxy chain termination method (Sanger et al., 1977).

Screening of Amebocyte cDNA Library—To determine the complete gene coding sequence for LEBP-PI, a cDNA library was constructed from the poly(A)-RNA of *Limulus* amebocytes by Clontech using λgt10 as a vector (Huynh et al., 1985). The 550-bp band from PCR was nick-translated using [³²P]dCTP (Du Pont-New England Nuclear), and the radioactive probe was used to screen the cDNA library.

Iodination of the LEBP-PI—Three hundred micrograms of purified LEBP-PI were radiolabeled with 1.3 mCi of Na¹²⁵I using IODO-BEADS (Pierce Chemical Co.) (Markwell, 1982). The iodinated LEBP-PI was separated from the free ¹²⁵I with a Sephadex GD-10 column equilibrated with PBS containing 2% BSA. The radiolabeled LEBP-PI had a specific activity of 0.3–0.9 μCi/μg of protein, and >90% of the radioactivity was precipitable with 10% cold trichloroacetic acid. The radiolabeled LEBP-PI migrated as a single band of 12 kDa on SDS-PAGE under either reducing or nonreducing conditions. Analysis of the autoradiogram from the gel showed 98% of the radioactivity was associated with the 12-kDa band.

Binding of the Radiolabeled LEBP-PI to LPS—The binding studies were performed using 96-well polyvinyl microtiter plates according to the methods of Tobias et al. (1989). Each well was coated with 100 μl of LPS (100 μg/ml) in 0.1 M Na₂CO₃, pH 9.6, 20 mM EDTA, for 3 h at 37 °C. The LPS-coated wells were blocked with 200 μl of 10% BSA in binding buffer (50 mM HEPES, pH 7.4, 0.15 M NaCl, and 50 mM CaCl₂) for 30 min at room temperature. After the excess BSA solution was removed, radiolabeled LEBP-PI was added in a total volume of 50 μl in binding buffer and incubated at room temperature for 3 h with constant agitation. The unbound materials were removed and the wells were washed with three changes of binding buffer. The data were analyzed according to the method of Chamness and McGuire (1975). The K_d values were calculated by using the program LIGAND (Munson and Rodbard, 1980). For inhibition experiments, various competitors were added to the wells of the microtiter plate 30 min

prior to the addition of radiolabeled LEBP-PI. The wells were washed and dried following incubation. The radioactivity of the bound material was determined by counting individual wells in an LKB γ counter.

Binding of Radiolabeled LEBP-PI to Bacteria—For the binding studies, the overnight culture of *E. coli* (LE392) was harvested, washed twice in 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, and 10 mM CaCl_2 (binding buffer), and resuspended in the binding buffer to a final concentration of 2×10^8 cells/ml (Filipssen *et al.*, 1990; Paulsson and Wadström, 1990). Fifty microliters of cell suspension containing 0.1% BSA were preincubated with 20 μl of unlabeled LEBP-PI over a concentration range of 0–2 μM at room temperature for 30 min and 20,000 cpm of ^{125}I -LEBP-PI was added, and the resultant mixture was incubated for 1.5 h at room temperature. The reaction mixtures were quenched by the addition of 200 μl of cold binding buffer and centrifuged for 10 min in an Eppendorf microcentrifuge. For cell concentration dependence studies, different bacterial concentrations were used (0–8 $\times 10^8$ cells/ml). The pellets were washed three times in 500 μl each of PBS, and the amounts of the bound radioactive LEBP-PI were measured in an LKB γ counter.

Protease Inhibition—The inhibitory activities of LEBP-PI were tested against trypsin, chymotrypsin, thrombin, and Factor Xa with either [^{14}C]casein or the respective specific synthetic substrates as described by Armstrong and Quigley (1985) with some modifications. Ten microliters of [^{14}C]casein (0.89 μM) were added to 90 μl of 0.05 M Tris-HCl, 0.05 M CaCl_2 , pH 8.0, protease (0.025–0.5 μM), and different concentrations of purified LEBP-PI (0.025–2 μM). The reaction was stopped by the addition of 30% trichloroacetic acid and 200 mg/ml BSA and centrifuged in an Eppendorf microcentrifuge. Aliquots of the supernatants were monitored for radioactivity using a Beckman scintillation counter. Amidolytic activity was measured in microtiter plates in the presence of 0.5 mM concentrations of the appropriate synthetic substrate. The absorbances were recorded through use of a Titertek Multiskan apparatus at 405 nm.

Exocytosis of the Amebocytes—Freshly collected amebocytes were washed and resuspended in 0.5 M NaCl, 10 mM EDTA, 0.1 M glucose, 0.05 M citrate buffer, pH 4.6. The cells were washed twice with 0.5 M NaCl, containing 10% dimethyl sulfoxide, and the exocytosis was initiated by adding a solution containing 0.5 M NaCl, 0.01 M CaCl_2 , and 10 μM ionophore A23187 (Sigma) to the cells. The tube was quickly mixed and allowed to incubate at room temperature for 1 h. The tube was centrifuged to separate cell aggregates from the cytoplasmic fluid. Lactate dehydrogenase activity (Sigma), a cytoplasmic marker, was used to measure the extent of cell lysis (Armstrong and Quigley 1985).

Electron Microscopy and Immunocytochemistry—*Limulus* blood was collected in an equal volume of 0.05 M HEPES, pH 7.4, containing 0.5 M *N*-ethylmaleimide, 0.165 M NaCl, 0.01 M EDTA, and 1% glutaraldehyde. Cells were pelleted by centrifugation and resuspended in the same buffer for 1 h for fixation. After several washes with PBS, the cells were dehydrated, embedded in "LR White" resin (Ted Pella, Inc.), and sectioned (Newman *et al.*, 1982). The nickel grids containing the amebocyte sections were placed in PBS solution containing 1% BSA for 30 min, followed by a solution of rabbit anti-LEBP-PI or anti-coagulogen (1:50) in PBS containing 20% normal goat serum. After 1 h, the grids were washed three times in PBS, and placed in PBS containing 20% normal goat serum and goat anti-rabbit IgG serum gold conjugate (1:10) (AuroProbe, 10 nm gold-labeled, Amersham) for 1 h. The grids were subsequently washed in distilled water and stained by conventional methods with uranyl acetate (De May *et al.*, 1981). The sections were analyzed in a JEOL-100CX electron microscope with 8000 $\times$ magnification.

Computer Analysis for Sequence Homology—FASTA and Wisconsin programs (Devereaux *et al.*, 1984) were used to compare the protein sequence homology between LEBP-PI and other proteins of known sequence.

RESULTS

Purification of LEBP-PI—Application of the amebocyte acetic acid extracts to an LPS-Affi-Gel column results in the retention of a 12-kDa protein which is subsequently eluted as one of the most abundant components among other bound proteins by either sodium citrate or LPS. In an alternative purification procedure using a Sephacryl S-100 gel filtration column, the last peak is comprised primarily of LEBP-PI which is further purified by Mono S cation exchange chro-

matography. The LEBP-PI-rich fractions from the affinity column and the Mono S column are then purified on a C-4 reverse-phase column yielding a single peak with an apparent molecular weight of 8000 on SDS-PAGE (Fig. 1). Amino-terminal sequence analysis reveals a single sequence (first 40 residues, Fig. 2) with an absolute yield of 97% and a repeated yield of 87%. Carbohydrate analysis indicates that the protein is not glycosylated.

Nucleotide and Deduced Amino Acid Sequence of LEBP-PI—The 550-bp band from PCR contains a sequence for most of the coding region of LEBP-PI including the amino acids near the amino terminus as determined by Edman degradation of the purified protein. A full length cDNA clone of 1068 bp was isolated from the amebocyte cDNA library using the 550-bp sequence as a probe. DNA sequence analysis indicates that this clone contains an open reading frame coding for 136 amino acids (Fig. 2). We assigned ATG at position 375 as the initiation codon which was followed by a 31 amino acid signal sequence. The serine residue at position 1 was assigned as the

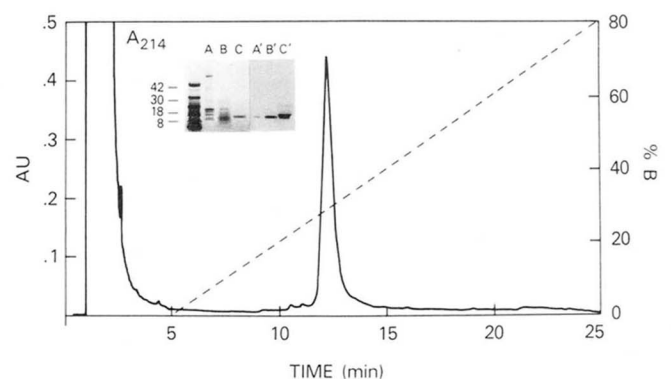


FIG. 1. Purification of LEBP-PI by reverse-phase HPLC on an Aquapore C-4 column (4.6 $\times$ 100 mm, Brownlee Laboratories). Mobile phase: 0.1% TFA in H_2O ; B = 0.1% TFA in CH_3CN ; flow rate = 0.5 ml/min; gradient = 0–80% in 25 min; temperature = 25 $^\circ\text{C}$. Proteins eluted from LPS affinity chromatography were applied to the column in 0.1% TFA. The inset shows the SDS-PAGE (A, B, and C) and Western blot analysis (A', B', and C') using rabbit anti-LEBP-PI. Lanes A and A', whole lysate; lanes B and B', acid extract; lanes C and C', HPLC-purified LEBP-PI.

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CAGGTAAACAC TGTCACTTAA TGCTAATGAT TTGCAACACA GTCATGTCAA CTAACATTAT AAACTTACT CAACCTTCCA
AATATTAAAT AAAATATAAT AACGTTCTAA GTATTAACTT AACGTTTTT TCAG ATG GTC AAA GGT ATA AAC TTC
-31
AGC TTC CTT TGC CTT CTG GGA TGT GTA CTT CTG GCT TCC ATC TCC CGC TGT CTC CGA ATT GTC
Ser Phe Leu Cys Leu Leu Gly Cys Val Leu Leu Ala Ser Ile Ser Pro Cys Leu Gly Arg Ile Val
CGC CGA AGT AAC ATC CAG TGT CAG TCT TGG AAG AGC AGT GAG ATA CGA TGT GGT AAG GAC GAT TGG
Arg Arg Ser Asn Ile Gln Cys Gln Ser Trp Lys Ser Ser Ser Glu Ile Arg Cys Gly Lys Asp Asp Trp
1 20
CCA TCG AAT CGA ATT AAG ATC ATA AAC AAG TCC TCT GCT AGT GTC ACA TTT GTT GTT GAC CAG TGG
Pro Ser Asn Arg Ile Lys Ile Ile Asn Lys Ser Ser Ala Ser Val Thr Phe Val Val Asp Gln Trp
40
ATA AGC TCG TGC GGT TGG CCA GGA TCG AAG TAT TCT TCT AGC CAG TAC ACA TTG AAC TCT TAT CAA
Ile Ser Ser Cys Gly Trp Pro Gly Ser Lys Tyr Ser Ser Arg Gln Tyr Thr Leu Asn Ser Tyr Gln
60
AAT ATC GAA CTA CAA TTT GCA CAT GCA TCG GGT GGT AAA TGT CGA GAA ATG TTT ATT ATT GAC TGT
Asn Ile Glu Leu Gln Phe Ala His Ala Ser Gly Gly Lys Cys Arg Glu Met Phe Ile Ile Asp Cys
80
CGT CAA CAT GGA ACT TAT ATT AAT TGT CTT CAT GTT TTG AAA GGT TAT CCT GTT GGC TAA CTTTACT
Arg Gln His Gly Thr Tyr Ile Asn Cys Leu His Val Leu Lys Ala Tyr Pro Val Gly End
100
GTAAAGTAAC AATTGTGAGA AGCTATTTCG CCGCACTACA GTAAAGCATT ATCAAAATATG TTCTCCAAAT TTGATTAA
TTGTAATAT TGTATTATTT CTTTAACT ATATATTATT GCTTCATCTT ATTAAGTGCTT GTATAATGAT GTACGTTTA
TTGATAATT TTATAATTAT GTTCTGTGT TGGATTATTC AGTCACCTTT GATTITGATT CATTTAATAA GAGTGTAA
ACACTAGAAA TAAAGCACTT TATAACCCA AAAAAAAA

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FIG. 2. Nucleotide sequence of the gene and the deduced amino acid sequence of LEBP-PI. The arrow indicates the possible cleavage site for the signal sequence and leaves serine as the amino-terminal amino acid which was confirmed by Edman degradation of the mature protein. The initiation codon (ATG), termination codon (TAA), and poly(A) addition signal are underlined. The matching sequences of amino acids determined by Edman degradation of the native protein, a CNBr I fragment, and a peptide derived from endoproteinase Lys-C digestion of CNBr fragment II with the exception of half-cysteine residues which cannot be determined by the Edman degradation method are also underlined. The disulfide bonds connecting cysteines are indicated. The sequence in bold type is the region from which the oligonucleotide was synthesized as primer for PCR.

amino terminus of the mature protein based on the amino acid sequence analysis of the purified protein. The first 40 amino acids predicted from the DNA sequence match perfectly with the amino acid sequence determined via Edman degradation. The DNA sequence predicts a total of 105 amino acids for the mature protein, consisting of 33.3% nonpolar, 46.67% polar, 6.67% acidic, and 13.33% basic amino acids, with a pI of 9.62. Compositional analysis of the purified protein agrees reasonably well with that deduced from cDNA sequencing (Table I). The cDNA also contains noncoding sequences at both the 3' and 5' ends, a polyadenylation site, and a poly(A) tail.

Determination of Disulfide Bonds—LEBP-PI consists of a single polypeptide chain, and there are no free SH groups in the molecule as indicated by the absence of carboxymethylation of the denatured protein with [¹⁴C]iodoacetate. The six half-cystines present in the protein must therefore exist in disulfide linkages. To establish the positions of the disulfide bonds, the native protein was initially treated with CNBr to cleave at the single methionyl residue in the molecule. Edman degradation of the purified CNBr fragment I (Fig. 3 and Table II) established Cys⁸⁶-Cys⁹⁶. Endoproteinase Lys-C digestion of CNBr fragment II yielded a peptide CNBr-II-endoproteinase Lys-C-6 and Edman degradation of this peptide estab-

lished the Cys⁵-Cys¹⁵ linkage (Table III). Since there are no free SH groups in this protein, the remaining two half-cystines, Cys⁴⁶ and Cys⁷⁸ must be linked via a disulfide bond.

Binding of LEBP-PI to LPS—Fig. 4A shows the saturation curve obtained from the binding to LPS to increasing concentrations of ¹²⁵I-LEBP-PI. The nonspecific binding was calculated from the "B/F limit" (Chamness and McGuire, 1975), which was obtained by a plot of B/F versus B. Subtraction of nonspecific binding from each point of the saturation curve allowed the generation of the Scatchard plot (Fig. 4A, inset). From the Scatchard plot, a K_d value of 0.6 × 10⁻⁷ M was obtained. Fig. 4B shows the ability of unlabeled LEBP-PI to displace the binding of labeled LEBP-PI to LPS. The displacement is concentration-dependent. The data were analyzed by using the program LIGAND (Munson and Rodbard, 1980) and a K_d of 3.2 × 10⁻⁷ was obtained.

The inhibition of LEBP-PI binding to immobilized LPS by polymyxin B (IC₅₀ = 10⁻⁹ M), LPS (IC₅₀ = 10⁻⁵ M), lipid A (IC₅₀ = 5 × 10⁻⁴ M), and glucosamine (IC₅₀ = 10⁻¹ M) are shown in Fig. 5. Although the lipid A moiety appears to be important for the interaction with LEBP-PI, the binding data suggest that components of the LPS molecule other than lipid A are essential for optimum binding to LEBP-PI. Polymyxin B, a cyclic decapeptide with antibiotic activity known to bind to the lipid A portion of LPS, competes most effectively for the binding of LEBP-PI to LPS.

Binding of LEBP-PI to Bacteria—Fig. 6 shows the binding of ¹²⁵I-LEBP-PI to *E. coli*. The binding is proportional to the cell number (Fig. 6A) and can be competitively displaced by the addition of unlabeled LEBP-PI (Fig. 6B). However, a substantial portion of the binding appears to be irreversible for reasons that are not known.

Protease Inhibitory Activities of LEBP-PI—After dialysis against Tris-HCl, 0.05 M, pH 8.0, the purified LEBP-PI inhibited trypsin against *N*-*t*-Boc-Leu-Gly-Arg-*p*-nitroanilide and [¹⁴C]casein substrate with an IC₅₀ value of 1.66 and 1.25 × 10⁻⁷ M, respectively (Fig. 7). The presence of LPS (100 μg/ml) in the assay mixture appeared not to interfere with this inhibitory activity. The inhibitory activity was abolished completely when the protein preparation was treated with the specific antibody and the antigen-antibody complex was removed by protein A Sepharose 4B. Successive purification steps including dialysis and gel permeation chromatography did not remove the inhibitory activity, supporting the conclusion that the inhibitory activity is associated with the protein. LEBP-PI inhibits also the proteolytic activity of chymotrypsin against casein, but a hundredfold excess of inhibitor is required. The amidolytic activity of thrombin or Factor Xa against their respective synthetic substrates was not inhibited by LEBP-PI.

Electron Microscopy and Immunocytochemistry—Fig. 8 indicates that *Limulus* amoebocytes are filled with large, dense secretory granules. Both LEBP-PI (Fig. 8A) and coagulogen (Fig. 8B) are present uniformly in all secretory granules. Neither protein was observed in the cytoplasm, nuclei, or membranes of the cells. When the first antibody was omitted or used in low concentrations, the second antibody did not stain any of the granules.

Exocytosis—When amoebocytes were treated with the ionophore A23187, LEBP-PI was released into the extracellular fluid as detected by Western blot analysis. Under the conditions of our experiment, the amoebocytes were not lysed as indicated by the lack of lactic dehydrogenase activity in the exocytosis fluid (data not shown). This result confirms the earlier report of Armstrong and Quigley (1985) and suggests that LEBP-PI is a granular protein.

TABLE I

Amino acid composition of LEBP-PI

Amino acid	Predicted ^a	Found ^b
Aspartic acid + asparagine	10	10.2
Threonine	3	2.75
Serine	15	14.9
Glutamic acid + glutamine	10	9.82
Proline	3	2.78
Glycine	7	6.91
Alanine	4	4.00
½Cys	6	5.50 ^c
Valine	5	4.65
Methionine	1	0.92 ^c
Isoleucine	10	7.75
Leucine	5	4.84
Tyrosine	5	5.20
Phenylalanine	3	2.94
Lysine	7	7.00
Histidine	2	3.10
Arginine	5	5.06
Tryptophan	4	3.68

^a Predicted values were derived from the amino acid sequence as deduced from the cDNA coding.

^b Hydrolysis performed at 150 °C for 90 min in a microcapillary tube in 4 M methanesulfuric acid containing 0.2% tryptamine. Reported values are averages of three analyses. Correction values used for threonine and serine are 90% and 80%, respectively.

^c ½Cys and methionine were determined as cysteic acid and methioninesulfone, respectively, after performic acid oxidation.

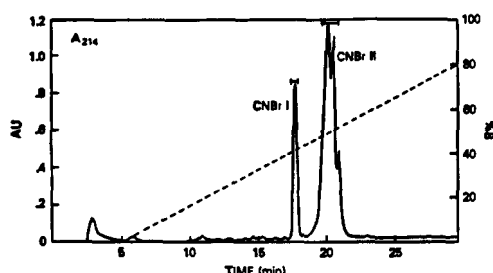


FIG. 3. Separation of CNBr fragments by reverse-phase HPLC on an aquapore C-4 column (4.6 × 100 mm, Brownlee Laboratories). Mobile phase: 0.1% TFA in H₂O; B = 0.1% TFA in CH₃CN; flow rate = 0.5 ml/min; gradient = 30–80% in 25 min. Temperature = 25 °C.

TABLE II
Amino acid sequence of CNBr I (1 nmol applied), containing disulfide linkage Cys⁸⁶-Cys⁹⁵

Cycle number	1	5	10	15
Residue number	20	25	91	96
	101	106	111	
Amino acids	Phe-Ile-Ile-Asp-Cys-Arg-Gln-His-Gly-Thr-Tyr-Ile-Asn-Cys-Leu			
	His-Val-Leu-Lys-Ala-Tyr-Pro-Val-Gly			
Picomoles	836	449 662 495	X ^a 276 275 153 246	64 159 130 117 X 93
	40	47 33 20	23 24 16 12 8	

^a X, blank. Position of cysteine predicted from DNA sequence analysis. Analysis of performic acid-oxidized sample yielded 1.90 mol of cysteic acid per mol of peptide.

TABLE III

Amino acid sequence of disulfide-linked (Cys⁶-Cys¹⁵), peptide CNBrII endoproteinase Lys-C-6 (220 pmol applied)

Cycle number	1	5	10
Residue number(s)	1	5	10
	11	16	19
Amino acid(s)	Ser-Asn-Ile-Gln-Cys-Gln-Ser-Trp-Lys		
	Ser-Ser-Glu-Ile-Arg-Cys-Gly-Lys		
Picomoles	277 163 188 180	X ^a 157 48 42 75	
	+ ^b 119 142 171 165	X 81 83	

^a X, blank. Position of Cys predicted from DNA sequence analysis. Analysis of performic acid-oxidized sample yielded 2.0 mol of cysteic acid per mol of peptide.

^b Combined recovery of serine at positions 1 and 11.

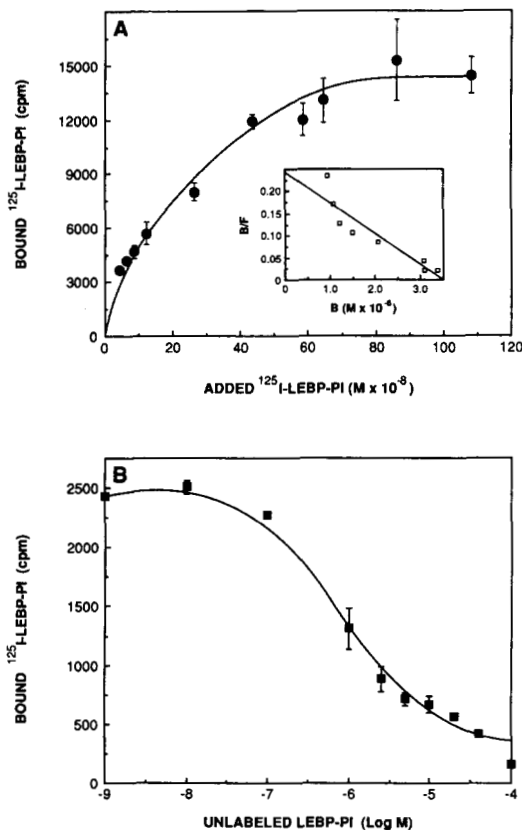


FIG. 4. A, binding of the radiolabeled LEBP-PI to LPS. The binding reaction was carried out with increasing concentrations of ¹²⁵I-LEBP-PI. Each point is the mean $\pm$ S.D. of three determinations. The inset is a Scatchard plot obtained after determination of specific binding in each point (see "Results"). B, displacement of binding of radiolabeled LEBP-PI to LPS by increasing the concentrations of unlabeled LEBP-PI. Each point is the mean $\pm$ S.D. of two determinations.

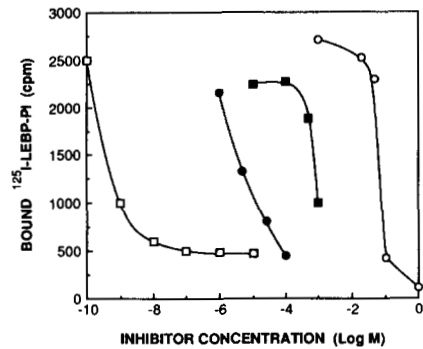


FIG. 5. Competition of the binding of radiolabeled LEBP-PI to LPS by polymyxin B (□), LPS (●) assuming the molecular weight to be 20,000, lipid A (■), and D-glucosamine (○). Each point is the mean of two determinations.

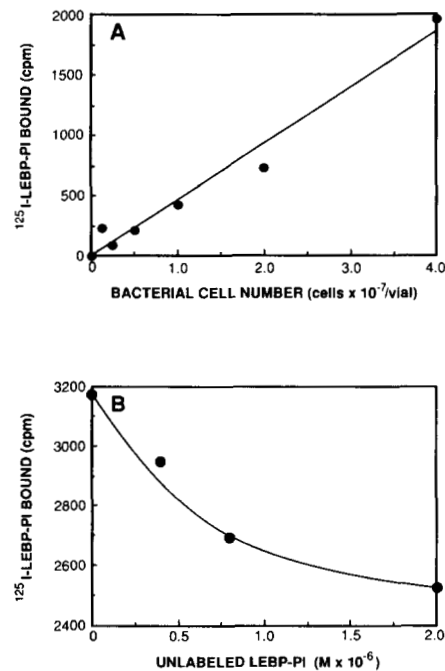


FIG. 6. A, binding of ¹²⁵I-LEBP-PI to increasing concentrations of *E. coli* cells. Radiolabeled LEBP-PI (20,000 cpm) was added to varying amounts of bacterial cells. After a 1.5-h incubation, cells were centrifuged, washed, and counted for radioactivity as described under "Experimental Procedures." B, inhibition by unlabeled LEBP-PI of ¹²⁵I-LEBP-PI binding to *E. coli*. The experiment was performed similarly to that of A, except that a fixed amount of bacteria (10^8) was incubated with increasing concentrations of unlabeled LEBP-PI 30 min before the addition of ¹²⁵I-LEBP-PI (20,000 cpm).

Concentration of LEBP-PI in Amebocyte Lysate and in Serum—Using enzyme-linked immunosorbent assay with biotinylated anti-LEBP-PI, the concentration of LEBP-PI in

the amebocyte lysate was estimated to be 10 $\mu\text{g}/\text{mg}$ total protein, whereas the serum contains only 10 ng/mg (250 ng/ml).

Computer Analysis of Sequence Homology—Statistically significant sequence homologies with LEBP-PI were not found in the computer search of all proteins in the GenBank. This includes the LPS-binding protein, anti-LPS factor (Aketa-gawa *et al.*, 1986), tachyplesin (Nakamura *et al.*, 1988b), a 16-kDa protease inhibitor (Donovan and Laue, 1991), the acute phase reactant from rabbit and human (Schumann *et al.*,

1990), and the bacterial permeability increasing protein (Gray *et al.*, 1989). Limited sequence homologies were found between LEBP-PI and fibronectin, complement proteins, and some members of the immunoglobulin superfamily (CD4 and Fc surface protein). Homologies were restricted to short stretches of less than 5 residues with the total number of similar residues comprising less than 35%.

DISCUSSION

Purification and Cellular Localization of LEBP-PI—A 12-kDa LEBP-PI has been isolated and purified from the acid extract of *Limulus* amebocytes by two methods, LPS affinity column chromatography and gel filtration followed by ion exchange chromatography. It is conceivable that LEBP-PI is a secretory granular protein for the following reasons: 1) *in vitro* induction of amebocyte exocytosis with the ionophore A23187 results in extracellular release of LEBP-PI; 2) immunocytochemical studies (Fig. 8) using highly purified rabbit anti-LEBP-PI and gold-labeled anti-rabbit IgG demonstrate that LEBP-PI is uniformly distributed in the secretory granules of the amebocytes together with coagulogen, which is known as a granular protein of the amebocytes (Armstrong and Rickles, 1982). This protein therefore differs from the membrane-associated endotoxin-binding protein previously isolated from *Limulus* amebocytes (Liang *et al.*, 1980) found solely in the insoluble membrane components of the amebocytes and not in the lysate containing soluble intracellular components. In the serum, the LEBP-PI concentration was estimated to be 10 ng/mg protein or 250 ng/ml serum, 1000-fold less than that in the cell. The low level of LEBP-PI in serum could arise from cell lysis during the collection of blood or residual LEBP-PI from exocytosis when amebocytes encounter external stimulators.

Properties of LEBP-PI—Sequence analysis of the cDNA reveals that the protein is probably synthesized in a precursor form with a leader sequence of 31 amino acids that possesses typical features of a signal peptide. LEBP-PI is not glycosylated and may be found in both the intra- and extracellular compartments. Although there are only minute amounts of LEBP-PI in the serum of *Limulus*, the ionophore-induced release of LEBP-PI upon exocytosis suggests that a high level of LEBP-PI could be secreted and subsequently delivered to

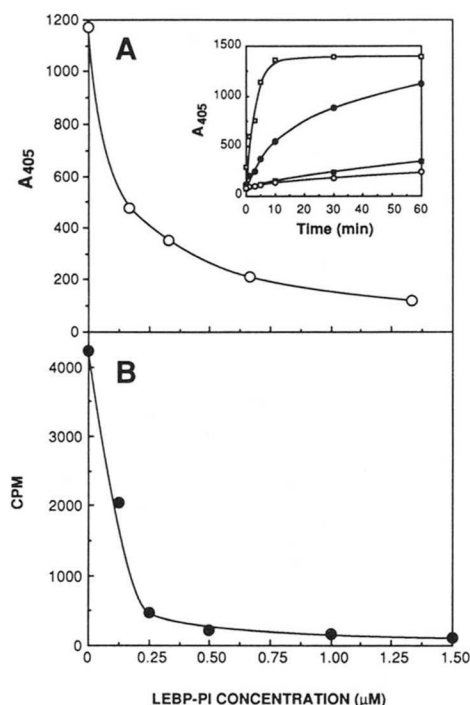


FIG. 7. Concentration dependence of anti-tryptic activity of LEBP-PI. A, substrate Boc-Leu-Gly-Arg-*p*-nitroanilide (0.5 mM); B, substrate [^{14}C]casein (0.89 μM). The inset in A shows the time course of the tryptic activity in the presence of different amounts of LEBP-PI (μM), as follows: $\square$, control (no inhibitor); $\bullet$, 0.16; $\blacksquare$, 0.32; $\circ$, 0.64 μM . In all experiments, each point is the average of two determinations.

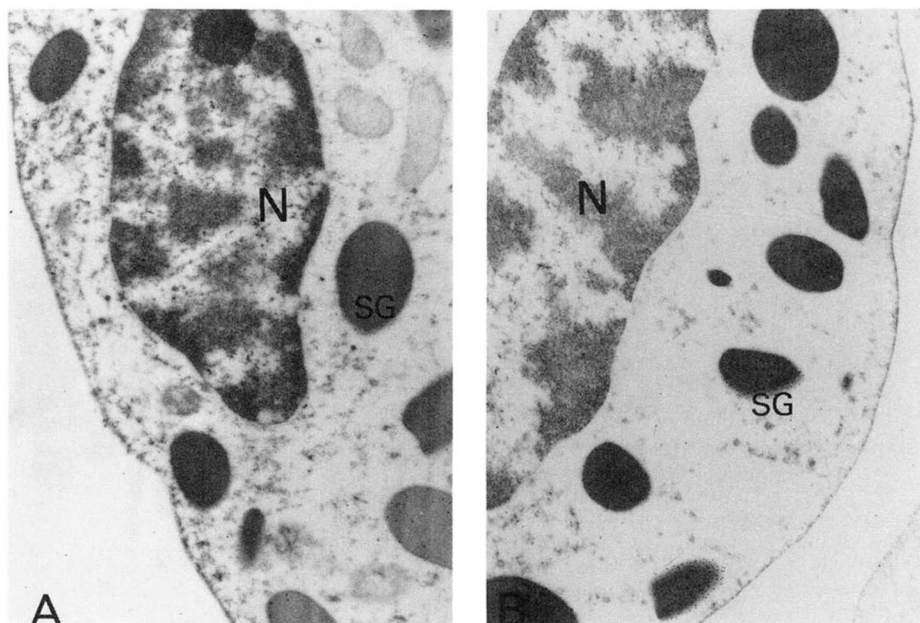


FIG. 8. Electron micrographs of *Limulus* amebocytes with immunogold staining. First antibody used was an affinity-purified rabbit anti-LEBP-PI (A) and rabbit anti-coagulogen (B). The second antibody was a gold-labeled rabbit anti-IgG. SG = secretory granule; N = nucleus. Final magnification: 8000 $\times$.

the "critical sites" upon infection, for instance, by Gram-negative bacteria. Minute quantities of endotoxin from Gram-negative bacteria are capable of provoking massive exocytosis of amoebocytes. The use of the ionophore in our studies instead of endotoxin for the induction of exocytosis provides an advantage of keeping the secreted components soluble.

The reduced and nonreduced forms of LEBP-PI exhibit an apparent molecular weight of about 8,000 (Fig. 1) in SDS-PAGE, whereas the minimum molecular weight calculated from the amino acid sequence deduced from the cDNA coding was 12,289. The amino-terminal and total amino acid analysis of the purified protein indicated that the mature protein has not been subjected to post-translational proteolytic cleavages. The discrepancy observed between the molecular weight determined by SDS-PAGE and that calculated from the amino acid composition probably reflects the intrinsic properties of the protein. Highly globular proteins have been shown to exhibit lower molecular weight than expected in SDS-PAGE.

The protein has been shown to bind specifically to LPS from Gram-negative bacteria. This binding was the basis for the purification procedure employing affinity chromatography as described under "Experimental Procedures." The binding is apparently Ca^{2+} -dependent and can be disrupted by Ca^{2+} -chelating agents such as citrate. Experiments were performed to determine whether LEBP-PI interacts directly and specifically with LPS. The results in Fig. 4 show specific binding of LEBP-PI to LPS with K_d on the order of 10^{-7} M. Kinetic studies of the binding process indicate that the equilibrium is attained after 3 h of incubation (data not shown). The binding is saturable and inhibited by the addition of excess amounts of unlabeled LEBP-PI, LPS, or lipid A. However, the latter is required in larger quantities (500 times more) to compete for the binding of LEBP-PI to LPS. Monosaccharides such as D-(+)-glucosamine HCl can also displace the binding at higher concentration levels (Fig. 5). Other sugars such as D-glucose, D-glucuronic acid, D-(+)-mannose, and D-(+)-galactose are not effective in competing for the binding. These results indicate that lipid A could be the major site, but not the only site, for the binding of LEBP-PI to LPS. It is interesting to note that polymyxin B, a peptide known to bind to the lipid A moiety in LPS, completely abrogates the binding of LEBP-PI to LPS with an IC_{50} as low as 10^{-9} M.

Among the LPS-binding proteins, the sites of interaction with LPS have not been identified although significant sequence homologies were observed among some of these proteins (Schumann *et al.*, 1990). While LEBP-PI does not share any homology with other LPS-binding proteins whose sequences are known, it is a basic protein ($\text{pI} = 9.6$) similar to most LPS-binding proteins. The basic nature of these proteins could be an important factor in their interaction with the negatively charged LPS molecule.

During the purification of LEBP-PI, we observed that the activity associated with the protein emerged from the Sephacryl S-100 column after salt and other low molecular weight components. When the purified LEBP-PI protein was subjected to chromatography on a Superose 12 column using a buffer containing 0.5 M KCl (the physiological ionic strength in *Limulus*), the protein eluted at a position following vitamin B₁₂ ($M_r = 1350$) (data not shown). These observations indicate that LEBP-PI interacts strongly with the polysaccharide gel matrix of the agarose column. The lectin-like property of LEBP-PI is most likely responsible for its binding to bacterial cells, but the nature of this interaction appears to be far more complex than its binding to LPS. The binding of ^{125}I -LEBP-PI to bacterial cells may be inhibited by the addition of excess amounts of unlabeled LEBP-PI although saturation kinetics

could not be demonstrated due, in part, to a substantial amount of irreversible binding. In this respect, LEBP-PI binding to bacterial cells resembles that of tachyplesin, but unlike this peptide, LEBP-PI is not bactericidal.

Possible Physiological Function of LEBP-PI—In this study, we have identified a major constituent of the *Limulus* amoebocyte granules, LEBP-PI, which possesses unusual bi-functional characteristics (*i.e.*, it binds specifically to LPS and selectively inhibits tryptic activity). The inhibition of proteolytic activity is specific and selective since LEBP-PI inhibits trypsin more effectively than chymotrypsin and exhibits no effect on papain, thermolysin, or Factor Xa (data not shown). LEBP-PI is capable of increasing the clotting time of the thrombin-elicited coagulation of human plasma by 2-fold when a 100:1 ratio of inhibitor:enzyme is used (data not shown). However, there is no inhibitory effect on thrombin at the same inhibitor:enzyme ratio when a synthetic substrate is used. Interestingly, the binding of LEBP-PI to LPS neither inhibits nor enhances the activation of the clotting system by LPS. Likewise, the anti-trypsin activity of LEBP-PI is not affected by the presence of LPS. Intracellularly, LEBP-PI probably acts as an inhibitor of trypsin-like enzyme(s) to prevent the onset of the coagulation cascade. Minute amounts of LPS as well as trypsin are known to be capable of initiating the coagulation process in the *Limulus* lysate leading to the formation of an insoluble clot (Levin and Bang, 1964; Tai and Liu, 1977). In the absence of LPS, spontaneous degranulation and gelation occur *in vitro* in isolated amoebocytes, albeit very slowly (Armstrong, 1980). The latter may be due to the presence of trypsin-like activity in the *Limulus* amoebocytes. The presence of high concentrations of LEBP-PI in the granules may serve to ensure that the proteolytic enzymes remain in their inactive form under normal physiological conditions. At the time of cell lysis or exocytosis, LEBP-PI is expelled from the amoebocytes together with other cellular components including the proteins in the coagulation cascade system.

Although the exact function of LEBP-PI binding to LPS and to bacteria is not known at present, it might serve to recruit LPS for the activation of the coagulation cascade system. Binding of LEBP-PI to bacterial cells could target these microorganisms for the phagocytic activities of amoebocytes (Armstrong and Levin, 1979). In this manner, LEBP-PI may act in concert with other components to serve as part of the defense mechanism of the horseshoe crab against invading microorganisms. However, LEBP-PI differs from anti-LPS factor (Aketagawa *et al.*, 1986) and tachyplesin (Nakamura *et al.*, 1988b) in terms of its ability to inhibit LPS from activating the clotting cascade. It is conceivable that the binding of LEBP-PI to LPS could down-regulate further exocytosis of granules from the amoebocytes and terminate the clotting process after the invading microorganisms are sequestered in the clot.

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