β-Glucosyl and α-Galactosyl Yariv Reagents Bind to Cellulose and Other Glucans†

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Yariv reagent [1,3,5-tris(4-β-D-glucopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene] has been used in the purification/quantification of arabinogalactan-proteins and as a histochemical reagent to localize arabinogalactan-proteins in plant tissues. When developing cotton fiber cells were stained with β-glucosyl Yariv reagent, interference from other cell wall polymers, especially cellulose, was suspected. In order to examine this potential interference, we have developed two dye-binding assays to measure the binding of Yariv reagent in the presence of various carbohydrate polymers. Reaction conditions such as pH, ionic strength, and exposure time in the standard binding assays were investigated. The results show that β-glucosyl and α-galactosyl Yariv reagents bind to cellulose and several other glucans. Treatments with excess glucose in the assay to inhibit binding were only partially effective in preventing β-glucosyl Yariv reagent from binding to cellulose.

Keywords: *Arabinogalactan protein; cellulose; glucan; histochemical stain; Yariv reagent*

INTRODUCTION

Arabinogalactan-proteins (AGPs) are proteoglycans ubiquitously found in the plant exocellular matrix, in intercellular spaces, and in certain tissue exudates. In plant cell cultures, AGPs are abundantly secreted into the culture medium. Typically, AGPs contain greater than 90% carbohydrate by weight and less than 10% of a hydroxyproline-rich protein. The carbohydrate portion of the molecule is mainly a β-D-(1,3)-galactopyranosyl backbone with side chains of β-(1,3)- or β-(1,6)-D-galactopyranosyl and L-arabinofuranosyl residues (Keegstra *et al.*, 1973; Buchala and Meier, 1981; Fincher *et al.*, 1983).

For many years, AGPs have been operationally defined by their ability to bind to β-glucosyl Yariv reagent [1,3,5-tris(4-β-D-glucopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene]. While the precise mechanism of binding between Yariv reagent and arabinogalactan-proteins has not been rigorously characterized, the reagent has been used extensively as an aid in the purification and identification of arabinogalactan-proteins (Yariv *et al.*, 1967; Jermyn and Yeow, 1975; Anderson *et al.*, 1977; Clarke *et al.*, 1979; Fincher *et al.*, 1983; Van Holst and Clarke, 1985, 1986). In addition, β-glucosyl Yariv reagent is reported to be a useful histochemical reagent for identifying the subcellular location of AGPs (Anderson *et al.*, 1977; Gleeson and Clarke, 1979; Schopfer, 1990; Zhu *et al.*, 1993). The α-galactosyl Yariv reagent [1,3,5-tri(α-D-galactosyloxyphenylazo)-2,4,6-trihydroxybenzene] is often used as a control reagent since it does not precipitate AGPs.

Arabinogalactan-proteins are found in cotton fibers (*Gossypium arboreum* L.) (Buchala and Meier, 1981), especially during the early elongation phase of fiber development. In an effort to continue our characterization of cell wall-associated polymers in cotton fiber cells (Timpa and Triplett, 1993), we have used Yariv reagents and monoclonal antibodies to AGPs to characterize cotton fiber proteoglycans. During these studies, the histochemical staining intensity of immature cotton fibers with β-glucosyl Yariv reagent appeared to parallel the deposition of cellulose in the secondary cell walls of the fiber cells. That is, more mature fibers that had already entered into the secondary cell wall phase of fiber development stained more intensely with Yariv reagent, while less mature fibers from stages where elongation growth was maximal were less intensely stained. As a control, a sample of highly pure cellulose was treated with Yariv reagent under conditions mimicking histochemical staining. Surprisingly, under these conditions Yariv reagent bound tenaciously to cellulose. We report in this study a characterization of Yariv reagent binding to cellulose and other glucan polymers by measuring the amount of dye released in an optimized dye-binding assay.

METHODS AND MATERIALS

Chemicals. β-Glucosyl and α-galactosyl Yariv reagents and gum arabic were purchased from Biosupplies Australia Pty. Ltd. (Parkville, Victoria, Australia). Solutions of Yariv reagents were freshly prepared for each experiment. Gum arabic was used as a reference standard to demonstrate that the β-glucosyl Yariv reagent binds to arabinogalactan-proteins and that α-galactosyl Yariv reagent does not bind. The carbohydrate polymers tested for binding to β-glucosyl and α-galactosyl Yariv reagents and their commercial sources are listed in Tables 1 and 2. Phosphate-buffered saline (PBS) tablets were purchased from Sigma Chemical Co. (St. Louis, MO). Water used for all solutions was purified by a pyrogen-free Milli-Q water purification system (Millipore Corp., Bedford, MA).

Yariv Reagent Dye-Binding Assay for Insoluble Polymers. Assays were performed in triplicate. Carbohydrate polymers (1 mg) were suspended in 1.0 mL of PBS, pH 7.4. Only polymers that are insoluble in aqueous solution at room temperature were tested using this assay. The polymers were collected by centrifugation at 10000*g* (Eppendorf 5415C microcentrifuge) for 5 min. Liquid was aspirated from the polymer pellet, and a fresh 1.0-mL aliquot of PBS was added.

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Table 1. Amount of β-Glucosyl and α-Galactosyl Yariv Reagent Bound to Aqueous-Insoluble Carbohydrate Polymers and Several Crystalline Forms of Cellulose[a]

polymer	common name	source	μmol of β-glucosyl Yariv/g of cellulose	μmol of α-galactosyl Yariv/g of cellulose
β-D-(1,4)-glucan	cellulose	FMC Avicel PH-101	4.52	4.84
β-D-(1,4)-glucan	cellulose	*Acetobacter*	3.93	3.65
β-D-(1,4)-glucan	cellulose II	Avicel PH101-mercerized	4.3	*
β-D-(1,4)-glucan	rayon	regenerated cellulose II	0	*
β-D-(1,4)-glucan	cellulose III	from cellulose I	0	*
β-D-(1,4)-glucan	cellulose III	from cellulose II	0	*
α-D-(1,4)-glucan	amylose	Sigma A-7043	0	0
α-D-(1,4)-glucan	amylopectin	Sigma A-7780	0	0
β-D-(1,3)-glucan	pachyman	Biosupplies Australia	0	0
β-D-(1,4)-*N*-acetylaminoglucan	chitin	V-Labs, Inc.	3.58	2.73

[a] Cellulose II and III allomorphs were prepared as described by Weimer *et al*. (1991). Samples denoted with an asterisk (*) did not quantitatively release the Yariv reagent by elution with DMF even after overnight incubation at 50 °C. The chitin sample was purified and decalcified (Muzzarelli, 1977).

Table 2. Assessment of β-Glucosyl and α-Galactosyl Yariv Reagents Binding to Carbohydrates Dried on Immobilon-P Membranes[a]

carbohydrate	source	β-glucosyl Yariv reagent stain intensity	α-galactosyl Yariv reagent stain intensity
alginic acid	Sigma A-7003	0	0
arabinogalactan	Roth	0	0
ι-carrageenan	Sigma C-1138	0	0
chitin oligosaccharide	V-Labs, Inc.	+	0
CM-cellulose	Sigma C-4888	0	0
dextran	Pharmacia	0	0
glycogen	Sigma G0885	0	+
guar gum	Sigma G-4129	++	+
gum arabic	Biosupplies Australia	+++	0
laminarin	Sigma L-9634	0	0
locust gum	Sigma G-0753	+	+
mannan	Sigma M-7504	0	0
pectin-apple	Sigma P-2157	+	0
pectin-citrus	Sigma P-9135	0	0
pullulan	Pfanstiehl 12476	0	0
xanthan gum	Sigma G-1253	0	0
xylan	Sigma X-0376	+	+

[a] Staining intensity was scaled from 0 for no staining above background to +++ for the darkest staining.

The carbohydrate polymers were prewashed three times with PBS to remove possible water-soluble low molecular weight contaminants. After the last wash, the pellet was resuspended in 0.1 mL of Yariv reagent (0.1 mg mL^{-1}) in PBS. The sample was gently mixed by rocking on a LabQuake Shaker for at least 2 h at room temperature. Carbohydrate polymers were collected by centrifugation and washed three times with 1.0 mL of PBS as described for the prewashing step. Yariv reagent was released from the polymers by adding 0.5 mL of dimethylformamide (DMF) and gently shaking the tube for 3 min. The liquid was transferred to a fresh test tube, and the polymer pellet was washed with a second 0.5-mL aliquot of DMF. The amount of Yariv reagent eluted in the pooled DMF washes was measured with a Hewlett-Packard diode array spectrophotometer Model 8450A at 510 nm. For microscale assays, the volumes were decreased proportionately, and the released Yariv reagent was measured at 490 nm in a Molecular Devices Thermo-Max microtiter plate reader.

Yariv Reagent Interactions with Other Carbohydrates. A method for determining if carbohydrates that are soluble in aqueous buffers could bind to β-glucosyl Yariv reagent was based on drying the compounds on a PVDF membrane. Carbohydrates were dissolved in PBS to a final concentration of 5 mg mL^{-1}. Some of the samples needed to be heated to 80 °C to ensure complete dissolution. Carbohydrates (5 μg) were applied in triplicate to Immobilon-P membranes (Millipore) using a slot blot manifold (Schleicher and Schuell, Keene, NH). After air drying overnight, the membrane was incubated in 10 μg mL^{-1} β-glucosyl or α-galactosyl Yariv reagent in PBS at room temperature for 8 h. The membrane was washed three times with PBS for 15 min. A qualitative estimate of Yariv reagent binding to the carbohydrate polymer spots was determined by visual inspection.

RESULTS AND DISCUSSION

When β-glucosyl Yariv reagent is incubated with cellulose in PBS, the staining reagent becomes tightly associated with the cellulose samples. Even after washing the cellulose sample several times with PBS or incubating in PBS overnight with gentle shaking, the β-glucosyl Yariv reagent does not dissociate from cellulose. Treatment of the Yariv-reagent-stained cellulose samples with organic solvents such as acetone, butanol, ethanol, or 2-propanol did not release the dye from the cellulose. Raising the ionic strength of the washing solution to 5.0 M NaCl was also ineffective in releasing the β-glucosyl Yariv reagent. In contrast, organic nucleophiles such as DMF, dimethyl sulfoxide, and pyridine were all successful in releasing the β-glucosyl Yariv reagent from cellulose samples. Some aspects of the binding mechanism may be inferred from this differential solubility of Yariv reagent bound to cellulose. In summary, hydrophobic and ionic interactions between Yariv reagent and cellulose are less important than hydrogen bonding interactions.

Since β-glucosyl Yariv reagent could be completely recovered from cellulose samples by treatment with DMF, a dye-binding assay was developed to measure quantitatively the association of the stain with cellulose and other polymers. The absorption spectrum of pure β-glucosyl Yariv reagent in DMF had a λ_{max} at 510 nm from which the molar absorption coefficient at this wavelength was calculated to be 48 500. From the absorption coefficient, the number of moles of Yariv reagent bound per gram of cellulose could be determined. The amount of cellulose necessary to bind 10 μg of β-glucosyl Yariv reagent was determined in order to perform the standard dye-binding assay under conditions where the Yariv reagent would be in excess. When 10 μg of β-glucosyl Yariv reagent was present in each 100-μL assay, over 10 mg of cellulose was necessary to completely remove the dye from solution (Figure 1). Accordingly, the standard dye-binding assay used 1 mg of cellulose or other glucan per replicate sample. The amount of β-glucosyl Yariv reagent present in the dye-binding assay is 20 times less concentrated than the recommended concentration for histochemical staining (Schopfer, 1990). The more dilute concentration of β-glucosyl Yariv reagent permitted more dye-binding

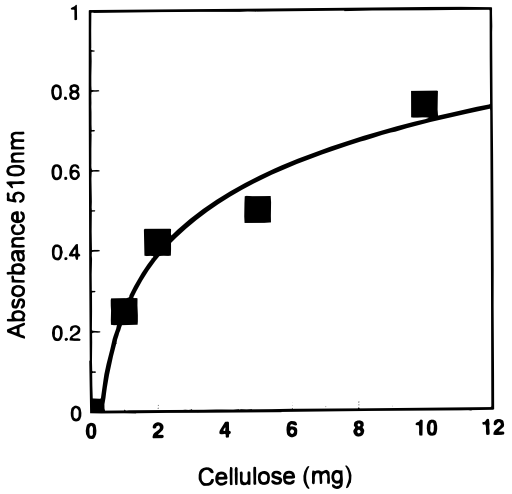

Figure 1. Cellulose content in the standard assay necessary to completely bind a fixed amount of β-glucosyl Yariv reagent. Avicel PH-101 was used as the cellulose standard. The binding step was conducted at room temperature for 16 h with gentle mixing.

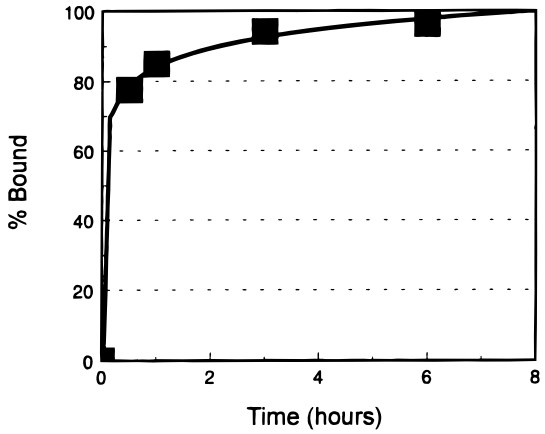

Figure 2. Kinetics of Avicel PH-101 cellulose binding to β-glucosyl Yariv reagent at room temperature under standard assay conditions.

assays to be conducted for a reasonable cost. At the same time, this concentration of β-glucosyl Yariv reagent was sufficient to yield an acceptable level of absorbance at 510 nm when the dye was released from 1 mg of cellulose with DMF.

The kinetics of β-glucosyl Yariv reagent binding to cellulose in PBS are shown in Figure 2. In 15 min, nearly 80% of the stain was associated with the cellulose sample. Since the reaction conditions are similar to those conditions recommended for histochemical staining tissue sections for AGPs, these results suggest that cellulose interferes with the histochemical localization of AGPs with Yariv reagent. Prior observations that both α-galactosyl and β-glucosyl Yariv reagents stained cell walls (Schopfer, 1990) must be reviewed in light of the possibility that cellulose in the walls was responsible for some of the observed staining.

The binding of insoluble carbohydrate polymers to β-glucosyl and α-galactosyl Yariv reagents (Table 1) was measured in the standard dye-binding assay. Cellulose [β-D-(1,4)-glucan] samples bound both β-glucosyl and α-galactosyl Yariv reagents in nearly equal molar amounts. Avicel PH-101 and *Acetobacter* celluloses were chosen for this study since these cellulose samples are highly pure and fairly well-characterized in the literature. Cellulose samples from seven other commercial sources also exhibited similar levels of binding to both β-glucosyl and α-galactosyl Yariv reagents (data not shown). Furthermore, extraction of Avicel PH-101 with acetic−nitric reagent (Updegraff, 1969) did not eliminate the binding of β-glucosyl and α-galactosyl Yariv reagents to the sample. Acetic−nitric reagent hydrolyzes all cell wall-associated polymers except crystalline cellulose; therefore, the binding of Yariv reagent to cellulose does not appear to be the result solely of contamination with other carbohydrate polymers. In addition, PH-101 was subjected to glycosyl composition analysis and found to consist of 93 mol % glucose, 2 mol % xylose, and 4.6 mol % mannose with no other monosaccharides in the sample.

Since α-galactosyl Yariv reagent is designed to be a negative control reagent for the binding of β-glucosyl Yariv reagent, this result suggests that the association between the Yariv reagents and cellulose is somewhat different from the association with AGPs. When β-glucosyl Yariv reagent was preincubated with 1 mg of gum arabic prior to addition to Avicel, the staining of the cellulose sample was inhibited by 93.7%. This result suggests that once Yariv reagent has bound to gum arabic, the dye cannot become associated with cellulose.

Pretreatment of Avicel with histochemical fixatives (Schopfer, 1990) prior to incubation with Yariv reagents had no effect on the ability of the dyes to bind cellulose. Interestingly, chitin, a β-D-(1,4)-*N*-acetylaminoglucan, also bound β-glucosyl and α-galactosyl Yariv reagents. In contrast, pachyman (a β-D-(1,3)-glucan), insoluble amylose, and amylopectin [α-D-(1,4)-glucan] samples did not bind either Yariv reagent under the standard dye-binding assay conditions.

In muro, cellulose molecules contain both crystalline and less crystalline or amorphous regions. In addition, the native crystalline cellulose (cellulose I) can be induced to assume several different crystalline structures. These allomorphs of cellulose differ in the crystallite unit cell dimensions, the amount of hydrogen bonding within the unit cell, and the polarity of cellulose molecules within the crystallite (Sarko, 1986). In dye-binding experiments with cellulose allomorphs, the β-glucosyl Yariv reagent bound to cellulose I and cellulose II from mercerized but not regenerated cellulose II (Table 1). These results are consistent with previous observations that the microstructure of cellulose II from mercerization and regeneration are different (Rowland and Howley, 1988). As a result, it may be possible to use Yariv reagents as probes into structural details of cellulose allomorphs. Studies have been initiated to examine if there is a relationship between cellulose crystallinity index, a measure of crystalline versus amorphous structure, and Yariv reagent binding.

The β-glucosyl and α-galactosyl Yariv reagents can bind to other carbohydrate polymers as well when the compounds are dried on a PVDF membrane (Table 2). In addition to gum arabic, β-glucosyl Yariv reagent bound to guar gum, apple pectin, xylan, and a chitin oligosaccharide. In a similar assay, the α-galactosyl Yariv reagent bound glycogen, guar gum, locust gum, and xylan. The binding of other glucan polymers

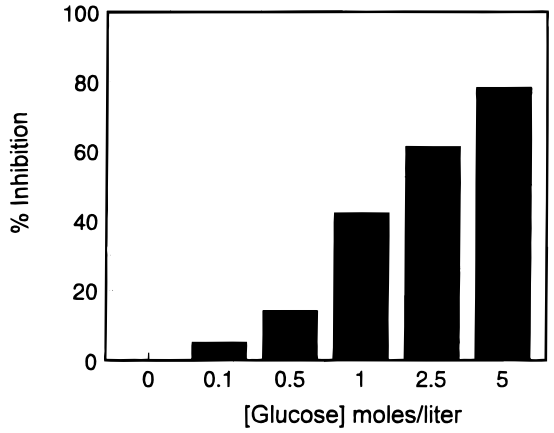

Figure 3. Inhibition of β-glucosyl Yariv reagent binding to Avicel PH-101 in the presence of glucose under standard assay conditions.

(namely, β-D-(1,3)-glucans), to aniline blue fluorochome was found to be sensitive to the presence of phosphate ions in the reaction mixture (Evans *et al.*, 1984). Since the standardized conditions used phosphate-buffered saline, the Yariv reagent dye-binding assay was repeated in water titrated to pH 7.4. There was no difference in the binding of β-glucosyl Yariv reagent to the glucan samples in the absence of phosphate ions (data not shown).

Excess glucose in the standard dye-binding assay inhibited the association between β-glucosyl Yariv reagent and cellulose (Figure 3). This observation suggests that carbohydrate−carbohydrate interactions mediated through hydrogen bonding may participate in the binding of Yariv reagents to cellulose. Even at high glucose concentrations (5 M), however, some Yariv reagent became bound to the cellulose. Until more is known about the mechanism for Yariv reagent binding to AGPs and cellulose, it is unclear which incubation conditions would be optimal to prevent binding to cellulose without also influencing the binding of Yariv reagent to AGPs as well.

Pachyman and callose are both linear β-D-(1,3)-glucans, differing only in molecular weight. Differences in the binding of β-glucosyl Yariv reagent with cellulose (β-D-(1,4)-glucan) as compared with pachyman suggest that a Yariv reagent-based assay could be developed to differentiate between the *in vitro* synthesis of cellulose and callose. Similarly, the difference between the binding ability of cellulose I and cellulose II with β-glucosyl and α-galactosyl Yariv reagents coupled with differences in the release of the dye with DMF offers the possibility of distinguishing between the two cellulose allomorphs with a simple chemical test.

The results of this study suggest that experiments conducted with β-glucosyl Yariv reagent must always include appropriate control experiments with α-galactosyl Yariv reagent to avoid potential artifactual interactions with other carbohydrate polymers.

ABBREVIATIONS USED

AGP, arabinogalactan-protein; DMF, dimethylformamide; PBS, phosphate-buffered saline.

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