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Inhibition of high-molecular-weight-(1 → 3)-β-D-glucan-dependent activation of a limulus coagulation factor G by laminaran oligosaccharides and curdlan degradation products

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ABSTRACT

Extensive surveys for the effects of various β-D-glucans on the coagulation cascade in horseshoe crab amebocyte lysates showed that low-mol-wt-(1 → 3)-β-D-glucans and laminaran oligosaccharides inhibit the activation of a limulus coagulation factor G by high-mol-wt-(1 → 3)-β-D-glucans. The inhibitory properties are exclusively dependent upon their number-average mol wt (M_n) in a range of 342–58 100, which correspond to a degree of polymerization (dp) range of 2–359. The most effective is a laminaran dextrin of M_n 5800 (dp of 35–36), which causes 50% inhibition of factor G activation at a concentration of 3.16 ng/mL. The inhibition of the activation of factor G proportional to the concentration of the inhibitor, and the adsorption of factor G by inhibitory β-D-glucan-conjugated cellulose suggested a high affinity of the inhibitory saccharides for the activator-recognition site of factor G. Branched (1 → 6), (1 → 3)-β-D-glucans, laminarans, mixed linkage (1 → 3), (1 → 4)-β-D-glucans, and partially substituted curdlan and laminaran were found to be inhibitory, possibly owing to clusters of consecutive (1 → 3)-β-D-glucopyranosyl residues as intrachain units. The inhibition appears to be related to the inability of the inhibitory (1 → 3)-β-D-glucans to form ordered conformations and to their tendency to take a random-coil structure in aqueous solution.

INTRODUCTION

Two microbial polysaccharides, e.g., lipopolysaccharides (LPSs, endotoxins) and (1 → 3)-β-D-glucans, act as the discrete initiators that activate the coagulation cascade leading to the coagulin clot formation in the amebocyte lysates of horseshoe crabs[1–3]. Linear and branched (1 → 3)-β-D-glucans, major polysaccharide

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components of fungal cell walls, have been suggested to play a crucial role for activating factor G, the first protease zymogen of the alternative coagulation pathway in the amebocyte lysate[4,5]. Recent investigations of various saccharides differing in sugar composition and type of linkage have demonstrated that linear (1 → 3)-β-D-glucans, mixed linkage (1 → 4), (1 → 3)- and (1 → 6), (1 → 3)-β-D-glucans are effective activators of factor G[6]. The activating property of linear (1 → 3)-β-D-glucans was expressed as the number-average mol wt (M_n) exceeding a critical M_n of 6800; it increases progressively with increasing M_n[6]. Conformational analysis of ordered structure by high-resolution, solid-state ^{13}C NMR spectroscopy suggested the necessity of a single helical conformation for the factor G-activating property of (1 → 3)-β-D-glucans[7]. Studies using partially degraded products of curdlan indicated that low-mol-wt-(1 → 3)-β-D-glucans are able to inhibit the activation of factor G by higher-mol-wt-(1 → 3)-β-D-glucans. We report herein the characterization of β-D-glucans acting as the inhibitor for factor G activation with respect to their structural feature and M_n (dp), and discuss the mode of the inhibition and the usefulness of the inhibitory property for the discrimination of the two limulus coagulation pathways.

EXPERIMENTAL

Materials and sources. – Laminaran oligosaccharides (dp 2–7) and Pregel-M, *Tachypleus tridentatus* amebocyte lysate; Seikagaku Corp., Tokyo. D-Glucose and curdlan, a linear (1 → 3)-β-D-glucan from *Alcaligenes faecalis*, and Limulus HSII-Test wako, *Limulus polyphemus* amebocyte lysate; Wako Pure Chemical Industries, Osaka. *Laminaria digitata* laminaran, baker's yeast β-D-glucan, barley β-D-glucan, and *Cetralia islandica* and *Usnea barbata* lichenans; Sigma Chemical Co., St. Louis, MO, USA. Lentinan medicine; Ajinomoto Co., Tokyo. Sonifilan medicine (schizophyllan); Kaken Chemical Co., Tokyo. Lipopolysaccharide from *Escherichia coli* 0111 : B4; Difco Laboratories, Detoroit, MI, USA). TSK gel GPW_{XL} series and Toyopearl HW65F; Tosoh, Co., Tokyo. Factor G and proclotting enzyme were prepared from *T. tridentatus* amebocyte lysate[8]. A suspension of curdlan (50 g) in water (2.5 L) was stirred at room temperature for 1 h and centrifuged at 16000*g* for 20 min. The resulting precipitate was washed repeatedly with water and dried (insoluble fraction, 48.3 g). The supernatant and washings were combined, concentrated in vacuo, ultrafiltered through a Millipore membrane (0.22 μm), and lyophilized (soluble fraction, 7.6 mg).

Formolysis of curdlan (50 g), followed by deacylation and exhaustive water extraction, afforded insoluble and soluble fractions (32.6 and 0.2 g), respectively[9]. Gel permeation chromatography (GPC) of the soluble fraction on tandem columns of TSK gel $G3000PW_{XL}$ and $G2500PW_{XL}$ using water as a mobile phase provided six (1 → 3)-β-D-glucans with the following M_n and polydispersities [ratios of weight-average mol wt (M_w) to M_n shown in parentheses]: 2370 (1.20), 3400 (1.20), 4800 (1.20), 5800 (1.20), 6800 (1.20), and 9800 (1.22). Separation of the insoluble glucans by GPC using the same system as described above and 0.3 M NaOH as the

mobile phase gave two β-D-glucans, M_n 14500 (1.24) and M_n 27500 (1.26). Sonication[10] of curdlan (50 g) in a sonicator (Ohtake Works, 5202PZT, Tokyo) and GPC of the degradation products on tandem columns of TSK gel G6000PW$_{XL}$ and G5000PW$_{XL}$ as described above gave eight (1 → 3)-β-D-glucans having the following M_n and polydispersities: 20700 (1.27), 28300 (1.18), 50200 (1.26), 58100 (1.29), 76300 (1.26), 92600 (1.23), 171000 (1.19), and 216000 (1.19).

Eisenia bicyclis laminaran was prepared from pulverized fronds by the procedure of Usui et al.[11]. *Laminaria japonica* laminaran was prepared from the dry fronds by the method of Connell et al.[12]. A *Sclerotinia libertiana* glucan was extracted from defatted sclerotia with 7% NaOH (w/v) and purified by $CuSO_4$ precipitation[13]. A *Schizophyllum commune* β-D-glucan [schizophyllan (Sonifilan medicine)] was sonicated in saline solution for 10 h and resolved into three components differing in M_n by GPC under the conditions used for the separation of sonically degraded curdlan. *Saccharomyces cerevisiae* β-D-glucan (baker's yeast β-D-glucan) was dissolved in water and centrifuged; the soluble glucan in the supernatant was used for the assay. A barley β-D-glucan (Sigma) was dissolved in 0.3M NaOH or hot water at 80°C, and the solubilized β-D-glucans were purified by GPC as described above. Carboxymethylated (CM) curdlan (25 mg) having a degree of substitution (ds) of 0.63[14,15], was dissolved in 0.1 M NH_4OAc (5 mL), fractionated on a Toyopearl HW65F column (5 × 100 cm) equilibrated with 0.1 M NH_4OAc, and fractions (10 mL) were collected at a flow rate of 5.8 mL/min. The major fractions were combined and subjected to GPC on tandem columns of TSK gel G6000PW$_{XL}$ and G3000PW$_{XL}$ (7.8 × 300 mm, each) using 0.1 M NH_4OAc as the mobile phase at a flow rate of 0.6 mL/min to yield a fraction (M_n, 231000). The CM-curdlan (300 mg) was sonicated in water (30 mL) with a sonicator (Insonator Model 201M, Kubota Works, Tokyo) at 9 kHz for 1 h, and two partially degraded products (M_n 42400, 0.42 mg; M_n 77300, 0.55 mg) were resolved by GPC as described above. To the gelatinized CM-curdlan (10 g) in 10.5 M NaOH (25 mL), under N_2 at 0°C, was added $CH_2ClCOOH$ (10 g/12 mL) and the mixture was heated at 60°C under stirring for 4 h followed by addition of 2 M HCl (30 mL). The resulting highly carboxymethylated curdlan (ds 1.20) was precipitated by addition of ethanolic HCl (200 mL; 1:4 HCl–EtOH), washed with 70% and 99.5% EtOH, ether, and dried (yield, 9 g). CM-laminaran (ds 0.06) was prepared from *L. digitata* laminaran as described above. Preparation of partially methylated curdlan with ds of 0.16[16], was performed according to the method of Samec[17]. Dried *L. digitata* laminaran (500 mg) was suspended in dry pyridine (50 mL), treated with SO_3–pyridine complex (1 g, Wako Pure Chem. Ind.) at 60°C for 1 h and cooled. After addition of water (100 mL) and neutralization, the sulfated laminaran was dialyzed against water, in an alkali-washed Spectrapore tubing (mol wt 1000 cutoff), concentrated, precipitated with acetone (2 vol), washed with acetone, and dried (yield, 380 mg; ds 0.14, as determined by the method of Croon and Manley[18]).

Determination of molecular weight. – Weight-average mol wt (M_w) and number-

average mol wt (M_n) were determined by GPC as described previously[6]. Polydispersity (M_w/M_n) was calculated from the M_w and M_n values of each sample.

Enzymatic assay for the inhibition of factor G activation. – The property to inhibit the activation of factor G was monitored by measuring the amidolytic activity of the clotting enzyme catalyzing the hydrolysis of *tert*-butyloxycarbonyl(Boc)Leu-Gly-Arg-4-nitroanilide (4-NA). The enzyme reaction for the inhibitor assay was carried out in a mixture (total volume, 200 μL) consisting of tris · HCl buffer, pH 8.0 (20 μmol), $MgCl_2$ (20 μmol), the chromogenic substrate (0.13 μmol), factor G (20 μL, A_{280} 0.9), the proclotting enzyme (30 μL, A_{280} 2.5), the β-D-glucan to be tested for the inhibitory ability, and a (1 → 3)-β-D-glucan (M_n 216000; M_w/M_n 1.19; 10 pg) which was designated as GA and used as the standard activator for factor G. The amount of 4-nitroaniline released after incubation at 37°C for 30 min was determined at A_{545} after diazo-coupling[8]. The saccharides to be tested were dissolved in water or 0.3 M NaOH and centrifuged at 3000 rpm for 10 min. The supernatants were serially diluted and assayed directly or after neutralization with HCl. The sugar content of each sample was estimated by the phenol–H_2SO_4 method[19] using D-glucose as the standard. The concentration (I_{50}) of each saccharide capable of inhibiting the activation of factor G by the standard activator (GA) at 50% was determined by plotting the concentrations of saccharides against their inhibition ratios. The inhibition ratio (%) was calculated according to the following equation:

$$\text{Inhibition ratio} = 100 - \frac{[A_{545}\text{ of sample with GA}] - [A_{545}\text{ of control without GA}]}{[A_{545}\text{ of control with GA}] - [A_{545}\text{ of control without GA}]} \times 100,$$

where A_{545} is the absorbance due to 4-nitroaniline released and the control mixture contained water instead of the sample.

Mechanism for inhibition of factor G activation. – The step susceptible to inhibition in the (1 → 3)-β-D-glucan-driving coagulation cascade was investigated by the following assay system (Table II, see below). The reaction mixture (total volume, 200 μL) consisted of laminaroheptaose (G_7, 5 μg) or a (1 → 3)-β-D-glucan (50 ng; M_n 5800; M_w/M_n 1.20, which was hereafter designated as GI) as the inhibitor, GA (3 pg), factor G (20 μL, A_{280} 0.9), the proclotting enzyme (30 μL, A_{280} 2.5), Tris · HCl buffer, pH 8.0 (20 μmol), $MgCl_2$ (20 μmol) and the chromogenic substrate (0.13 μmol).

The mode of inhibition of factor G activation was evaluated by measuring the amidolytic activity of the clotting enzyme due to the activation of a defined amount of factor G (20 μL) by varying the concentrations of GA (2 pg–20 μg), and GI (3–150 ng) in the enzyme assay system described above. The influence of various amounts of GI (0.34–5.42 ng) and factor G (5–60 μL) upon the amidolytic activity was investigated in the presence of a fixed amount of GA (2.5 pg).

Effects of the inhibitor on the coagulation cascade. – The amidolytic activity of the clotting enzyme was assayed to determine the inhibitory effect of GI which was used as the standard inhibitor on the coagulation cascade in *T. tridentatus*

amebocyte lysate. The reaction was carried out in a mixture (total volume, 200 μL) consisting of the lysate (40 μL), $MgCl_2$ (16 μmol), the chromogenic substrate (0.13 μmol), Tris · HCl buffer, pH 8.0 (20 μmol), GI (150 ng), and various amounts of GA or of an *E. coli* 0111:B4 LPS. 4-Nitroaniline released was determined after incubation at 37°C for 30 min. For a control, water was added to make a total volume of 200 μL.

Assay for the inhibition of clot formation. – The ability of saccharides to inhibit clot formation in limulus amebocyte lysates was examined by use of two commercial preparations, Pregel-M (a *T. tridentatus* amebocyte lysate) and Limulus HSII-Test wako (a *L. polyphemus* amebocyte lysate). For the coagulation test, *E. coli* 0111:B4 LPS or GA was diluted serially 10-fold to concentrations of 0.004–40 and 0.4–4000 ng/mL, respectively. To aliquots (100 μL) of Pregel-M (dissolved in 2.6 mL of water) in test tubes (10 × 75 mm), were added GA or the LPS (25 μL), and GI (150 ng) in 50 μL; the total volume was made up to 200 μL with water. For the control, water (100 μL) was added. Reaction mixtures of the same dilution series were prepared for Limulus HSII-Test wako (100 μL, dissolved in 5.0 mL of water). The reaction mixtures were incubated at 37°C, and the tubes were turned upside-down after incubation for 60 min. The formation of a stable gel was defined as a positive reaction, whereas either the disintegration of the gel or no noticeable formation of a gel was defined as a negative reaction.

Binding of factor G to immobilized inhibitor. – An affinity method using GI as a ligand was employed for a binding-test of factor G. GI was conjugated with hydrazine–cellulose powder (100–200 mesh, Toyo Roshi Co., Tokyo) by the method of Matsumoto et al.[20]. The resulting GI-conjugated cellulose powder was washed with water, phosphate-buffered saline, and used as an affinity gel. The affinity cellulose powder (0.4 mL, wet volume) was mixed with *T. tridentatus* amebocyte lysate (1 mL) and stirred at 0°C for 5 min, and then filtered through a sintered-glass filter. The amidolytic activity of the clotting enzyme was assayed to detect the activation of factor G remaining in the filtrate (20 or 80 μL) under the standard condition, and compared to the control that used the untreated lysate.

RESULTS AND DISCUSSION

Effect of molecular weight of (1 → 3)-β-D-glucans on factor G activation. – Fig. 1 shows a comparison of the factor G-activating property vs. concentrations [log(g/mL)] of six (1 → 3)-β-D-glucans having different M_n. The curves illustrated in panels A–E indicate a decrease in the minimal amount of the glucan capable of activating factor G as the M_n exceeds a critical value ($M_n \geqslant 6800$) as well as a reduction in the activating property of lower-mol-wt-β-D-glucans (M_n 6800–28300) with increasing concentration. However, such a decrease in the activating property was not observed for high concentrations of a β-D-glucan having a M_n 216000 (GA), the standard activator for factor G (panel F). These results raise the possibility that the low-mol-wt-β-D-glucans have the property to inhibit the activa-

tion of factor G by higher-mol-wt-β-D-glucans. Consequently we investigated the inhibitory property of β-D-glucans and their derivatives with respect to their M_n or ds.

Relationship between molecular weight and type of glycosidic linkage, and the inhibitory activity. – Table I summarizes the inhibitory properties of β-D-glucans differing in mol wt and type of glycosidic linkage. Curdlan (M_n > 136000) and its insoluble fraction (M_n > 159000) obtained after exhaustive water extraction, were equally ineffective as the inhibitor, as revealed by their log I_{50} value of > −3.00, although they are potent activators of factor G[6]. The water-soluble fraction from curdlan (M_n 3270, M_w/M_n 2.49) and a β-D-glucan obtained from curdlan by GPC (M_n 3050, M_w/M_n 1.29) had inhibitory potencies (log I_{50}) of −7.35 and −7.00, respectively, demonstrating that commercial curdlan contains an appreciable amount of low-mol-wt-(1 → 3)-β-D-glucan acting as the inhibitor. Such contamination of the inhibitory (1 → 3)-β-D-glucans suggests that the estimation of the factor G-activating property of curdlan that has been frequently employed as the activator for the (1 → 3)-β-D-glucan-driving coagulation cascade may be inaccurate.

A survey of the inhibitory property of D-glucose, laminaranoligosaccharides (dp 2–7), and 16 samples of degradation products (M_n 2370–216000), obtained from formolyzed or sonicated curdlan, revealed that the activation of factor G by GA is inhibited exclusively by the oligo- and poly-saccharides consisting of 2–359 consecutive (1 → 3)-β-D-glucopyranosyl residues, which correspond to M_n of 342–58100. Conversely, higher-mol-wt-(1 → 3)-β-D-glucans exceeding a critical M_n of 58100 were essentially devoid of inhibitory property while their ability to activate factor G increased[6].

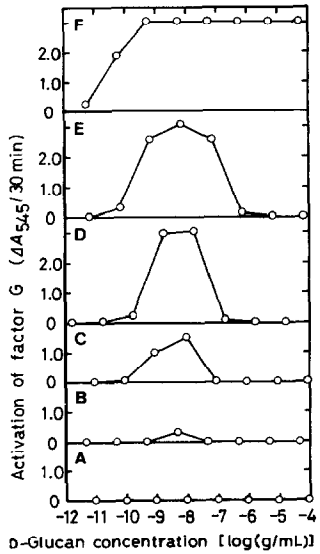

Fig. 1. Effect of the concentrations of (1 → 3)-β-D-glucans having different number-average mol wt on their property to activate factor G. M_n: (A) 5800; (B) 6800; (C) 9800; (D) 14500; (E) 28300; (F) 216000.

TABLE I

Inhibitory property of various β-D-glucans

Saccharide	Structure [a]	M_n [b]	M_w/M_n	log I_{50} [log(g/mL)]
D-Glucose		180		> −3.00
Laminaran oligosaccharides [c]				
(dp 2–7)	A	342–1153		−3.33 ~ −6.01
Curdlan	A	> 136000	> 2.76	> −3.00
Insoluble fraction	A	> 159000	> 2.50	> −3.00
GPC Fraction	A	3050	1.29	−7.00
Soluble fraction	A	3270	2.49	−7.35
Curdlan formolysates [d]				
Fractions 1–8 [c]	A	2370–27500	1.20–1.26	−6.10 ~ −8.50
Curdlan sonicates [d]				
Fractions 1–4 [c]	A	20700–58100	1.18–1.29	−6.81 ~ −3.37
Fractions 5–8 [c]	A	76300–216000	1.19–1.26	> −3.00
CM-curdlan (ds 0.63) [d]				
Fraction 1	A	42400	1.14	−6.07
Fraction 2	A	77300	1.10	−5.96
Fraction 3	A	231000	1.10	−5.90
CM-curdlan (ds 1.20)	A	> 329000	> 1.27	> −3.00
Methylated curdlan	A	78200	1.10	−5.97
E. araborea laminaran [e]	B-1	16800	1.49	−4.83
E. araborea laminaran [f]	B-1	11200	1.55	−5.47
E. bicyclis laminaran	B-1	22500	1.27	−5.81
L. digitata laminaran	B-1	5850	1.16	−7.85
L. japonica laminaran	B-1	17700	3.98	−5.60
CM-laminaran	B-1	8170	1.21	−7.56
Sulfated laminaran	B-1	10300	2.04	−6.07
Schizophyllan [d]				
Fraction 1	B-2	6750	3.14	−6.14
Fraction 2	B-2	23600	2.37	−5.07
Fraction 3	B-2	27500	1.49	−5.70
Lentinan	B-2	94700	1.46	−5.00
Sclerotan	B-2	16800	2.77	−5.42
Baker's yeast β-D-glucan				
(soluble fraction)	B-3	11600	5.14	−5.06
C. islandica lichenan	C	22000	4.72	−4.55
U. barbata lichenan	C	23000	4.07	−3.08
Barley β-D-glucan [d]				
Fraction 1	C	54000	1.16	−5.49
Fraction 2	C	129000	1.09	−5.07
Fraction 3	C	200000	1.13	−5.61

[a] The structures of β-D-glucans are classified as A, B, and C, based on the types of linkage as follows: (A) oligosaccharides and linear β-D-glucans consisting of consecutive (1 → 3)-D-glucopyranosyl residues; (B-1) (1 → 6), (1 → 3)-β-D-glucans containing (1 → 6)-D-glucopyranosyl residues as intrachain group; (B-2) (1 → 3)-β-D-glucans, to which single β-D-glucopyranosyl residues or side chains consisting of (1 → 6)-β-D-glucopyranosyl residues are attached at O-6; (B-3) mixed linkage (1 → 3), (1 → 6)-β-D-glucan; (C) (1 → 3), (1 → 4)-β-D-glucans containing (1 → 4)-β-D-glucopyranosyl residues as intrachain group. [b] Mol wt of D-glucose and laminaran oligosaccharides are theoretical values; number-average mol wt (M_n) of β-D-glucans were determined by GPC using poly(ethyleneoxides) and poly(ethyleneglycols) as the standards. [c] See Fig. 2 for details. [d] Separated into fractions with different M_n by GPC. [e] A product of Nakarai Chemical Co. [f] A product of Tokyo Kasei Co.

Interestingly, three CM-(1 → 3)-β-D-glucans, obtained from partially carboxymethylated curdlan (ds 0.63) after sonic degradation and separation by GPC, turned out to be inhibitory, although originally curdlan lacked the inhibitory property because of their high M_n. Such conversion of the (1 → 3)-β-D-glucans to the inhibitory form is probably due to the formation of an alignment of the unsubstituted (1 → 3)-β-D-glucopyranosyl residues distributed as intrachain units, which might be responsible for the inhibitory property. Indeed, the inhibitory potency of CM-curdlan decreased markedly with an increase in the ds value to 1.2. Laminarans, which are mixed linkage (1 → 6), (1 → 3)-β-D-glucans, were all effective as inhibitors with relatively high inhibitory potencies. The potency (log I_{50} −7.85) of *L. digitata* laminaran is comparable to that of a low-mol-wt-(1 → 3)-β-D-glucan (M_n 6800, log I_{50} −7.80). However, partial carboxymethylation or sulfation converted *L. digitata* laminaran to products with reduced potencies. Three fractions, separated from barley (1 → 4), (1 → 3)-β-D-glucan by GPC were found to be inhibitory at comparable concentrations with those of laminaro-tetraose or -pentaose. However, lichenans were poor inhibitors, probably because of their structures[21]. Water-soluble, branched (1 → 6), (1 → 3)-β-D-glucans such as schizophyllan, lentinan, and sclerotan were moderately active as inhibitors. It can be postulated that the inhibitory property of laminarans, and branched and mixed linkage β-D-glucans might be ascribed to consecutive alignments of (1 → 3)-β-D-glucopyranosyl residues that are distributed *en bloc* in the chains[22–25].

It is evident from the curve (Fig. 2) plotting the mol wt of (1 → 3)-β-D-glucans and D-glucose against their inhibitory potency, as expressed by the I_{50} values (g/mL), that β-D-glucans having a M_n range of 3400–14 500 have high inhibitory potencies (log I_{50} − 7.26 ~ −8.50). The most potent inhibitor (log I_{50}, −8.5) is a (1 → 3)-β-D-glucan (M_n 5800 and M_w/M_n 1.20), and the I_{50} values of the samples tend to increase progressively with increasing or decreasing mol wt. D-Glucose and (1 → 3)-β-D-glucans with M_n exceeding 58 100 did not cause any appreciable inhibition.

The following saccharides were shown to be inactive as inhibitors when assayed under the standard condition, even at 10^{-3} g/mL: CM-cellulose, gyrophoran [a (1 → 6)-β-D-glucan from a lichen, *Gyrophora esculenta*], nigeran, yeast α-D-mannan, (1 → 3)-β-D-galactan, (1 → 3)-β-D-xylan, ethylene glycol-chitin, chondroitin sulfates A and C, dermatan sulfate, heparin, and keratan sulfate. Lipopolysaccharides from five Gram-negative bacteria and *Salmonella minnesota* R595 lipid A also were not inhibitory.

Mechanism of inhibition of factor G activation. – As shown in Table II (Expts. 1, 2, and 3) complete inhibition of the factor G activation by GA occurred with simultaneous addition of either laminaroheptaose or GI and an activator (GA), or by prior addition of the inhibitor to GA. In contrast, the GA-activated factor G no longer reacted with I added afterward (Expts. 4 and 5). These results suggest strongly that a preferential binding of I to the GA-recognition site on factor G

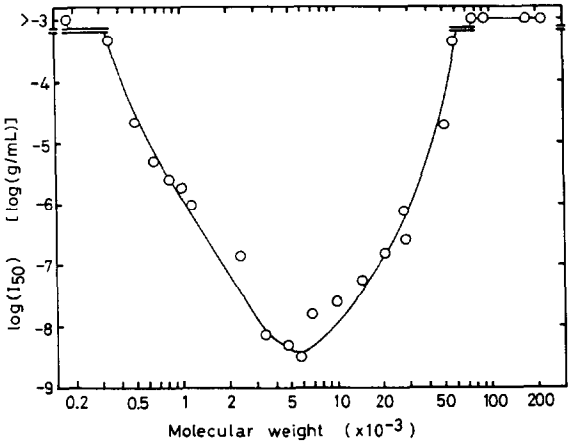

Fig. 2. Relationship between the number-average mol wt of (1 → 3)-β-D-glucans and their I_{50} values. I_{50}: concentration (g/mL) of β-D-glucans capable of inhibiting the activation of factor G by the standard (1 → 3)-β-D-glucan (GA) at 50%. Mol wt of D-glucose and laminaran oligosaccharides (dp 2–7) are theoretical values.

results in a complete stop of the (1 → 3)-β-D-glucan-driving coagulation cascade at the initial step.

Fig. 3A shows the effect of various amounts of GI (3–150 ng) and GA (2 pg–20 μg) on the activation of a defined amount of factor G (20 μL), as assayed by measuring the amidolytic activity of the clotting enzyme. The activation of factor G by GA was completely inhibited with increasing concentrations of GI, even though

TABLE II

Procedure to demonstrate the inhibition of the coagulation cascade by the preferential binding of laminaroheptaose and a standard inhibitor (GI) to factor G [a]

Expt.	Procedure [b]	Inhibition (%) [c]	
		G_7	GI
1	FG + GA + I + ProCE + S (37°C, 10 min)	100	100
2	FG + GA + I(37°C, 30 min) → + ProCE + S (37°C, 3 min)	100	100
3	FG + I(37°C, 10 min) → + GA(37°C, 10 min) → + ProCE + S(37°, 10 min)	100	100
4	FG + GA(37°C, 30 min) → + I(37°C, 10 min) → + ProCE + S(37°C, 10 min)	1.7	1.5
5	FG + GA + ProCE(37°C, 30 min) → + I(37°C, 10 min) → + S(37°C, 3 min)	0	0

[a] Abbreviations: FG, factor G; GA, an activator, a (1 → 3)-β-D-glucan (M_n 216000); I, inhibitor, laminaroheptaose (G_7) or GI (M_n 5800, M_w/M_n 1.20); ProCE, proclotting enzyme; S, substrate, Boc-Leu-Gly-Arg-4-NA. [b] Factor G was first mixed at 0°C with other components according to the composition of reaction mixture in each experiment and incubated at 37°C. After the addition of the substrate, the reaction mixture was incubated at 37°C and monitored for the amidolytic activity of the clotting enzyme. [c] Relative inhibition (%) of the enzyme activity in each experiment was calculated, based on that obtained in the absence of the inhibitor as the control.

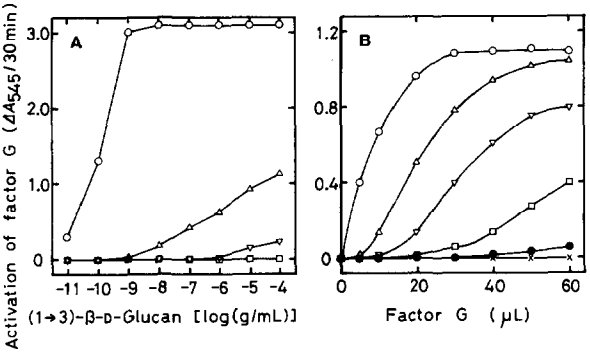

Fig. 3. Inhibition of factor G activation. (A) Effect of various amounts of GI on factor G activation by varying concentrations of GA. Activation of factor G by various concentrations of GA was determined by measuring the amidolytic activity of the clotting enzyme in the presence of the following amounts of GI: (○) control; (△) 3 ng; (▽) 30 ng; (□) 150 ng. (B) Effect of various amounts of GI on the activation of various amounts of factor G. Activation of various amounts of factor G by a fixed amount of GA (2.5 pg) was determined enzymatically as described above, in the presence of the following amounts of GI: (○) control; (△) 0.34 ng; (▽) 0.68 ng; (□) 1.36 ng; (●) 2.71 ng; (×) 5.42 ng.

sufficient amounts of GA to convert factor G to its fully active form were present in the reaction mixture. Fig. 3B shows the effect of various amounts of GI (0.34–15.4 ng) on the activation of various amounts of factor G (5–60 μL) in the presence of a fixed amount (2.5 pg) of GA. It is evident that the amount of GI required for inhibiting the activation of factor G is correlated to the concentrations of factor G. Thus, GI would be able to bind to a defined amount of factor G, and thereby inhibit the conversion of the proenzyme to the activated form by a defined amount of GA.

The binding of factor G to GI immobilized on cellulose was demonstrated by a complete loss of factor G from *T. tridentatus* amebocyte lysate when this was mixed with GI-conjugated cellulose powder, kept at 0°C for 5 min, filtered, and then assayed for the amidolytic activity of the clotting enzyme in the filtrate. No appreciable activity could be detected in the filtrate after addition of GA. On the other hand, most of the enzyme activity could be recovered when LPS or a mixture of factor G and GA were added to the filtrate separately. This indicated that the proenzymes involved in the coagulation cascade in the lysate, except factor G, remain intact after filtration. For control, the lysate was mixed with cellulose powder, kept as described above and filtered. The assay for amidolytic activity after addition of GA to the filtrate revealed that factor G in the lysate was fully recovered, thus indicating that no interaction of cellulose with factor G had taken place. The data indicate the effectiveness of GI-conjugated cellulose to adsorb factor G specifically, and imply a tight binding of GI to the GA-recognition site on factor G, such that GA is unable to activate factor G.

Effects of the inhibitor on the coagulation cascade. – Table III summarizes the effect of the inhibitors on clot formation in two amebocyte lysates. The clot-forming property was totally suppressed when GI (150 ng) was added to Pregel-M (0.1

TABLE III

Inhibitory effect of a (1 → 3)-β-D-glucan (GI) on clot formation in limulus amebocyte lysates

GA [a] (ng/mL) [d]	LPS [b] (ng/mL) [d]	Clot formation [c]			
		T. tridentatus [e]		*L. polyphemus* [f]	
		−GI [g]	+GI [g]	−GI [g]	+GI [g]
0	0	−	−	−	−
0.05	0	−	−	−	−
0.5	0	−	−	−	−
5.0	0	+	−	+	−
50	0	+	−	+	−
500	0	+	−	+	−
0	0.0005	−	−	−	−
0	0.005	−	−	+	+
0	0.05	+	+	+	+
0	0.5	+	+	+	+
0	5	+	+	+	+
0.05	5	+	+	+	+
0.5	0.5	+	+	+	+
5	0.05	+	+	+	+
50	0.005	+	−	+	+
500	0.0005	+	−	+	−

[a] The standard activator, a (1 → 3)-β-D-glucan (M_n 216000). [b] LPS, *E. coli* 0111:B4 lipopolysaccharide. [c] (+) Clot formed; (−) clot not formed. [d] Concentration (ng/mL) in the reaction mixture. [e] Pregel-M, a *T. tridentatus* amebocyte lysate. [f] Limulus HSII − Test wako, a *L. polyphemus* amebocyte lysate. [g] 150 ng/ml of GI (M_n 5800).

mL, a *T. tridentatus* amebocyte lysate) or HSII-Test wako (0.1 mL, a *L. polyphemus* amebocyte lysate), even in the presence of GA, indicating a complete inhibition by GI of the GA-driving coagulation pathway. In contrast, the inhibitor (GI) added did not disturb the clot formation due to the activation of factor C by *E. coli* LPS in the coagulation cascade. Hence, it left no doubt that the LPS-driving coagulation cascade in both the amebocyte lyates alone could be independently activated to form a clot. The activation of the LPS-driving coagulation pathway occur exclusively, with the simultaneous addition of *E. coli* LPS and GI to the reaction system (Fig. 4A). Moreover, the addition of an excessive amount of GI (150 ng) completely neutralized the factor G-activating property of increasing concentrations of GA (Fig. 4B). These results indicate that (1 → 3)-β-D-glucans with a limited range of M_n inhibit specifically the coagulation cascade that is initiated through the activation of factor G by GA, but are entirely without effect on the LPS-driving coagulation cascade involving factor C.

Kakinuma et al.[3] reported an optimal concentration of CM-curdlan for causing clot formation and the activation of a proclotting enzyme in *T. tridentatus* and *L. polyphemus* amebocyte lysates, and a marked decrease in its ability to activate the coagulation cascade at concentrations exceeding the optimum. Similarly, by assaying the amidolytic activity in the presence of several β-D-glucans, Morita et al.[26]

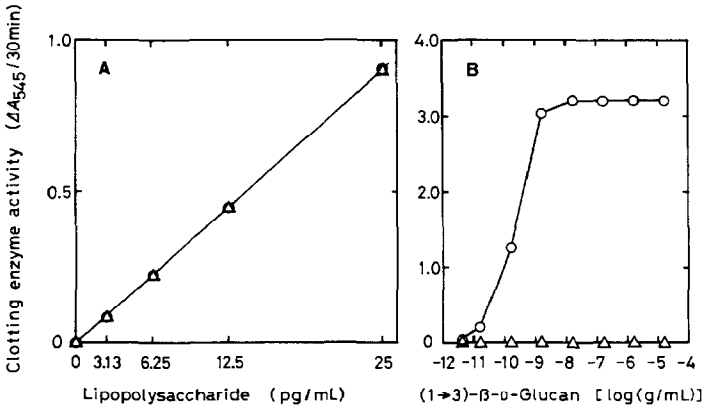

Fig. 4. Effects of an inhibitory (1 → 3)-β-D-glucan on the limulus coagulation pathway. The lipopolysaccharide is *E. coli* 0111:B4 lipopolysaccharide and the (1 → 3)-β-D-glucan (GA) has M_n 216 000: (A) Activation of the LPS-driving cascade involving factor C. (B) Activation of (1 → 3)-β-D-glucan-driving coagulation cascade involving factor G. The amidolytic activity of the clotting enzyme was assayed in the absence (○) or in the presence (△) of an inhibitor (GI, 150 ng), as described in Experimental section.

found the maximum respective concentrations to activate factor G in *T. tridentatus* amebocyte lysate. Such inhibitory effect of the activators in excess on the β-D-glucan-driving coagulation cascade have been interpreted as contamination with traces of low-mol-wt-β-D-glucans that act as inhibitors capable of binding to factor G specifically.

In summary, we present herein evidence that (1 → 3)-β-D-glucans having a M_n range of 342–58 100 (dp 2–359) and substituted, branched, and mixed-linkage β-D-glucans having consecutive (1 → 3)-β-D-glucopyranosyl residues act as inhibitors of factor G activation as the initial step of the coagulation cascade. This type of inhibition is essentially induced by the competition of structurally related β-D-glucans for their binding site on factor G, i.e., the ones having chain length that are large enough to activate the zymogen, and the others capable of binding to factor G but devoid of an activating property because of insufficient chain length to cover the recognition site(s) specific for the activation of factor G. Moreover, both activating and inhibitory properties appear to be closely related to the conformation of the (1 → 3)-β-D-glucan chains in aqueous solution, since laminaran dextrins of dp < 25 have been reported to be devoid of ordered structure, whereas the high-mol-wt-polymers capable of activating factor G tend to take a single helical conformation[7,9,27]. A well-characterized soluble inhibitor, such as GI, may be a useful component of a sensitive assay system for the specific detection of endotoxins (LPSs) by complete inhibition of the (1 → 3)-β-D-glucan-driving coagulation pathway with GI.

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