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Limulus ANTI-LPS FACTOR: AN ANTICOAGULANT WHICH INHIBITS THE ENDOTOXIN-MEDIATED ACTIVATION OF Limulus COAGULATION SYSTEM

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SUMMARY: Exposure of Limulus amebocytes to bacterial endotoxins (lipopolysaccharides, LPS) results in the activation of the coagulation system, which consists of several protein components. During the separation of these components, a potent anticoagulant, named tentatively anti-LPS factor, which inhibits the endotoxin-mediated coagulation reaction, was found in both amebocytes from the hemolymphs of Tachypleus tridentatus and Limulus polyphemus. The principle purified partially from Tachypleus amebocyte lysate had a molecular weight less than 10,000, as judged with the ordinary gel-filtration experiment. It inhibited specifically the activation of factor B, which has recently been characterized to be a coagulation factor highly sensitive to LPS, but it did not inhibit the activities of the active factor B and the active clotting enzyme separated from the lysate. The inhibitory activity of anti-LPS factor disappeared almost completely by the treatments with pronase-P and subtilisin, suggesting its polypeptide-like substance, but it resisted to a boiling treatment. A possible site of the anticoagulant action on the Limulus coagulation system was discussed.

Lipopolysaccharides (LPS) located in the outer membrane of Gram-negative bacteria have a variety of biological activities (1). One of them is a biological effect which is known to induce the clot formation of Limulus amebocyte lysate. The principle of so-called "Limulus test" for detection of endotoxin is based on the LPS-induced coagulation reaction, using amebocyte lysate (2-5). We have studied biochemically the LPS-mediated coagulation system in amebocytes (6-10) and recently found that it consists of several clotting factors similar with the mammalian blood coagulation system, which involves the sequential activation of many zymogens (11,12). In the course of these studies, a potent anticoagulant, which inhibits specifically the LPS-mediated activation of one of the coagulation factors, factor B, was found in Limulus amebocytes. This paper will describe the occurrence, partial purification and a possible mode of action of the anticoagulant.

MATERIALS AND METHODS

The amebocyte lysate from Tachypleus tridentatus was prepared by the previous method (6). Lyophilized powder of Limulus polyphemus amebocyte

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lysate was a generous gift from Dr. Jack Levin, The Johns Hopkins University. Chromogenic substrate, Boc-Leu-Gly-Arg-p-nitroanilide (pNA), was a product of the Protein Research Foundation, Minoh, Osaka. A lipopolysaccharide prepared from E. coli O111-B4 was a product of Sigma Chemical Co., St. Louis. A pyrogen-free carboxymethylated (1→3)-β-D-glucan (CMPS) used here was kindly supplied by Dr. Atsushi Kakinuma, Central Research Division, Takeda Chemical Industries, Ltd., Osaka. Heparin-Sepharose CL-6B was prepared by the method of March et al (13). Sepharose CL-6B was purchased from Pharmacia Fine Chemicals, Uppsala. Bovine α-chymotrypsin (65 units/mg) and porcine elastase (8.5 units/mg) were products of Worthington Biochemical Corp. Freehold, New Jersey. Pronase-P, 750 Tyr units/mg, was purchased from Kaken Chemical Co., Ltd. Subtilisin (7-15 units/mg) was a product of Sigma Chemical Co., St. Louis. All glasswares and buffer solutions were sterilized according to the method previously described (5,6). All procedures for column chromatography were performed at 4°C under the sterilized condition.

Methods for the activity measurements of Limulus coagulation factor:

a) Factor B; The reaction mixture containing 50 µl sample, 30 µl LPS (600 ng/ml) and 100 µl 0.2 M Tris-HCl buffer, pH 8.0, containing 13 mM $MgCl_2$ (buffer A) was preincubated at 37°C for 15 min. Then, 20 µl 5 mM chromogenic substrate and 50 µl fraction A in Fig. 1 was added to the reaction mixture and incubated at 37°C for 10 min. After incubation, 0.8 ml of 0.6 M acetic acid was added to terminate the reaction and the absorbance at 405 nm was measured.

b) Proclotting enzyme; The reaction mixture containing 50 µl sample, 50 µl activated factor B and 100 µl buffer A, was preincubated at 37°C for 30 min. Then, 50 µl 2 mM chromogenic substrate was added to the reaction mixture and the appearance of amidase activity was measured. Activated factor B was prepared by incubating 400 µl of fraction B_2 in Fig. 1 with 200 µl LPS (400 ng/ml) and 200 µl 0.4 M Tris-HCl-26 mM $MgCl_2$ buffer, pH 8.0, (buffer B) for 15 min at 37°C.

c) Factor G; The reaction mixture containing 50 µl sample, CMPS (final 20 ng/ml) and 0.4 mM chromogenic substrate and 100 µl of buffer A, in a total volume of 250 µl, was incubated at 37°C for 30 min. After incubation, 0.8 ml of 0.6 M acetic acid was added and the absorbance at 405 nm was measured.

d) Anti-LPS factor; The mixture containing 50 µl sample, fractions A and B and buffer B was mixed with 20 µl 5mM chromogenic substrate and 30 µl LPS (400 ng/ml). After incubation at 37°C for an appropriate time, the appearance of the amidase activity was measured. One unit of anti-LPS factor is defined as the amount of inhibitor which inhibits 50 % of the activation of factor B mediated with 2 ng of LPS.

RESULTS

A stepwise elution pattern of Tachypleus amebocyte lysate on a heparin-Sepharose CL-6B column is shown in Fig. 1. Proclotting enzyme which was activated by factor B previously reported (11) was eluted in three fractions (fractions A, G and B in Fig. 1). Factor G sensitive to (1→3)-β-D-glucan derivative (12) and coagulogen (6) were found in fraction G. On the other hand, factor B sensitive to LPS was eluted in the two separate fractions, B_1 and B_2, and the result suggested that an inhibitor, which disturbs the LPS-mediated activation of factor B, may be contained in the fractions between fractions B_1 and B_2. We named tentatively this principle as anti-LPS factor.

To remove proclotting enzyme and factor B coexisted in the fractions containing anti-LPS factor, the fractions indicated by solid bar in Fig. 1 were collected and applied to a column of Sepharose CL-6B. The result is shown in Fig. 2. On this molecular sieving, anti-LPS factor appeared in the retarded fractions and was completely separated from proclotting enzyme and factor B.

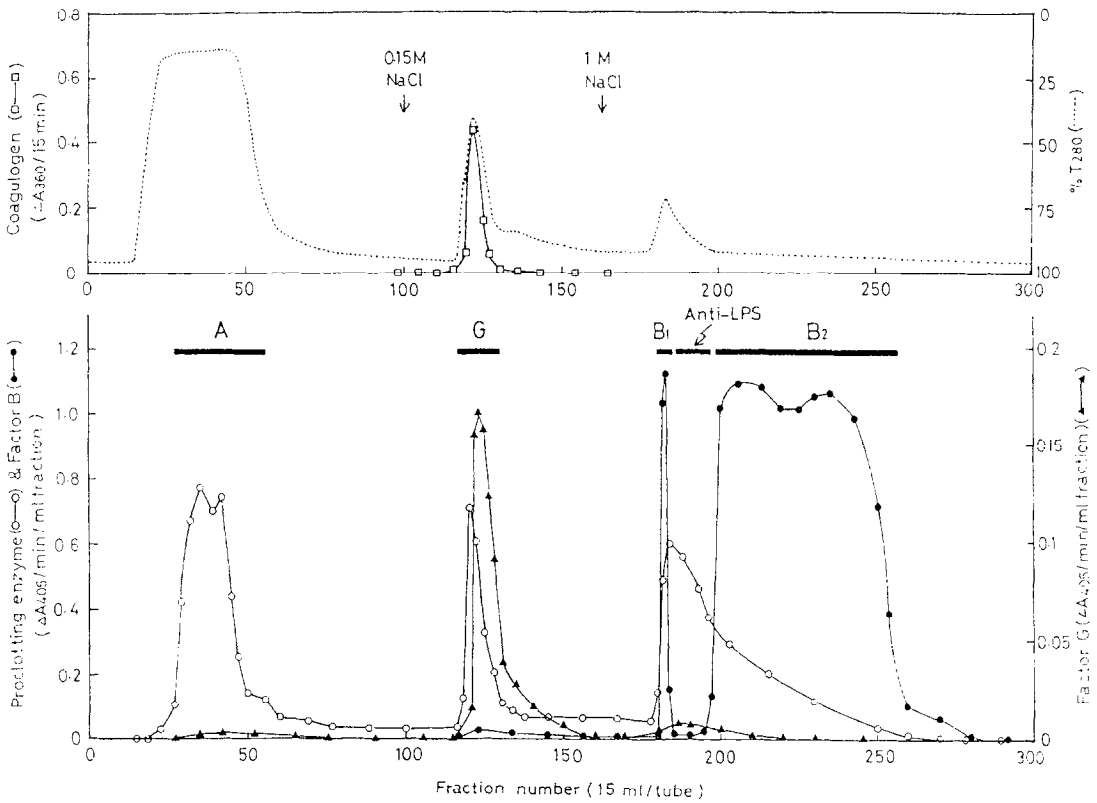

Fig. 1. Heparin-Sepharose CL-6B column chromatography of Tachypleus tridentatus amebocyte lysate. The lysate (340 ml) was applied to a column (5 x 17 cm), pre-equilibrated with 0.05 M Tris-HCl buffer, pH 7.2. The stepwise elution was performed at 4°C firstly with the equilibration buffer, secondly with the buffer containing 0.15 M NaCl and finally with the buffer containing 1.0 M NaCl. Fractions of 15 ml were collected at a flow rate of 83 ml per hr, and the fractions A, G, B_1, anti-LPS and B_2 indicated by solid bars were collected. The activities of proclotting enzyme (O), factor G (▲), factor B (●), coagulogen (□) and transmittance at 280 nm (---) were measured, and the detailed procedures were described in MATERIALS AND METHODS.

The fraction Nos. 195-204, in Fig. 2 were combined, lyophilized and desalted by a column of Sephadex G-50. As shown in Fig. 3, anti-LPS factor was found as a single peak in the fractions before a salt was eluted. From this result, anti-LPS factor appeared to have a molecular weight less than 10,000.

The same experiments as made for the lysate from T. tridentatus was performed using the lysate from Limulus polyphemus. The elution pattern of L. polyphemus lysate on a heparin-Sepharose CL-6B column was almost identical to that shown in Fig. 1, and the anti-LPS factor activity was found in fraction B, which was eluted with 1 M NaCl (data not shown). Thus, a substance similar with the Tachypleus anti-LPS factor must be contained in L. polyphemus amebocyte lysate.

Table 1 shows the effect of the partially purified anti-LPS factor on LPS- and CMPS-induced activation of Limulus coagulation system previously

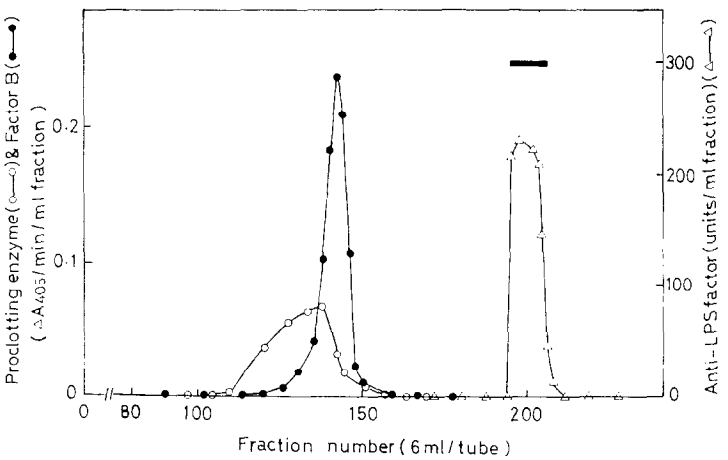

Fig. 2. Gel filtration of anti-LPS fraction on Sepharose CL-6B. Pooled fraction (fraction Nos. 184 to 196 in Fig. 1) was applied to a column (3.5 x 122.5 cm) of Sepharose CL-6B. Elution was performed with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl at a flow rate of 47 ml per hr. The activities of proclotting enzyme (○), factor B (●) and anti-LPS factor (△) were measured by the methods described in text.

reported (11,12). In these experiments, a recombination system with proclotting enzyme (fraction A in Fig. 1) and factor B (fraction B_2) or a system with proclotting enzyme and factor G (fraction G) was used, in addition to Limulus amebocyte lysate, which contains all the coagulation factors sequentially activated in the presence of LPS or CMPS. As shown in experiment 1, the appearance of LPS-induced amidase activity due to the clotting enzyme in the lysate was strongly inhibited in the presence of anti-LPS factor. The same inhibitory effect of anti-LPS factor on the recombination system with fractions A and B was observed (exp. 2). However, there was no inhibitory

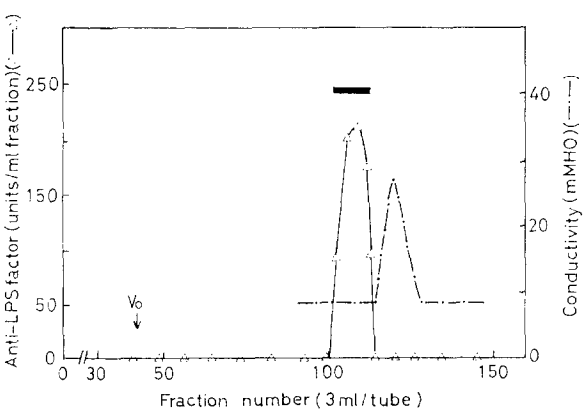

Fig. 3. Desalting of anti-LPS fraction on Sephadex G-50 (fine). Anti-LPS fraction (fraction Nos. 195-204 in Fig. 2) was collected, lyophilized and desalted on a column (2.2 x 95 cm) of Sephadex G-50. Elution was carried out with 0.02 M HCl at a flow rate of 38 ml per hr. The activity of anti-LPS factor was measured by the methods described in text.

Table 1

Effect of anti-LPS factor on LPS- and CMPS-induced activation of Limulus coagulation system

Exp.	Sample	Trigger	Amidase activity		Inhibition (%)
			Without anti-LPS	With anti-LPS	
			μmoles pNA released/30 min/ml (x 10^{-4})		
1	Amebocyte lysate	LPS	682	115	83.1
2	Fr A+B	LPS	335	30	91.0
			μmoles pNA released/17 min/ml (x 10^{-4})		
3	Amebocyte lysate	CMPS	288	315	0
4	Fr A+G	CMPS	295	316	0
			μmoles pNA released/18 min/ml (x 10^{-4})		
5	Active clotting enzyme		165	170	0
6	Activated factor B + proclotting enzyme (Fraction A)		210	220	0

The reaction mixture containing 20 μl Tachypleus amebocyte lysate or the mixtures containing 50 μl each of fractions in Fig. 1 and LPS (final, 24 ng/ml) was incubated at 37°C for 30 min in the presence of 30 μl of anti-LPS fraction in Fig. 2. After incubation, the LPS-induced amidase activity was measured using chromogenic substrate. The same experiments as above were made by using CMPS (final, 24 ng/ml) instead of LPS.

effect of anti-LPS factor on the CMPS-induced activation of Limulus coagulation system (exp. 3). This result was also confirmed by testing the system reconstituted with proclotting enzyme and factor C (exp. 4). Moreover, anti-LPS factor did not any effect on the activities of clotting enzyme and factor B in their activated forms (exp. 5 and 6).

These results indicate that anti-LPS factor inhibits only the activation of Limulus coagulation system mediated with LPS but not with CMPS.

Table 2 shows the stability of partially purified anti-LPS factor treated under various conditions. On heat treatments at neutral and acidic media, the anti-LPS factor was highly stable, whereas it was very unstable at alkaline medium. The inhibitory activity disappeared almost completely by treatments with pronase P and subtilisin, but it relatively resisted to those with α-chymotrypsin and elastase. Thus, the anti-LPS factor seemed to contain a polypeptide portion, which is essential to develop its biological activity.

DISCUSSION

The amebocytes of Limulus hemolymph contain a coagulation system highly sensitive to LPS (2). This system seems to participate not only in hemostasis

Table 2

Effect of various treatments on the anti-LPS factor

Treatment	Relative activity (%)
Without treatment	100
Heat in buffer at 100°C for 5 min[a)]	97.4
Heat in 0.1 N HCl at 100°C for 5 min[a)]	100
Heat in 0.1 N NaOH at 100°C for 5 min[a)]	0
Pronase P at 37°C for 1 hr[b)]	0
Subtilisin Carlsberg; subtilopeptidase A at 37°C for 1 hr[b)]	7.2
α-Chymotrypsin at 37°C for 1 hr[b)]	79.7
Elastase at 37°C for 1 hr[b)]	82.5

a) Anti-LPS fraction (270 µl) in Fig. 1 was treated under the conditions described in Table, and then rapidly cooled and neutralized with 30 µl of 1 N NaOH or 1 N HCl, respectively. The aliquots (30 µl each) were taken for the assay of anti-LPS activity.

b) Anti-LPS (200 µl) fraction in Fig. 1 was incubated, with 20 µl each of pronase-P (1.13 mg/ml), subtilisin (2 mg/ml), α-chymotrypsin (0.5 mg/ml) or elastase (2 mg/ml). Then, the reaction was stopped by boiling for 5 min, and the remaining anti-LPS activity was measured.

but also in defence against invading microorganisms (2,3). In the amebocyte lysate, LPS seems to induce the activation of factor B and its active form mediates the activation of proclotting enzyme, which results in the conversion of coagulogen to coagulin gel (11). On the other hand, there exists another component, factor G, sensitive to (1→3)-β-D-glucan, and its active form mediates also the activation of proclotting enzyme (12). Thus, Limulus amebocytes contain two independent coagulation pathways, LPS-mediated and (1→3)-β-D-glucan-mediated pathways, both of which result in the gel formation (12).

Anti-LPS factor found in Tachypleus amebocyte lysate appears to inhibit exclusively the LPS-mediated coagulation pathway (Table 1). Its inhibitory effect is presumed upon the activation of factor B mediated with LPS, because no such effect was observed on both active forms of proclotting enzyme and factor B. Although we have not reconstituted the LPS-mediated Limulus coagulation system using highly purified components, the following possible mode of action of anti-LPS factor could be considered based on the present data: 1) its neutralizing effect on bacterial endotoxins, 2) its competitive binding effect on an LPS-interaction site with factor B, and 3) its degradation

effect on LPS. Moreover, a bactericidal action of anti-LPS factor in biological defence of Limulus against invading microorganisms can be considered. To resolve these problems, further experimentation will be required, in addition to the chemical characterization of anti-LPS factor.

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