

Fractionation of Xyloglucan Fragments and Their Interaction with Cellulose¹

Jean-Paul Vincken, Arie de Keizer, Gerrit Beldman, and Alphons Gerard Joseph Voragen*

Department of Food Science (J.-P.V., G.B., A.G.J.V.) and Department of Physical and Colloid Chemistry (A.d.K.), Wageningen Agricultural University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

Tamarind seed xyloglucan was partially degraded with a purified endoglucanase (*endoV*) from *Trichoderma viride*. Analysis by high-performance anion-exchange chromatography showed that this digest was composed of fragments consisting of 1 to 10 repeating oligosaccharide units ($[xg]_1$ – $[xg]_{10}$). To study the adsorption of xyloglucan fragments to cellulose in detail, this digest was fractionated on BioGel P-6. Fragments were separated satisfactorily up to 5 repeating oligosaccharide units ($[xg]_5$). The galactose substitution of the fragments increased with increasing molecular weight. The BioGel P-6 pools, as well as polymeric xyloglucan ($[xg]_\infty$), were tested for their ability to interact with Avicel crystalline cellulose. Quantitative binding to cellulose occurred for sequences consisting of (at least) 4 repeating units. The adsorption of $[xg]_4$ to Avicel was very high relative to that of $[xg]_\infty$. The dimensions of these fragments were such that they could also penetrate the smaller pores of cellulose. Apparently, the effective surface area for the polymers is much smaller. Adsorption isotherms of $[xg]_\infty$ and $[xg]_4$ showed a pattern that is typical for polydisperse systems. However, the mechanisms underlying these patterns were different. At high xyloglucan concentrations, this polydispersity resulted in preferential adsorption of the larger molecules in the case of $[xg]_\infty$ and a more extensive colonization of the smaller pores of cellulose in the case of $[xg]_4$. The pH influenced the interaction between xyloglucan (fragments) and cellulose to only a small extent.

Cellulose and xyloglucan are important structural components of the primary cell walls of plants (McCann and Roberts, 1994). Both polysaccharides are composed of a β -(1 \rightarrow 4)-glucan backbone. In cellulose, these chains associate laterally by hydrogen bonding to form microfibrils, but self-association of xyloglucan molecules is prohibited by a large number of side chains (Hayashi, 1989). Although cellulose and xyloglucan are assembled at different locations, an intimate interaction between these two polysaccharides exists in vivo. In the primary cell wall, cellulose microfibrils are extensively coated with xyloglucans (Hayashi, 1989), which prevent their aggregation into even larger cellulose complexes (McCann et al., 1990; Acebes et al., 1993); only small amounts of "naked" cellulose occur (Hayashi and Maclachlan, 1984). Strong chaotropic reagents are needed to swell cellulose and solubilize xyloglucan from cell wall material (Edelmann and Fry, 1992),

which suggests that binding of xyloglucan to cellulose is mediated by hydrogen bonds (Hayashi and Maclachlan, 1984; McCann and Roberts, 1994). Recently, two endogenous proteins (expansins) were found that are thought to interfere in the noncovalent binding between cellulose and xyloglucan, thereby inducing cell wall extension (McCann and Roberts, 1994; McQueen-Mason and Cosgrove, 1994).

The xyloglucan-binding capacity of cellulose in native cell walls surpasses by many times that found in in vitro binding experiments (Hayashi et al., 1987; Baba et al., 1994). In addition to these studies on the interaction of $[xg]_\infty$ and cellulose, Valent and Albersheim (1974) showed that xyloglucan oligosaccharides (XXXG and XXFG) do not bind to cellulose in aqueous conditions. Recently, Hayashi et al. (1994) demonstrated that a minimum length of at least five consecutive glucosyl residues is required to enable binding of xyloglucan molecules to cellulose. Cello-oligosaccharides of similar backbone length were found to bind to a larger extent under the same conditions. Structural aspects might also be important. Based on computer simulations, Levy et al. (1991) demonstrated that fucosylated side chains can facilitate the adoption of a flat backbone conformation. They speculated that these straightened regions trigger the binding of xyloglucan to cellulose. In vitro modification of xyloglucans by a β -galactosidase increased self-association of the backbone, ultimately leading to gel formation (Reid et al., 1988). Hisamatsu et al. (1992) reported the existence of endoglucanase-resistant fragments that contained a glucosyl residue with additional branching at the C₂ position. These structural elements were hypothesized to destabilize xyloglucan-cellulose interactions. The examples indicate that, in addition to size, the side chain configuration also might play an important role in the adsorption behavior of xyloglucans.

Abbreviations: DP, degree of polymerization; *endoV*, endoglucanase V; Γ , adsorbed amount; HPAEC, high-performance anion-exchange chromatography; HPSEC, high-performance size-exclusion chromatography; K_{av} , (elution volume – void volume) ÷ (total volume of the packed bed – void volume); NaOAc, sodium acetate; PAD, pulsed amperometric detection; $[xg]_n$, population of xyloglucan fragments composed of n repeating xg units; $[xg]_\infty$, polymeric xyloglucan; XXXG, XLXG, XXLG, and XLLG, xyloglucan oligosaccharides according to the nomenclature of Fry et al. (1993), where G, X, and L represent different substituted β -D-glucosyl residues of xyloglucan: G, β -D-Glcp; X, α -D-Xylp-(1 \rightarrow 6)- β -D-Glcp; L, β -D-Galp-(1 \rightarrow 2)- α -D-Xylp-(1 \rightarrow 6)- β -D-Glcp (p indicates pyranose).

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* Corresponding author; e-mail fons.voragen@lmc.lmt.wau.nl; fax 31–8370–84893.

Efficient enzymic degradation of cell wall-embedded cellulose requires a preceding solubilization of the xyloglucan coating (Vincken et al., 1994). Although the data of Hayashi et al. (1994) demonstrated that the adsorption equilibrium shifts toward more bound material with increasing chain length, these data do not predict to what extent xyloglucans have to be degraded before "lift-off" from cellulose microfibrils occurs. In other words, what is the minimum length of a xyloglucan molecule for quantitative binding to cellulose? The present paper reports the fractionation of such molecules and compares their *in vitro* adsorption on cellulose with that of polymeric xyloglucan.

MATERIALS AND METHODS

Enzymes and Chemicals

EndoV (EC 3.2.1.4) was purified to homogeneity from a commercial preparation from *Trichoderma viride* (Maxzyme CI, Gist-Brocades, Delft, The Netherlands) as described by Beldman et al. (1985).

Avicel crystalline cellulose (type SF) was purchased from Serva (Heidelberg, Germany). Cotton linters cellulose powder was obtained from Fluka Chemie (Buchs, Switzerland). Tamarind seed xyloglucan was kindly provided by Dainippon Pharmaceutical (Osaka, Japan). Standards of xyloglucan oligosaccharides (XXXG, XLXG, XXLG, and XLLG) were obtained by enzymic degradation of apple fruit xyloglucan and subsequent fractionation by BioGel P-2 and HPAEC. Their characterization will be described elsewhere.

Preparation of Xyloglucan Fragments

Xyloglucan fragments were prepared by partial degradation of 250 mg of tamarind seed xyloglucan with 5 μ g (approximately 53 milliunits) of endoV in 25 mL of a 25-mM NaOAc buffer, pH 5.0 (40°C, 9 h). The degradation was monitored using HPSEC. When the molecular weight distribution was such that mainly dimers, trimers, and tetramers of xyloglucan oligosaccharides were present, the incubation was stopped by pouring the mixture into 475 mL of boiling water, followed by 10 min of heating (100°C). The digest was concentrated under reduced pressure to a final volume of 4 mL. Two milliliters were then applied to a column (100 \times 2.6 cm, i.d.) of BioGel P-6 (200–400 mesh, Bio-Rad) at 60°C and eluted (20 mL h⁻¹) with distilled water. Fractions (2.6 mL) were assayed for total neutral sugar content. Appropriate fractions were combined and designated as I, II, III, IV, V, and VI. The column was calibrated using a mixture of Dextran T150 (Pharmacia) and Glc (K_{av} = 0 and 1, respectively). Chromatography of a completely digested (endoV) tamarind seed xyloglucan was conducted in a similar way on BioGel P-2.

Adsorption of Xyloglucan (Fragments) to Cellulose

In a series of experiments, some aspects of the interaction between xyloglucan and cellulose were studied. The length of xyloglucan molecules, the pH, and the origin of cellulose were parameters that were varied. Also, the oligosaccha-

ride (subunit) composition of bound xyloglucan was investigated. All incubations were done in 1 mL of 25 mM buffer (specified for the individual experiments) containing 10 mM CaCl₂ and appropriate amounts of cellulose and xyloglucan (fragments). After 6 h of incubation at 40°C (head-over-tail mixing), the samples were centrifuged (1 min, 20,000g), and the amount of unbound material in the supernatants was quantified by either HPSEC or determination of total neutral sugar content. The adsorption was calculated from the difference in soluble material before and after incubation with cellulose.

To determine their critical length for quantitative binding to cellulose, 1 mg of an unfractionated xyloglucan digest was incubated with Avicel (50 mg) in NaOAc buffer (pH 5.8) and analyzed by HPSEC. In another experiment, Avicel (25 mg) was incubated with the same buffer containing 250 μ g of purified xyloglucan fragments (BioGel P-6 pools I–VI) or [xg]_∞ in triplicate, and analyzed in a similar way.

Adsorption isotherms were obtained by treating 50 mg of Avicel or cotton linters in NaOAc buffer (pH 5.8) with varying concentrations of [xg]_∞ (25–1000 μ g). In a similar way, pools IV and VI were mixed with 1 or 5 mg of Avicel. The unbound material was quantified by total neutral sugar analysis. To study the effect of the pH on the interaction of xyloglucan and cellulose, adsorption isotherms of [xg]_∞ to Avicel were also made using NaOAc buffers of different pH levels (5.0 and 4.0) and a citrate buffer of pH 3.0 under conditions similar to those described above. Additionally, the adsorption of pool VI (500 μ g) to Avicel (5 mg) was investigated at different pH levels using the four buffers described previously.

The oligosaccharide (subunit) composition of material that was bound to cellulose was determined as follows. Cellulose (1 mg) was incubated with NaOAc buffer (pH 5.8) containing 200 μ g of pool IV as described above. After centrifugation the supernatant was removed and the pellet (cellulose with bound xyloglucan fragments) was resuspended in 1 mL of a similar buffer. This suspension was then treated with 50 μ g of endoV for 24 h (head-over-tail mixing; 40°C). The parental material of pool IV was treated with endoV in a similar way. The resulting oligosaccharides were analyzed by HPAEC.

Analytical Methods

Analysis of Xyloglucan Fragments

Quantitative analyses of xyloglucan fragments was performed by HPSEC as described previously (Vincken et al., 1994). In addition, samples were analyzed by HPAEC using a Dionex (Sunnyvale, CA) Bio-LC GPM-II quaternary gradient module equipped with a Dionex CarboPac PA-100 column (250 \times 4 mm, 20°C). Samples (20 μ L) were injected using a SP8780 autosampler (Spectra Physics, San José, CA) equipped with a Tefzel rotor seal in a 7010 Rheodyne (Cotati, CA) injector valve. Solvents were degassed and stored under helium using a Dionex eluent degassing module. The eluate (1 mL min⁻¹) was monitored using a Dionex pulsed electrochemical detection detector in the PAD

mode. A reference Ag/AgCl electrode was used with a working gold electrode with the following pulse potentials and durations: E_1 , 0.1 V and 0.5 s; E_2 , 0.6 V and 0.1 s; E_3 , -0.6 V and 0.1 s. The xyloglucan fragments were analyzed by application of the following gradient: 0 to 8 min, linear gradient of 80 to 170 mM NaOAc in 100 mM NaOH; 8 to 30 min, linear gradient of 170 to 220 mM NaOAc in 100 mM NaOH. After each analysis the column was rinsed for 5 min with 1000 mM NaOAc in 100 mM NaOH and equilibrated in 80 mM NaOAc in 100 mM NaOH for 15 min.

Total Neutral Sugar Content

The total neutral sugar content was determined colorimetrically with an automated orcinol/sulfuric acid assay (Tollier and Robin, 1979). Glc was used as a standard.

Sugar Composition

Tamarind seed xyloglucan and fragments thereof were hydrolyzed using 2 N TFA; crystalline cellulose was pre-treated with 72% (w/w) H_2SO_4 for 1 h at 30°C, followed by hydrolysis with 2 N H_2SO_4 for 3 h at 100°C. The released neutral sugars were converted to their alditol acetates and analyzed by GC as described previously (Vincken et al., 1994).

Protein Content

The protein content of enzyme preparations was determined according to Sedmak and Grossberg (1977). BSA was used as a standard.

RESULTS

Fractionation of Xyloglucan Fragments

Tamarind seed xyloglucan has been shown to consist of four repeating units: XXXG (approximately 13 mol%), XLXG (approximately 9 mol%), XXLXG (approximately 28 mol%), and XLXG (approximately 50 mol%) (York et al., 1990). In our study, tamarind seed xyloglucan was completely degraded by endoV, and the resulting oligosaccharides were fractionated and quantified by BioGel P-2 chromatography (data not shown). The fractions were analyzed by HPAEC and the retention times of the four products were compared with those of standards derived from apple fruit xyloglucan. Our results were in close agreement with those reported by York et al. (1990). This collection of four oligosaccharides will be further referred to as $[xg]_1$.

The degradation of tamarind seed xyloglucan by endoV was monitored by HPSEC, and continued until a pattern as shown in Figure 1A (solid line) was obtained. The major part of this digest is composed of molecules that were later shown to contain one, two, or three repeating oligosaccharide units ($[xg]_1$, $[xg]_2$, and $[xg]_3$, respectively). Elution of material with a retention time less than 29.5 min indicated that larger molecules were also present; however, no complete separation of these fragments was obtained using HPSEC. When cellulose was added to this digest, very few molecules larger than $[xg]_2$ were found in the supernatants after 6 h of incubation and subsequent centrifugation (Fig. 1A, dashed line). This demonstrated that chain length largely determines the adsorption behavior of xyloglucan

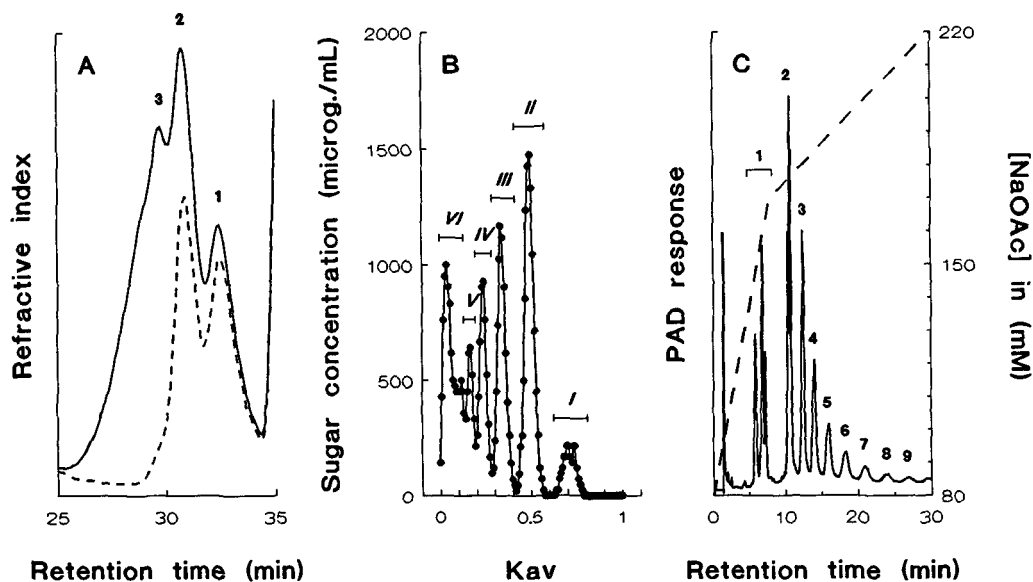


Figure 1. Elution profiles of a digest of tamarind seed xyloglucan analyzed by HPSEC (A), BioGel P-6 chromatography (B), and HPAEC (C). A, The degradation of xyloglucan by endoV was monitored using HPSEC, and the enzyme was inactivated when the profile shown as a solid line (—) was obtained. Incubation (25 mM NaOAc buffer [pH 5.8] containing 10 mM $CaCl_2$) of this mixture of xyloglucan fragments (1 mg) with Avicel crystalline cellulose (50 mg) for 6 h yielding the profile shown as a dashed line (- -). B, The partially digested tamarind seed xyloglucan was fractionated using BioGel P-6 (for experimental details, see text). C, The same sample was also analyzed by HPAEC (—, PAD response; - - -, NaOAc gradient). Digits 1 to 9 indicate the number of repeating oligosaccharide units ($[xg]_1$ to $[xg]_9$). Roman numerals I to VI are used to designate BioGel P-6 pools.

molecules. Subsequently, the xyloglucan digest was fractionated on BioGel P-6, and six pools were obtained, as indicated in Figure 1B. The K_{av} values of pools I and II were similar to those reported by Hayashi and Maclachlan (1984) for pea xyloglucan oligosaccharides ($[xg]_1$) and dimers ($[xg]_2$) thereof, respectively. When it was assumed that pools III to V correspond to $[xg]_3$ to $[xg]_5$, respectively, a linear relationship between $\text{Log}(\text{DP})$ and K_{av} was obtained in the range $[xg]_1$ to $[xg]_5$. Fractions I to III were analyzed by HPSEC and corresponded to peaks 1 to 3 (Fig. 1A), respectively.

The same digest was also analyzed using HPAEC. Figure 1C shows that this method gives a much higher resolution than HPSEC and BioGel P-6; the sample is composed of 10 products. Pools I to IV corresponded with $[xg]_1$ to $[xg]_4$, respectively, in the pattern of Figure 1C. Pool V contained primarily $[xg]_5$ and some slight contamination of $[xg]_6$. Typically, pool VI did not contain only products larger than $[xg]_5$; relatively large amounts of smaller fragments, including $[xg]_1$, were also present. Even when taking into consideration the decreasing PAD response for fragments with increasing molecular weights, approximately 35% (w/w) of pool VI consisted of $[xg]_{n<6}$. Self-association with larger xyloglucan fragments might be an explanation for this phenomenon. Figure 1C suggests that xyloglucan molecules consisting of at least 10 repeating oligosaccharides ($[xg]_{10}$) can be separated.

Both $[xg]_1$ and $[xg]_2$ showed multiple signals upon HPAEC (Fig. 1C), which demonstrated that these were heterogeneous fractions. Fraction I ($[xg]_1$) contained primarily XXXG and XXLG building units, but also some XLXG and XLLG (Fig. 2A). $[xg]_2$ probably contains any combination of the above forms of $[xg]_1$ oligosaccharides, which renders 16 possibilities. For $[xg]_3$ and $[xg]_4$, the number of possibilities increases dramatically. Incomplete resolution of the different compounds within one population by HPAEC results in an apparent homogeneity of populations larger than $[xg]_2$.

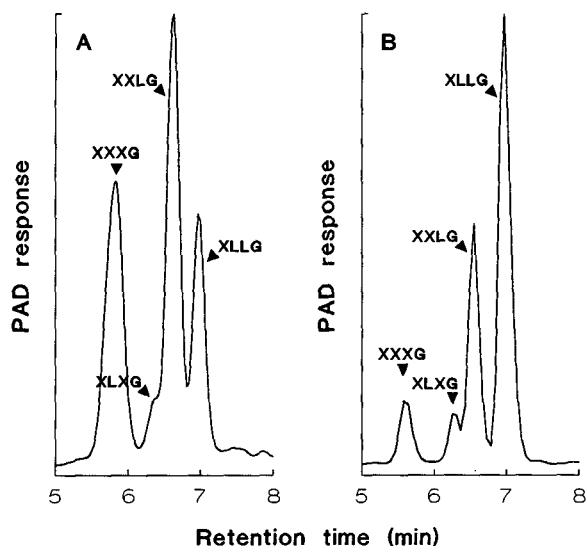


Figure 2. HPAEC elution profiles of BioGel P-6 pool I ($[xg]_1$, A) and an endoV digest of BioGel P-6 pool IV ($[xg]_4$, B).

Sugar analysis of the BioGel P-6 pools showed that the fragments were gradually enriched in Gal with increasing molecular weight (data not shown). This suggested that larger fragments were composed of different building blocks than the smaller molecules. To verify this, the fragments of pool IV were degraded to their building units with endoV. Figure 2B demonstrates that XLLG is the major building block of $[xg]_4$, in contrast to $[xg]_1$, where XXXG and XXLG are the most abundant oligosaccharides (Fig. 2A).

Adsorption of Xyloglucan Fragments of Different Length to Cellulose

The combination of the results of Figure 1, A and B, suggested that fractionation on BioGel P-6 enabled the purification of fragments of critical length in relation to adsorption on cellulose. Therefore, pools I to VI, as well as $[xg]_{\infty}$, were tested for their ability to interact with cellulose. This is shown in Figure 3. $[xg]_1$ did not bind to cellulose in aqueous conditions, which is in accordance with the results of Valent and Albersheim (1974). Binding of only a small amount of $[xg]_2$ and a larger amount of $[xg]_3$ occurred, which is in agreement with results reported by Hayashi et al. (1994). Apparently, $[xg]_5$ molecules possessed the minimum length required for quantitative binding to cellulose under the conditions used. It should be noted that $[xg]_{6<n<11}$ is a part of pool VI, and $[xg]_1$ and $[xg]_2$, which are also present in this fraction, did not adsorb. Typically, the adsorption of $[xg]_{\infty}$ was much lower compared to the adsorption of $[xg]_5$. Obviously, the effective specific surface area is much smaller for the polysaccharides.

The above findings indicated that relative to $[xg]_{\infty}$, large amounts of $[xg]_4$ and pool VI were needed to saturate the cellulose surface. This was further substantiated by the adsorption isotherms in Figure 4, A and B. Initially, all xyloglucan bound to cellulose, which is illustrated by the steep part of the isotherms. At higher xyloglucan concentrations, Γ increased less sharply and tended to a constant value. A difference in adsorption behavior between $[xg]_4$ and pool VI became prominent here, because only for pool VI was this "plateau value" realized. In accordance with Figure 3, the adsorption of $[xg]_{\infty}$ was much smaller relative to, for instance, that of $[xg]_4$.

The origin of the cellulose was shown to influence the adsorption behavior of $[xg]_{\infty}$ (Fig. 4A; compare Δ to \blacksquare). The adsorption of $[xg]_{\infty}$ to cotton linters cellulose was much lower than that to Avicel. Both cellulose preparations seemed to contain a small amount of hemicellulosic and/or pectic material. Apart from Glc (approximately 92 mol%), minor amounts of uronic acid (approximately 4 mol%), Xyl (approximately 2 mol%), and Man and Rha (each approximately 1 mol%) were found. Apparently, cellulose has some ionizable groups, and these might affect the adsorption of xyloglucan to cellulose. Therefore, the adsorption of $[xg]_{\infty}$ and pool VI were studied as a function of pH. Figure 4A shows that adsorption of $[xg]_{\infty}$ decreased from pH 5.8 to pH 4.0. The xyloglucan adsorption at pH 3.0 did not follow this tendency; the adsorption at pH 3.0 was similar to that at pH 5.0. This might be due to different buffer ions;

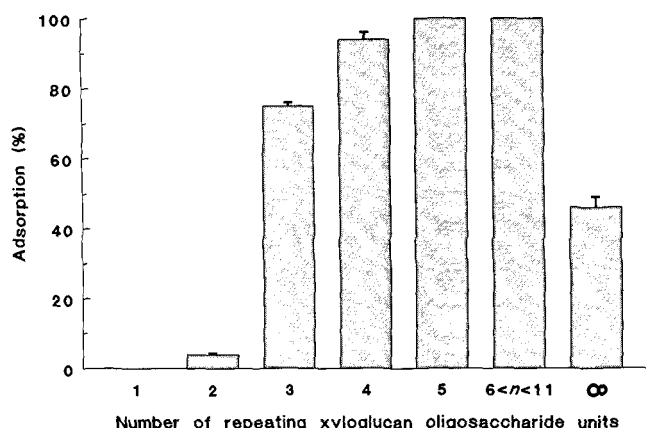


Figure 3. Adsorption of xyloglucan fragments of varying DP to Avicel crystalline cellulose. Various xyloglucan fragments (250 μ g) were incubated (40°C) with 1 mL of 25 mM NaOAc buffer (pH 5.8) containing 10 mM CaCl_2 and 25 mg of cellulose. After 6 h of incubation the samples were centrifuged (1 min, 20,000g) and the supernatants were analyzed by HPLC. The adsorbed amount was calculated from the ratio of the peak areas before and after incubation. Error bars indicate deviations of the average of three measurements.

another explanation might be protonation of xyloglucan at low pH values, but this was not further substantiated. Similar effects were observed for the fragments of pool VI.

The possible heterogeneity of the $[\text{xg}]_4$ population was discussed above. To investigate whether some fragments in this population adsorbed preferentially to cellulose, the

oligosaccharide composition of adsorbed $[\text{xg}]_4$ was determined. To this end, $[\text{xg}]_4$, which had previously been adsorbed on Avicel, was degraded by endoV after the unbound $[\text{xg}]_4$ had been removed by centrifugation. Analysis of the resulting oligosaccharides by HPAEC showed that the adsorbed $[\text{xg}]_4$ had a higher portion of the XXXG and XXLG building blocks compared to a digest of the total population $[\text{xg}]_4$ (data not shown). This experiment demonstrated that the adsorbed $[\text{xg}]_4$ material was enriched in smaller building blocks and that subtle differences in molecular structure of $[\text{xg}]_4$ influence their adsorption: substitution with Gal probably decreases the ability of fragments to penetrate the smaller pores in cellulose.

DISCUSSION

The present paper discusses several chromatographic techniques aimed at fractionation and analysis of large xyloglucan fragments. HPSEC was used for monitoring the degradation of $[\text{xg}]_\infty$ and allowed simple quantification of xyloglucan by the refractive index. Rather pure fragments of up to five repeating oligosaccharide units could be obtained by fractionation of a partially degraded tamarind seed xyloglucan using BioGel P-6 chromatography. HPAEC served merely as an analytical tool to check the purity of BioGel P-6 pools and the oligosaccharide composition of certain fragments. However, from the results presented here it might be anticipated that this technique could be used successfully for preparative fractionation of fragments (much) larger than $[\text{xg}]_5$. This was not further substantiated, since a sequence of three or four xyloglucan oligosaccharides appeared to be of critical length for interaction with cellulose.

The observation that polymeric xyloglucan binds to cellulose whereas its individual building blocks ($[\text{xg}]_1$) do not suggests that the interaction between xyloglucan and cellulose is a reversible process. Adsorption of $[\text{xg}]_\infty$ to cellulose can be envisaged as is indicated in Figure 5 (Fleer et al., 1993). A number of building blocks ("trains") will interact with the cellulose surface. The so-called "loops" and "tails" stick out into solution. Our results suggest that binding of $[\text{xg}]_\infty$ involves at least five oligosaccharides; however, it is

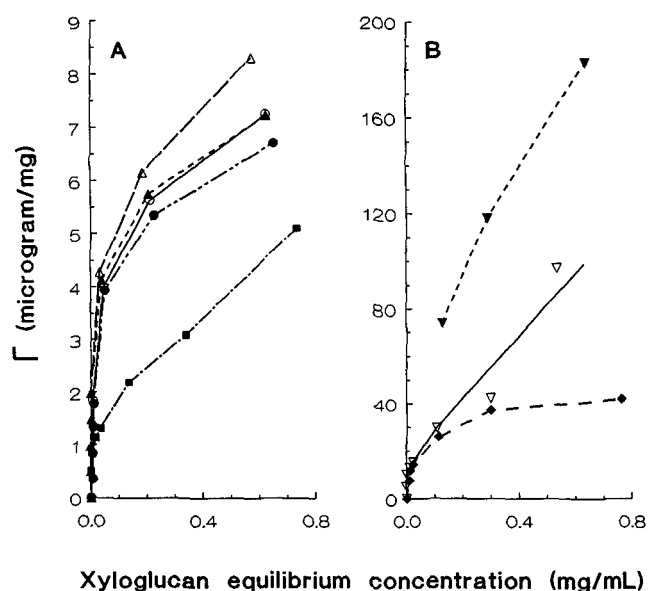


Figure 4. Adsorption isotherms of polymeric xyloglucan $[\text{xg}]_\infty$ (A) and two BioGel P-6 fractions (B) under various conditions. A: \circ , Avicel (50 mg), pH 3.0; \bullet , Avicel (50 mg), pH 4.0; \blacktriangle , Avicel (50 mg), pH 5.0; \triangle , Avicel (50 mg), pH 5.8; \blacksquare , cotton linters (50 mg), pH 5.8. B: ∇ , pool IV ($[\text{xg}]_4$), Avicel (5 mg), pH 5.8; \blacktriangledown , pool IV ($[\text{xg}]_4$), Avicel (1 mg), pH 5.8; \blacklozenge , pool VI ($[\text{xg}]_{6 < n < 11}$), Avicel (5 mg), pH 5.8. After 6 h of incubation the samples were centrifuged (1 min, 20,000g) and the supernatants were analyzed for their neutral sugar content, from which the adsorbed amount was calculated.

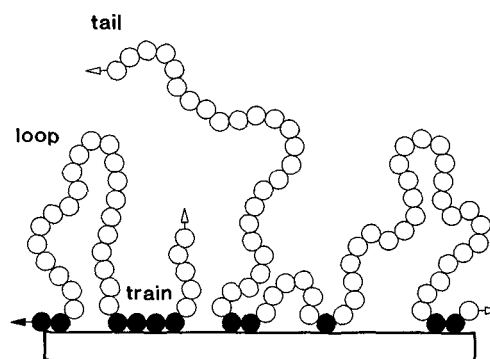


Figure 5. Schematic representation of the interaction of polymeric xyloglucan ($[\text{xg}]_\infty$) and cellulose. \circ , Xyloglucan oligosaccharide ($[\text{xg}]_1$) in so-called loops and tails; \bullet , xyloglucan oligosaccharides that interact with cellulose (trains); \square , cellulose.

unknown whether these should occur contiguously. In principle, adsorption of xyloglucan oligosaccharides to cellulose is a cooperative effect. For instance, it is thought that $[xg]_{\infty}$ can bind equally well by a train of six oligosaccharides or by three trains of two oligosaccharides.

The first part of the adsorption isotherm represents a situation in which all xyloglucan molecules are bound to the cellulose surface: Γ increases dramatically with increasing $[xg]_{\infty}$ concentration. The steepness of this part of the isotherm emphasizes the high-affinity character of xyloglucan adsorption on cellulose. Finally, this surface is saturated with xyloglucan, and for homodisperse systems Γ will reach a constant value. However, HPSEC analysis of $[xg]_{\infty}$ (data not shown) showed a rather broad molecular weight distribution, which indicates that we are dealing with a polydisperse system. When the supply of $[xg]_{\infty}$ exceeds the binding capacity of the cellulose, preferential adsorption of larger molecules occurs because their binding is more favorable in terms of entropy compared to smaller molecules. As a result of this, none of the isotherms (Fig. 4A) reach their plateau value instantaneously; rather, the isotherms level off until, finally, the cellulose surface is covered with the largest molecules. Thus, with increasing supply of $[xg]_{\infty}$, loops and tails will contribute more and more to the adsorbed amount, although the coverage (train density) of the cellulose remains the same.

Adsorption isotherms show that the origin of cellulose influences the amount of xyloglucan that can adsorb. Similar observations were made by Hayashi et al. (1987). The lower adsorption of $[xg]_{\infty}$ to cotton linters cellulose compared to Avicel suggests a larger effective surface area for the latter. This seems to be in accordance with the higher crystallinity index for cotton linters reported by Hoshino et al. (1993). Stone and Scallan (1968) demonstrated that cellulose is a porous matrix in which small pores contribute the major part of the total surface area of the cellulose. The $[xg]_{\infty}$ s are too large to reach the smaller pores. The stiffness of the glucan chain in solution, which is partially due to its extensive branching (Gidley et al., 1991), probably reinforces their poor penetration. Apparently, the surface of many of these small pores can be colonized by $[xg]_4$, considering the much higher adsorption (approximately 10-fold) of these fragments compared to $[xg]_{\infty}$.

Maclachlan et al. (1992) showed that an important part of tamarind seed xyloglucan is composed of $XXLG \rightarrow XXLG \rightarrow XLLG$ fragments that were found to be reasonably enzyme resistant. Additionally, substitution with Gal at the penultimate position of an oligosaccharide seems to hinder the release of such oligosaccharides by endoV (J.-P. Vincken, G. Beldman, A.G.J. Voragen, unpublished results). These observations suggest that the degradation of xyloglucan is not completely random, but rather that cleavage occurs at linkages adjacent to an XXXG subunit. This explains why $[xg]_1$ contains predominantly XXXG and XXLG and why larger fragments, such as $[xg]_4$, are enriched in Gal-containing oligosaccharides. Theoretically, $[xg]_4$ can adopt 256 different permutations, but this number of possibilities is narrowed down to some extent due to the mode of action of endoV. All $[xg]_4$ structures

containing only XXXG and/or XLXG are very improbable; molecular masses will range from approximately 4.8 to 5.5 kD. These results demonstrate that $[xg]_4$, like $[xg]_{\infty}$, is a polydisperse population, but on a miniature scale.

The polydispersity of $[xg]_4$ finds expression in its adsorption isotherm, but because these molecules are too small to form loops and tails, a mechanism different from that described for $[xg]_{\infty}$ must underlie this pattern. The Γ of $[xg]_4$ seems to depend on the amount of Avicel (Fig. 4B), which suggests that not only the dimensions of $[xg]_4$ but also the pore size distribution of cellulose plays an important role here. At low $[xg]_4$ concentrations, the surface of the smallest pores of cellulose is probably only partly covered with xyloglucan due to the limited supply of the smaller molecules (built mainly from XXXG and XXLG) within the $[xg]_4$ population. At higher concentrations, more of these smaller molecules are present, and the smaller pores of cellulose can be further colonized. Our observation suggesting that relatively more small molecules are adsorbed is in accordance with this. The adsorption isotherm of pool VI was the only one achieving a constant value under the conditions used. This value was much lower than that found for $[xg]_4$, which emphasized that the smaller pores contribute an important part to the total surface area of cellulose.

Figure 4A further suggests that the pore size can be manipulated to a small extent by changing the pH. Both cellulose preparations contained approximately 8 mol% of sugar residues other than Glc. The Xyl and part of the uronic acid probably originate from 4-O-methyl-glucuronoxylans, which are known to be closely associated with cellulose just like xyloglucans (Sjöström, 1989). Depending on the pH, the carboxyl groups (pK_a of approximately 4.5) of these glucuronoxylans can introduce a negative charge to the cellulose. Repulsion of these ionized groups is probably attended by an enlargement of the cellulose pores. A larger portion of the cellulose surface would be accessible for xyloglucan molecules, which might explain why more $[xg]_{\infty}$ is adsorbed at increased pH.

We have shown previously that enzymic degradation of cell-wall-embedded cellulose is enhanced by stripping the xyloglucan coating (Vincken et al., 1994). The present paper demonstrates that $[xg]_5$ adsorbs quantitatively to cellulose. This means that xyloglucans have to be degraded extensively (preferably to $[xg]_2$) before cellulose becomes accessible to a cellulolytic enzyme combination. It should be realized that this study was done with commercially available Avicel cellulose and tamarind seed xyloglucan instead of apple fruit cellulose and xyloglucan. It was shown by solid-state ^{13}C NMR that Avicel and apple cellulose are not alike (Newman et al., 1994); further, the presence of Fuc in apple fruit xyloglucan might influence the binding of these molecules to cellulose (Levy et al., 1991). Apart from this, we would like to put forward some thoughts on possible implications of these results in cell-wall processes.

First, the DP of xyloglucans has never been considered an important factor in the assembly of the cellulose-xyloglucan complex. It could very well be that the secretory vesicles (budded off from Golgi) contain a large collection

of xyloglucan molecules (ranging, for instance, from $[xg]_4$ to $[xg]_{\infty}$). Smaller molecules might occupy the space between cellulose-synthesizing terminal complexes (Delmer and Stone, 1988) more easily than larger ones and serve as a kind of fixture to which larger molecules can be attached by, for instance, transglycosylation (Fry et al., 1992). This might explain the much higher xyloglucan levels in native cellulose-xyloglucan complexes than in reconstituted ones (Hayashi et al., 1987).

Second, Coimbra et al. (1994) reported a pectic fraction that was enmeshed in the cellulose microfibrils of olive. Such a pectin (after removal of methyl groups) could influence the pore size of cellulose in a way similar to a glucuronoxylan. Ricard and Noat (1986) postulated a role for pectin methyl esterase in locally modulating the cell wall pH for "growth enzymes." Swelling of cellulose might also be triggered by these enzymes.

Finally, and probably most importantly, this work provides a method for manufacturing, purifying, and analyzing a homologous series of xyloglucan fragments. These defined fragments and cellulose can be used to build model systems for testing a number of hypotheses that have recently been put forward: facilitated binding of fucosylated fragments (Levy et al., 1991), prohibited binding of endoglucanase-resistant fragments (Hisamatsu et al., 1992), and disruption of noncovalent interactions by expansins (McQueen-Mason and Cosgrove, 1994).

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