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Sodium hydroxide-induced conformational change in schizophyllan detected by the fluorescence dye, aniline blue

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Abstract

Molecular conformation is considered to be an important factor in determining the biological activity of glucans; however, a simple method to detect the conformation change for glucans in solution has not been developed. We found that the fluorescence intensity of aniline blue bound to schizophyllan (SPG) can be used to estimate the relative amount of single helix converting to triple helix during different stages of a denature–renature cycle. This observation provides a method to monitor conformational change that is simpler and easier to perform than other techniques (such as solid-state ^{13}C NMR spectroscopy). The native conformation for SPG [a branched β-(1→3) glucan] is a rigid, closed triple helix. Treatment with NaOH, followed by neutralization, produces a single helix-rich preparation. We observed that aniline blue does not stain native SPG, but will stain the renatured NaOH-treated SPG. This suggests that aniline blue binds only to single helix forms of SPG. Further supporting evidence is that the fluorescence intensity is decreased on consecutive days after neutralization, which is consistent with the report that NaOH-treated SPG gradually lost 77% of their single helix component in 1 week (N. Nagi, N. Ohno, Y. Adachi, J. Aketagawa, H. Tamura, Y. Shibata, S. Tanaka, and T. Yadomae, *Biol. Pharm. Bull.*, 16 (1993) 822–828). The single helix is the conformation which activates the *Limulus* amebocyte lysate (LAL). The biological reactivity of renatured SPG, stabilized with aniline blue at different days, was evaluated using a glucan sensitive LAL. The activity of LAL toward SPG was decreased over time, suggesting that the conformation of glucan detected by fluorescence intensity correlated with the LAL activity.

Keywords: Schizophyllan; Conformation; Fluorescence spectroscopy; Aniline blue

1. Introduction

β-(1→3)-D-Glucans exhibit a variety of biological and immunopharmacological activities. The important factors for determining the biological activity are molecular weight (MW), degree of branching (DB), and conformation [1]. Three conformations of glucans have been reported: the triple-helix, the single helix and the random coil [2]. The biologically active form of glucan is unclear. Several studies have suggested that the triple-helix

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is the most biologically active conformation [3,4]. More recently, reports suggest that the single helix is more potent [5,6]. Most of these structure–activity studies have used solid-state ^{13}C NMR spectroscopy to determine conformation [7,8]. However, this technique is not applicable for determining conformation in solution, which may be more relevant to in-situ biological activity. Furthermore, because the two peaks assigned to the triple and single helix by solid-state ^{13}C NMR spectroscopy are very close (86 and 89 ppm, respectively), most studies report a single broad peak, which corresponds to the average amount of the single and triple helix present.

Previous studies have reported that the dye aniline blue contains an impurity, sirofluor [sodium carbonylbis(4-(phenyleneamino)benzenesulfonate)], that binds specifically to β-(1→3)-glucans [9–13]. When bound to β-(1→3)-glucans, sirofluor fluoresces (ca. 140 times increase). The marked enhancement of fluorescence induced by binding to β-(1→3)-glucans is thought to occur via H-bonding rather than by hydrophobic bonding [13].

One method of preparing the single helix-rich forms from triple-helix glucan is to treat it with NaOH, followed by neutralization and dialysis against distilled water [1,5]. Over time, the single helix will revert to the triple helix, which is a more stable conformer than the single helical form [1].

The details of this transformation are not well understood. The objectives of this study were to evaluate the interaction between dye aniline blue with SPG, a soluble triple helix, after treatment with NaOH. Specifically, we treated SPG with NaOH, neutralized the preparation, and added aniline blue at different times after neutralization to determine the state of conformation. We also evaluated the effect of the different conformations of SPG, stabilized with aniline blue, to activate a glucan-responsive LAL assay.

2. Results

Aniline blue fluoresces only when it binds to glucan in solution. However, the fluorescence intensity of aniline blue–glucan complexes can be affected by several factors of the solution, such as ionic strength, pH and temperature. In order to correlate the fluorescence changes of the dye with conformational changes of the glucan, we have studied fluorescence of glucan–aniline blue complexes under different experimental conditions.

Effect of ionic strength and pH.—Ionic strength of the solution was found to increase the fluorescence intensity of laminarin–aniline blue complexes (Fig. 1). As the ionic strength changes with the denaturation and renaturation of the glucan solution by the acid-base neutralization, we performed the present studies near saturation to avoid the effects of ionic strength on the fluorescence changes (about 0.5 M).

Evans et al. [10] reported that laminarin binding to aniline blue enhanced the fluorescence intensity of the dye, and this florescence enhancement was pH dependent. As the pH of the solution increased from 3 to 10. the fluorescence increased and reached a maximum at about pH 11.5, then dropped to zero at pH levels higher than 13. This is consistent with our observation for laminarin (Fig. 2). However, SPG in neutral solution did not bind with aniline blue and showed very low fluorescence intensity. Even in pH 11.5 buffer solution, where aniline blue had maximum sensitivity with laminarin, only a very low amount of fluorescence intensity was observed. These data suggested that the native SPG containing the triple

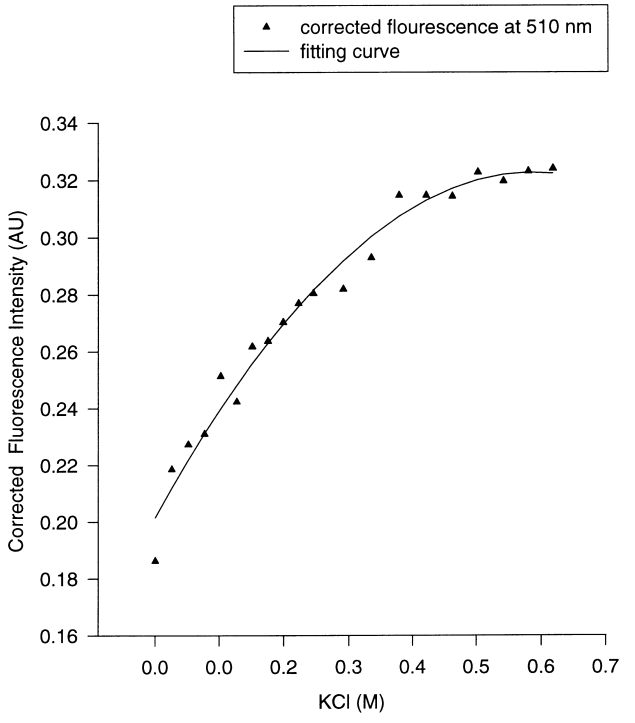

Fig. 1. Effect of ionic strength on laminarin–aniline blue fluorescence. Laminarin 1.5 mL (0.5 mg/mL) + 10 μL aniline blue (0.436 mg/mL) in pH 7 buffer. The ionic strength of the solution was changed by adding ~10–20 μL of 3.95 M KCl. The peak intensity at 510 nm has been corrected for change in total volume.

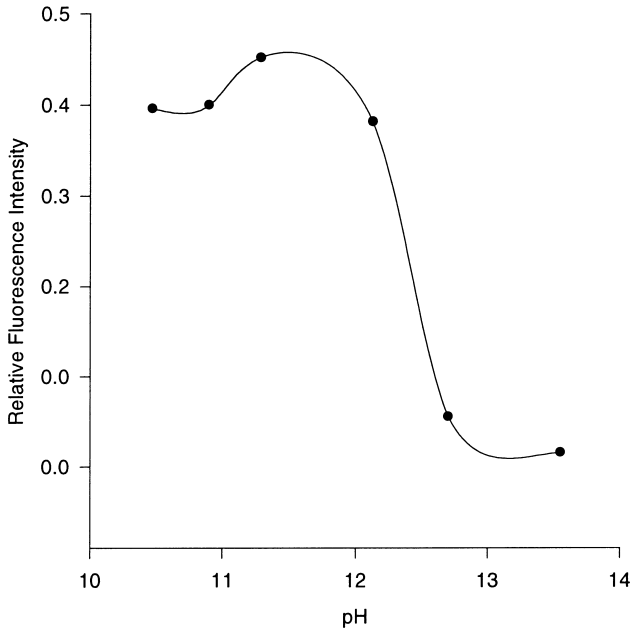

Fig. 2. pH Dependence of laminarin–aniline blue fluorescence observed at 510 nm. To three mg of laminarin in six different pH buffer solutions (pH 10 to 12.9) was added 10 μL of aniline blue (0.436 mg/mL), and the fluorescence intensity was measured at 510 nm.

helix conformation does not bind with aniline blue. However, when the sample was treated and denatured with NaOH ($\geq$0.25 M), then adjusted to pH 11.5, a high fluorescence intensity was observed. This indicated that an open conformation of SPG was formed after NaOH treatment. This open conformation, similar to laminarin, bound to the dye aniline blue, thus enhancing the observed fluorescence. The observed fluorescence changes (Fig. 3) also suggested that the denaturation of the glucan is NaOH-concentration dependent. Higher NaOH concentrations caused a higher degree of denaturation of the glucan and, therefore, resulted in a higher fluorescence intensity of glucan–aniline blue complex.

Interaction between denatured glucan and aniline blue.—The interaction between the dye aniline blue and denatured glucan, monitored by fluorescence changes, shows a time dependence. Fig. 4 shows the time course of fluorescence of denatured SPG–aniline blue complex at 20 °C. This fluorescence change can be described by a nonlinear equation expressed as:

$$F = Ae^{-kt} + B,$$

where F is the fluorescence intensity of the solution, A and B are constants, t is time (s) and k is the overall reaction rate constant for the interaction between the dye and the glucan.

At 20 °C the interaction rate of aniline blue with SPG is very slow (3.5 h). If the temperature is increased to 25 °C, the rate is much faster. Table 1 lists the reaction rate constants (k) for laminarin and SPG after denaturization treatment with different NaOH concentrations. The rate constant k is in the range of 1.1–$\sim$2.0$\times10^{-3}$ (1/s), indicating that

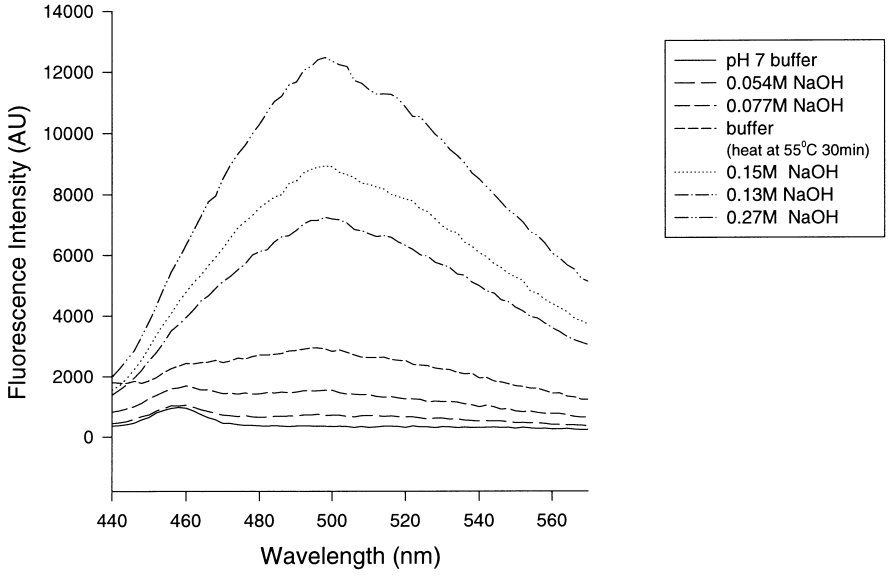

Fig. 3. Fluorescence intensities of SPG denatured by different NaOH concentrations. SPG 100 μL (0.03 mg/mL) was treated with different concentrations of NaOH solution, neutralized in pH 7 buffer, 10 μL of aniline blue (0.436 mg/mL) was added, and the mixture was continuously stirred for 1 h. The fluorescence intensity was measured after 1–2 days.

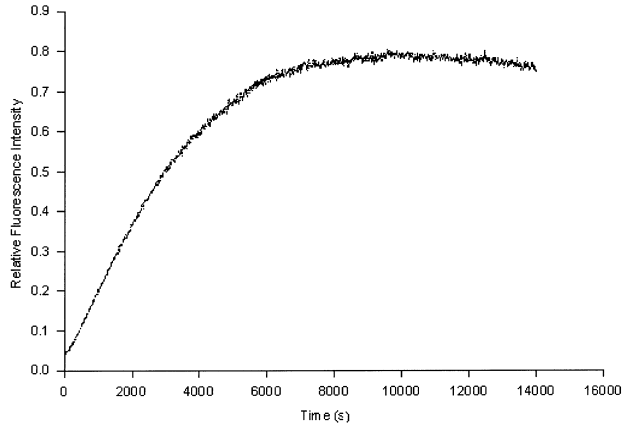

Fig. 4. SPG renatured in pH 11.5 buffer at 20 °C with 10 μL of aniline blue (0.436 mg/mL). The slow kinetics of SPG was measured immediately after neutralization by addition of HCl.

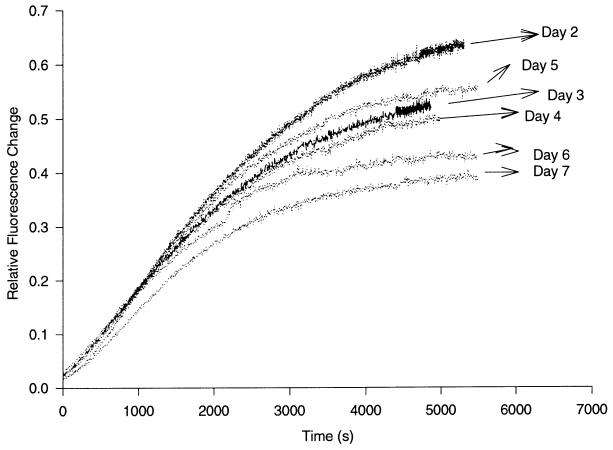

Fig. 5. Days of adding dye versus fluorescence intensity of SPG. The slow kinetics were measured immediately after SPG renatured (at 510 nm).

the binding of aniline blue with glucans is slow. Normally, dye staining is expected to be completed within minutes. The rate-determining step for aniline blue staining with glucan may be related to the slow dynamic motion of glucan molecule at 20 °C, since at 25 °C we observed a much faster binding rate.

Renature of the denatured glucan.—Denatured triple–helix glucan undergoes a slow renaturing process in the solution (Figs. 5 and 6). Fig. 5 shows the slow kinetics of fluorescence after adding aniline blue on consecutive days following renaturation of SPG, while Fig. 6 shows the intensity of the fluorescence emission spectrum of dye added to renatured SPG at different days. The time course for development of fluorescence in Fig. 5 is similar to the pattern observed in Fig. 4. For all but day 5, the relative height of fluorescence intensity at a fixed time (for example, 4500 s) decreased with the increasing number of days. This is consistent with Fig. 6, showing there is a decreasing trend of fluorescence intensity with the increasing number of days. These results suggest a slow renaturation process of the open conformation of glucan after denaturation treatment with NaOH. The reason for the anomaly in fluorescence intensity at day 5 is unclear. It may be related to the small differences in the pattern of the SPG–aniline blue fluorescence for days 3–5, which would suggest a similarity in the number of binding sites available over these times.

An interesting observation in this study is that aniline blue can stabilize the open conformation of lucan for a certain period of time. After the addition of aniline blue to denatured SPG, the fluorescence intensity of the SPG–aniline blue complex remained reasonably constant for 2 weeks. For

Table 1
Staining rate for aniline blue for same concentration of glucan but with different concentrations of NaOH, followed by neutralization at 20 °C

Laminarin		Schizophyllan	
NaOH (M)	Rate (1/s)	NaOH (M)	Rate (1/s)
0	1.19×10^{-3}	0.077	1.50×10^{-3}
0.09	1.46×10^{-3}	0.135	1.46×10^{-3}
0.177	1.53×10^{-3}	0.15	1.23×10^{-3}
0.33	1.53×10^{-3}	0.18	1.45×10^{-3}
		0.33	2.08×10^{-3}

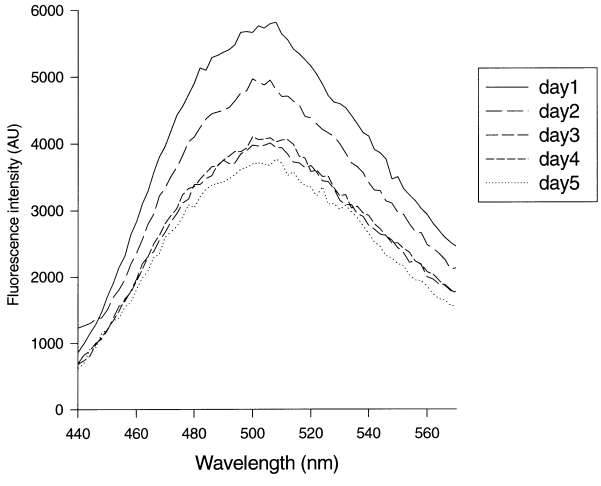

Fig. 6. To determine the decrease of fluorescence intensity for renatured SPG, aniline blue was added from day 1 to day 5. Aniline blue was added to a set of same concentration of SPG solution at different days. The fluorescence intensity of this set samples was measured at the same time to avoid the possible variation of lamp output.

example, the maximum fluorescence intensity at 20 °C was 0.45, 0.44, 0.44 and 0.50 at day 2, 5, 7 and day 15, respectively. This suggests that the reaction is irreversible at 20 °C. Since only the single helix-rich portion of glucan is capable of binding with aniline blue, we would have observed a decrease in fluorescence intensity as the glucan renatured if the staining reaction were reversible.

Reactivity of glucan–aniline blue complexes with LAL.—The concentration of endotoxin in the glucan–aniline blue complexes was evaluated using a non-glucan responsive LAL lysate (WB). Table 2 shows that while there was a dose-dependent response to increasing levels of native SPG at a concentration of 3∼30 ng/mL in the glucan-insensitive lysate from WB, the corresponding levels of endotoxin, expressed as lipopolysaccharide (LPS) (ng/mL), were low relative to the same concentration level with a glucan-sensitive lysate (ACC). These data demonstrate that we could assess the glucan concentration using the ACC lysate.

To determine if glucan conformation affected LAL reactivity, denatured SPG that had been stabilized with aniline blue (0–105 h) at different times after neutralization was evaluated with both glucan-sensitive and insensitive lysate (Table 3). There was a minimal response by the LAL from WB, indicating that the samples were not contaminated with endotoxin and had limited responsiveness to SPG. For the glucan-sensitive lysate, the response to SPG was much higher than the WB lysate and decreased from 68.8 LPS ng/mL at zero h (a higher proportion of single helix SPG) to 10.6 LPS ng/mL at 105 h (a higher proportion of renatured triple helix SPG).

Table 2
Effect of SPG concentration (native form) on the reactivity of LAL from WB and ACC

SPG concentration (μg/mL)	0.03	0.3	3	30
Corresponding LPS ng/mL (from WB)	< 0.0005	< 0.0005	0.0056	0.0430
Corresponding LPS ng/mL (from ACC)	0.101	0.337	4.094	18.117

To rule out the possibility that the observed decrease of LAL responsiveness was caused by adding the aniline blue, we compared the responsiveness of glucan sensitive LAL to different preparations of glucan with and without aniline blue. The results show that the effect of adding aniline blue was to decrease the overall responsiveness of glucan to LAL activity (Table 4). However, the denatured glucan was more reactive than native glucan both with and without aniline blue, confirming that the glucan sensitive lysate is more responsive to single helix conformation.

3. Discussion

β-(1→3)-Glucans have been shown to exhibit a variety of biological and immunopharmacological activities, including the activation of macrophage through interaction with a specific cell-surface receptor [14]. This specific interaction suggests that molecular conformation is an important parameter in understanding the structure–activity relationships of glucans. Although three conformations of glucans have been reported (the triple helix, the single helix and a random coil [21]), little information is available on their conformation in solution. The conformation of glucans in solution is difficult to study because of their low water solubility. In general, treatments such as adding strong acid or base or Me_2SO are needed to enhance the water solubility. However, these treatments may change the conformation of glucan, which complicates the interpretation of studies evaluating structure–activity relationships. To avoid these problems, we used glucans with good water solubility to study their conformation in solution and their structure–activity relationships.

The triple helix conformer of glucan is formed by three hydrogen bonds (H-bonding) to oxygen in the C-2 position [15,16]. While these bonds are relatively strong, the H-bonding can be broken by high temperature, high pH, or with selected solvents. For example, the temperature required to break the triple helix structure of SPG is 135 °C

Table 3
Effect of conformational change on the LAL reactivity of SPG after denaturation–renaturation (SPG, 9.23 μg/mL pH 7.8)

Time added aniline blue	0 h	17 h	32 h	43 h	60 h	73 h	105 h
Corresponding LPS ng/mL (from WB)	0.34	0.53	0.39	0.26	0.20	0.13	0.02
Corresponding LPS ng/mL (from ACC)	68.84	58.34	45.99	39.07	34.35	29.55	10.55

Table 4
Effect of adding aniline blue on the responsiveness of glucan toward LALs (from ACC). Expressed in corresponding LPS concentration (ng/mL)

	Native glucan	Native glucan + aniline blue	Denatured glucan	Denature glucan + aniline blue
Laminarin	8.9	2.4	77.6	16.8
SPG	8.0	3.8	51.0	21.0

[17]. At high pH (> 0.24 M NaOH), SPG becomes a random coil conformation, and in Me_2SO SPG has been shown to have a single-chain conformation [18]. Among those three methods, the addition of a strong base, such as NaOH, provides a convenient method to break the H-bonding at room temperature, which can be reversed by neutralization.

Our experimental results show that aniline blue does not bind with native conformation of SPG (triple helix), but it does bind with NaOH-treated SPG, a single helix-rich conformation. The denatured single helix-rich form of SPG, once neutralized, is not stable and will slowly revert to triple helix forms. This process can be monitored by observing the fluorescence signal change of SPG–aniline blue complexes when aniline blue is added at different times after neutralization. Furthermore, the fluorescence of the SPG–aniline blue complexes is stable over 15 days, suggesting that the aniline staining is an irreversible reaction at the conditions used in this study.

Based on these observations, we studied and evaluated the relationship of structure and activity of the glucan SPG after NaOH treatment using the LAL assay. We used the *Limulus* amebocyte lysate (LAL) assay in this study to evaluate one type of biological activity of the different stabilized conformations of glucan. This assay, derived from the horseshoe crab, is commonly used to evaluate Gram-negative bacterial endotoxins. Endotoxin activates a specific enzymatic pathway, via a component designated as factor C, which results in the formation of a clot. The *Limulus* reagent also contains an alternative pathway that is sensitive to glucans, via a component designated as factor G. Single-helix glucans have been reported to be a better stimulant of G factor than triple helix glucans [5,6,19]. Commercially, there are LAL tests that have eliminated either factor G or factor C, which are specific for endotoxin and glucans, respectively. At the time of these studies the glucan specific lysate was not readily available in the United States, and therefore an alternative LAL experiment was done. Different commercial LALs have different responses to glucan [20]. These differences may be due either to species variability of the horseshoe crabs or from specific commercial procedures to alter the glucan sensitivity of the lysate. For example, chloroform extraction in LAL preparation has been shown to increase the sensitivity of LAL toward glucan and is a patent of ACC [20,21], and LAL from WB has been treated to reduce the response to LAL-reactive material, which includes glucans. For our experiments, the background endotoxin contamination was evaluated by the glucan-insensitive LAL from Whittaker Bioproducts, and the glucan levels were evaluated by the endotoxin and glucan sensitive ACC lysate.

Since there is no standard glucan available for these LALs, we used endotoxin as the calibration standard for comparison of the responsiveness of LAL. In separate experiments, serial dilutions of both SPG and endotoxin had a linear relationship in log concentration versus log onset time to the LAL from ACC. In order to compare the day-to-day variation in LAL responsiveness and avoid the lot-to-lot LAL variation, we used aniline blue to stabilize the conformation. To rule out the possibility that the change in LAL responsiveness was caused by aniline blue added to stabilize the different glucan conformation, we compared the responsiveness of LAL to different glucan preparations with and without adding aniline blue. The results showed that aniline blue decreased the overall responsiveness of glucan to LAL activity. The day-to-day decrease in fluorescence intensity in the SPG–aniline blue system was correlated with the decrease in LAL activity (Fig. 5 and Table 3). These data demonstrated that the biological activity as determined by the activation of the enzyme cascade of the LAL assay was correlated to conformational change found in the fluorescence spectrum.

Nagi et al. used the G test to evaluate the activity of SPG at day 1 and day 7 [19]. They suggested that about 77% of the single-helix conformer would be changed to a triple helix once during

seven days by measuring the decrease in reactivity of glucan in G-test. We also observed the same trend in this experiment. Comparing results from the fluorescence experiment from day 2 to day 7, about 39% of the single helix was converted to a triple helix (Fig. 5), whereas comparing results from the LAL experiment, about 84% of the activity was lost from 0 h to 105 h (about 4 days, Table 3).

Both laminarin and SPG have been reported to have a triple-helix conformation; however, in pH 7 buffer, only laminarin shows fluorescence upon being complexed with aniline blue. If aniline blue only stains the single-helix portion, then native laminarin should not fluoresce. This discrepancy may result from the dependence of conformation on MW. Saito et al. have reported that only linear β-(1→3)-D-glucans of intermediate chain length assume a triple-helix conformation on lyophilization from aqueous solution [22]. They suggest that neither the glucans of shorter chains (dp <14) nor longer chains (dp >250) are able to form the triple-helix conformation under the condition of ambient temperature. Although the conformation of laminarin is not clear at this time, they may form a loosely triple helix in solution, which can bind with the aniline blue. After denaturing with NaOH, laminarin also shows a higher fluorescence intensity than native laminarin, which supports the idea of more opening of the triple helix. Experimental data from laminarin and SPG suggested that high concentrations of NaOH are required to denature the rigid triple-helix conformation of SPG, and that aniline blue is only able to bind with the single-helix conformer.

In summary, NaOH treatment of triple-helix glucans caused the formation of single helix-rich conformers which gradually revert to the triple-helix conformer after neutralization. Only single-helix forms of glucan were observed to bind with aniline blue. and the rate of binding was dependent on temperature and the concentration of NaOH used to denature triple helix glucans. The reaction between aniline blue and glucan formed a stable complex, and stable ratios of single helix to triple-helix glucan conformers could be prepared by adding aniline blue at different times after neutralizing NaOH-treated triple-helix glucans. Glucan–aniline blue conformers with a higher proportion of single helix conformers were more reactive with a glucan sensitive LAL than a conformer with a higher proportion of triple helix.

4. Materials and methods

Chemicals.—Laminarin was obtained from Sigma Chemical Co. (MO, USA). SPG in sterile water (10 mg/mL) was kindly provided by Dr. David L. Williams of the East Tennessee State University. SPG normally exists as a rigid, rod-like triple helix in aqueous solution and has good water solubility [3]. The molecular weight of the same SPG as used in this study has been measured by Muller et al. [23] to be 3.06×10^5 g/mol with a narrow polydispersity ($I=1.08$, M_w/M_n). Aniline blue was purchased from Polysciences, Inc. (Warrington, PA). Limulus amebocyte lysate (LAL) reagents were purchased from Associates of Cape Cod (ACC, Pyrotell-T, Woods Hole, MA) and Whittaker Bioproducts (WB, Walkersville, MD). Lipopolysaccharide was purchased from Sigma.

Due to the limited source of SPG, laminarin, a commercially available triple-helix glucan, was used to evaluate some of the variables that will affect the fluorescence intensity of aniline blue–glucan complexes. Laminarin has a MW of 7.7×10^3 g/mol, a polydispersity of 1.17, and an intrinsic viscosity of 0.07 dL/g (D. Williams, pers. comm.). SPG and laminarin are composed of β-(1→3)-D-linked glucopyranose backbone with degree of β-(1→6) side chain of 0.33 and 0.05 for SPG and laminarin, respectively [23,24].

Steady-state fluorescence measurements.—Steady-state fluorescence measurements were carried out on a SLM 8000C or ISS photon-counting spectrofluorometer. Spectral measurements were made at 395 nm excitation, and emission (maximum at 500 nm) spectra were corrected for solvent background and Raman scattering. To avoid the inner filter effect, solution absorbance was controlled below 0.05. Temperature was controlled at 20 ± 0.1 °C unless otherwise mentioned. The slow kinetic fluorescence intensity was measured while constantly stirring. The rate of aniline blue binding with glucan was calculated by a nonlinear curve fitting equation.

Denaturation–renaturation process of glucans.—Glucans were treated with NaOH to pH 13. The increase of fluorescence intensity was measured after adjusting the solution pH to 7 for laminarin and 11.5 for SPG.

The experimental procedure for SPG fluorescence denaturation–renaturation was as follows: SPG (25 μL) was added to 1 mL of 0.25 M NaOH (in 0.5 M NaCl), after 20 min the pH was adjusted

to about 11.6 by adding 1 N HCl. After 2 min this solution was diluted with 10 mL pH 11.5 Na_2HPO_4–NaOH buffer (in 0.5 M NaCl). For binding with aniline blue, 3 μL of 5.52×10^{-4} M (0.436 mg/mL) aniline blue was added to 1 mL of the SPG buffer preparation at different times.

LAL assay from ACC.—The kinetic turbidimetric procedure was used to evaluate the LAL reactivity of glucan [25]. LAL assays were performed in flat-bottomed 96-well microtiter plates as follows: 80 microliters of each glucan preparation and 20 μL of pyrotell T (Associates of Cape Cod) was added to each well; the plates were incubated at 37 °C, and the absorbance of each well was measured every 30 s at 340 nm for 40 min, using a microplate reader (Kinetic-QCL, Whittaker Bioproducts) and communication software (Anthos reader V. 2); the onset time, defined as a change of optical density (ΔOD) of 0.02, was determined for each sample; a standard endotoxin curve (0.0005 to 50 LPS ng/mL) was prepared using a commercial lipopolysaccharide (*E. coli*) from Sigma; then the reactivity of glucans was converted to the corresponding lipopolysaccharide concentration. In each run, lipopolysaccharide 0.5 ng/mL and sterile water were used as the positive and negative controls, respectively.

LAL assay (from WB).—The estimation of possible background endotoxin contamination in glucan solutions was performed by the LAL reagent from WB. Lipopolysaccharide samples were prepared by making a 10-fold serial dilution from the 50 ng/mL lipopolysaccharide solution. LAL reagent water blank (50 μL), endotoxin standards or samples were put into the appropriate wells of a 96-well microplate. The plate was preincubated for 10 min. To each of the solution wells, 50 μL of LAL was added. The onset time for WB, was defined as a change of optical density (ΔOD) of 0.2. The absorbance of each plate at 405 nm was measured continuously using a microplate reader (Kinetic-QCL, Whittaker Bioproducts).

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