PURIFICATION AND PROPERTIES OF THE CLOTTING ENZYME FROM LIMULUS LYSATE[1]

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Received August 6,1975

SUMMARY: The clotting enzyme from Limulus lysate which is involved in the gelation reaction of lysate with endotoxin has been purified and some of its properties determined. It was isolated from endotoxin-treated lysate and purified by gel filtration, ion exchange chromatography, and disc gel electrophoresis. Reaction of clotting enzyme with lysate clottable protein produces a clot or gel such as occurs with the gelation of lysate by endotoxin. Purified clotting enzyme has an approximate molecular weight of 84,000 (subunit MW 43,000), is isoelectric at pH ca. 5.5, trypsin-like, heat labile and pH sensitive.

INTRODUCTION: Reaction of Limulus lysate with endotoxin results in formation of a gel. The active constituents of lysate responsible for its gelation by endotoxin are proteins. Young et al. (1) using Sephadex column chromatography have shown the lysate to contain a high molecular weight enzyme which after activation by endotoxin reacts with a clottable protein to produce a gel. Kinetics of this reaction have been discussed by Levin and Bang (2). The lysate enzyme has been reported heat labile and sensitive to inhibitors affecting serine hydroxyl and sulfhydryl groups while the clottable protein is heat stable and has a molecular weight of 27,000 (1). Optimal conditions for the reaction are pH 7.5 and 37°C (1). The clottable protein and gel protein have been purified and characterized by Solum (3,4). This report describes the purification and properties of the trypsin-like clotting enzyme (CE) in

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Abbreviations:

CE, clotting enzyme
SDS, sodium dodecylsulfate
BANA, N-benzoyl-arginine naphthylamide
TAME, p-tosyl arginine methyl ester
pI, isoelectric point

Limulus lysate which reacts with clottable protein to form a gel. Whether endotoxin directly activates the CE from an inactive form such as a zymogen is not known. The possibility remains that endotoxin acts on another enzyme which in turn activates the CE. Efforts to determine the mechanism of endotoxin activation have no doubt been hampered by isolation of the active lysate constituents under pyrogen (endotoxin)-free conditions, a situation difficult to control using conventional protein purification techniques. The CE described herein has been purified from endotoxin-treated lysate. After breaking the formed clot and centrifugation to remove the gel protein, CE present in the supernatant can be purified with no need for pyrogen-free conditions.

MATERIALS AND METHODS: Pyrogen-free water (Travenol Labs); Klebsiella pneumoniae endotoxin (Food and Drug Administration); Salmonella minnesota mR595 endotoxin (Dr. O. Luderitz, Max Planck Inst.); Bio Gel A-0.5m (Bio-Rad Labs); ultrafiltration equipment (Amicon); horse, bovine, and sheep erythrocytes (Gibco Diagnostics); DEAE-Sephadex A-50, Coomassie blue R, Fast Garnet GBC, p-tosyl L-arginine methyl ester, ovomucoid trypsin inhibitor, pepsin, ribonuclease, cytochrome c, chymotrypsin (Sigma Chemical); ovalbumin (Worthington); trypsin, papain, protease (Calbiochem); blue dextran 2000 (Pharmacia Fine Chemicals); D-amino acid oxidase, bovine serum albumin, N-benzoyl arginine naphthylamide (Nutritional Biochemicals). Other chemicals used were reagent grade.

Limulus lysate was prepared according to the method of Sullivan and Watson (5). Horseshoe crabs (Limulus polyphemus) were collected locally in the Cape Cod, Massachusetts area during the summer months. The lysate used for isolation of the CE clotted with less than 0.1 ng endotoxin per ml (Klebsiella pneumoniae). Maximum sensitivity was obtained by treatment of the lysate with chloroform and addition of calcium as previously described (5).

Polyacrylamide disc gel electrophoresis was performed according to the method of Ornstein (6) and Davis (7) at pH 9.5 using 7% gels. Gel electrophoresis in the presence of SDS was done by the method of Weber and Osborn (8). Gels were stained with Coomassie blue (0.25% in methanol:water:acetic acid, 5:5:1) and destained electrophoretically with a Canalco gel destainer using 7.5% acetic acid containing 5% methanol. Some disc gels were stained for proteinase activity, specifically trypsin, using the post-incubation coupling procedure described by Hagenmaier (9) with BANA as substrate and Fast Garnet GBC as the stain. Reddish bands against a yellow-orange background indicated the presence of trypsin-like enzymes.

Determination of trypsin esterase activity was based on the method of Hummel (10) using TAME as substrate. Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as the standard. Hemagglutination assays were performed as described by Marchalonis and Edelman (12) using horse, bovine, and sheep erythrocytes.

Assay of Limulus CE was done by reacting 0.1 ml CE (or dilution therefrom) with 0.1 ml of undiluted 80°C treated lysate in a 10 x 75 mm test tube at 37°C 60 min. Enzyme activity was shown by the appearance of a solid gel. Lysate

which had been treated at 80°C 15 min served as a source of clottable protein for assay purposes and was itself devoid of CE activity.

The molecular weight of CE was estimated from SDS gel electrophoresis using bovine serum albumin, pepsin, trypsin, and ribonuclease as standards and gel filtration using a 1.5 x 50 cm column of Bio-Gel A-0.5m equilibrated with 0.05 M Tris-HCl buffer pH 8 containing 0.154 M NaCl and 0.01 M $CaCl_2$. Protein markers for gel filtration included cytochrome c, ovalbumin, bovine serum albumin, and D-amino acid oxidase. The void volume of the Bio-Gel column was determined with blue dextran 2000.

The isoelectric point (pI) of CE was determined by isoelectric focusing using an LKB 8100 (110 ml column) with a 1% ampholine pH gradient of 3-10. Operational procedure was followed according to the manufacturer's (LKB) instructions. Fractions (ca. 2 ml) were collected from the cooled column after 22 hr when the voltage had reached 700 V and the current was 1 mA. Each fraction was assayed for CE activity by reaction with 80°C lysate as described earlier. The pH of each fraction was determined with an Orion 801 pH meter. From the linear pH gradient obtained and region of CE activity, the pI was approximated.

Isolation of the Limulus CE was done by reacting 30 ml of Limulus lysate with 0.5 ml of Salmonella minnesota mR595 endotoxin (0.1 mg/ml) at 37°C 60 min. The solid gel produced by the reaction was broken, an equal volume of pyrogen-free was added and the mixture was centrifuged at 12,000 x g 10 min. The supernatant containing the CE was recovered and the precipitate discarded. Addition of more endotoxin to the supernatant neither improved the CE activity nor produced further clotting. All steps during purification of the CE were carried out between 0 and 5°C unless stated otherwise. The 12,000 x g supernatant (54 ml) was dialyzed overnight against 2,000 ml distilled water. NaCl (0.154 M) and $CaCl_2$ (0.01 M) were added to the retentate previously clarified by centrifugation (12,000 x g 10 min). This solution (57 ml) was concentrated to 6 ml by ultrafiltration using an Amicon 52 stirred cell equipped with a PM-10 membrane. The 6 ml concentrate was applied to a Bio-Gel A-0.5m column, 2.5 x 58 cm, which had been equilibrated with 0.05 M Tris-HCl buffer pH 8 containing 0.154 M NaCl and 0.01 M $CaCl_2$. A single peak of CE activity was obtained by this gel filtration step (Figure 1). The pooled sample (ca. 45 ml) from the Bio-Gel column was dialyzed overnight initially against 1,000 ml distilled water and finally against 1,000 ml 0.02 M Tris-HCl pH 8. The 47 ml sampled was applied to a 1.5 x 18 cm column of DEAE-Sephadex A-50 equilibrated with the dialysis buffer. The ion exchange column was eluted with a linear gradient of NaCl composed of 65 ml 0.02 M Tris-HCl pH 8 and 65 ml of same buffer containing 0.4 M NaCl. The CE lacking detectable A_{280} was eluted at ca. 0.3 M NaCl (Figure 2). Pooled sample (35 ml) from the DEAE-Sephadex column was dialyzed overnight against two changes of 1,000 ml distilled water. The retentate was concentrated to 10 ml by ultrafiltration and then lyophilized. Final purification was done by disc gel electrophoresis at pH 9.5. The lyophilized sample was resuspended with 2 ml distilled water and 0.6 ml applied to each of 3 gels. A fourth gel received 0.2 ml sample and was reserved for staining with Coomassie blue. After electrophoresis for ca. 3 hr at 1.5 mA/gel, the 3 gels were then individually sectioned at 2-3 mm intervals and the respective pooled sections homogenized with a glass stirring rod in 2 ml distilled water.

RESULTS: That the CE had been purified to homogeneity was shown by the appearance of a single band stained with Coomassie blue following re-electrophoresis under the same conditions for final purification and also by SDS gel electro-

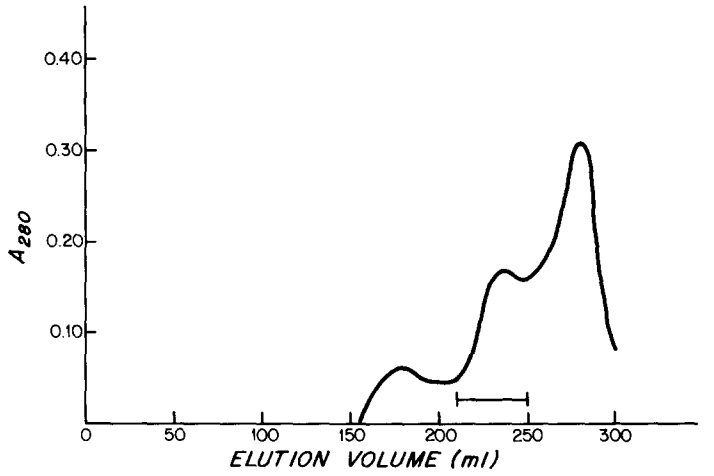

Figure 1. Gel filtration of Limulus lysate clotting enzyme (CE) on a 2.5 x 58 cm column of Bio-Gel A-0.5m. The 12,000 x g supernatant was concentrated to 6 ml by ultrafiltration (Amicon PM-10) and applied to the agarose column. Elution buffer was 0.05 M Tris-HCl pH 8 containing 0.154 M NaCl and 0.01 M $CaCl_2$. Fraction volume was 3-4 ml with a flow rate ca. 10 ml per hr. The CE designated by the bracket produced gelation of 80°C treated lysate.

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phoresis (Figure 3). Although not illustrated, disc gels containing samples of CE before final purification reflected multiple bands of proteinase (trypsin) activity. A single band, however, giving the trypsin color reaction with Fast Garnet GBC was found with the purified CE at the same mobility of gel C in Figure 3 stained with Coomassie blue.

A summary of the purification of CE is shown in Table 1. Approximately 3% (not corrected for small usage in assays) of the original CE activity was recoverable and nearly 20-fold purification obtained. Higher recoveries of activity were found on other occasions when the amount of lysate used was twice or greater than reported herein.

No loss in CE activity was found after 47 days at 5°C in unbuffered salt solution (0.154 M NaCl and 0.01 M $CaCl_2$). Treatment of CE at 60°C 5 min resulted in a 75% loss in activity. At pH 5, 50% of CE activity was lost and complete irreversible loss occurred at pH 4.7. CE was active between pH 5.5 and 10.3.

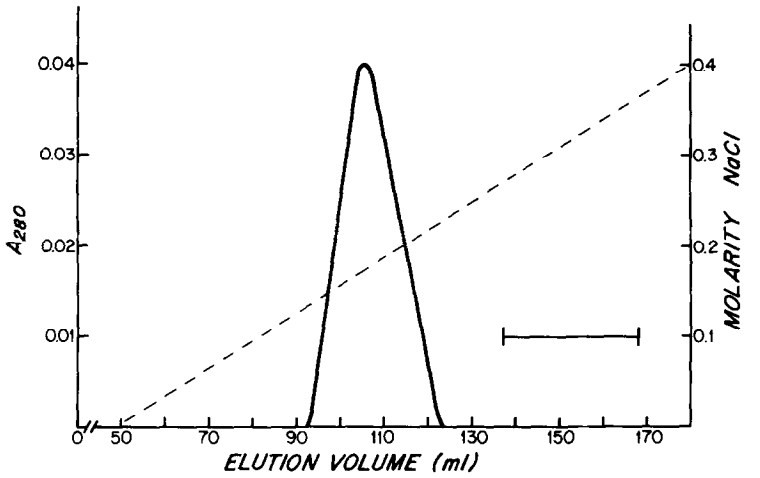

Figure 2. DEAE-Sephadex A-50 chromatography of Limulus lysate clotting enzyme (CE). The 47 ml sample of CE from the Bio-Gel A-0.5m column which had been dialyzed against 1,000 ml 0.02 M Tris-HCl pH 8 was applied to a 1.5 x 18 cm column of DEAE-Sephadex equilibrated with 0.02 M Tris buffer. The CE was released from the column by using a linear gradient of NaCl from 0 to 0.4 M prepared in 0.02 M Tris-HCl pH 8. Fraction volume was 3-5 ml with a flow rate ca. 15 ml per hr. The bracket represents enzyme activity detectable by its gelation of 80°C treated lysate. The A_{280} peak lacked CE activity.

Trypsin was reported by Solum (4) to clot Limulus lysate and the gelation reaction blocked by trypsin inhibitor. These findings have been confirmed in this study. Proteolytic enzymes such as papain, chymotrypsin, and protease unlike trypsin did not cause gelation of 80°C treated lysate. Incomplete gelation occurred with reactions of 80°C treated lysate using either chymotrypsin or protease while papain showed no gelation activity. The slight clotting activity of chymotrypsin and protease was not affected by ovomucoid trypsin inhibitor. Trypsin-induced clotting of 80°C treated lysate was blocked by trypsin inhibitor. The presence of trypsin inhibitor had no effect on gelation of 80°C treated lysate by CE. Clotting of 80°C treated lysate could be promoted by a minimal concentration of 7.5 µg/ml for trypsin and 10 µg/ml for CE (see Table 1)

CE lacked trypsin esterase activity as no change in A_{247} occurred during its reaction with TAME. Trypsin, however, under the same conditions showed a pronounced change (increase) in A_{247}.

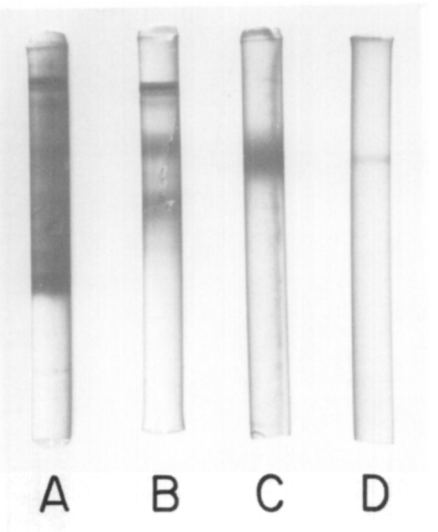

Figure 3. Disc gel electrophoresis at pH 9.5, 7% gels: (A) clotting enzyme, CE, from Bio-Gel A-0.5m column, (B) CE from DEAE-Sephadex column, and (C) purified CE obtained by semi-preparative disc gel electrophoresis. Gel (D) represents SDS gel electrophoresis of purified CE. Gels were stained with Coomassie blue and destained electrophoretically. Direction of migration is from top to bottom.

The activity of CE was not affected by treatment with trypsin at 37°C 60 min. That no loss in activity occurred was shown by reaction with 80°C treated lysate following addition of trypsin inhibitor to prevent interference by trypsin.

CE as well as Limulus lysate showed no hemagglutinating activity when tested with either horse, bovine, or sheep erythrocytes while significant activity was associated with the hemolymph as previously reported (12). The amebocytes present in the hemolymph from which Limulus lysate is obtained do not appear to contain hemagglutinins.

The molecular weight of CE as determined by gel filtration was 84,000 and by SDS gel electrophoresis, 43,000. A single band obtained by SDS gel electrophoresis (Figure 3) suggested that CE contained two identical subunits. Column isoelectric focusing indicated a pI of ca. 5.5 for the CE. In a separate ex-

Table 1

Purification of the clotting enzyme from Limulus lysate

Stage of purification	Volume (ml)	Protein (mg/ml)	Total protein (mg)	80°C titer[1]	Specific activity[2]	Fold-purification	Yield (%)[3]
12,000 x g supernatant	57	0.68	38.8	1:4	5.9	1.0	100
Bio-Gel A-0.5m column	47	0.14	6.6	1:2	14.3	2.4	41
DEAE-Sephadex A-50 column	10	0.08	0.8	1:4	50.0	8.5	18
Disc electrophoresis	6	0.01	0.06	1:1	100.0	16.9	3

[1]80°C titer determined by two-fold serial dilution of enzyme solution with distilled water. For example, a titer of 1:1 referred to undiluted enzyme while 1:2 and 1:4 were two-fold and four-fold dilutions respectively. The titer was the greatest dilution at which 0.1 ml of enzyme would form a solid gel when incubated with 0.1 ml of 80°C treated lysate at 37°C 60 min.

[2]Specific activity was expressed as the 80°C titer per mg protein.

[3]Recovery of enzyme activity was a conservative approximation as no corrections were made for small aliquots removed at each stage for determination of protein and 80°C titer

periment, isoelectric focusing of 80°C treated lysate gave a pI of ca. 9.0 for the clottable protein.

DISCUSSION: An enzyme from Limulus lysate involved in the gelation reaction between lysate and endotoxin has been purified and partially characterized. Referred to as the clotting enzyme (CE), it has a molecular weight of 84,000, contains two identical subunits (MW 43,000), is heat labile and pH sensitive and is an acidic protein (pI ca. 5.5). Reaction of CE with 80°C treated lysate (clottable protein free of CE) produces a gel. Gelation of clottable protein can also be promoted by trypsin. Unlike trypsin, CE is a high molecular weight acidic protein and is unaffected by trypsin inhibitor. CE lacks esterase activity but exhibits proteinase activity suggestive of a trypsin-like enzyme based on its hydrolysis of BANA (see Methods: enzyme staining). Whether CE is activated by endotoxin is not known. CE could exist in lysate as a zymogen in a non-activated state. Endotoxin could act directly on CE or another enzyme which in turn activates the CE.

ACKNOWLEDGEMENTS: This investigation was supported by a research grant from the Arthur Vining Davis Foundation.

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