A NEW (1 → 3)-β-D-GLUCAN-MEDIATED COAGULATION PATHWAY FOUND IN *LIMULUS* AMEBOCYTES

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1. Introduction

The amebocytes of horseshoe crab (*Limulus*) hemolymph contain a coagulation system highly sensitive to bacterial endotoxins (lipopolysaccharides) [1,2]. This system seems to participate not only in haemostasis but also in defence against invading microorganisms [3]. In the amebocyte lysate, endotoxin has been reported to mediate directly the activation of a proclotting enzyme resulting in the transformation of coagulation to coagulin gel [4]. However, our recent studies have indicated that both amebocyte lysates from *Limulus polyphemus* and *Tachypleus tridentatus* contain at least two components beside coagulogen, all of which are associated with the coagulation system [5]. One of them is a new component sensitive to endotoxin, tentatively named factor B, and the other is a component corresponding to the known proclotting enzyme but insensitive to endotoxin. During these studies, we were informed that, in addition to endotoxin, a water-soluble antitumor carboxymethylated (1 → 3)-β-D-glucan (CMPS) activated the *Limulus* coagulation system and induced the clot formation [16]. This result prompted us to examine which component associated with the coagulation system is activated by CMPS. We report here that neither factor B nor proclotting enzyme previously identified is sensitive to CMPS but that there exists a third component (tentatively named factor G) sensitive to CMPS, which mediates the activation of the proclotting enzyme to its active form. The results also suggest that the *Limulus* amebocytes contain two independent coagulation pathways, endotoxin-mediated and (1 → 3)-β-D-glucan-mediated pathways, both of which result in the transformation of coagulogen to coagulin. The latter pathway seems to correspond to an alternative pathway found in the mammalian complement system.

2. Materials and methods

The amebocyte lysate from *Tachypleus tridentatus* (Japanese horseshoe crab) was prepared according to the method in [7]. A pyrogen-free carboxymethylated (1 → 3)-β-D-glucan (CMPS) used here was kindly supplied by Dr A. Kakinuma, Central Research Division, Takeda Chemical Industries (Osaka). Boc-Leu–Gly–Arg–*p*-nitroanilide (pNA) used as substrate for the *Limulus* clotting enzyme assay was a product of the Protein Research Foundation (Minoh, Osaka) and the amidase activity was measured by the method in [8,9]. Factor B used for the assay of preclotting enzyme was a preparation obtained in [5]. Endotoxin prepared from *E. coli* 0111-B4 was a product of Sigma Chemical Co. (St Louis MO). Heparin–Sepharose CL-6B was prepared as in [10]. All glassware and buffer solutions were sterilized by heating at 200°C for 2 h and autoclaving at 120°C for 1.5 h, respectively.

3. Results and discussion

Fig.1 shows chromatographic separation of the lysate on a heparin–Sepharose CL-6B column. When each fraction was incubated with factor B and Boc-Leu–Gly–Arg–pNA in the presence of endotoxin, a strong amidase activity appeared in both fractions A (proclotting enzyme fraction [5]) and G, and the latter fraction contained coagulogen, which was detected by treating it with TPCK-trypsin. However, factor B sensitive to endotoxin [5] was eluted in fraction B in

an inactive form and its activity was easily detected after incubation with fraction A and Boc-Leu–Gly–Arg–pNA in the presence of endotoxin.

The fractions A, G and B indicated by solid bars in fig.1 were collected and their amidase activities and clot-forming abilities were measured after treatments of the pooled fractions with CMPS instead of endotoxin. As shown in exp. 1–3 of table 1, these activities were found only in fraction G, indicating that the fraction contains a CMPS-sensitive factor (tentatively named factor G). This fraction G also developed the amidase activity in the presence of a native (1 → 3)-β-D-glucan (not shown). These results indicate that factor G found in fraction G is a new component sen-

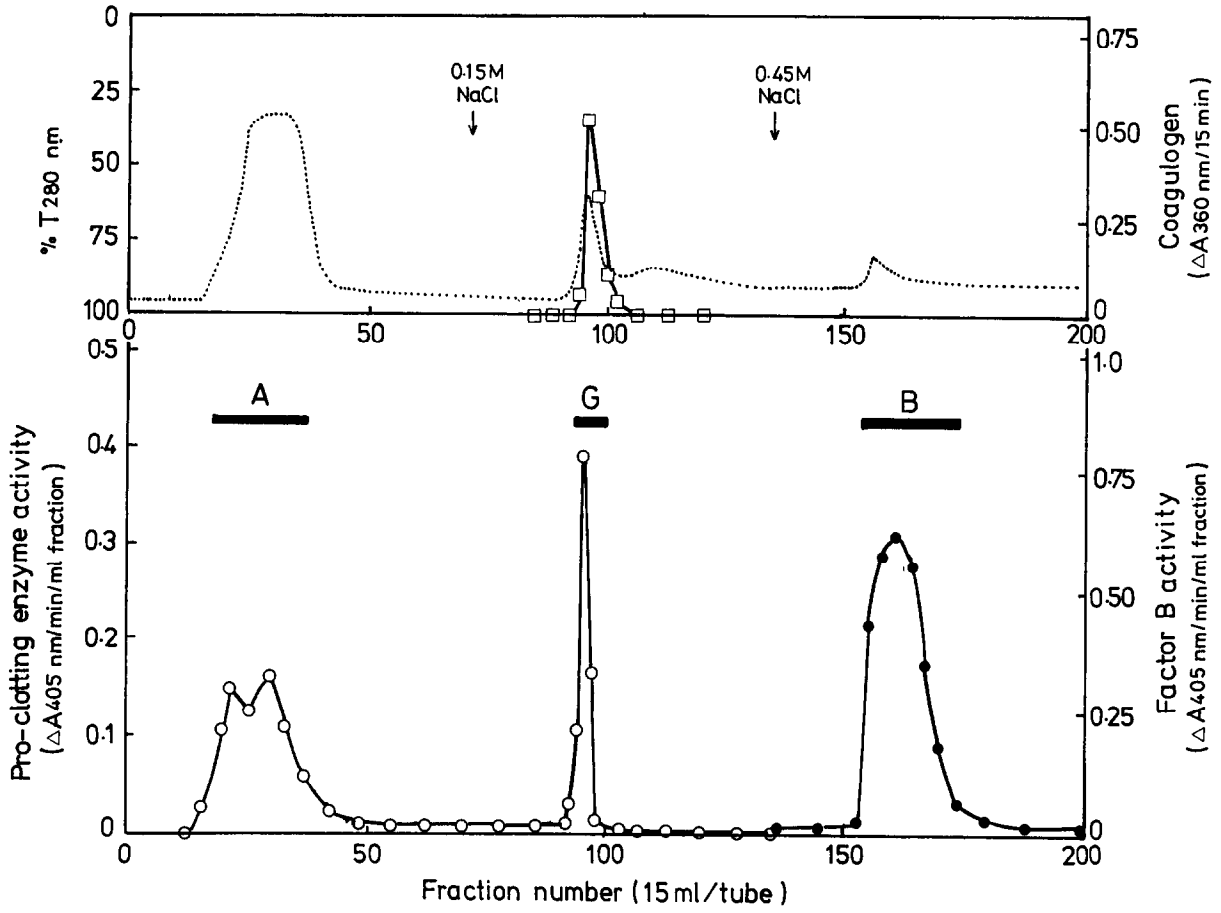

Fig.1. Heparin–Sepharose CL-6B column chromatography of *Tachypleus tridentatus* amebocyte lysate. The lysate (50 ml) prepared as in [7] was diluted 3-fold, using 0.05 M Tris–HCl buffer (pH 7.2) and applied to a column (5.0 × 1.7 cm), pre-equilibrated with the same buffer. The stepwise elution was performed at 4°C firstly with the equilibration buffer, secondly with the buffer containing 0.15 M NaCl, and finally with the buffer containing 0.45 M NaCl. Fractions of 15 ml were collected at a flow rate of 73 ml/h, and the fractions A, G and B indicated by solid bars were collected. The amidase activity of the proclotting enzyme (○) was measured by activating it to the clotting enzyme in the presence of factor B and endotoxin. The reaction mixture contained 50 μl of each fraction, 50 μl factor B (A_{280} = 0.085), 0.4 mM Boc-Leu–Gly–Arg–pNA, endotoxin (final, 80 ng/ml), 80 mM Tris–HCl buffer (pH 8.0) and 5.2 mM $MgCl_2$, in a total volume of 250 μl. After incubation at 37°C for 20 min, 0.8 ml 0.6 M acetic acid was added to terminate the reaction and the absorbance at 405 nm was measured. Factor B activity (●) was detected by incubating each fraction with fraction A in the presence of endotoxin and the chromogenic substrate. The reaction mixture was the same as that above, except that 50 μl of fraction A instead of factor B was added. Coagulogen (□) was detected by testing the clot formation induced by trypsin; the mixture containing 500 μl fractions, 20 μl TPCK-trypsin (1 mg/ml) and 100 μl 0.2 M Tris–HCl/20 mM $CaCl_2$ buffer (pH 8.0) was incubated at 37°C for 15 min, and then 1.0 ml saline was added. The turbidity was measured spectrophotometrically at 360 nm. Transmittance at 280 nm (····).

Table 1
Recombination experiments of fractions A, G and B in the presence of carboxymethylated (1 → 3)-β-D-glucan (CMPS)

Exp.	Fraction	Amidase activity ($\times 10^{-4}$) (μmol pNA released/ml)	Clot-forming ability
1	Fraction A	0	–
2	Fraction G	284	+
3	Fraction B	0	–
4	Fractions A + G	455	+
5	Fractions A + B	0	–
6	Fractions G + B	373	+
7	Fractions A + G + B	491	+

The reaction mixture containing 50 μl each of fraction, CMPS (final, 24 ng/ml), 0.4 mM chromogenic substrate, 80 mM Tris–HCl buffer (pH 8.0) and 5.2 mM $MgCl_2$, in a total volume of 250 μl, was incubated at 37°C for 30 min. Then, 0.8 ml 0.6 M acetic acid was added to terminate the reaction and the absorbance at 405 nm was measured [9]. In recombination experiments, 50 μl each of fractions A, G or B was mixed for each other and the amidase activity was measured under the same conditions as above. A clot-forming ability of each fraction was tested by using a highly purified coagulogen [12] as follows. The reaction mixture contained 50 μl each of fractions A, G and B or their combined mixture, 30 μl CMPS (400 ng/ml), 100 μl *Tachypleus* coagulogen (2 mg/ml) and 50 μl 0.4 M Tris–HCl buffer (pH 8.0) containing 26 mM $MgCl_2$, in a total volume of 330 μl. When a clot appeared within 1 h at 37°C, it was judged that the sample showed a positive reaction

Table 2
Recombination experiments of fractions A, G and B in the presence of *E. coli* 0111-B4 endotoxin

Exp.	Fraction	Amidase activity ($\times 10^{-4}$) (μmol pNA released/ml)	Clot-forming ability
1	Fraction A	0	–
2	Fraction G	0	–
3	Fraction B	57	–
4	Fractions A + G	0	–
5	Fractions A + B	326	+
6	Fractions G + B	414	+
7	Fractions A + G + B	662	+

The reaction conditions in these experiments were those of table 1, except that endotoxin (final, 24 ng/ml) instead of CMPS was used

sitive to CMPS, and that it differs from coagulogen, proclotting enzyme and factor B all associated with the *Limulus* coagulation system.

To elucidate further the relationship among the components in fractions A, G and B, a recombination experiment was performed, using CMPS as a mediator for the coagulation reaction. The results are also shown in table 1. A mixture of fraction G with fraction A (exp. 4) or fraction B (exp. 6) in the presence of CMPS showed a stronger amidase activity, as compared with fraction G alone. However, there was neither amidase activity nor clot-forming ability in the combination of fractions A and B (exp. 5). The maximum amidase activity was obtained in the mixture of fractions A, G and B (exp. 7). These results suggest again that a CMPS-sensitive factor, factor G, must be contained in fraction G and that the factor induces the activation of the known proclotting enzyme eluted in fraction A. However, there is a possibility that factor G activates a hitherto unknown proclotting enzyme eluted in fraction G, since the fraction itself treated with CMPS showed a strong amidase activity in the absence of fraction A (exp. 2). It is also possible to presume that active factor G itself may have a clot-forming activity.

Table 2 shows the recombination experiments in the presence of endotoxin. There was little or no amidase activity and clot-forming ability in fractions A, G and B alone (exp. 1–3). However, on incubation of the reaction mixture of fractions A and B, a strong amidase activity, in addition to the clot-forming ability, appeared, as shown in exp. 5 (table 2), and the result was the same as that in [5]. Moreover, the combined mixture of fractions G and B showed a stronger amidase activity and formed a clot (exp. 6).

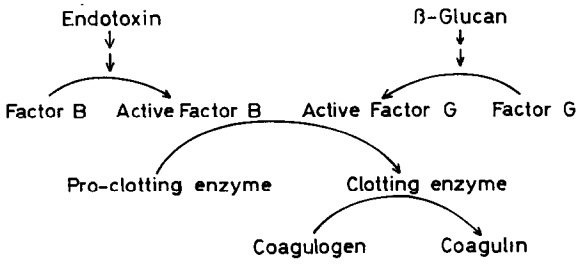

Fig.2. Tentative mechanism for the coagulation cascade found in *Limulus* amebocyte lysate. Doubtless, this cascade sequence will need modification as new factors and cofactors are discovered, and further studies will be required for the identification of each component.

On the contrary, the combined mixture of fractions A and G did not show any activities, indicating that both were insensitive to endotoxin (exp. 4). The maximum amidase activity was obtained in the recombination of fractions A, G and B (exp. 7). These results indicate that factor G differs completely from factor B in its inability to respond to endotoxin.

Based on the results described above, we would suggest new (1 → 3)-β-D-glucan-mediated coagulation pathway in *Limulus* amebocytes, as shown in fig.2. Thus, CMPS activates factor G and the active factor G converts the known proclotting enzyme to the clotting enzyme, which then catalyzes the transformation of coagulogen to coagulin gel. To establish the glucan-mediated pathway, in addition to the endotoxin-mediated pathway postulated in [5,11], detailed biochemical studies on the isolation and characterization of each component will be required.

Acknowledgements

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