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COMPLEX FORMATION OF GEL-FORMING BACTERIAL (1→3)-β-D-GLUCANS (CURDLAN-TYPE POLYSACCHARIDES) WITH DYES IN AQUEOUS SOLUTION

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ABSTRACT

The complex formation of Polysaccharide 13140 (**1**) or 13127 (**2**) [curdlan-type polysaccharides: gel-forming bacterial (1→3)-β-D-glucans] with dyes in aqueous solution was investigated. The (1→3)-β-D-glucans reacted specifically with Aniline Blue (water soluble) (**3**) to form stable color complexes. The rate of interaction between **1** or **2** and **3** in aqueous solutions was shown to be proportional to both concentration and gel-forming ability of **1** and **2**. This complex formation is to be applied to the quantitative analysis and the determination of gel-forming ability of curdlan-type polysaccharides.

INTRODUCTION

A gel-forming (1→3)-β-D-glucan (curdlan, Polysaccharide 10C3K), was first isolated from the culture of *Alcaligenes faecalis var. myxogenes* 10C3K by Harada *et al.*[1] and its gel-forming characteristic attracted attention for its useful application to the field of food manufacture[2]. Subsequently, Nakanishi *et al.*[3,4] reported that Polysaccharide 13140 can be prepared from an efficient polysaccharide-producing mutant derived from the strain 10C3K and that the polysaccharide is gel-forming but slightly different from curdlan in nature. Nakanishi *et al.*[3,4] also reported that some strains of *Agrobacterium radiobacter* produced a similar gel-forming (1→3)-β-D-glucan, which they designated Polysaccharide 13127. Polysaccharides 13140 and 13127 were shown to be linear polymers consisting of (1→3)-linked β-D-glucose residues and to contain no other linkages, such as internal (1→6)-linkages and branch points[4].

As a property of these curdlan-type polysaccharides, the complex formation of Polysaccharides 13140 and 13127 with dyes, especially with Aniline Blue (water soluble), is described in this paper.

EXPERIMENTAL

Materials. — Polysaccharides 13140 and 13127 were obtained from cultures[3] of *Alcaligenes faecalis var. myxogenes* IFO 13140 and of *Agrobacterium radiobacter*

IFO 13127. The average numbers of degrees of polymerization ($\overline{DP}$) of the respective D-glucans, determined by the method of Manners *et al.*[5], were 420 and 450. Partially depolymerized samples of Polysaccharide 13140 were prepared from long-period cultures by the same procedure that gave the standard sample. The depolymerization of Polysaccharide 13140 would be effected by the action of (1→3)-β-D-glucanase produced in low yield by the microorganism.

Pachyman and yeast glucan were prepared from Bukuryo (*Poria cocos*) and yeast cells (*Torula utilis*) according to the method described by Saito *et al.*[6] and Peat *et al.*[7], respectively. Laminaran was obtained from Tokyo Chemical Industry Co. Ltd., Tokyo, and Xanthan gum from Kelco Co., San Diego, California 92101. Other polysaccharides were purchased from Wako Pure Chemical Industries, Ltd., Osaka.

All the dyes employed were reagent grade and used without further purification. Brilliant Blue was purchased from Tokyo Chemical Industry Co. Ltd. Other dyes, including Aniline Blue (water soluble), were obtained from Wako Pure Chemical Industries, Ltd.

Measurement of gel-forming ability (gel strength). — A well homogenized, aqueous suspension containing 20 mg of a D-glucan sample per ml in a test tube of 15 mm in diameter was heated in a boiling water bath for 10 min to form a gel. Strength at breaking point of the gel (1.0-cm thick disc) was measured by a curd tension meter M301A (Iio Electric Co.) with a cylinder of 5.6 mm in diameter and expressed as dyne per cm^2.

Staining of polysaccharides with dyes. — A mixture (6 ml) containing a given polysaccharide (100 mg) and a given dye (0.05 mg) was kept for 30 min at room temperature. The water-insoluble polysaccharides were collected by centrifugation and washed with water several times. The water-soluble polysaccharides were precipitated with methanol, collected by centrifugation, and washed with methanol several times. The formation of color complex was determined visually and is reported in Table I.

Absorption spectra. — A Hitachi 124 spectrophotometer (Hitachi Seisakusho Co. Ltd.) was employed for the determination of the absorbance.

RESULTS

Specificity of color-complex formation. — Color-complex formation with various polysaccharides in the presence of several dyes was investigated (Table I). Curdlan-type polysaccharides, as well as pachyman and yeast glucan, were stained with dyes including Aniline Blue, Brilliant Blue, Trypan Blue, and Congo Red in neutral aqueous media at room temperature. The color complexes were stable and were not decolorized by repeating washes with water or methanol. As shown in Table I, other polysaccharides, such as starch, dextrin, cellulose, agar, and laminaran, were also stained with Brilliant Blue, Trypan Blue, and Congo Red but not with Aniline Blue. From these results, it was concluded that Aniline Blue reacted specifically with polysaccharides having a β-D-(1→3) linkage to form color complexes, laminaran

being the only (1→3)-β-D-glucan not stained with Aniline Blue. Xanthan gum, an acidic polysaccharide, was only stained by Toluidine Blue O and Methylene Blue, as reported by Moraine and Rogovin[8].

TABLE I

STAINING OF POLYSACCHARIDES WITH DYES

Polysaccharide	*Dye*[a]					
	Aniline Blue	*Brilliant Blue*	*Trypan Blue*	*Congo Red*	*Toluidine Blue O*	*Methylene Blue*
Polysaccharide 13140	+	+	+	+	−	−
Polysaccharide 13127	+	+	+	+	−	−
Pachyman	+	+	+	+	−	−
Yeast glucan	+	+	+	+	−	−
Laminaran	−	+	+	+	−	−
Cellulose	−	+	+	+	−	−
Starch[b]	−	+	+	+	−	−
Dextrin	−	+	+	+	−	−
Dextran[c]	−	−	−	−	−	−
Agar[d]	−	+	+	+	−	−
Xanthan gum	−	−	−	−	+	+

[a]Stained +; not stained −. [b]From potato. [c]Mol. wt. 60 000–90 000. [d]In powder form.

Spectrophotometric behavior of Aniline Blue towards (1→3)-β-D-glucans. — Figure 1 shows absorption spectra of Aniline Blue in neutral aqueous media in the presence and absence of Polysaccharide 13140. The presence of the D-glucan caused not only the shift of absorption maximum of Aniline Blue from 575 to 580 nm but also the increase in absorbance of the dye. The difference of absorbance between the dye–glucan mixture and the plain dye solution was maximal at the 590 nm wave-

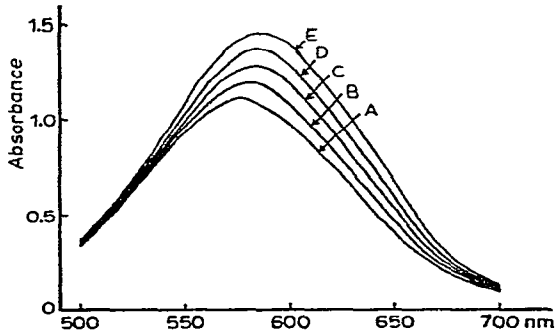

Fig. 1. Influence of concentration of Polysaccharide 13140 on the absorption spectra of Aniline Blue. A solution of 0.5M phosphate buffer (10 ml, pH 7.0) containing 2.5 mg of Aniline Blue and a solution of 5mM sodium hydroxide (10 ml) containing the following concentrations of Polysaccharide 13140 were mixed and kept for 120 min at room temperature, and their visible absorption spectra measured: (A) 0 μg (control), (B) 50 μg, (C) 100 μg, (D) 150 μg, and (E) 200 μg per ml.

length. Polysaccharides not stained with Aniline Blue, *i.e.* dextran and cellulose, neither caused this red shift of the absorption maximum nor increased the absorbance of the dye.

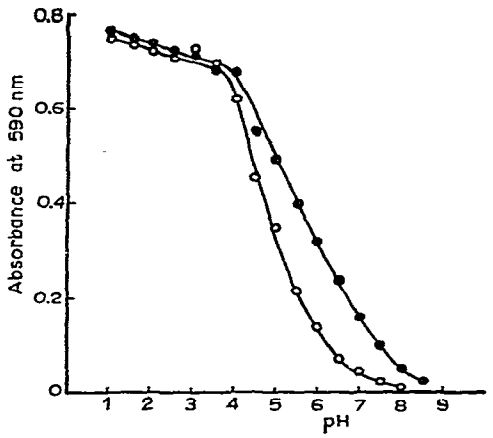

Fig. 2. Influence of pH on the absorbance of the Polysaccharide 13140–Aniline Blue complex. A 5mM sodium hydroxide solution (10 ml) with and without Polysaccharide 13140 (1 mg) and 10 ml of each buffer solution containing 0.2 mg of Aniline Blue were mixed and kept for 120 min at room temperature, and their absorbances at 590 nm measured. Buffer solutions were as follows: 0.2M KCl–HCl, pH 1.0–2.5; 0.2M acetate, pH 3.0–4.0; and 0.2M phosphate, pH 4.5–8.5. ○ Aniline Blue, ● Aniline Blue–Polysaccharide 13140 complex.

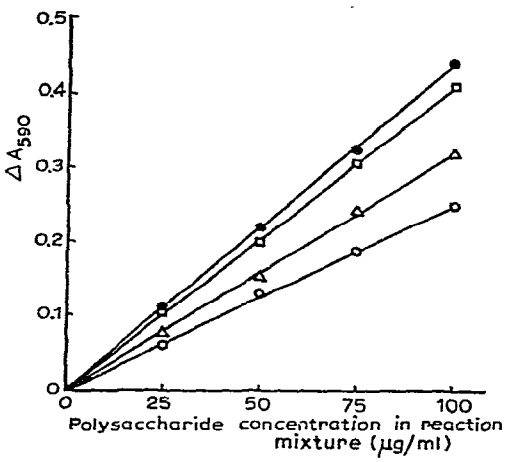

Fig. 3. Relationship between absorbance difference and concentration of the (1→3)-β-D-glucans. The absorbance differences at 590 nm of the reaction mixtures of pH 7 were measured by the same procedure as described in the legend of Fig. 1. Gel strength (GS) and average number of degrees of polymerization ($\overline{DP}$) of the D-glucans used in this experiment were as follows: Polysaccharide 13140 (□, GS 1000×10^3 dyn/cm^2, $\overline{DP}$ 420); depolymerized Polysaccharide 13140 (△, GS 670×10^3 dyn/cm^2, $\overline{DP}$ 310; ○, GS 500×10^3 dyn/cm^2, $\overline{DP}$ 260); and Polysaccharide 13127 (●, GS 1100×10^3 dyn/cm^2, $\overline{DP}$ 450).

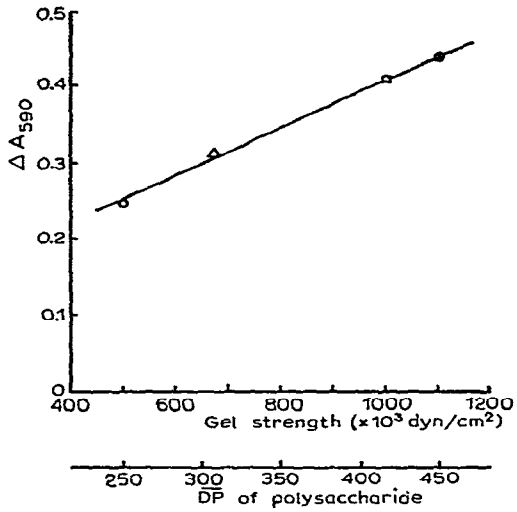

Fig. 4. Relationship between absorbance difference and gel-forming ability (gel strength), and between absorbance difference and $\overline{DP}$ of the (1→3)-β-D-glucans. The correlation curves between gel strength (GS) and absorbance difference at 590 nm were calculated from the experimental results shown in Fig. 3. The concentration of each D-glucan in the reaction mixture was 100 μg per ml. The symbols are the same as those of Fig. 3.

The difference of absorbance depended on the pH of the medium, as shown in Figure 2 where absorbances of Aniline Blue at 590 nm in the presence and absence of Polysaccharide 13140 are reported for pHs ranging from 1 to 8.5. The difference of absorbance was observed in the pH range from 4 to 8.5 and was maximum at about pH 6.

As shown in Figs. 3 and 4, the difference of absorbance at 590 nm was proportional to the concentration of the D-glucan and to its gel-forming ability. The linear relationship between the difference of absorbance and either the D-glucan concentration or the gel strength is expressed by the equation: $\Delta A_{590} = k(C)(GS) + b$, where ΔA_{590} is the absorbance difference at 590 nm, (C) is the D-glucan concentration, (GS) is the gel strength of the D-glucan, and k and b are the constants.

DISCUSSION

Some poly- and oligosaccharides have been found to form complexes with azo dyes. For example, α- and β-cyclodextrins include azo dyes within their void space to form color complexes in aqueous solutions[9,10]; similarly amylose forms complexes with azo dyes[10]. The distribution of callose substance in plant materials has been detected by staining with Aniline Blue and Resorcine Blue[11–13], and recently Ogawa *et al.*[14] reported the formation of a complex by Polysaccharide 13140 with Congo Red in alkaline solution.

The present study shows that Aniline Blue, reacts specifically with (1→3)-β-D-glucans, including curdlan-type polysaccharides, pachyman, and yeast glucan, to

form stable color complexes (Table I). The lack of reaction of laminaran, although it has a (1→3)-β-D-glucoside linkage, may be due to the small size of the molecule. This explanation is supported by the reduction of both properties of the curdlan-type (1→3)-β-D-glucan to form a gel by heating and to form a color complex with Aniline Blue with decreasing degree of polymerization (Figs. 3 and 4).

The specificity of the Aniline Blue stain could be valuably applied not only to the study of the conformation of (1→3)-β-D-glucans but also to the identification of this type of glucans in natural sources. Furthermore, the equation derived from the results shown in Figs. 3 and 4 may be applied to the quantitative analysis as well as to the determination of the gel strength of the curdlan-type polysaccharides.

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