A NEW ENDOTOXIN SENSITIVE FACTOR ASSOCIATED WITH HEMOLYMPH COAGULATION SYSTEM OF HORSESHOE CRAB (LIMULIDAE)

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1. Introduction

The amebocytes in horseshoe crab hemolymph contain a coagulation system [1], which is activated by 10^{-9} g Gram-negative bacterial endotoxins (lipopolysaccharide). This system is associated with cell aggregation, degranulation and release of a clottable protein, coagulogen [2]. The principle of the so-called 'Limulus test' for detection of endotoxin is based on the endotoxin-induced coagulation reaction, using amebocyte lysate [3]. In [4] the amebocyte lysate endotoxin activated directly a proclotting enzyme resulting in the transformation of coagulogen to coagulin (fig.1A). To establish the molecular events in such a reaction system, we have reported on the gelation mechanism of coagulogen to coagulin using a purified active clotting enzyme [5,6], on the complete amino acid sequence of coagulogen [7,8], and on the development of a new method for determination of endotoxin, using chromogenic or fluorogenic peptide substrate of the clotting enzyme [9–12].

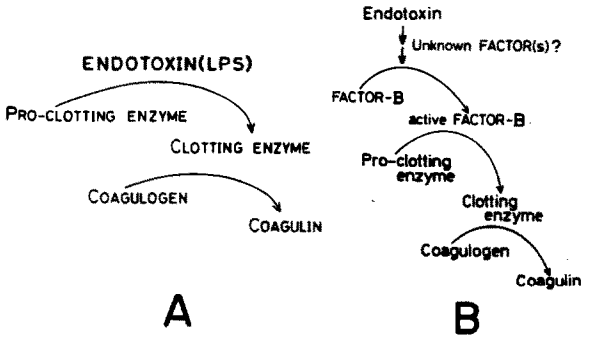


Fig.1. Schematic representation (A, old and, B, new) of coagulation system in horseshoe crab amebocyte lysate.

During these investigations, we found that proclotting enzyme shown in fig.1A is not an endotoxin-sensitive protein but that another unknown factor (tentatively named factor B) sensitive to endotoxin is involved in the reaction sequence which mediates the activation of proclotting enzyme. Evidence for a new reaction scheme (fig.1B) of the endotoxin-induced cascade reaction will be presented here.

2. Materials and methods

Boc-Leu–Gly–Arg–*p*-nitroanilide (pNA) was a product of the Protein Research Foundation, Minoh, Osaka. A lipopolysaccharide prepared from *Escherichia coli* 0111-B4 was a product of Difco Labs., and heparin–Sepharose CL-6B was from Pharmacia Fine Chemicals, Uppsala. All glass ware and buffer solutions were sterilized by heating at 200°C or autoclaving for 3 h [5]. The amebocyte lysate from Japanese horseshoe crabs *(Tachypleus tridentatus)* was prepared by the method in [5]. Lyophilized powder of *Limulus polyphemus* amebocyte lysate was a generous gift from Dr Jack Levin, The John Hopkins University. Amidase activity for active clotting enzyme was measured using the chromogenic peptide substrate as in [9,10].

3. Results

3.1. *Heparin–Sepharose column chromatography of amebocyte lysate from* T. tridentatus

To isolate proclotting enzyme, 5 ml lysate was applied to a column of heparin–Sepharose. The result is shown in fig.2. In the breakthrough fraction (frac-

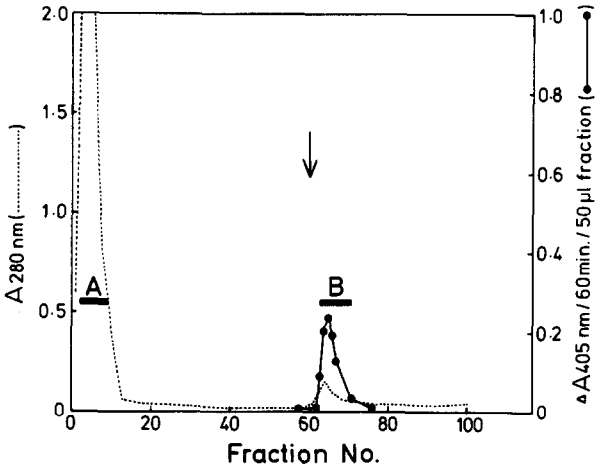


Fig.2. Heparin–Sepharose column chromatography of *T. tridentatus* amebocyte lysate. Lysate (5 ml) was applied to a column (1.4 × 2.3 cm), equilibrated with 0.05 M Tris–HCl buffer (pH 7.2) containing 0.154 M NaCl and 1 mM $CaCl_2$. The elution was performed at 4°C first with the equilibration buffer and then with the buffer containing 0.454 M NaCl and 1 mM $CaCl_2$. Fractions of 3.4 ml were collected at a flow rate of 8.4 ml/h and the fractions indicated by solid bars were collected.

tion A), no amidase activity was detected even after pre-incubation with endotoxin for 1 h. On the other hand, a weak amidase activity was found in the adsorbed fraction (fraction B) after preincubation with endotoxin for 1 h, and this amidase activity was not demonstrated in the absence of endotoxin. However, total amidase activity recovered from the column was <5% of that of the lysate used. This low recovery suggested that an unknown factor required for the activation of proclotting enzyme may have been separated by this column. To test this possibility, a reconstitution experiment with fractions A and B was performed under the conditions described in table 1. A strong amidase activity appeared only in the combined mixture of fractions A and B, indicating that both fractions are essential to develop the amidase activity in the presence of endotoxin.

3.2. *Evidence for unknown factors sensitive to endotoxin*

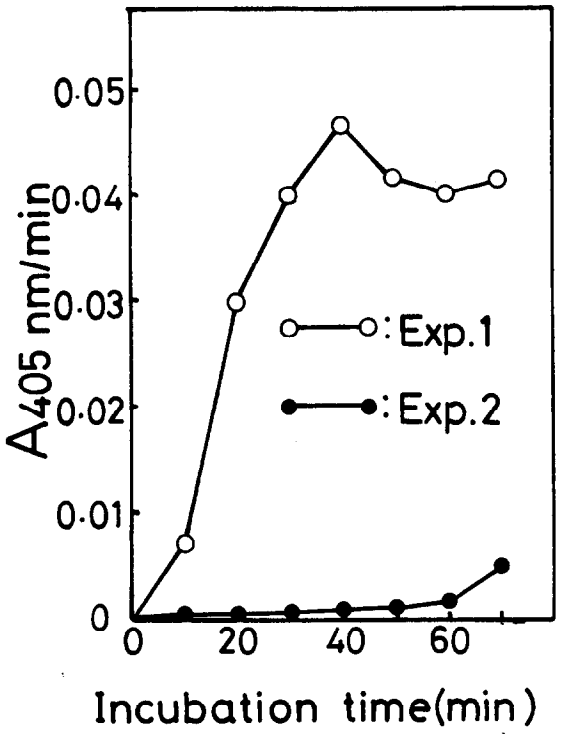
Fig.3 shows the amidase activity produced in the mixture of fractions A and B after treatment of each fraction, independently, with endotoxin. The experimental procedures are shown in the same figure. A strong amidase activity was found only when fraction B was pre-incubated with endotoxin (expt 1). Experiment 2 was the control to expt 1, in which fraction B was pre-incubated without endotoxin. On the contrary, when fraction A was preincubated with endotoxin and then incubated with fraction B (expt 3), the amidase activity was almost identical to that of the control mixture shown in expt 4. These results suggest that an endotoxin-sensitive factor must be contained in fraction B, and the proclotting enzyme in fraction A.

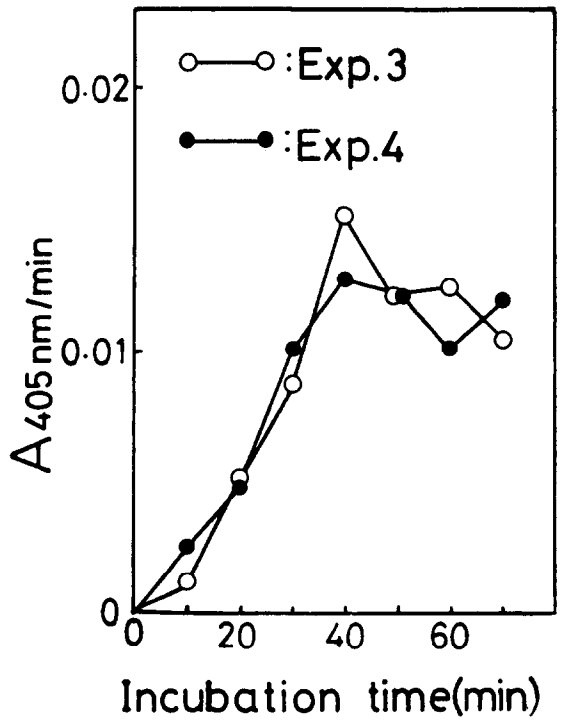
To confirm this point, fraction A or B was incubated, respectively, with varying amounts of fraction B or A, and the amidase activity developed in the mixture was measured. The results are shown in fig.4A,B. The increase of endotoxin-mediated amidase activity was proportional to the amount of fraction A added to the reaction mixture (fig.3A), while the amidase activity reached a plateau when increasing amounts of fraction B were added to a constant amount of fraction A. These results indicate that a component contained in fraction A is the known

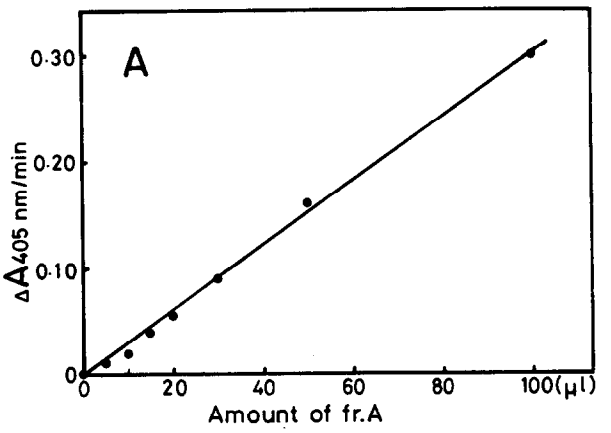
Table 1
Recombination experiments of fractions A and B obtained from fig.2

Fractions from heparin–Sepharose column	Amidase activity (μmol pNA released . min^{-1})	
	Without LPS	With LPS
Breakthrough fraction no. 3(A)	0	0
Adsorbed fraction no. 65(B)	0	0.009
Fraction A + B	0	1.182

The reaction mixture containing 50 μl fraction A or 125 μl fraction B, 200 μl 0.4 M Tris–HCl–40 mM $MgCl_2$ buffer (pH 8.0), 275 μl saline and 50 μl endotoxin (1 μg/ml), was pre-incubated at 37°C for 20 min. Then, 200 μl mixture was taken and the amidase activity was measured. In recombination experiment, 75 μl fraction A and 50 μl fraction B were mixed and pre-incubated in the presence of endotoxin under the same conditions as above







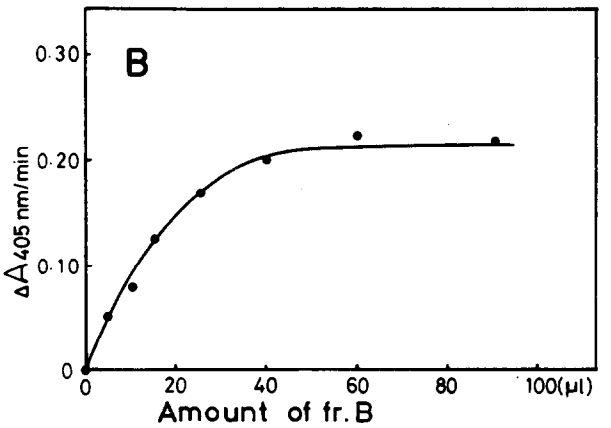


Fig.4. Amidase activity in the reaction mixtures when varying amounts of fraction A or B was mixed with a constant amount of fraction B or A. The mixture containing 250 μl fraction B, 75 μl saline and 125 μl 0.4 M Tris–HCl containing 40 mM $MgCl_2$, was pre-incubated with 50 μl LPS (1 μg/ml) at 37°C for 15 min. In expt A, 50 μl of this endotoxin-treated fraction B and the indicated amounts of fraction A were mixed and incubated at 37°C for 60 min. Then, 20 μl aliquots of the incubation mixture were taken and the amidase activity was measured. In expt B, 25 μl of fraction A and the indicated amounts of endotoxin-treated fraction B were used.

Fig.3. Amidase activity produced in the reaction mixture of fractions A and B after treatment of each fraction with endotoxin. In expt 1, the mixture containing 200 μl fraction B, 125 μl 0.4 M Tris–HCl–40 mM $MgCl_2$ buffer (pH 8.0), 50 μl LPS (1 μg/ml) and 125 μl saline, was pre-incubated at 37°C for 30 min. Then, 50 μl aliquots were taken and mixed with 200 μl fraction A, and the amidase activity was measured after incubation for the time indicated. In expt 2, the same amount of fraction B as used for expt 1 was pre-incubated without LPS and the subsequent procedures were identical to that of expt 1. The procedures in expt 3 and 4 were as in expt 1 and 2, except that 325 μl fraction A was used.

proclotting enzyme and a component in fraction B, named tentatively factor B, is its activator.

The same experiments as made for the lysate from *T. tridentatus* was performed using the lysate from *L. polyphemus*. The elution pattern of *L. polyphemus* lysate on a heparin–Sepharose column was completely identical to that shown in fig.2, and the proclotting enzyme and endotoxin-sensitive factor were separated, respectively, in the breakthrough fraction and adsorbed fraction. Furthermore, the same results as in the reconstitution experiments shown in table 1 were obtained using fractions A and B from *L. polyphemus* lysate.

4. Discussion

In 1977, a proclotting enzyme purified from *L. polyphemus* amebocyte lysate was reported to be activated by Ca^{2+} and endotoxin, thereby effecting gelation of the clottable protein, coagulogen [4]. However, the present data do not support these results. Our results indicate that both amebocyte lysates from *T. tridentatus* and *L. polyphemus* contain at least two components beside coagulogen, all of which are associated with the coagulation system. One of them is a new component sensitive to endotoxin, named factor B, and the other is a component corresponding to the known proclotting enzyme but insensitive to endotoxin. This proenzyme shows a strong amidase activity after incubation with factor B. On the other hand, factor B shows a very weak endotoxin-dependent amidase activity and seems to be an endotoxin-sensitive proteinase, which activates the proclotting enzyme. Thus, all the data presented here indicate that the endotoxin-mediated coagulation system in the amebocytes consists of a multienzyme system shown in fig.1B, and that this cascade system may provide an extremely high sensitivity of the lysate to bacterial endotoxin. Further experimentation will be required to establish whether factor B activates directly the proclotting enzyme or whether factor B is a single component or not.

Acknowledgements

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References

[1] Levin, J. and Bang, F. B. (1964) Bull. John Hopkins Hosp. 115, 265–274.
[2] Mürer, E. H., Levin, J. and Holme, R. (1975) J. Cell Physiol. 86, 533–542.
[3] Yin, E. T., Galanos, C., Kinsky, S., Bradshaw, R. A., Wessler, S., Lüderitz, O. and Samiento, M. E. (1972) Biochim. Biophys. Acta 261, 284–289.
[4] Tai, J. Y., Seid, R. C. jr, Huhn, R. D. and Liu, T. Y. (1977) J. Biol. Chem. 252, 4773–4776.
[5] Nakamura, S., Iwanaga, S., Harada, T. and Niwa, M. (1976) J. Biochem. 80, 1011–1021.
[6] Nakamura, S., Takagi, T., Iwanaga, S., Niwa, M. and Takahashi, K. (1976) J. Biochem. 80, 649–652.
[7] Nakamura, S., Takagi, T., Iwanaga, S., Niwa, M. and Takahashi, K. (1976) Biochem. Biophys. Res. Commun. 72, 902–908.
[8] Takagi, T., Hokama, Y., Morita, T., Iwanaga, S., Nakamura, S. and Niwa, M. (1979) in: Biomedical Applications of the Horseshoe Crab (Limulidae) (Cohen, E. ed) pp. 169–184, Alan R. Liss, New York.
[9] Nakamura, S., Morita, T., Iwanaga, S., Niwa, M. and Takahashi, K. (1977) J. Biochem. 81, 1567–1569.
[10] Iwanaga, S., Morita, T., Harada, T., Nakamura, S., Niwa, M., Takada, K., Kimura, T. and Sakakibara, S. (1978) Haemostasis 7, 183–188.
[11] Harada, T., Morita, T., Iwanaga, S., Nakamura, S. and Niwa, M. (1979) in: Biomedical Applications of the Horseshoe Crab (Limulidae) (Cohen, E. ed) pp. 209–220, Allan R. Liss, New York.
[12] Harada, T., Morita, T. and Iwanaga, S. (1978) J. Med. Enzymol. (in Japanese) 3, 43–60.