Application of Limulus Test (G Pathway) for the Detection of Different Conformers of (1→3)-β-d-Glucans

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The reactivity of factor G mediated coagulation pathway in limulus amoeocyte lysate which is triggered by (1→3)-β-d-glucans is thought to depend on the structure of the glucans, especially on the ultrastructure: triple helix, single helix and random coil. We used Sonifilan (SPG) and grifolan (GRN) as parent compounds to compare the reactivities of these three conformers. Under a neutral condition, alkaline treated SPG (SPG-OH, single helix) and polycarboxylated SPG (PC-SPG, random coil) showed significantly stronger reactivity than untreated SPG (triple helix). After the alkaline treatment, all three conformers showed comparable reactivities. It is suggested that the pretreatment of the glucan preparations by sodium hydroxide is quite important to compare quantitatively the reactivity of the glucans by limulus test, and comparing the data of untreated and alkaline treated glucans would provide information about their conformations. Using this approach, it was found that after heat treatment at around 150°C, the conformation of GRN was changed to rich in the triple helix, and that following sodium hydroxide treatment and dialysis of GRN, the conformation of GRN was changed to single helix rich conformer. About half of the single helix conformer was gradually changed to triple helix conformer over one week at 4°C.

Keywords limulus test; β-glucan triple helix; β-glucan single helix; Sonifilan; conformation; factor G

The amoeocyte lysate from horseshoe crab (limulus) coagulates in the presence of gram-negative bacterial endotoxins (lipopolysaccharides, LPS) or (1→3)-β-d-glucans. Initiation of LPS and (1→3)-β-d-glucan triggered reactions is mediated by specific zymogens called factor C and factor G, respectively.1 Gelation reaction of the lysate, so-called limulus test, has been applied to determine the concentration of LPS in medicine and biological fluids. The limulus test is a simple and very sensitive method for the determination of LPS, and has been included in the Japanese Pharmacopoeia since the X1th edition. Each of these pathways involves several serine proteases that are activated sequentially. Some of these proteases are common in both pathways. Measurement of the amido-lytic activity of clotting enzyme with chromogenic substrate instead of gelation assay improved the sensitivity of the reaction.2

(1→3)-β-d-Glucans are present in a variety of sources, including fungi, yeast, algae, bacteria, and higher plants, and in comparison with LPS, their structures are also significantly varied depending on molecular weight, degree of branching, solubility in water, and ultrastructure. In addition to the structural variability, (1→3)-β-d-glucans show a variety of biological activities. Mechanisms of their mediated antitumor activities have been established in strong relation to the host defense. Thus, the structure-activity relationship of (1→3)-β-d-glucan toward parameters of the host defense mechanisms has warranted investigation. Interestingly, the structure essential to show each activity was different,3 i.e. antitumor activity against the solid form of sarcoma 180 does not require helix conform but activation of hematopoietic responses does; the classical pathway of complement has been activated mainly by single helix conformer and the alternative pathway of complement by the triple helix. It is already established that the factor G mediated pathway is completely dependent on the presence of (1→3)-β-d-glucosyl linkage. In a previous paper, we characterized the reactivity of limulus factor G from several points of view and suggested that the reactivity of factor G is, in addition to the (1→3)-β-d-glucosyl residues, dependent on molecular weight, conformation, and degree of branching of the glucans.4,5 We also suggested that the single helix conformer is important to the reactivity of factor G when the reconstructed (1→3)-β-d-glucan specific system.5 However, conformation of (1→3)-β-d-glucans is reversible, at least in part, and the stable conformation would be different in each glucan. Thus, it is not enough to establish the structure-activity relationship of limulus factor G.

Because of the increasing incidence of fungal infection, especially in relation to the host immunocompromised for various reasons,6,7 measurement of the β-glucan concentration in the blood using factor G is critically important for an early judgment of the fungal infection.7 In order to apply the test properly, establishment of the structure-activity relationship of factor G mediated pathway and optimization of the reaction condition are important. Clarification of the structure-activity relationship of structurally defined β-glucans and its derivatives would be the best way to establish the concept. In this paper, we will show the structure-activity relationship for (1→3)-β-d-glucans in the factor G mediated (coagulation) pathway in the limulus test using kinetic measurement.

Materials and Methods
Grifolan (GRN) was prepared from mycelia of Grifola frondosa as described previously.8 SPG (Sonifilan prepared from schizophyllan) was generously provided by Kaken Pharmaceutical Co., Ltd. (Tokyo). Polycarboxylated SPG (PC-SPG) was prepared by the literature procedure.9 Distilled water was purchased from Osaka Co., Ltd. and sodium hydroxide was purchased from Wako Pure Chemical Industries, Ltd.

Formation of Complexes with Aniline Blue10 The glucans were dissolved in aniline blue solution (10 μg/ml; 0.1 or 0.3 N NaOH), the intensity
of fluorescence was measured at an excitation wavelength of 400 nm and emission was scanned from 450 to 550 nm using a Hitachi 650-40 fluorescence spectrophotometer.

Heat Treatment of GRN
GRN suspended in distilled water (2.5 mg/ml, 10 ml) was heated to 150°C using a glass tube with a screw cap in an aluminum-block heater for 30 min. Insoluble material was centrifuged and the supernatant was heated again under the same conditions for an appropriate period. The molecular weight of each fraction was determined by elution from a column of TSK-GEL HW-65F with 0.3 N sodium hydroxide. Dextran T10—T2000 (Pharmacia) were used as standards. The carbohydrate contents of fractions were monitored by phenol-sulfuric acid method.

Limulus Test
The activation of factor G by glucans was measured by the colorimetric method using an endotoxin quantitative kit (Toxicolor LS-1, lot 310128, Seikagaku Corporation, Tokyo) which contained factors C and G. Glucans were diluted in three different ways: condition a: with distilled water; condition b: with 0.01 N NaOH; and condition c: treated with 0.5 N NaOH and diluted with 0.01 N NaOH. 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 or 0.01 N alkali, was placed in the plate and the reagent (LS-1 25 μl) was added to each well. The plate was incubated for 40 min at 37°C, and during the incubation period, the absorbance at 405 nm was measured using a microplate reader (Wellreader SK601, Seikagaku Corp.) every 5 min. Re-LPS (Sigma, L-9764), prepared from Salmonella minnesota Re595, was used as the reference endotoxin. Disposable plastic for the tissue culture or in the clinic were used, and all glassware was sterilized at 260°C for 2 h. All operations were performed under aseptic conditions.

Results

Kinetics of Factor G Mediated Pathway
Until recently we have used conventional endpoint assay to determine the concentration of (1→3)-β-D-glucan in various solutions. However, especially in the case of factor G mediated pathway, higher concentration of the glucan solution does not always show higher reactivity, because a certain inhibition mechanism of the factor G mediated cascade exist. We felt that measurement of kinetics might give additional informations.

Figure 1 shows representative data of a dose response curve of sodium hydroxide treated Sonifilan (SPG-OH) by kinetic measurement. Optical density of the reaction was increased dose dependently in the range of 100 pg/ml—1 μg/ml. To analyze the kinetics of SPG-OH more precisely, cross sections of Fig. 1 made by absorbance and reaction time are shown in Fig. 2. Compared with the endpoint analysis, one can choose optimum cross sections to analyze each point of data. We used cross sections of 15 min or A 0.1 to analyze the following data.

Effect of Sodium Hydroxide Treatment of SPG on the Reactivity of Limulus Test
It is noteworthy that, under the appropriate condition, the limulus test could determine the concentration as well as conformation of the glucan. First of all, to learn the optimum condition for determination of the concentration of the glucan, we measured the limulus reactivity under various reaction conditions. SPG was used for this experiment, because its conformation was determined to be triple helix by a couple of methods. Figure 3 shows the reactivity of limulus test by various dilution methods of SPG. It is known that 150°C, sodium hydroxide, or urea treatment changes the conformation of SPG. In the present section, we used the sodium hydroxide treatment. Using more than 0.25 N sodium hydroxide solution, conformation of the glucan changed from helix to random coil, and neutralization or dialysis to lower the sodium hydroxide concentration returned the conformation to the helix. Interestingly, however, the content of triple helix conformation was not the same as that before treatment, and the alkaline treatment was
suggested to increase the single helix conformer assessed by solid state nuclear magnetic resonance (NMR) spectroscopy. Because we wish to learn the optimum reaction condition under which to determine the concentration of the glucan without any concern about the conformation, we treated glucans with several dilution conditions as follows: distilled water (condition a), 0.01M NaOH (condition b), first dissolved in 0.5M then diluted with 0.01M NaOH (condition c). As stated, Fig. 1 shows the kinetics of SPG treated with condition c and Fig. 3 summarizes the dose response obtained at 15 min. Figure 3 indicates that the reactivity is clearly separated into higher (conditions c) and lower (conditions a, b) depending on the conditions of pretreatment. SPG treated with 0.5M NaOH showed significantly higher reactivity than that without treatment. This can be explained as follows: when SPG was treated with 0.5M NaOH, its conformation was turned from triple helix into random coil. Dilution of the treated SPG by 0.01M NaOH (SPG-OH) changed the conformation from random coil to single helix and kept its reaction site to limulus factor G. The sodium hydroxide treatment is thus be the best to show the strongest reactivity.

**Relationship between Conformations of (1→3)-β-D-Glucans and Their Reactivity to Limulus Test**

As described above, (1→3)-β-D-glucan possesses at least three distinct conformers, triple helix, single helix, and random coil in aqueous solution. In high molecular weight glucan, random coiled conformer could be stable only under special conditions such as in dimethyl sulfoxide (DMSO) solution, in urea solution, or in NaOH solution. Thus, the reactivity of the random coiled conformer has not yet been clearly demonstrated. There is previous evidence obtained using carboxymethylated GRN (CM-GRN) prepared from *Grifola frondosa* that CM-GRN (degree of substitution of CM; 0.78) significantly reacted with limulus test, but these data were not compared quantitatively. On the contrary, in the course of study on β-glucan in yeast cell wall, we identified a segment which could detected by conventional, aqueous $^{13}$C-NMR spectroscopy. It is assumable that at least a part of the yeast β-glucan contained random coiled segment. In view of the clinical use of the limulus test, it is quite important to demonstrate the reactivity of this segment. We therefore prepared a polycarboxylated derivative of SPG (PC-SPG) to clearly demonstrate the reactivity of random coiled conformer of considerably high molecular weight (MW) glucan. We previously prepared the PC-derivatives of GRN, and characterized the physicochemical as well as immunopharmacological properties. All the physicochemical data obtained by $^{13}$C-NMR spectral analysis, dye binding, and viscosity have confirmed the conformation of PC-GRN as random coiled conformer under the physiological condition (in neutral aqueous solution). Immunopharmacological data of PC-GRN, especially significantly reduced ability to induce acute phase responses, confirmed the conformation of PC-GRN as the random coil. PC-GRN has shown strong reactivity to limulus lysate (data not shown). To quantitatively evaluate the reactivity of limulus reagent to the high molecular weight random coiled conformer, we prepared the PC-derivatives of SPG and compared the reactivity with that of SPG. Conformation of PC-SPG was assessed by the fluorescence of aniline blue. As shown in Fig. 4, SPG induced significant fluorescence in the presence of aniline blue in 0.1M NaOH solution and the fluorescence was significantly reduced in 0.3M NaOH solution. PC-SPG did not induce any fluorescence in the presence of aniline blue either in 0.1 or 0.3M NaOH solution, suggesting it as random coiled conformer.

As shown in the above section, we optimized the sample pretreatment condition to show the glucan mediated limulus reaction. Thus we used conditions a and c to compare the reactivity. Under the conditions employed, gel forming glucan showed stronger activity under condition c. As shown in Fig. 5, in PC-SPG, reactivities under both conditions showed comparable reactivity. These data strongly suggested that random coiled conformer reacts with limulus reagents comparable to the single helix conformer. As discussed below, we prepared smaller MW GRN by heat degradation method (HD-GRN), and these MW were dependent on the reaction time. Fluorescence intensity of HD-GRN in the presence of aniline blue was also reduced dependent on the reaction time. After 2–4 h of reaction, reactivity to aniline blue was significantly reduced, but the limulus reactivity was still substantial. The previous data that random coiled conformer does not activate limulus reagent may be related to the MW of the products.

**Estimation of the Ratio of Triple and Single Helices by Limulus Reaction**

(1→3)-β-D-Glucons were obtained from various sources by various preparation and purification methods to develop a biological response modifiers. As described above, ratio of triple and single helices in various glucans vary for a variety of reasons, such as structure, sample preparation, and molecular weight. It is generally accepted that sodium hydroxide treatment increased the ratio of the single helix conformer and 150°C treatment increased the triple helix one. The relationship of conformation and biological activity is quite an interesting subject, and several biological activities in addition to the limulus reactivity have been found to be influenced by the conformation: complement activation, plasma clotting,
Fig. 4. Fluorescence Intensity of SPG or PC-SPG Mixed with Aniline Blue

Solutions of SPG (200 μg/ml) or PC-SPG (200 μg/ml) were mixed with equal volume of aniline blue (20 μg/ml) and fluorescence intensity was measured in 0.1 or 0.3 N NaOH solution as described in Materials and Methods. a, SPG; b, SPG-OH; c, PC-SPG; d, PC-SPG-OH. ----, 0.1 N; ---, 0.3 N.

Fig. 5. Reactivity of SPG and PC-SPG Diluted under Conditions a and c

Solutions of SPG and PC-SPG were diluted under conditions a and c and the limulus reactivity measured: SPG diluted with condition a (○); SPG diluted with condition c (●); PC-SPG diluted with condition a (□); PC-SPG diluted with condition c (■).

and antitumor activity. Until now we could only use solid state NMR to determine the conformation of the glucan.15) The data shown in this paper suggests that even in the glucan preparation of mixed conformations, it might be possible to measure the maximum reactivity of the preparation by sodium hydroxide treatment, and thus be able to determine the ratio of conformation without using a huge apparatus and large quantity.

To test the applicability of the limulus test to determine the ratio of triple and single helix segments in natural β-glucan, the mixtures of single helix (SPG-OH) and triple helix (SPG) conformers of SPG were prepared and their reactivity compared after dilution with distilled water. As shown in Fig. 6, the reactivity was significantly proportional to the content of the single helix conformer, and the pattern of reaction was shifted to the corresponding dose of single helix conformer. These facts suggested that the ratio of the triple and the single helix conformers would be measurable by limulus test.

As the first application of the procedure, we estimated the changes of conformation of SPG-OH in 0.01 N sodium hydroxide solution while keeping it in a refrigerator for a week. As shown in Fig. 7, compared with the reactivity of freshly prepared solution, that of the preparation kept 7d in the refrigerator was lower. The reduced reactivity would correspond to the increasing concentration of the triple helix segment. To estimate the ratio of the single helix conformer, we calculated the concentration to show absorbance of 0.1 (A 0.1). The concentration of the single helix part in freshly prepared and 7d old preparations was $10^{-7.60}$ g/ml (25.1 ng/ml) and $10^{-8.24}$ g/ml (5.75 ng/ml), respectively. This suggested that about 77% of single helix conformer would be changed to triple helix one during a week.

Sodium hydroxide treatment, neutralization, and dialysis
were used to prepare single helix rich conformer for determination of the conformation dependency of the glucan on various biological activities. As shown above, the single helix conformer of the branched glucan was gradually changed to the triple helix one, thus, we then compared the reactivity of sodium hydroxide treated GRN after dialysis. As shown in Fig. 8, these data suggested that about 87% of single helix conformer was changed to triple helix one during dialysis.

We previously reported that the reactivity of limulus test was significantly related to the MW of the glucan\(^4\), to demonstrate this dependency, we prepared, using heat degradation method\(^{10}\) derivatives of GRN having a variety of MW. We found that increasing the incubation time resulted in lowering the MW, and in reduction of the reactivity of limulus test. Although we knew the conformation dependency of the limulus reactivity, until recently we did not clearly recognize the importance of conformation on this reactivity. We found earlier that the triple helix conformer was abundant after heat degradation, as assessed by solid state NMR spectroscopy\(^{10}\). However, in that report we were not concerned about the pretreatment of the sample and used distilled water for the sample dilution (condition a in this paper). It is notable that the previous data were influenced by both MW and conformation. Thus we examined again the molecular weight dependency of limulus test using heat degraded GRN. Heat degradation products of GRN (HD-GRN) were prepared after period of incubation of 30 min, 1, 2, 4 and 6 h. Average MW of these fractions was calculated as 250, 148, 40, 15 and 6.4 kDa. Figures 9a and b show the dose response of these derivatives under conditions of a and c. Similar to the previous results, the reactivity under condition a was significantly reduced in relation to the heating period. Interestingly, the reactivity under condition c was significantly higher than that under condition a, confirming the existence of a significant proportion of triple helix conformer in HD-GRN. The data under condition c suggested that MW higher than about 10 kDa would show comparable reactivity. As shown before, the fluorescence intensity of aniline blue in the presence of HD-GRN was significantly reduced dependent on the reaction time (Fig. 10). The fluorescence was significantly reduced in the case of HD-GRN (2 h) and almost disappeared in the case of HD-GRN (4 h). In contrast, limulus reactivity of these fractions under condition c was still significant. These data suggested that not only single helix conformer but also random coiled conformer could activate factor G mediated pathway.

**Discussion**

In this paper we attempted to clarify the structure–activity relationship of limulus test on the different conformers of \((1\rightarrow3)\)-\(\beta\)-d-glucans, and applied a method to determine
the ratio of conformers in the glucan preparations. Two observations were interesting. The first is that the triple helix conformer did not react with limulus test, but the single helix and the random coiled conformers reacted equally. The second is that limulus test could be applied to determine the ratio of conformers in the glucan preparations.

It is of great importance to develop a method to determine the conformations of (1→3)-β-D-glucans, because many of the glucan mediated immunopharmacological activities such as complement activation and macrophage activation are dependent on the conformation. Gel formable (1→3)-β-D-glucan, especially that extracted from mushrooms under severe conditions, contains such microheterogeneities as inequable distribution of branches and branching in the main chain producing a tree-like structure, even after its purification to homogeneity. To clarify the structure–activity relationships of these glucans, comparison of the data of different research groups on the conformations must be done by an easier methods than solid state NMR, in addition to compare the primary structure. We believe that the limulus reactivity would be a suitable parameter to compare the conformation of the glucans. It might be possible to determine the ratio of conformers by comparing the limulus reactivities of the glucans with and without sodium hydroxide treatment using a minute quantity.

During studies of the gel-sol transition of (1→3)-β-D-glucans by conventional aqueous 13C-NMR spectroscopy, we found that the signals attributable to the helix conformers were observable under a sodium hydroxide solution even below 0.25 N, which is the critical transition point of the concentration of gel to sol. However, no signals attributable to the helix conformers were observed under the neutral aqueous solution. Using this approach, we found a significant quantity of the β-glucan signals on the 13C-NMR spectra of the yeast suggesting the presence of random coiled conformer in the cell wall of yeast. It is quite important to clarify the structure and conformation of the β-glucans measurable by limulus test in order to clinically apply the test to monitor fungal infection. During the series of experiments on the structure–activity relationship of CM derivatives of (1→3)-β-D-glucans, we found by conventional aqueous 13C-NMR spectroscopy that the derivatives having higher than 1/6 substitution of CM groups in the main chain glucose (1 CM group per 6 main chain glucose units) have lost their gel formability. The derivatives higher than 1/6 substitutions of CM group also showed significant reactivity to limulus test. However, the possibility cannot be denied that the distribution of substitution group was not uniform and that the derivative contained a helix segment. Thus we used a strategy to oxidize branched glucosyl group to carboxyl group (poly-carboxylated (PC) derivative). Using model compounds, like PC-SPG, we found that the random coiled conformer showed comparable reactivity with the single helix. Tanaka et al. reported using reconstructed factor G mediated coagulation pathway that in addition to the 6-branched (1→3)-β-D-glucan, glucans showing low helix formable one, lichenan and barley glucan (1,3-, 1,4-glucan), showed significant reactivity. These results strongly supported our conclusion. It is noteworthy that the β-glucan having
sol conformer might also contribute to the reactivity of limulus test in clinical materials like blood and specimen of organs. It should be noted that the conclusion that the random coiled conformer could activate factor G could be strongly related to the method of assessment and any limitation of that method.

As discussed above, the conformation of (1→3)-β-D-glucan is significant for its biological activities. The alternative pathway of complement was activated by triple helix conformer.\(^3\) Antitumor activity against the solid form of sarcoma 180 was shown by all of the triple helix, single helix, and random coiled conformers.\(^3\) Several methods have been utilized to determine the solution conformation of (1→3)-β-D-glucans: conventional \(^{13}\)C-NMR spectroscopy, dye binding (congo red and aniline blue), and viscosity measurement. It should be noted that each of the analytical methods has limitations. NMR spectroscopy is utilized based on the mobility of the glucan segment, which is closely related to the inter- and intrachain interactions due to the presence of helix conformation. Dye binding is utilized based on the micro-environment where a helical conformation can be produced. Application of limulus test to estimate conformation of the β-glucan is quite useful as discussed above. However, it must be noted that higher concentration of the glucan inhibited the factor G-mediated pathways. Usually the β-glucans thought to be acted as both activator and inhibitor. In order to use limulus test to estimate conformation of the glucan, optimization of the reactive conditions is important.

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References


