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GELATION OF LIMULUS AMOEBOCYTE LYSATE BY AN ANTITUMOR (1→3)-β-D-GLUCAN

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SUMMARY

An antitumor carboxymethylated (1→3)-β-D-glucan (CMPS) was found to have a potent ability to cause gelation of the amoebocyte lysate of horseshoe crab at concentrations as low as 10^{-6} mg/ml. The gelation of the lysate and the activation of the proclotting enzyme in the lysate caused by CMPS were unique in that these reactions occurred at CMPS concentrations ranging from 10^{-6} to 10^{-3} mg/ml. The optimum concentration was 10^{-5} - 10^{-4} mg/ml and at concentrations above 10^{-2} mg/ml no gelation occurred. This gelation pattern was also observed in common with other antitumor polysaccharides. The mechanism of the gelation caused by CMPS was revealed to be distinctly different from that working in the gelation caused by endotoxins.

INTRODUCTION

The Limulus test is now widely used as a rapid, simple, and sensitive in vitro method for the detection of minute amounts of endotoxins (pyrogens, bacterial lipopolysaccharides) in pharmaceuticals (1,2). The method is based on the gelation of amoebocyte lysate of horseshoe crab by reaction with endotoxins (3). Knowledge on the molecular events involved in the gelation reaction is rapidly accumulating and the currently accepted mechanism of the gelation is summarized as follows. The lysate contains a proclotting enzyme that is transformed to the active form in the presence of endotoxins. The clotting enzyme thus generated then coagulates a clottable protein, coagulogen, also contained in the lysate (4,5). Recently, the presence of a new endotoxin-sensitive component named factor B, has been proposed; it is transformed to active factor B, which then mediates the activation of the proclotting enzyme (6).

In the course of testing the pyrogenicity of a water-soluble antitumor carboxymethylated (1→3)-β-D-glucan (CMPS) (7,8), we have observed that its pyrogen-free preparation gave a positive Limulus test at a concentration as low as 10^{-6} mg/ml. Moreover, the gelation occurred only at CMPS concentrations of

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Abbreviations: PS, a water-insoluble and thermogelable (1→3)-β-D-glucan produced by Alcaligenes faecalis var. myxogenes IFO 13140; CMPS, carboxymethylated PS; pNA, p-nitroanilide.

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10^{-6} - 10^{-3} mg/ml. Detailed investigations have revealed that the gelation caused by CMPS is of a hitherto unknown type distinctly different from that caused by endotoxins.

MATERIALS AND METHODS

CMPS (7,8) was synthesized by carboxymethylation of curdlan 13140 (PS) (9), a water-insoluble and thermogelable linear (1→3)-β-D-glucan produced by Alcaligenes faecalis var. myxogenes IFO 13140. CMPS has a strong antitumor activity against Sarcoma-180 implanted in mice (8). In the present investigation, a CMPS preparation with the number-average degree of polymerization 540 and the average degree of substitution (the average number of carboxymethyl groups per anhydroglucose unit) 0.68 was used after it was freed from pyrogens by treatment with 0.1 N NaOH at 4°C for 16 h, followed by neutralization with acetic acid, precipitation with ethanol and washing with ethanol. As endotoxin, lipopolysaccharide of E. coli O111:B4 (Difco Laboratories, Detroit) was used. CMPS and endotoxin were dissolved in saline before use.

As amoebocyte lysate, Pregel® purchased from Seikagaku Kogyo Co., Ltd. (Tokyo) was used. It contains the lysate from the Japanese horseshoe crab, Tachypleus tridentatus. Gelation of the lysate was tested as follows. The content of a Pregel® ampoule was dissolved in 0.1 ml of distilled water, and 0.1 ml of a CMPS solution was added. After being shaken gently, the mixture was incubated in a water bath at 37°C for 1 h and then at room temperature for 5 min, and the degree of gelation was observed. Activation of the proclotting enzyme in the lysate was estimated by measuring the amidase activity of the clotting enzyme generated by incubation of the lysate with CMPS or endotoxin according to the chromogenic substrate method of Iwanaga et al. (10), using Boc-Leu-Gly-Arg-p-nitroanilide (pNA) (Protein Research Foundation, Minoh, Osaka) as substrate. The proclotting enzyme solution was prepared by dissolving the content of a Pregel® ampoule in 0.3 ml of 0.4 M Tris·HCl-0.04 M $MgCl_2$ (pH 8.0).

Distilled water, 0.4 M Tris·HCl-0.04 M $MgCl_2$ (pH 8.0) and saline were used after they were autoclaved at 120°C for 20 min, filtered through a PM 10 membrane filter (Amicon Far East Ltd., Tokyo), and confirmed not to contain pyrogens by the above-described chromogenic substrate method. All glassware was freed from pyrogens by heating at 250°C for 30 min and all procedures were performed aseptically.

The rabbit pyrogen test was performed according to the method described by Minami et al. (11).

RESULTS AND DISCUSSION

Table 1 shows that CMPS, which gave a negative pyrogenicity in rabbits when injected intravenously at a dose as high as 10 mg/kg, caused gelation of the amoebocyte lysate at 10^{-6} mg/ml. A variety of substances are known to have ability to cause the gelation. Except for endotoxins, however, CMPS seems to be by far more potent than these substances. To our knowledge, there have been no reports describing the gelation of amoebocyte lysate caused by polysaccharides that do not contain a lipid moiety. It has been reported that the polysaccharide moiety is not involved in the biological activities (reactivity with the lysate, pyrogenicity, etc.) of lipopolysaccharides (12), and polysaccharides

Table 1

Gelation of amoebocyte lysate of horseshoe crab and rise of temperature in rabbit pyrogen test by varing concentrations of CMPS

CMPS (mg/ml)	Gelation of amoebocyte lysate[a]	Rise of temperature in the rabbit pyrogen test (°C)[b]
1×10^{-7}	-	
1×10^{-6}	+	
1×10^{-5}	++	
1×10^{-4}	++	0.20 ± 0.10
1×10^{-3}	+	
1×10^{-2}	-	0.13 ± 0.09
1×10^{-1}	-	
1	-	0.23 ± 0.03

[a] The degree of gelation was scored as follows: -, no or little viscosity detectable; +, soft gel with fluidity; ++, firm gel remaining adherent to the tube when inclined 45°.

[b] Three male albino rabbits, each weighing 2.0-2.7 kg, were used in a group. A CMPS solution was injected intravenously at a dose of 10 ml/kg and the maximum rise of body temperature during 3 h after injection was recorded and expressed as mean ± s.e.m.

including glycogen, dextran, hyaluronic acid, chondroitin, chondroitin sulfate, heparin, alginic acid were reported not to be reactive with the lysate (13).

Unexpectedly, gelation of the lysate did not occur at CMPS concentrations higher than 10^{-2} mg/ml (Table 1), suggesting that a definite concentration of CMPS was necessary for the gelation. This was confirmed by quantitative measurement of the amidase activity of the clotting enzyme generated in the lysate after its incubation with varying concentrations of CMPS. Activation of the proclotting enzyme occurred at CMPS concentrations of 10^{-6} - 10^{-3} mg/ml, and no substantial activation was observed above or below this concentration range. The optimum concentration was 10^{-5} - 10^{-4} mg/ml (Fig. 1). Similar results were obtained when the amoebocyte lysate of Limulus polyphemus (Limulus test Wako® from Wako Pure Chemical Ind., Ltd., Osaka) was used as source of the proclotting enzyme. In contrast, no optimum concentration was observed in the activation of the proclotting enzyme by endotoxin; the activation paralleled the increase of concentation (Fig. 1).

The gelation of the lysate and the presence of an optimum concentration of the activation of the proclotting enzyme were also observed in common with other antitumor polysaccharides. All the tested antitumor polysaccharides including PS, lentinan, PSK, pachyman and carboxymethylparamylon caused the

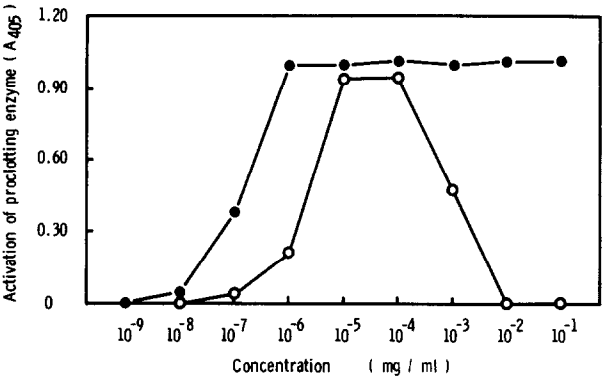

Figure 1. Activation of proclotting enzyme in amoebocyte lysate of horseshoe crab by varing concentrations of CMPS and endotoxin. A reaction mixture consisting of the proclotting enzyme solution (0.025 ml), a 2 mM Boc-Leu-Gly-Arg-pNA solution (0.025 ml) and either a CMPS (-o-o-) or an endotoxin (-●-●-) solution (0.05 ml) was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.4 ml of 12.5 % acetic acid and the absorbance at 405 nm was read.

gelation and had an optimum concentration for the activation at around 10^{-4} mg/ml. In contrast, those polysaccharides which did not show any antitumor activity against Sarcoma-180 in our test did not cause the gelation at concentrations ranging from 10^{-7} to 10^{-1} mg/ml. The detailed results will appear elsewhere (14). The presence of these glucans may raise a serious problem as to the practical use of the Limulus test as a convenient in vitro method for the detection of endotoxins; their possible contamination must be taken into account when the positive results are obtained with unknown samples

The addition of 10^{-2} mg/ml of CMPS inhibited the activation of the proclotting enzyme by 10^{-4} mg/ml of CMPS but not the activation by 10^{-5} mg/ml of endotoxin. Further, 10^{-4} - 10^{-1} mg/ml of CMPS did not affect the amidase activity of the clotting enzyme once-generated from the proclotting enzyme by 10^{-4} mg/ml of CMPS. These results led us to the conclusion that the absence of activated amidase activity in the presence of 10^{-2} mg/ml of CMPS (Fig. 1) was not due to the inhibition by CMPS of the activity of once-generated clotting enzyme but to the inability of CMPS at 10^{-2} mg/ml to activate the proclotting enzyme. The reason for this difference in activating ability of CMPS at 10^{-4} mg/ml and 10^{-2} mg/ml must await further investigation. Some specific concentration-dependent change in its higher structure in aqueous solutions might be expected.

Figures 2(A) and 2(B) show that the addition of 1 mg/ml of endotoxin or 10^{-4} mg/ml of CMPS to the reaction mixture, in which the activation by 10^{-4} mg/ml of CMPS (A) or 10^{-6} mg/ml of endotoxin (B) had been completed, resulted

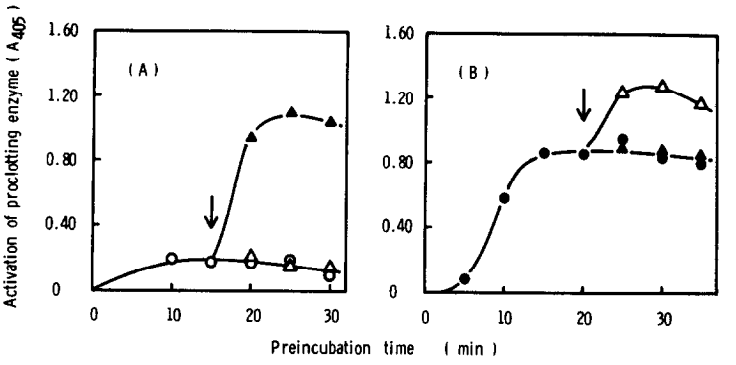

Figure 2. Effect of the addition of endotoxin or CMPS on the activation of the proclotting enzyme after its primary activation by 10^{-4} mg/ml CMPS or 10^{-6} mg/ml endotoxin. (A) A reaction mixture consisting of the proclotting enzyme solution (0.8 ml) and a 1.5 x 10^{-4} mg/ml CMPS solution (1.6 ml) was incubated at 37°C. At indicated times, an aliquot (0.05 ml) was removed and assayed for activity of the clotting enzyme generated (—o—o—). After 15 min incubation (at the time indicated by the arrow), three 0.6 ml-aliquots were taken from the mixture. To each of the three, 0.2 ml of a 4 mg/ml endotoxin solution, a 4 x 10^{-4} mg/ml CMPS solution, or saline was added and incubation was continued. At intervals, an aliquot (0.05 ml) was removed and assayed for activity of the clotting enzyme generated in the presence of added endotoxin (—▲—▲—) or CMPS (—△—△—) and in their absence (—o—o—). For assay of the activity of the clotting enzyme, each aliquot (0.05 ml) was incubated with a 4 mM Boc-Leu-Gly-Arg-pNA solution (0.025 ml) and a 2 x 10^{-2} mg/ml CMPS solution (0.075 ml) (to stop the activation of the proclotting enzyme by 10^{-4} mg/ml CMPS during the assay) at 37°C for 5 min. The reaction was stopped by the addition of 0.35 ml of 15 % acetic acid and the absorbance at 405 nm was read. (B) A reaction mixture consisting of the proclotting enzyme solution (0.8 ml) and a 1.5 x 10^{-6} mg/ml endotoxin solution (1.6 ml) was incubated at 37°C. At indicated times, an aliquot (0.1 ml) was removed and assayed for activity of the clotting enzyme generated (—●—●—). After 20 min incubation (at the time indicated by the arrow), three 0.6 ml-aliquots were taken. To each of the three, 0.2 ml of a 4 x 10^{-4} mg/ml CMPS solution, a 4 x 10^{-6} mg/ml endotoxin solution, or saline was added and incubation was continued. At intervals, an aliquot (0.1 ml) was removed and assayed for activity of the clotting enzyme generated in the presence of added CMPS (—△—△—) or endotoxin (—▲—▲—) and in their absence (—●—●—), in the same way as described in (A).

in a further activation of the remaining proclotting enzyme. This effect was not brought about as a result of supply of the exhausted activators, because the addition of 10^{-4} mg/ml of CMPS (A) or 10^{-6} mg/ml of endotoxin (B) did not cause further activation.

These results clearly indicate that the proclotting enzyme in the lysate is activated by CMPS by way of a mechanism different from that working in the activation by endotoxins. In view of the recent finding of endotoxin-sensitive factor B by Ohki et al. (6), our results are suggestive of the presence of some CMPS-sensitive component(s) in the lysate.

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