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STUDIES ON THE LIMULUS COAGULATION SYSTEM: INHIBITION OF ACTIVATION OF THE PROCLOTTING ENZYME BY DIMETHYL SULFOXIDE

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Dimethyl sulfoxide inhibits the endotoxin-induced clotting activity of Limulus amoebocyte lysate. The inhibition is dependent upon the concentration of dimethyl sulfoxide and is competitive with endotoxin. Further studies showed that dimethyl sulfoxide inhibited the clot formation by reversibly inhibiting the activation of the proclotting enzyme to the clotting enzyme. Dimethyl sulfoxide had no effect on the active form of the clotting enzyme. Based on these findings, a procedure has been developed for the isolation of the proclotting enzyme from Limulus amoebocyte in the presence of dimethyl sulfoxide.

In 1964, Levin and Bang discovered that endotoxin could rapidly induce gelation of Limulus amoebocyte (1). Because of this unique property and its extreme sensitivity, Limulus lysate has been used to detect endotoxin in a variety of biological fluids. Our laboratory has been investigating the mechanism of the lysate gelation. We have purified a proclotting enzyme (2) a coagulogen (3), a clotting enzyme (4), and an endotoxin binding protein (5) that possesses calmodulin-like activity (6) from the Limulus amoebocyte. The mechanism of clotting in Limulus amoebocyte lysate involves Ca^{++} and endotoxin dependent activation of the proclotting enzyme to the clotting enzyme followed by the proteolytic cleavages of the coagulin to form a clot (3, 7).

Since minute amounts of endotoxin cause the activation of the proclotting enzyme, the isolation of the proclotting enzyme necessitates endotoxin-free conditions (2). However, it has been found that activation of the proclotting enzyme occurs frequently during the purification procedure (8). This endotoxin-induced activation of the proclotting enzyme has resulted in very poor yields or even failure in isolating the proclotting enzyme (9). In this communication, we report that dimethyl sulfoxide (DMSO)

Abbreviations: EDTA, Ethylenediamine tetraacetic acid DMSO, Dimethyl sulfoxide

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inhibits this endotoxin-induced activation of the proclotting enzyme. By adding DMSO to the buffer solution, the proclotting enzyme has been purified in two chromatographic steps.

MATERIALS AND METHODS

Pyrogen-free water (Abbott labs); Escherichia coli 0113:H10 endotoxin (Dr. Donald Hochstein, Food and Drug Administration); dimethyl sulfoxide (Pierce labs); frozen Limulus amoebocytes and pyrotell Limulus amoebocyte lysate (Association of Cape Cod); Sephadex G75 and DEAE Sephadex A-25 (Pharmacia Fine Chemicals); S-2222 (Kabi Diagnostica); glassware was freed from pyrogens by heating at 180°C for at least 4 h.

The amidase activity of the Limulus proclotting enzyme was monitored by using a synthetic chromogenic substrate, benzoyl-Ile-Glu-(γ-OCH_3)-Gly-Arg-p-nitroanilide (S-2222) (10). One hundred microliters of reconstituted lysate or an unknown amount of the proclotting enzyme was added to a buffer mixture containing 0.045M Tris-HCl pH 7.5, 3 mM Ca^{++}, 0.1 mM S-2222, and 0.3 ng of endotoxin in a total volume of 1 ml. After 30 min of incubation at 37°C, the reaction was stopped by the addition of 0.1 ml of glacial acetic acid and the released p-nitroaniline was estimated by its absorbance at 405 nm.

All steps involved in the purification of the proclotting enzyme were performed at 4°C. Packed frozen amoebocytes (10 ml) were thawed and washed with 3% sterile saline. The cells were then lysed by vortexing them in 30 ml of pyrogen-free water containing 5% DMSO and 1 mM EDTA. The mixture was blended in a Waring blender for 30 sec at high speed. After centrifugation at 1000 Xg for 20 min, the supernatant was applied to a G75 column (2.6 X 90 cm) and eluted with a buffer containing 0.05M NaCl, 5% DMSO, and 1 mM EDTA in pyrogen-free water. Eluent fractions containing the amidase activity were pooled, applied to a DEAE Sephadex A25 column (2 x 40 cm), previously equilibrated with 0.05M Tris-HCl pH 6.8, 1 mM EDTA, and 5% DMSO. The column was washed with the same buffer and eluted with a linear gradient of 0 to 1.0 M NaCl in the same buffer. The active fractions were analyzed by gel electrophoresis.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according to the method of Laemmli (11) using a 7.5% gel.

RESULTS

The initial objective of this study was to improve the isolation of the proclotting enzyme from the amoebocyte. Contamination with even trace amounts of endotoxin induced the amoebocyte lysate to clot and thus rendered the isolation of the proclotting enzyme difficult. Since endotoxin is ubiquitous, the maintenance of endotoxin-free conditions during the entire purification procedure is time-consuming and prone to failure. We, therefore, approached this problem by searching for a reagent capable of reversibly inhibiting the endotoxin-induced activation. After screening many potential reagents, we found that DMSO was the most suitable. DMSO inhibited both the Limulus clotting activity and the amidase activity (Fig. 1). The inhibition

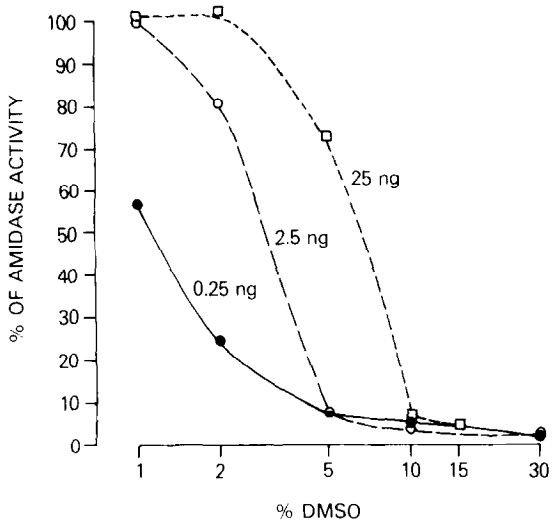

Fig. 1 Effect of DMSO on amidase activity of the Limulus amoebocyte lysate. The amidase activity of the lysate was plotted versus the concentration of DMSO on a logarithmic scale. The amidase activity assay was performed as described under Methods. The concentrations of endotoxin were 0.25 ng/ml (●), 2.5 ng/ml (o) and 25 ng/ml (□) respectively. The points plotted were the means of duplicate assay.

was dependent upon the concentration of DMSO and was competitive with endotoxin. In the presence of 0.25 ng/ml endotoxin, 1% DMSO inhibited 57% amidase activity; while at 25 ng/ml endotoxin, 6.5% DMSO was required to achieve the same magnitude of inhibition. The inhibition was reversed after the removal of DMSO by dialysis.

If the Limulus lysate was preincubated with endotoxin for 1 hr to convert the proclotting enzyme to the clotting enzyme, then the amidase activity of the clotting enzyme was not inhibited by even high concentrations of DMSO (up to 30%) (Fig. 2). Since simultaneous addition of DMSO with endotoxin blocked the amidase activity of the lysate (Fig. 1 & 2) it is apparent that DMSO inhibited the activation of the proclotting enzyme but had no effect once the enzyme became active. It was concluded, therefore, that DMSO may faciliate the isolation of intact proclotting enzyme.

By adding 5% DMSO and 1 mM EDTA to all buffer solutions, the purification process of the proclotting enzyme from Limulus amoebocyte was simplified and reduced to only two successive chromatographic steps, the first being gel permeation on Sephadex G75 and the second being ion-exchange

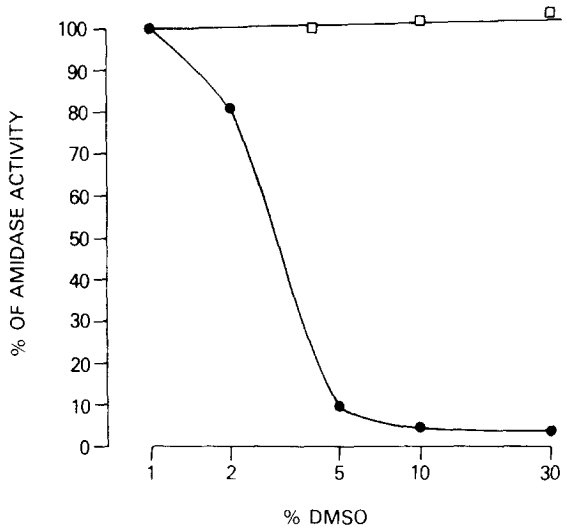

Fig. 2 Effect of DMSO on the endotoxin-preincubated Limulus amoebocyte lysate. The amidase activity of the lysate was plotted versus the concentration of DMSO on a logarithmic scale. Endotoxin (2.5 ng/ml) was added to the lysate 1 hr before (□) or simultaneously with (●) the addition of DMSO.

chromatography on DEAE Sephadex A25. The elution profile of the crude Limulus amoebocyte lysate on a Sephadex G75 column is shown in Fig. 3.

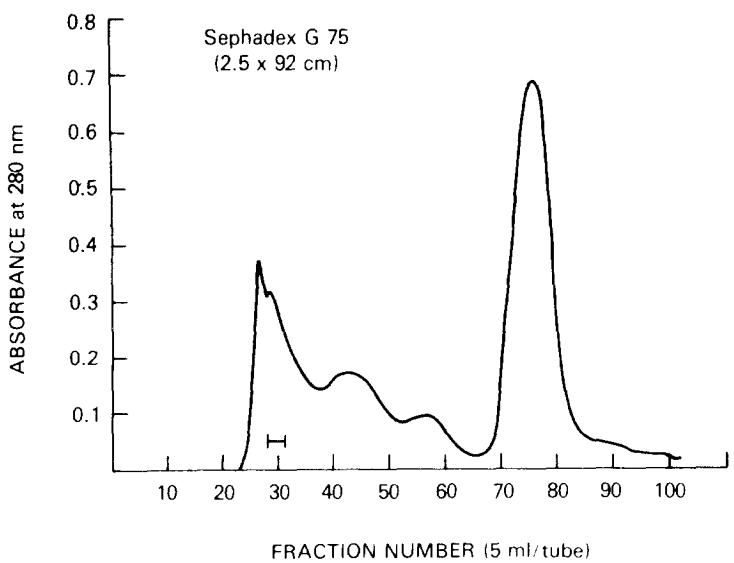

Fig. 3 Gel filtration of the crude amoebocyte lysate on a Sephadex G75 column. The column was eluted with 0.05 M NaCl, 5% DMSO and 1mM EDTA. Fractions of 5 ml were collected at a flow rate of 50 ml/hr. The portions which contained the amidase activity are indicated by the bar.

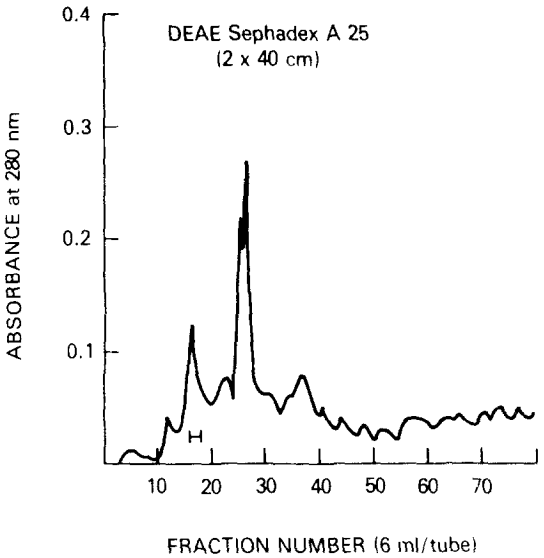

Fig. 4 DEAE Sephadex A25 chromatography of proteins from Sephadex G75 fractions. 15 ml sample was dialyzed against the eluting buffer, containing 0.05 M Tris-HCl pH 6.8, 1mM EDTA and 5% DMSO before application to the column. The column was first washed with 300 ml of eluting buffer at a flow rate of 15 ml/hr and then eluted with a linear gradient from 0 to 1.0 M NaCl. The fractions which contained the amidase activity are indicated by the bar.

Upon exposure to endotoxin, the eluates in the trailing shoulder of the first peak exhibited amidase activity indicating that this peak contained the proclotting enzyme. Subsequent chromatography of this fraction, containing the proclotting enzyme, on a DEAE Sephadex A25 column yielded two major peaks. The first peak contained a homogeneous protein having the proclotting enzyme activity (Fig. 4). SDS gel electrophoresis, molecular weight, and amino acid composition analysis of this homogeneous fraction indicated that this purified protein is the same as the proclotting enzyme isolated by Tai and Liu (2).

Using a Heparin Sepharose column, Ohki et al. (9) reported that they were unable to isolate fractions containing the proclotting enzyme activity from the Limulus amoebocyte lysate. Following the same procedure but with the addition of 5% DMSO, we found a peak containing the proclotting enzyme activity immediately after the void volume, as shown in Fig. 5. In addition, we also detected a small amidase activity in the absorbed fraction (Fractions 27-30) as reported by Ohki et al. (9). The proclotting enzyme fraction had 12.5 times more amidase activity than the absorbed fraction. In contrast to

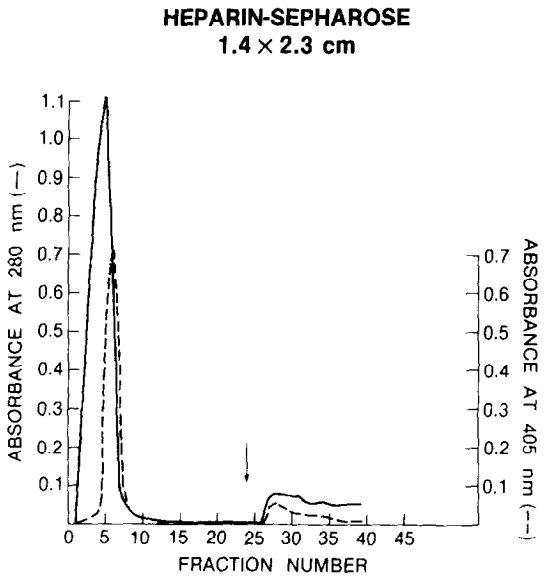

Fig. 5 Heparin-Sepharose column chromatogrphy of Limulus amoebocyte lysate. 10 ml of protein from the selected Sephadex G75 fractions were applied to the column, previously equilibrated with 0.05 M Tris-HCl pH 7.2, 1 mM $CaCl_2$, 0.15 M NaCl and 5% DMSO. The elution was performed at 4° C, first with the equilibration buffer and then with a linear gradient buffer from 0.154 M NaCl to 0.54 M NaCl in the same buffer. Arrow indicates the beginning of the linear gradient. Fractions of 3 ml were collected at a flow rate of 10 ml/hr. Solid line represents the absorbance at 280 nm and dotted line indicates the amidase activity.

the previous finding of Ohki et al. (9), addition of the absorbed fraction to the proclotting enzyme fraction did not increase the amidase activity, indicating that the activity of the proclotting enzyme is not dependent upon the absorbed fraction.

DISCUSSION

The endotoxin-induced activation of the proclotting enzyme initiates several subsequent enzymatic reactions that lead to the clot formation of the Limulus amoebocyte lysate (2-4). Reagents capable of inhibiting this endotoxin-induced activation and thus facilitating the isolation of the proclotting enzyme have not been found previoulsy. Schleef et al. (8) reported that 4M sodium chloride inhibited the activation of the proclotting enzyme. However, under this high salt condition, the proclotting enzyme was irreversibly denatured (8). In the present study, we found that DMSO inhibited clot formation in Limulus amoebocyte lysate by preventing the conversion of the proclotting enzyme to the enzyme without any concomitant

denaturation of the protein. It has been suggested that endotoxin plays a catalytic role in the conversion of the proclotting enzyme to the enzyme in the Limulus lysate system (4). The finding that DMSO had no effect on the active form of the enzyme is in agreement with this hypothesis. We suggest that DMSO rendered endotoxin ineffective as a catalyst for the conversion of the proclotting enzyme to the enzyme by preventing its association with its proclotting enzyme. Studies are underway to elucidate the mechanism of this inhibitory reaction by DMSO.

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