Studies on *Limulus* Lysate Coagulating System

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In 1964, Levin and Bang discovered that gram-negative bacterial endotoxin could rapidly induce gelation of *Limulus* amoebocyte lysate (1). Because of this unique property and its extreme sensitivity, *Limulus* lysate has been used to detect endotoxin in a variety of biological fluids. For the past three years, our laboratory has been engaged in the study of the *Limulus* lysate coagulating system. Our study is directed toward the understanding of the chemical and the physiological principles underlying the clotting processes. We have purified a proclotting enzyme (2), a coagulogen (3), and more recently, an active enzyme (4) and an endotoxin binding protein (5) from the *Limulus* amoebocytes.

The proclotting enzyme was purified by gel filtration of lysate on Sephadex G-75, followed by two steps of ion-exchange chromatography on DEAE-cellulose and DEAE-Sephadex. The purified proenzyme has a molecular weight of about 150,000 and requires both calcium and endotoxin for exhibiting maximum activity.

The active clotting enzyme(s) from the *Limulus* amoebocyte lysate has been purified to apparent homogeniety by gel filtration on BioGel A-5m (Fig. 1) followed by affinity chromatography using an endotoxin coated hydroxylapatite (Fig. 2). The *Limulus* enzyme(s) was found to be stabilized by the detergent Tween-20. Without the detergent, enzymatic activity decreased rapidly. Two active forms of the clotting enzyme were demonstrated to exist in the *Limulus* amoebocyte lysate. The enzymatic material existing as higher molecular weight aggregates was purified to give a single

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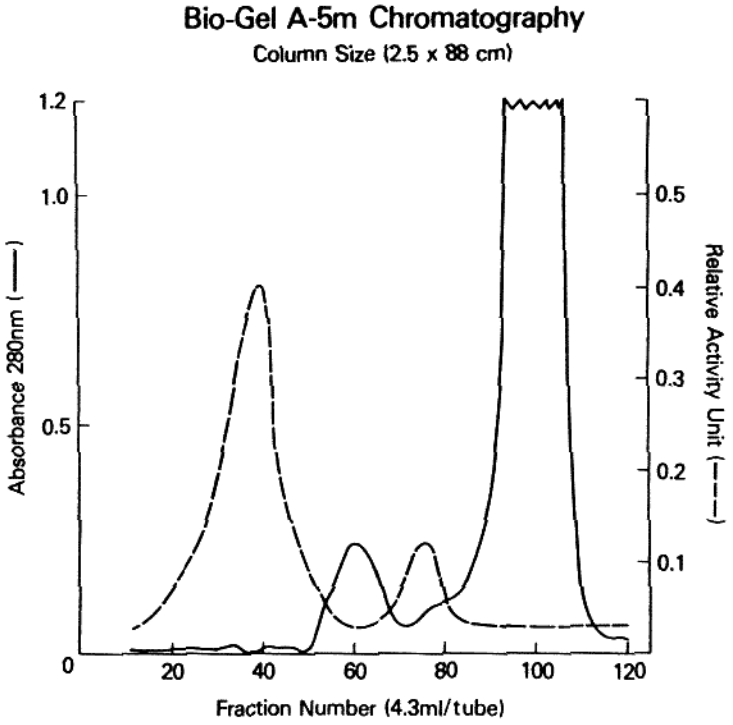


Figure 1. Gel permeation chromatography of *Limulus* clotting enzyme extract from lysate clot. The arrows indicate elution positions of markers for void volume (V_o) and total volume (V_t). Dashed line and solid bar represent amidase and clotting activities, respectively.

protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a molecular weight of 79,000. The enzymatic material eluting in the lower molecular weight region of the gel-permeation column yielded a protein with a molecular weight of about 40,000 (Fig. 3). The amino acid compositions of the two proteins were very similar indicating a "monomer"-"dimer" relationship but attempts to interconvert the 79,000 protein into the 40,000 protein have not been successful. Both enzymes have the same calcium and endotoxin requirement for exhibiting maximal clotting activity and share the same specificity toward synthetic substrates.

For the isolation of coagulogen, the amoebocytes were lysed and extracted with 30% acetic acid. This step removes

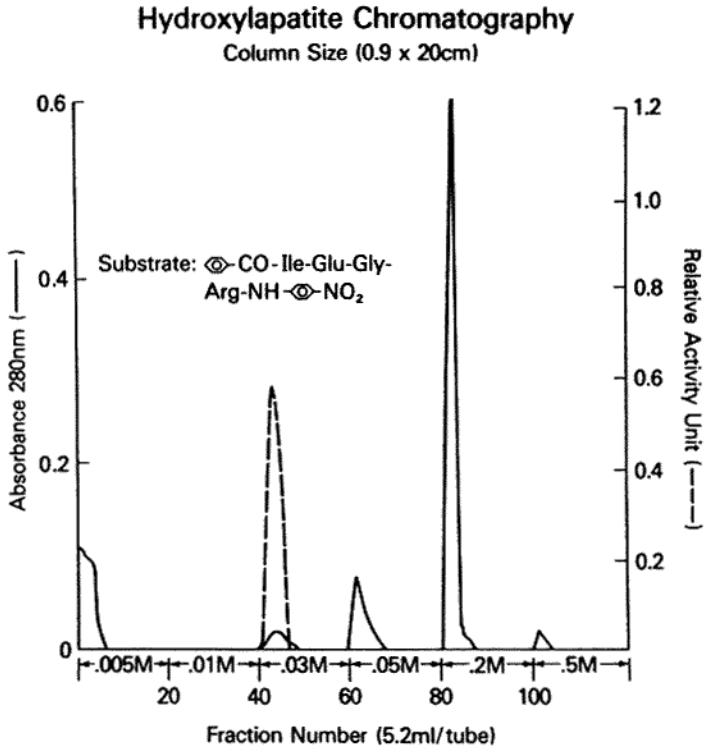


Figure 2. Affinity chromatography on endotoxin coated hydroxylapatite resin. *A*, chromatogram of *Peak I* material obtained from BioGel A-5m filtration, Figure 1. *B*, chromatogram of *Peak II* material. A stepwise increase in potassium phosphate was used to elute the clotting enzyme. Dashed line and solid bar indicate amidase and clotting activities, respectively.

contaminating proteins as insoluble precipitate and preserves the coagulogen as an intact protein. The clear acid-extract was placed on a Sephadex G-100 column in 30% acetic acid to yield five fractions (Fig. 4) Only fraction II gave a positive clot when the purified *Limulus* enzyme was added.

The purity of the protein in fraction II was ascertained by end-group analyses as well as by SDS-gel electrophoresis (Fig. 5). On SDS-gel electrophoresis both the native coagulogen and the reduced carboxymethylated coagulogen

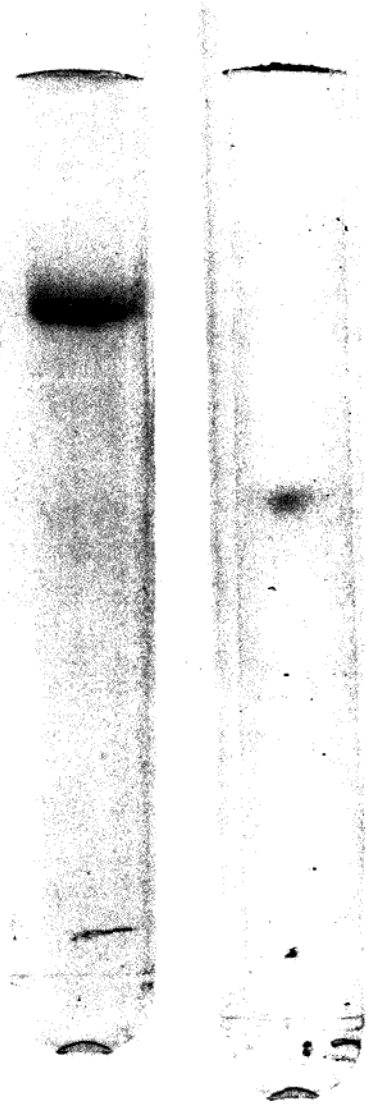


Figure 3. Acrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate, 0.1 mM dithiothreitol, 0.1M pH 7.2 phosphate buffer. *Gel 1* represent 0.03M clotting enzyme fraction, Fig. 2*A*, obtained from affinity chromatography; *Gel 2* represents 0.03M enzyme fraction of Figure 2*B*.

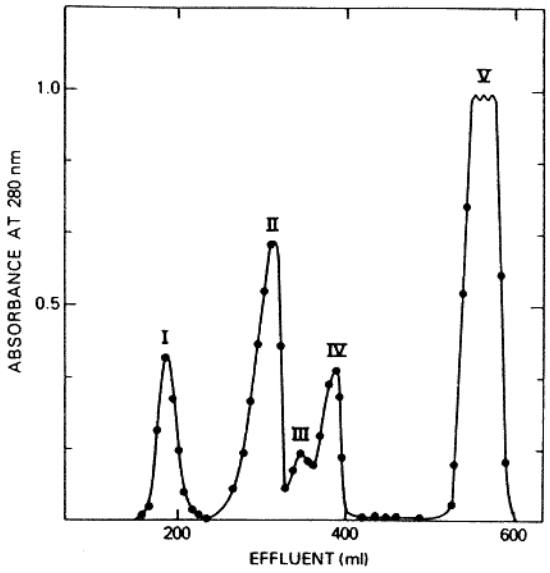


Figure 4. Gel filtration of 30% acetic acid extract of *Limulus* lysate on Sephadex G-100. The column (2 x 150 cm) was equilibrated and eluted with 30% acetic acid. A_{280}, absorbance of eluate at 280 nm.

migrated to similar positions, suggesting that the coagulogen is a single polypeptide chain. The coagulogen consists of about 220 amino acids, corresponding to a molecular weight of 24,500 which agrees with the result obtained from SDS-gel electrophoresis. The protein is devoid of methionine and has a high half-cystines content. There are a total of 18 half-cystines, all of which are involved in disulfide bond formation since free SH groups were not found by either titration with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) or alkylation with iodoacetamide in 6M guanidine-hydrocloride. No carbohydrate was found in the protein.

Amino terminal analyses showed glycine as the sole N-terminal residue. Carboxypeptidase A digestion of the reduced carboxymethylated protein indicated serine as its C-terminal residue.

Figure 6 shows the isolation of the coagulin and the soluble C-peptide. Purified coagulogen was incubated with clotting enzyme in the presence of calcium and endotoxin.

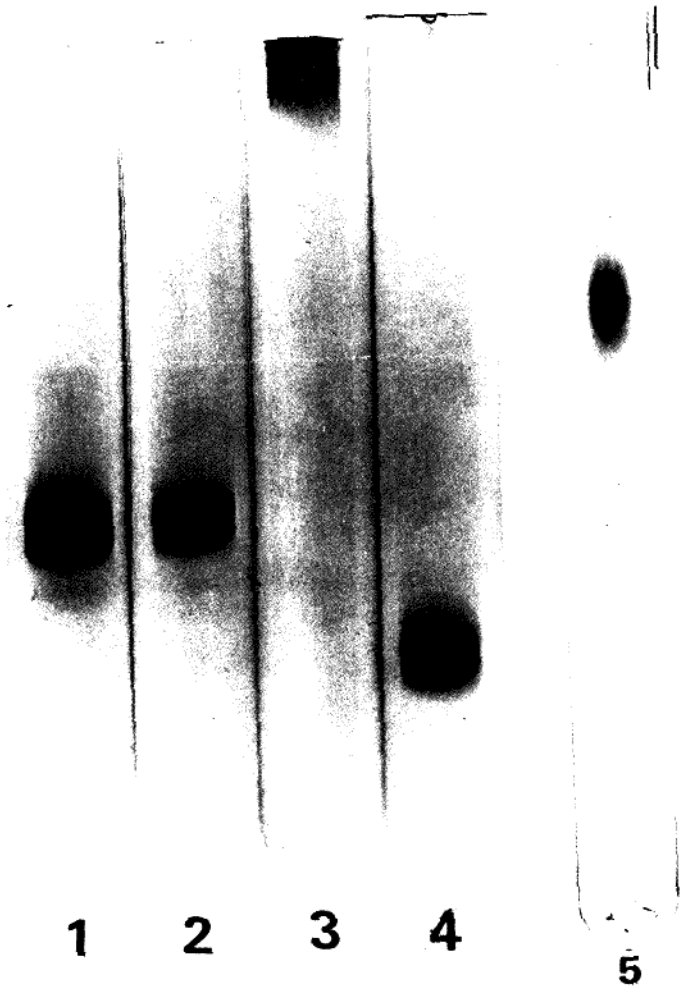


Figure 5. Acrylamide gel (7%) electrophoresis in 0.1% sodium dodecyl sulfate, 0.1M, pH 7.0, phosphate buffer of coagulogen, and the insoluble coagulin. *Gel 1*, coagulogen from *Peak II* in Fig. 4; *Gel 2*, reduced and carboxymethylated coagulogen; *Gel 3*, insoluble coagulin; *Gel 4*, reduced and carboxymethylated insoluble coagulin; *Gel 5*, 6M urea-acrylamide gel electrophoresis of C-peptide in 0.1% sodium dodecyl sulfate, 0.1M, pH 7.2, phosphate buffer. The acrylamide concentration is 12.5% and the ratio of acrylamide to methylenebisacrylamide is 10:1.

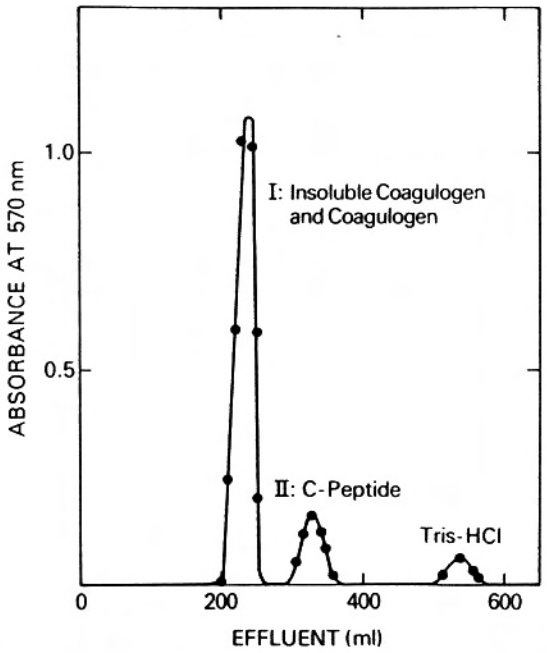


Figure 6. Gel filtration of the clotted coagulogen induced by the *Limulus* clotting enzyme through Sephadex G-50 in 30% acetic acid. Eluents were analyzed by ninhydrin after alkaline hydroylsis.

The resulting gel was dissolved in 30% acetic acid and placed on Sephadex G-50 column. Two fractions were obtained. The fraction at the void volume is a mixture of intact coagulogen and the coagulin or the N-peptide. The second fraction contained a small pure peptide of 45 amino acids termed the C-peptide. The first fraction was further purified on a Sephadex G-100 column in 30% acetic acid to yield the pure N-peptide (Fig. 7).

The amino acid composition of the N- and C- peptides is shown in Table I. The C-peptide is water soluble and consists of about 45 amino acids. It retains more than 50% of the charged amino acid residues, Lys, Arg, Asx and Gly from the parent coagulogen. The N-peptide is insoluble in aqueous solution. It retains the 9 disulfide bonds together with most of the hydrophobic amino acids.

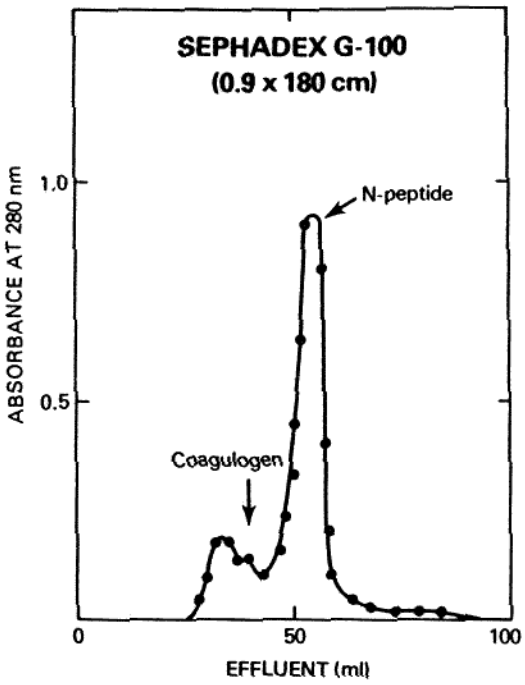


Figure 7. Gel filtration of *Fraction I* from Figure 6 through Sephadex G-100 in 30% acetic acid. A_{280}, absorbance of eluate at 280 nm.

The gelation of coagulogen can also be induced by trypsin to yield an identical N-peptide. However, the corresponding C-peptide is further hydrolyzed to several smaller peptides.

Some characteristics of the N- and C-peptides are summarized in Figure 8. The N-terminal sequence of the N-peptide was determined to be Gly-Asx-Pro which is identical to that of the intact coagulogen. The C-peptide gave the sequence Lys-Ile-Val, which is different from the coagulogen. However, its C-terminal serine is identical to the parent coagulogen. Thus, it is apparent that a single peptide bond of Arg-Lys is hydrolyzed by the clotting enzyme. Trypsin cleaves the same peptide bond. The N-peptides interact among themselves in a non-covalent fashion to form the insoluble clot.

TABLE I

AMINO ACID COMPOSITION OF LIMULUS COAGULOGEN, N-PEPTIDE AND C-PEPTIDE[a]

Amino Acid	Coagulogen	N-peptide		C-peptide CE-induced
		CE-induced	Trypsin-induced	
Lysine	11.9 (12)	6.0 (6)	6.0 (6)	6.0 (6)
Histidine	4.9 (5)	4.6 (5)	4.8 (5)	0.0
Arginine	16.8 (17)	13.8 (14)	14.1 (14)	3.4 (3)
Aspartic Acid	14.8 (15)	9.7 (10)	10.1 (10)	5.0 (5)
Threonine[b]	15.2 (15)	11.9 (12)	11.9 (12)	3.1 (3)
Serine[b]	13.7 (14)	10.8 (11)	10.9 (11)	3.1 (3)
Glutamic Acid	29.0 (29)	19.3 (19)	19.2 (19)	10.0 (10)
Proline	12.0 (12)	11.8 (12)	12.1 (12)	0.0
Glycine	16.6 (17)	14.0 (14)	14.3 (14)	1.8 (2)
Alanine	9.1 (9)	6.0 (6)	6.0 (6)	3.3 (3)
Half-Cystine[c]	18.2 (18)	17.8 (18)	17.9 (18)	0.0
Valine[d]	16.7 (17)	12.1 (12)	12.1 (12)	4.4 (5)
Methionine	0.0	0.0	0.0	0.0
Isoleucine[d]	7.7 (8)	2.6 (3)	2.5 (3)	5.2 (5)
Leucine	7.1 (7)	7.2 (7)	7.0 (7)	0.0
Tyrosine	5.6 (6)	5.9 (6)	6.1 (6)	0.0
Phenylalanine	15.0 (15)	14.7 (15)	14.9 (15)	0.0
Tryptophan	0.9 (1)	1.0 (1)	0.9 (1)	0.0
Total Residues	217.0	171.0	171.0	45.0

a. Average of duplicate analysis of 22, 48, and 72 hr. hydrolysates.

b. The values of threonine and serine were corrected for destruction during hydrolysis.

c. Half-cystine determined as S-sulfocystine is listed. Half-cystine determined as cysteic acid (Moore, S. (1963) *J. Biol. Chem. 238*, 235-237) gave a value of 18± 0.2.

d. The 72 hr. hydrolysate values for isoleucine and valine are listed.

e. The numbers given in the parentheses are nearest integer values.

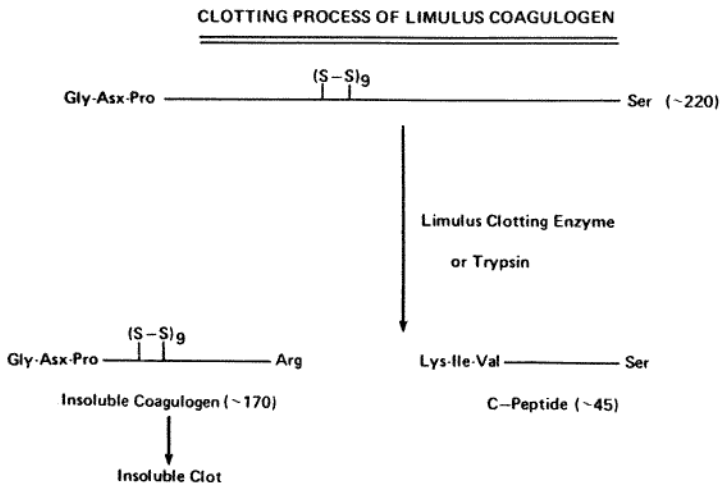


Figure 8. Schematic representation of the clotting process of *Limulus* coagulogen.

The specificity of the clotting enzyme was examined with the aid of a number of synthetic substrates (Table II). Benzoyl-Ile-Glu-(γ-OCH_3)-Gly-Arg-p-nitroanilide (-pNA) was hydrolyzed 30 times faster than Benzoyl-Phe-Val-Arg-pNA, whereas Benzoyl-Arg-pNA and Benzyloxycarbonyl-Gly-Pro-Arg-pNA were not hydrolyzed. More work is necessary to establish the clotting enzyme specificity which differs significantly from the specificity of trypsin.

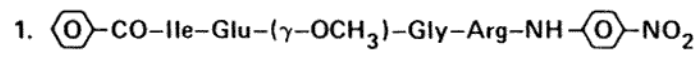
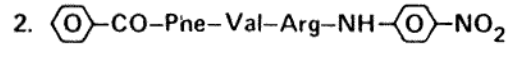
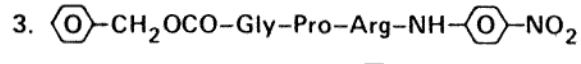
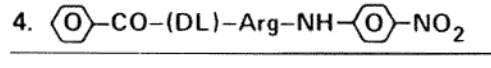
The existence of a cross-linking enzyme similar to that found in the mammalian coagulation system was investigated in collaboration with Dr. S.I. Chung of the National Institute of Dental Research, NIH (6).

Transglutaminase activity was demonstrated in the freshly prepared *Limulus* lysate by its ability to incorporate primary amines into the acetylated β-chain of oxidized insulin, casein, and other lysate proteins. The enzyme is activated maximally by Ca^{2+}, minimally by Mn^{2+}, Sr^{2+}, and totally inhibited by sulfhydryl reagents, such as iodoacetamide, mercury benzoate, and S-methyl-methyl sulfonate. The inhibitors to serine proteases showed no effect.

Finally, we would like to touch upon the possible physiological role of the *Limulus* clotting system. Minute amounts of endotoxin cause cellular degranulation and lysis,

TABLE II

RELATIVE SPECIFICITY OF LIMULUS CLOTTING ENZYME

Peptide Substrate	Relative Rate
1. 	100%
2. 	2.8%
3. 	~0%
4. 	~0%

followed by the formation of an extracellular gel. No enzymatic activity related to clotting occurs within the amoebocyte. All factors which eventually contribute to a clot must be expelled into the blood plasma when the cells degranulate or lyse. Activation of a zymogen by calcium and endotoxin is an early stage in the clotting process, in spite of the fact that endotoxin cannot enter the cell intact. Consequently, *in vivo*, endotoxin must mediate the degranulation and lysis of amoebocytes before the activation of zymogen occurs. We propose the existence of endotoxin receptor site(s) on the outer membrane of *Limulus* amoebocyte. A possible physiological advantage of such a system may be to localize and immobilize minute quantities of endotoxin in *Limulus* blood without the occurrence of massive intravascular coagulation. Receptor site bound endotoxin may stimulate the release of clotting zymogen which has an affinity for this endotoxin. The invading endotoxin is quickly engulfed by a clot.

A protein with a molecular weight of about 80,000 has been isolated and purified from amoebocyte membranes (Fig. 9). This protein binds with endotoxin and enhances the *Limulus* lysate coagulation process as effectively as about a 100-fold excess of endotoxin. It is possible that the "endotoxin-receptor protein" plays a role in the *Limulus* coagulating system.

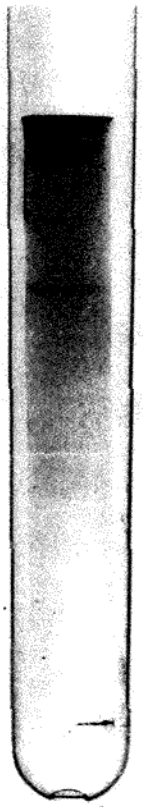


Figure 9. SDS-gel electrophoresis of the endotoxin membrane receptor of *Limulus* amoebocyte. The acrylamide concentration is 7.5% and the ratio of acrylamide to methylenebisacrylamide is 39:1.

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