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Reactivity of *Limulus* amoebocyte lysate towards (1→3)-β-D-glucans

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ABSTRACT

The structure–activity relationship for β-D-glucans for the gelation of the amoebocyte lysates of the horseshoe crab (*Limulus*) has been investigated. β-D-Glucans that had no (1→3) linkages induced little or no gelation. The (1→3)-β-D-glucans curdlan (unbranched), grifolan (~33% branched), schizophyllan (~33% branched), lentinan (~40% branched), SSG (~50% branched), and OL-2 (~66% branched) induced significant gelation. The optimum concentration for gelation was correlated with the content of branching. Single chain (rather than a triple helix) conformation and higher molecular weight were associated with higher reactivity.

INTRODUCTION

The amoebocytes of the horseshoe crab (*Limulus*) cause coagulation of the haemolymph in the presence of minute amounts of endotoxins (lipopolysaccharides, LPS) or (1→3)-β-D-glucans[1]. This property has been applied to determine the concentration of LPS in medicine and biological fluids[2]. The *Limulus* test[2] is one of the most sensitive methods for the determination of LPS and is included in the Japanese Pharmacopeia XI. *Limulus* amoebocytes also contain (1→3)-β-D-glucan-mediated (factor G) pathways of coagulation in addition to the endotoxin-mediated (factor C) pathways[3,4]. Each of these pathways involves several proteases that are activated sequentially. Some of these proteases are common to both pathways, and the cascades are similar to those in blood coagulation and the complement systems in mammals.

Activation of the endotoxin-mediated pathway (factor C) is induced only by the presence of Gram-negative bacteria and synthetic derivatives of lipid A. In contrast, (1→3)-β-D-glucans occur widely in the fruiting bodies and mycelia of Basidiomycetes and Ascomycetes, and also in waterweeds, lichens, and plants. Some of these (1→3)-β-

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TABLE I

Glycans and their structural components

Glycan	*Monomer*	*Linkage*	
Mannan from bakers' yeast	α-D-Mannose	(1→6)	Sigma Chemical Co.
Dextran	α-D-Glucose	(1→3),(1→6)	Tokyo Kasei Kogyo Co.
Dextran sulfate	α-D-Glucose	(1→3),(1→6)	Sigma Chemical Co.
Carboxymethylcellulose	β-D-Glucose	(1→4)	Daiichi Pure Chemical Co.
Ethylene glycol chitin	2-Acetamido-2-deoxy-β-D-glucose	(1→4)	Seikagaku Kogyo Co.
Soluble starch	α-D-Glucose	(1→4)	Koso Chemical Co.
Islandican	β-D-Glucose	(1→6)	
Curdlan (CRD)	β-D-glucose	(1→3)	Wako Chemical Co.
Grifolan (GRN) from *Grifola frondosa*	β-D-Glucose	(1→3),(1→6) (~33%)[a]	
Schizophyllan (SPG)	β-D-Glucose	(1→3),(1→6) (~33%)	Kaken Chemical Co.
Lentinan (LNT)	β-D-Glucose	(1→3),(1→6) (~40%)	Yamanouchi Pharmaceutical Co.
SSG from *Sclerotinia sclerotiorum* IFO 9395	β-D-Glucose	(1→3),(1→6) (~50%)	
OL-2	β-D-Glucose	(1→3),(1→6) (~66%)	
Laminarin from *Eisenia araborea*	β-D-Glucose	(1→3),(1→6)	Nakarai Chemical Co.
Carboxymethylated GRN	d.s. 0.22 and 0.78		

[a] Degree of branching.

D-glucans, such as lentinan (LNT)[5] and schizophyllan (SPG)[6], show antitumor activity, and are now used clinically as biological response modifiers (BRM). The culture media of fungi[7] also respond to the *Limulus* test. Thus, the (1→3)-β-D-glucan-mediated pathway is useful for monitoring fungal infections and the concentration of BRM in the blood.

The structure–activity relationships for the activation of factor C have been elucidated[8] using synthetic derivatives of lipid A, but those of factor G have not been investigated fully. During studies of the structure–activity relationships for antitumor activity, several (1→3)-β-D-glucans were isolated from fungi, namely, GRN[9–12], SSG[13–15], and OL-2[16], and derivatives were prepared (see Table I). We now report on the structure–activity relationships for the activation of factor G based on these glucans and their derivatives.

EXPERIMENTAL

Limulus *test.* — The activation of factor G by glucans was measured by a colorimetric method, using an endotoxin quantitative kit (Toxicolor LS-1, Seikagaku

Kogyo Co., Tokyo) which contained factors C and G (LS-1 No. 310065). Contamination by endotoxin was determined with an endotoxin specific kit (Endospecy, Seikagaku Kogyo Co.) which eliminated[7] factor G. Reactions were performed in flat-bottomed 96-well tissue culture plates (Sumitomo Bakelite Co., Tokyo) as follows. Each sample (25 μL), diluted with pyrogen-free distilled water (1 ng–1 mg/mL), was placed in a culture plate, and the reagent (LS-1 or Endospecy, 25 μL) was added to each well. The plate was incubated for 40 min at 37°, then 0.8M acetic acid (100 μL) was added to each well to stop the reaction, and the absorbance at 405 nm was measured using the microplate reader MTP-32 (Corona Electric). Re-LPS (Sigma, L-9764), prepared from *Salmonella minnesota* Re595, was used as the reference endotoxin. Disposable plastic for the tissue culture or the clinic was used, and all glassware was sterilised for 2 h at 250°. All operations were performed under aseptic conditions. The results are expressed as the absorbance relative to that of 100 ng/mL of Re-LPS, which was given an index of 10.

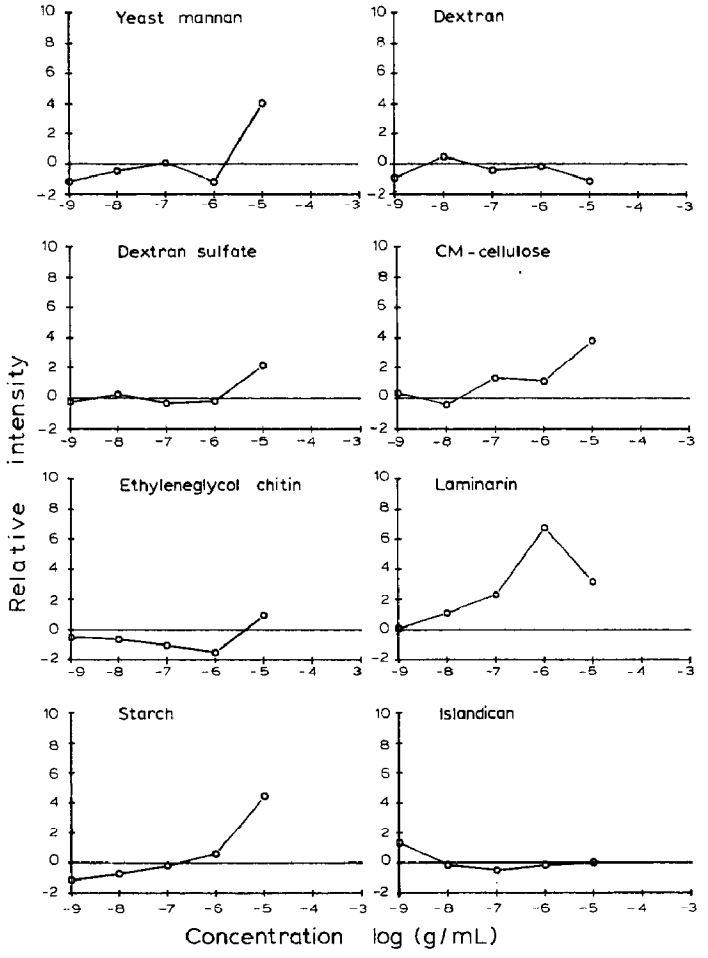

Fig. 1. Dose–response curves of LS-1 to various glycans (see Experimental).

Glucans. — The polysaccharides used are listed in Table I. LNT and SPG for hypodermic injection were generously provided by Yamanouchi Pharmaceutical Co. (Tokyo) and Kaken Chemical Co. (Tokyo), respectively. Curdlan (CRD) was purchased from Wako Chemical Co. (Osaka), and laminarin (from *Eisenia araborea*) from Nakarai Chemical Co. (Kyoto). The derivatives of GRN were prepared by the methods reported[17].

RESULTS AND DISCUSSION

Effect of the linkage of glucans on the reactivity of Limulus *lysate (LS-1).* — The reactivities of LS-1 to glucans with various linkages are shown in Figs. 1 and 2A. Each of the (1→3)-β-D-glucans gave a positive response (laminarin in Fig. 1 and the others in

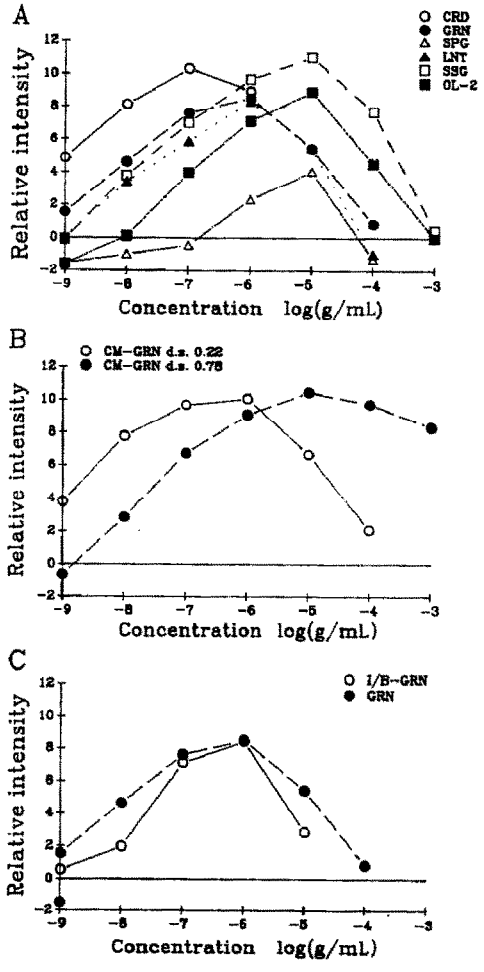

Fig. 2. Dose–response curves of LS-1 to *A*, (1→3)-β-D-glucans that have different degrees (%) of branching: CRD, 0; GRN, ~33; SPG, ~33; LNT, ~40; SSG, ~50; OL-2, ~66; *B*, carboxymethylated grifolan (CM-GRN) of d.s. 0.22 and 0.78; *C*, periodate oxidised–borohydride reduced GRN (I/B-GRN) and GRN.

Fig. 2A), dextran, dextran sulfate, ethylene glycol chitin, and islandican gave little or no response, and yeast mannan, CM-cellulose, and starch gave weak positive responses. Thus, LS-1 is specific for (1→3)-β-D-glucans. Of the (1→3)-β-D-glucans, SSG gave the strongest response and SPG gave the weakest.

Effect of branching of the β-D-glucans on the reactivity of LS-1. — The reactivity of LS-1 towards (1→3)-β-D-glucans that had different degrees of β-linked branching at position 6 was compared. The degrees of branching (%) were CRD, 0; GRN, ~33; SPG, ~33; LNT, ~40; SSG, ~50; and OL-2, ~66. Serial dilutions (1 ng–1 mg/mL) of each glucan were treated with LS-1. The concentrations for optimum reaction were CRD, 100 ng/mL; GRN, 1 μg/mL; LNT, 1 μg/mL; SPG, 10 μg/mL; SSG, 10 μg/mL; and OL-2, 10 μg/mL (Fig. 2A). Thus, the reactivity of LS-1 to (1→3)-β-D-glucans decreased with increase in the degree of branching.

The intensities of the reactions of SPG and laminarin were lower than those of the other glucans (Figs. 1 and 2A), reflecting lower molecular weights.

Laminarin and OL-2, which did not show any antitumor activity, also reacted with LS-1. Thus, the structure–activity relationship for reaction with the *Limulus* lysate was different from that for antitumor activity.

For carboxymethylated (1→3)-β-D-glucans, the degree of substitution is important for antitumor activity. Carboxymethylated grifolan (CM-GRN) of d.s. 0.22 was active but, with d.s. 0.78, was inactive. The optimum dose of CM-GRN with d.s. 0.78 was ~10 times higher than that of CM-GRN with d.s. 0.22, but the maximum reactivities of LS-1 to these derivatives were similar (Fig. 2B). These results reflected the suppressive effect of side chains or substituent groups on the reactivity towards LS-1.

The intensity of the reaction of periodate oxidised–borohydride reduced GRN (I/B-GRN) with LS-1 (Fig. 2C), as well as the optimum dose, were not significantly different from those of GRN, which also indicated the small contribution of the structure of side chains to the reactivity towards LS-1.

Effect of the molecular weight of the β-D-glucans on the reactivity towards LS-1. — GRN of low molecular weight was prepared by heating[18] solutions of GRN in distilled water for various periods at 150°. The degradation products gave symmetrical peaks on gel filtration and the peak widths were as narrow as those of the products prepared by conventional formolysis[19]. Methylation and n.m.r. spectroscopy established that the unit structures of the degradation products were indistinguishable from that of GRN. The molecular weights of the products were estimated by the Somogyi–Nelson method (Fig. 3A), and the intensity of fluorescence on interaction with Aniline Blue[18] also decreased as the mol. wt. decreased (Fig. 3A). The reactivity of these glucans towards LS-1 (Fig. 3B) was reduced significantly at mol. wt. 95 000 and almost disappeared at mol. wt. 26 000. These facts supported the low reactivity of LS-1 to SPG and laminarin as described above. The mol. wt. of the triple-stranded form of SPG was estimated to be 450 000, and it had reduced reactivity in comparison with that of GRN. Thus, the reactivity of LS-1 towards (1→3)-β-D-glucans was significantly dependent on the mol. wt.

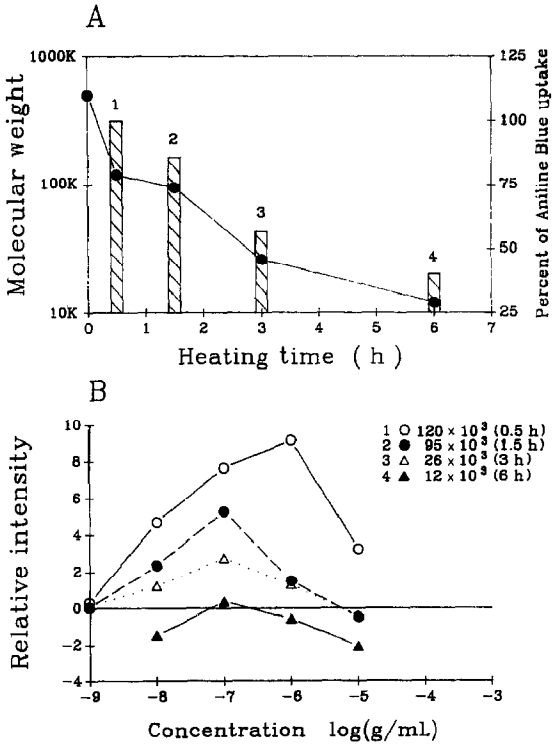

Fig. 3. *A*, Decrease in the molecular weight (–●–, estimation by the Somogyi–Nelson method) of GRN on heating at 150°. Aniline Blue solution (3 mL, 10 μg/mL in 0.1M NaOH) was mixed with each product (100 μg/mL) and the intensity of fluorescence was recorded. The value after heating for 30 min was taken as 100%. *B*, Dose–response curves for the reaction of LS-1 with the products in *A*.

Ultrastructure. — (1→3)-β-D-Glucans can form highly crystalline triple helices, single helices, and single chains[20–23]. The triple helix form was established by X-ray crystallography, but the extent of crystallisation was not high even after extensive annealing. (1→3)-β-D-Glucans produced by microorganisms and plants probably contain all three forms in various proportions. C.p.-m.a.s. ^{13}C-n.m.r. analysis can give information on the conformations of the crystalline and the non-crystalline parts of polysaccharides. Treatment of (1→3)-β-D-glucans with a high concentration of urea, with sodium hydroxide, or by heating above 140° caused a change in the equilibrium of the conformers[24].

After treatment with alkali, GRN contained mainly the single chain form and treatment at 150° increased the proportion of the triple helix form. The single chain form was more reactive towards LS-1 than the triple helix form (Fig. 4B). Commercially available CRD and LNT contain mainly the single chain form and each reacted strongly with LS-1.

The reactivity towards LS-1 of SPG, which contained mainly the triple helix form, was significantly increased by heating at 150° which increased the proportion of non-crystalline forms (Fig. 4A). A similar result was shown by GRN (Fig. 4B). Thus,

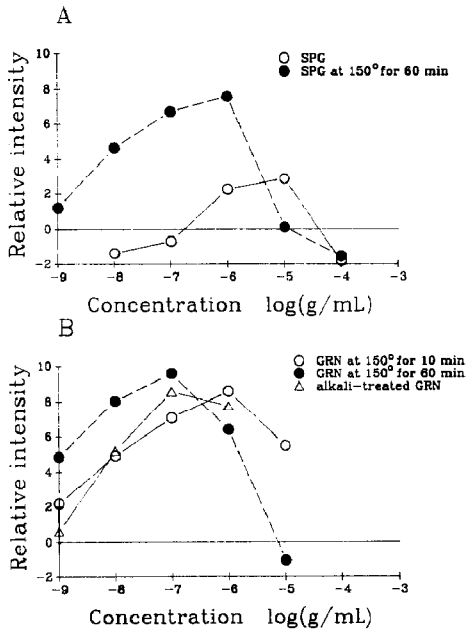

Fig. 4. Dose–response curves towards LS-1: *A*, triple helix conformer of SPG and the rearranged conformer (heating in distilled water at 150° for 60 min); *B*, triple helix conformer of GRN (150° for 10 min), rearranged conformer (150° for 60 min), and the single-chain conformer (alkali-treated GRN).

the single helical or non-crystalline forms of (1→3)-β-D-glucans contribute more to the reactivity towards LS-1 than the crystalline form.

The foregoing results show that reactivity of (1→3)-β-D-glucans towards *Limulus* lysate is influenced by several factors, including molecular weight, degree of branching, and conformation.

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