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Potato xyloglucan is built from XXGG-type subunits [1]

Jean-Paul Vincken [a], Alexandra J.M. Wijsman [a], Gerrit Beldman [a], Wilfried M.A. Niessen [b], Alphons G.J. Voragen [a,*]

[a] *Wageningen Agricultural University, Department of Food Science, P.O. Box 8129, 6700 EV Wageningen, The Netherlands*

[b] *Division of Analytical Chemistry, Leiden / Amsterdam Center for Drug Research, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

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Abstract

Extraction of potato cell-wall material with solutions of increasing strength of alkali yielded a xyloglucan-rich fraction which was further purified by anion-exchange chromatography and treatment with α-amylase and endogalactanase. Methylation analysis indicated that the purified xyloglucan contained a high percentage of unsubstituted glucosyl residues compared to, for instance, apple xyloglucan, and equal amounts of Xyl-(1 → 6)-, Gal-(1 → 2)-Xyl-(1 → 6)-, and Ara-(1 → 2)-Xyl-(1 → 6)-sidechains. This xyloglucan was degraded with endoglucanase (endoV), purified from *Trichoderma viride*. The resulting digest was fractionated by BioGel P-2 chromatography, followed by preparative high-performance anion-exchange chromatography of the pentamer to nonamer fractions. The purified oligosaccharides were characterized by monosaccharide analysis, mass spectrometry, and degradation with an exoglucanase. Degradation of potato xyloglucan by another endoglucanase (endoIV) of *Trichoderma viride* yielded a different set of products. EndoIV released predominantly oligosaccharides with two unbranched glucosyl residues at the reducing terminus, whereas endoV also released products containing unbranched glucosyl residues on both ends of the molecule. A difference in the mode of action of endoglucanases with xyloglucan-degrading activity is demonstrated. © 1996 Elsevier Science Ltd.

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* Corresponding author. Tel.: +31-317-482888; Fax: +31-317-484893.

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1. Introduction

Xyloglucans occur widely in the kingdom of plants [1], where they reinforce the primary cell wall by cross-linking cellulose microfibrils [2]. Their main structural characteristic is a (1 → 4)-β-D-Glc*p* backbone with α-D-Xyl*p*-(1 → 6)-sidechains. The presence of other glycosyl residues, like β-D-Gal*p*, α-L-Fuc*p* or α-L-Ara*f*, as well as sidechain density, depend on the plant species. Xyloglucans can be degraded to oligosaccharides by endoglucanase and usually a relatively crude cellulase preparation is used for this purpose. However, Vincken et al. [3] demonstrated that individual glucanases, derived from a *Trichoderma viride* cellulase preparation, can differ considerably in their ability to degrade apple fruit xyloglucan. It is unknown if these enzymes differ in their mode of action towards xyloglucan.

Apple fruit xyloglucan is built mainly from the structural elements XXXG, XLXG, XXFG, and XLFG [4,5] (for nomenclature, see ref. [6]), and hardly any sequences of consecutive unsubstituted Glc exist. Endoglucanases are 'forced' to cleave right next to a branched glucosyl residue (for instance -XXXG↓XXXG-). When using such heavily branched polysaccharides such as apple xyloglucan, possible differences in the mode of action of endoglucanases are likely to be masked. Therefore, we searched for a less branched xyloglucan substrate which would offer endoglucanases the possibility to hydrolyze their linkage of preference.

A number of these more linear xyloglucans have been described in literature. The xyloglucan of rice seedlings [7] and immature barley plants [8] are predominantly built from XXGGG structural units; the former also contains regions with an even lower degree of substitution. Contrary to apple fruit xyloglucan, these polysaccharides are poorly water soluble. Xyloglucan of potato has a slightly higher degree of branching than that of rice seedlings or barley plants (approximately 45% [9]), and is soluble in water; therefore, this polysaccharide seems a better candidate to study the mode of action of endoglucanases. Potato xyloglucan lacks fucosylated sidechains as in apple, but contains Ara-(1 → 2)-Xyl-(1 → 6)-sidechains instead.

Presently, the distribution of sidechains of potato xyloglucan is unknown. A degree of substitution of almost 50% allows structures like XGXGXG, in which case the endoglucanase is forced to cleave right next to a sidechain. However, by analogy to barley plants [8] and rice seedlings [7], clusters of unbranched glucosyl residues and xylosylated glucosyl residues (as in GXXGGXXG) seem more likely. This study investigates the branching pattern of potato xyloglucan. In addition, the different mode of action of two endoglucanases from *Trichoderma viride* (endoIV and endoV) towards this substrate will be discussed.

2. Experimental

Raw material.—Potatoes (*Solanum tuberosum* var. Bintje) were obtained at a local market. A mixture of cellodextrins (degree of polymerization 1 to 6) was obtained from Merck (Darmstadt).

Enzymes.—Two endoglucanases (endoIV and endoV) [E.C.3.2.1.4] and an exoglucanase (exoI) [E.C.3.2.1.91] were purified from a commercial enzyme preparation from *Trichoderma viride* (Maxazyme Cl, Gist-Brocades, Delft) by Beldman et al. [10]. Pancreas α-amylase was obtained from Merck and showed no activity towards cell wall polysaccharides. A purified endogalactanase was present in the laboratory of the authors [11].

Isolation of cell wall material.—Potatoes (4 kg) were peeled, cut (1 cm^3), and blanched (20 min) in water (70 °C) to inactivate endogenous enzymes and gelatinize starch. The potatoes were homogenized in a Waring Blendor with 3 L of 10 mM maleate buffer (pH 6.5) containing 1 mM $CaCl_2$, 10 mM NaCl, and 0.01% NaN_3. The potato mash was treated with 75 mg of α-amylase (approximately 15,000 U) for 16 h at 40 °C. After centrifugation (15 min, 16,300 *g*), the residue was extracted twice (1 h) with 3 L of distilled water. The first extraction was performed at 40 °C, the second one at 70 °C. The α-amylase treatment and subsequent extractions were repeated four times. The residual material was freeze-dried and ground in a Fritsch pulverisette (sieve 1.0 mm, Germany) and designated as WUS.

Extraction of WUS.—Potato WUS was subjected to a sequence of alkali extraction steps (0.05 M NaOH containing 5 mM CDTA, 1 M KOH containing 1% (w/w) $NaBH_4$ and 4 M KOH containing 1% (w/w) $NaBH_4$; 1 g of WUS per 200 mL of extractant), in a similar manner as described for apple [3]. Extracts were acidified to pH 5 with acetic acid, dialyzed extensively against distilled water and freeze-dried. In some cases tiny amounts of material precipitated, which were removed by centrifugation (20 min, 50,000 *g*).

Purification of the 4 M KOH extract.—Residual starch was removed by treating approximately 125 mg of the freeze-dried material with α-amylase (100 μg) in 250 mL of 50 mM NaOAc buffer (pH 6) containing 0.01% (w/w) NaN_3 at 40 °C. After 24 h a similar dose of enzyme was added and the incubation was continued for another 24 h. The incubation was stopped by heating the reaction mixture 10 min at 100 °C. The mixture was then dialyzed (2 times 12 h) against 50 mM NaOAc buffer (pH 5) containing 0.01% (w/w) NaN_3. Subsequently, the material was depectinized on a DEAE Sepharose CL-6B column (40 × 440 mm, Pharmacia, Uppsala) in a similar manner as described for apple xyloglucan [3]. The neutral fraction was concentrated (approximately two times) under reduced pressure, acidified to pH 4.5 with acetic acid, and treated with approximately 20 mU of endogalactanase for 48 h at 40 °C. The reaction mixture was heated for 10 min at 100 °C, dialyzed extensively against distilled water, and freeze-dried. The resulting material was designated POsxg.

Enzymic degradation of POsxg.—POsxg (50 mg) was dissolved in 5 mL of 40 mM NaOAc buffer (pH 5), containing 0.01% (w/w) of NaN_3 and 10 μg of endoV protein, and incubated for 48 h at 40 °C. The resulting digest was heated for 10 min at 100 °C to inactivate endoV, and freeze-dried. In a similar manner, POsxg was treated with endoIV (approximately 2 μg of protein).

BioGel P-2 chromatography.—The POsxg digests were dissolved in 2 mL of distilled water and applied to a column (100 × 2.6 cm, i.d.) of BioGel P-2 (200–400 mesh, Bio-Rad, Richmond) at 60 °C and eluted with distilled water (18 mL h^{-1}). Fractions (2.0 mL) were assayed for total neutral sugar content. Appropriate fractions

were combined. The column was calibrated using a mixture of glucose, cellobiose, raffinose, stachyose, and Dextran T150 (Pharmacia, Uppsala).

Preparative high-performance anion-exchange chromatography (HPAEC) of BioGel P-2 fractions.—The BioGel P-2 fractions **5**–**9** of the endoV digest and fraction **9** of the endoIV digest were subjected to high-performance anion-exchange chromatography (HPAEC) using a Spectra Physics P4000 pump (San José) equipped with a Dionex CarboPac PA-100 column (250 × 22 mm, Sunnyvale). Samples of 400 μL (containing 3 to 7 mg mL^{-1}) were injected with a Spectra Physics AS3000 autosampler, and eluted (25 mL min^{-1}) at 20 °C with 100 mM NaOH containing different concentrations of NaOAc. The NaOAc gradients, which were optimized for each BioGel P-2 fraction, were as follows: **5**, 0 → 30 min, linear gradient of 20 → 80 mM NaOAc; **6**, 0 → 20 min, linear gradient of 30 → 60 mM NaOAc; 20 → 40 min, linear gradient of 60 → 100 mM NaOAc; **7**, 0 → 60 min, linear gradient of 30 → 80 mM NaOAc; **8**, 0 → 50 min, linear gradient of 40 → 80 mM NaOAc; **9**, 0 → 15 min, linear gradient of 50 → 60 mM NaOAc; 15 → 20 min, linear gradient of 60 → 70 mM NaOAc; 20 → 50 min, linear gradient of 70 → 100 mM NaOAc. After each run, the column was cleaned for 5 min by elution with 100 mM NaOH containing 1000 mM NaOAc, and subsequently equilibrated for 15 min with the starting eluent. Solvents were degassed and stored under helium using a Dionex EDM module. The eluate was split post-column (ratio 1 to 9), and the smaller current was monitored using a Dionex PED detector in the pulsed-amperometric detection (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 eluate was neutralized by on-line addition of 1 M acetic acid and appropriate fractions (approximately 5 to 12 mL) were combined.

Desalting of oligosaccharides.—In order to remove NaOAc from the HPAEC pools, the oligosaccharides were adsorbed on Bakerbond SPE C_{18} disposable extraction columns (J.T. Baker, Phillipsburg), washed extensively with distilled water, and eluted with methanol. The most prominent purified oligosaccharides were characterized by monosaccharide analysis, mass spectrometry, and partial degradation with exoI (or endoV).

Reduction of oligosaccharides.—In order to label oligosaccharide **9.2** at its reducing terminus, this fraction (approximately 100 μg) was treated with 200 μL 1.5 M ammonia containing 75 mg mL^{-1} $NaBH_4$ for 1 h at 30 °C. After neutralization with HOAc, the reduced oligosaccharides were desalted batchwise using a mixture of Dowex 50W X8 (H^+) and AG3 X4A (OH^-) (Bio-Rad) in a ratio of approximately 0.6 (v/v), and subsequently dried in a stream of air.

Release of oligosaccharides from POsxg in time.—POsxg (2 mg) was dissolved in 1 mL of 50 mM succinate buffer (pH 4) containing 0.01% (w/v) NaN_3 and degraded with endoIV (approximately 140 ng) or endoV (approximately 5 μg) during 48 h at 40 °C. Samples of 50 μL were taken during the course of degradation. After inactivation of the enzyme (10 min, 100 °C), the products were analyzed by HPAEC.

Degradation of (mixtures of) oligosaccharides by exoI or endoV.—Monosaccharide analysis and mass spectrometry revealed that several oligosaccharides contained more than one unsubstituted glucose residue. In order to investigate whether these extra

residues were located on the reducing or non-reducing terminus, the oligosaccharides were treated with either exoI (active on the non-reducing end) or endoV (active on the reducing end). Approximately 10 μg of oligosaccharide (reduced **9.2** was also included in this series) or 100 μg of a POsxg digest of endoIV were treated (12 h, 40 °C) with exoI (approximately 100 ng of protein), endoV (approximately 1 μg of protein), or endoIV (approximately 200 ng of protein) in 100 μL of a 50 mM NaOAc buffer (pH 5) containing 0.01% (w/w) NaN_3. Mixtures were heated for 10 min at 100 °C to stop the reaction and the release of Glc (or glucitol in case of **9.2**) was analyzed by HPAEC.

Sugar analysis.—Uronic acids were estimated colorimetrically with an automated *m*-hydroxydiphenyl test [12] using concentrated sulphuric acid containing 0.0125 M $Na_2B_4O_7$ for hydrolysis. The total neutral sugar content was determined colorimetrically with an automated orcinol/sulphuric acid assay [13]. Glucose was used as a standard. For determination of the neutral sugar composition various methods were used. WUS was subjected to a 72% (w/w) H_2SO_4 prehydrolysis (1 h at 30 °C) followed, after dilution with water, by a 1 M H_2SO_4 hydrolysis (3 h at 100 °C). Alkali extracts from WUS and BioGel P-2 fractions were hydrolyzed (1 h, 121 °C) using 2 M TFA. The released neutral sugars were converted to their alditol acetates and analyzed by GC as described previously [3]. Oligosaccharides, obtained after fractionation on HPAEC, were hydrolyzed using 2 M TFA as above and the sugar composition was determined by HPAEC as described by De Ruiter et al. [14].

Glycosyl linkage composition.—POsxg was methylated according to a modification of the Hakomori method [15] and subsequently dialyzed against water and dried by evaporation (airstream, room temperature). This procedure was repeated once. Next, the methylated xyloglucan was hydrolyzed using 2 M TFA as described above. The released (partially methylated) sugars were converted to their alditol acetates, which were quantified by GC and identified by GC–MS as described before [3]. Sodium borodeuteride was used for reduction.

Starch content.—The amount of starch which was present in WUS was determined using a test-kit obtained from Boehringer (Mannheim).

Protein content.—The protein content of WUS (N × 6.25) was determined by a semi-automated micro-Kjeldahl method. Protein content of enzyme preparations was determined according to Sedmak and Grossberg [16]. Bovine serum albumin was used as a standard.

Determination of molecular mass of xyloglucan oligosaccharides.—The molecular mass of the purified xyloglucan oligosaccharides was determined with a Finnigan MAT TSQ-70 mass spectrometer (San José), equipped with a 20 kV conversion-dynode and a Finnigan MAT electrospray interface as described previously [17].

Analysis of xyloglucan oligosaccharides.—Xyloglucan oligosaccharides and their degradation products were analyzed by HPAEC (CarboPac PA-100 column; Dionex) combined with PAD-analysis as described previously [5]. Samples (20 μL) were eluted (1 mL min^{-1}) with the following NaOAc gradient in 100 mM NaOH: 0 → 5 min, linear gradient of 0 → 50 mM NaOAc; 5 → 30 min, linear gradient of 50 → 100 mM NaOAc; 30 → 45 min, linear gradient of 100 → 250 mM NaOAc. After each analysis, the column was rinsed for 5 min with 1 M NaOAc in 100 mM NaOH, and equilibrated in 100 mM NaOH for 15 min.

3. Results

Isolation and purification of xyloglucan.—Successive water extractions and α-amylase treatments of the potato mash yielded an insoluble fraction (WUS) which consisted of approximately 65% carbohydrates and approximately 25% proteins; approximately 2% of the peeled potato material was recovered as WUS. Approximately 25% (w/w) of the sugars in WUS could be attributed to starch. Several α-amylase treatments, preceded by an extraction at 70 °C, were insufficient to obtain an essentially starch-free WUS. DMSO, which is known as an effective starch extractant [9,18], was not used because some pectic polysaccharides, which were of interest to us in a different study, were solubilized as well. The WUS was subjected to a sequence of alkali extractions; the polysaccharides present in WUS were recovered in four fractions: 0.05 M NaOH [27% (w/w)], 1 M KOH [44% (w/w)], 4 M KOH [8% (w/w)], and the residue [21% (w/w)]. The sugar compositions of WUS and extracts are summarized in Table 1. Pectic polysaccharides accumulate in the 0.05 M NaOH extract. The relatively large amount of Glc in the 1 M KOH extract suggested that this fraction contained predominantly starch. This was confirmed by treatment with α-amylase.

The 4 M KOH extract was chosen as a source for purification of xyloglucan because this extract contained more xyloglucan and less starch than the 1 M KOH extract. Small amounts of contaminating polysaccharides were removed by α-amylase treatment (starch), anion-exchange chromatography (pectic material), and treatment by a purified endogalactanase (galactan). The selective degradation of starch and galactan was monitored by HPAEC; no typical degradation products of xyloglucan were observed. The resulting high molecular weight material was designated potato arabinoxyloglucan (POsxg) and comprised approximately 1% (w/w) of WUS. The sugar composition of POsxg is given in Table 1, which is in close agreement with the totals of the corresponding differently linked residues determined by methylation analysis (Table 2). The 1,4,6-linked glucose is indicative for the presence of xyloglucan; approximately 37% of the (1 → 4)-linked glucan backbone is branched at the C-6 position. This amount

Table 1
Sugar composition (mol%) of WUS, of fractions obtained by sequential extractions of WUS and of a purified potato xyloglucan

	Rha	Ara	Xyl	Man	Gal	Glc	GalA
Starting material							
WUS	tr [a]	5	2	1	24	51	17
Sequential extractions							
0.05 M NaOH	3	7	0	1	43	12	34
1 M KOH	1	4	2	0	16	68	9
4 M KOH	0	9	20	3	22	43	3
Residue	0	0	0	2	2	91	5
4 M KOH after several purification steps							
POsxg	0	8	25	4	9	54	0

[a] Traces.

Table 2
Glycosyl linkage composition of POsxg

Deduced linkage	Molar ratio	Total
L-Ara*f*-(1 → [a]	8	8
D-Gal *p*-(1 →	8	8
D-Xyl *p*-(1 →	9	
→ 2)-D-Xyl *p*-(1 →	12	
→ 4)-D-Xyl *p*-(1 →	1	22
→ 4)-D-Glc *p*-(1 →	34	
→ 4,6)-D-Glc *p*-(1 →	20	54
→ 4)-D-Man *p*-(1 →	6	
→ 4,6)-D-Man *p*-(1 →	2	8

[a] Determined as 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc.

of 1,4,6-linked Glc is in good agreement with that of the sum of terminal Xyl [D-Xyl *p*-(1 →] and 1,2-linked Xyl. Terminal and 1,2-linked xylosyl residues occurred in similar amounts. All Ara and Gal were present as terminal residues, and are probably attached to the C-2 position of Xyl. Detection of 1,4-linked Xyl suggests the presence of a small contamination of xylan in POsxg; short sidechains as observed in the xyloglucan of *Phaseolus coccineus* might be another possibility [19]. No 1,4-linked Gal or 1,4,6-linked Gal was detected which suggests that the β-(1 → 4)-galactan contamination was effectively removed by the endogalactanase treatment. Methylation analysis was not conclusive for the removal of starch. Table 2 further indicates the presence of a (1 → 4)-linked mannan of which approximately 25% of the mannose residues [→ 4,6)-D-Man *p*-(1 →] are branched with, most likely, Gal. As a result of this, potato xyloglucan will have more terminal arabinosyl than galactosyl residues. These data are in agreement with those reported by Ring and Selvendran [9] and Ryden and Selvendran [18].

Fractionation of xyloglucan oligosaccharides obtained by endoV.—The degradation of POsxg by two endoglucanases having a high xyloglucanase activity, was investigated. POsxg was completely degraded by endoV as is indicated by the elution profile of the digest on BioGel P-2 (Fig. 1). The degree of polymerization was later confirmed by mass spectrometry of the oligosaccharides. Pool **1** contained only Glc (data not shown), **2** and **3** were not analyzed; the sugar composition of **5–9** is given in Table 3. It should

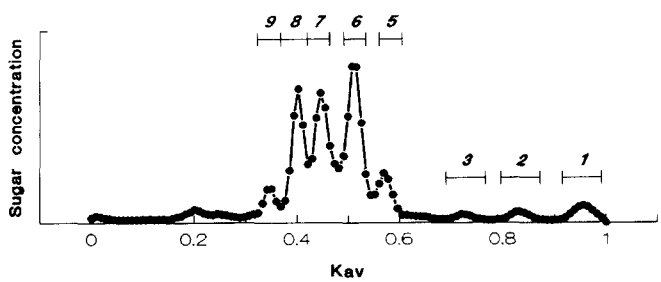

Fig. 1. Elution pattern on BioGel P-2 of a digest of POsxg with endoV.

Table 3
Sugar composition (mol%) of fractions obtained after enzymic degradation of POsxg by endoV. The POsxg digest was fractionated on BioGel P-2, and these fractions were further purified by HPAEC

	Glc	Xyl	Gal	Ara	Man
BioGel P-2 pentamer	54	40	1	3	2
5.1	61	39	0	0	0
BioGel P-2 hexamer	46	34	5	13	2
6.1	45	39	15	1	0
6.2	50	35	14	1	0
6.3	50	45	1	4	0
6.4	51	36	0	13	0
6.5	40	40	0	20	0
BioGel P-2 heptamer	51	29	8	11	1
7.1	46	39	15	0	0
7.2	55	29	13	3	0
7.3	54	30	13	3	0
7.4	57	29	2	12	0
7.5	60	27	0	12	0
7.6	56	28	1	15	0
BioGel P-2 octamer	48	26	15	9	2
8.1	45	29	26	0	0
8.2	46	42	12	0	0
8.3	52	26	10	12	0
8.4	50	25	12	13	0
BioGel P-2 nonamer	50	24	15	8	3
9.1	58	20	19	3	0
9.2	58	21	10	11	0
9.3	57	21	10	12	0
9.4	64	18	2	16	0

be noted that the Man-containing polysaccharide of POsxg was also degraded by endoV because hardly any material was detected in the void fraction. Apparently, endoV is able to cleave this polysaccharide or it contains a residual endomannanase activity. HPAEC analysis of **5–9** (Fig. 2) demonstrated that each pool (except **5**) consisted of at least four products. Pools **5–9** were subsequently fractionated by preparative HPAEC which yielded a large number of oligosaccharides with a sugar composition as is indicated in Table 3, and molecular masses as summarized in Table 4. Together with the known endoglucanase cleavage at the reducing side of an unsubstituted glucosyl residue, it can be deduced that each oligosaccharide contained two (adjacent) substituted glucosyl residues. The sidechains attached to these residues can have any arrangement: XX, LX, XL, LL, SX, XS, SS, LS, and SL. Fractions **6.3** and **8.2** are an exception to the above, because their large xylose content suggests the presence of three sidechains (discussed later).

Fractionation of a POsxg digest obtained by endoIV, and partial characterization of one of the degradation products.—The digest of POsxg with endoIV was fractionated

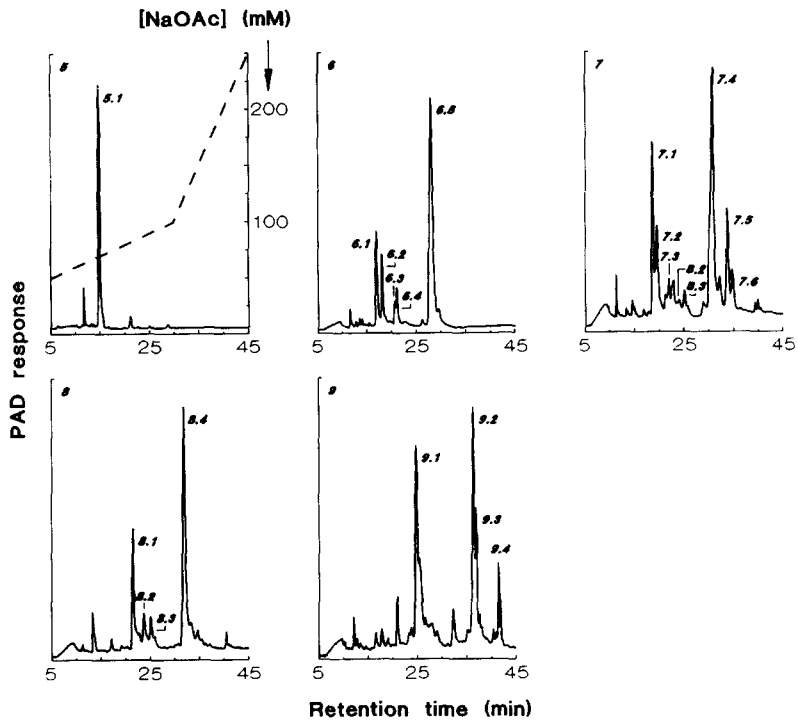

Fig. 2. Elution profiles on HPAEC of BioGel P-2 pools **5–9**, obtained after treatment of POsxg with endoV. Solid lines (————: **5–9**) and dashed line (– – –; **5**) indicate PAD response and NaOAc gradient, respectively.

by BioGel P-2 chromatography (data not shown). The void volume of the BioGel P-2 column contained similar amounts of Man, Glc, and Gal; no Xyl and Ara were found. Apparently, the Man-containing polysaccharide of POsxg was not degraded by endoIV. The presence of a large amount of Glc (in the absence of Xyl) indicates that POsxg still contained some residual starch. Whereas treatment of POsxg with endoV yielded predominantly hexa-, hepta-, and octasaccharides (**6**, **7**, **8**; see Fig. 1), the treatment with endoIV yielded mainly hepta-, and octasaccharides. Pool **9** from the digest obtained with endoIV was further fractionated by preparative HPAEC. This yielded an oligosaccharide with a Glc:Xyl:Gal:Ara ratio of approximately 6:3:1:1. The molecular mass of this oligosaccharide was 1386, which corresponds to $Glc_5Xyl_2Gal_1Ara_1$. Because this oligosaccharide contained many glucosyl residues compared to those obtained from apple xyloglucan [5], it was treated with exoI or endoV. HPAEC analysis showed that in both cases approximately 20% of the Glc was released from the oligosaccharide (data not shown); however, the oligomeric products that were formed, differed. This suggested that exoI and endoV removed one glucosyl residue from a different terminus. This means that $Glc_5Xyl_2Gal_1Ara_1$ corresponds to either GSLGG or GLSGG (for nomenclature, see ref. [6]; note that endoglucanases do not catalyze GSGL↓G). The exact position of arabinosyl and galactosyl residues is not known yet, and the structural

Table 4
Data on purified potato xyloglucan oligosaccharides obtained after enzymic degradation of POsxg by endoV

	Mol wt [a]	ExoI [b]	EndoV [c]	Tentative structure [d]	Peak No. [e]
5.1	768	n.d. [f]	n.d.	XXG	1
6.1	n.d.	n.d.	n.d.	[XL]G	2
6.2	930	n.d.	n.d.	[LX]G	3
6.3	900	n.d.	n.d.	?	6
6.4	n.d.	n.d.	n.d.	[SX]G	6 or 7 [g]
6.5	900	n.d.	n.d.	[XS]G	12
7.1	1092	+	n.d.	G[XL]G	4
7.2	1092	+	n.d.	G[LX]G	5
7.3	1092	−	+	[LX]GG	8
7.4	1062	+	n.d.	G[XS]G	14
7.5	n.d.	n.d.	n.d.	?	16
7.6	1062	−	+	[XS]GG	17
8.1	1254	+	n.d.	GLLG	7
8.2	1224	+	−	?	10
8.3	1062	+	−	?	11
8.4	1224	+	−	G[LS]G	15
9.1	n.d.	n.d.	n.d.	GLLGG	10 or 11 [g]
9.2	1386	+	+	G[LS]GG	19
9.3	n.d.	n.d.	n.d.	?	20
9.4	n.d.	n.d.	n.d.	GSSGG	21

[a] Determined by electrospray mass spectrometry.
[b] Oligosaccharides were treated with exoI to test for unsubstituted Glc on non-reducing terminus.
[c] Oligosaccharides with additional endoV treatment to test for two consecutive Glc on reducing terminus.
[d] Tentative structure based on sugar composition, methylation analysis, mol wt, and exoI/endoV degradation patterns. Nomenclature according to ref. [6]. Segments between brackets [..] are interchangeable because the exact position of Ara and Gal residues has not been determined. However, treatment of, for instance, **7.6** with endoV yields **6.5**, indicating a similar position of Ara (relative to Xyl) for both oligosaccharides.
[e] Numbers refer to peaks indicated in Fig. 3.
[f] Not determined.
[g] Exact location not known.

elements containing these residues will therefore be indicated between brackets, i.e. G[LS]GG. After reduction of the oligosaccharide, it was shown that exoI released Glc, whereas endoV released glucitol. Thus, endoV removed Glc from the reducing end of the molecule (G[LS]G↓G) while exoI acted on the other end (G↓[LS]GG). This example demonstrated that treatment with exoI or endoV can be used to determine the position of unbranched glucosyl residues in an oligosaccharide. Incubation of $Glc_5Xyl_2Gal_1Ara_1$ with endoIV also removes Glc from the reducing terminus, but this reaction was much slower than with endoV.

Further characterization of oligosaccharides by enzyme treatment.— Tables 3 and 4 show that many oligosaccharides (**7.1–7.6**, **8.1–8.4** and **9.1–9.4**) contained a relatively large proportion of Glc. To investigate whether these residues were located at non-reduc-

ing terminus, these oligosaccharides were treated with exoI. In the case of no Glc release by exoI, the oligosaccharides were treated by endoV, as a positive control. The results of these incubations are shown in Table 4. Based on sugar composition, mol wt, and incubations with exoI and/or endoV, tentative structures were proposed for the different oligosaccharides (Table 4). The order of the structural elements between brackets ([..]) is interchangeable. However, it should be noted that the order of letter codes between brackets is not completely arbitrary; degradation of **7.4** (G[XS]G) by exoI and **7.6** ([XS]GG) by endoV, both yielded **6.5** ([XS]G) as a product. Product **7.5** has a similar sugar composition as **7.6** ([XS]GG); its tentative structure might correspond to [SX]GG.

When assuming that the molar response factors are similar for oligosaccharides in one BioGel P-2 pool, it can be concluded from Fig. 2 that [XS]G (**6.5**), G[XS]G (**7.4**), and [XS]GG (**7.6**) are much more abundant than [SX]G (**6.4**; [SX]GG was not found). This suggests that arabinosyl substituents are predominantly attached to one of the two xylosyl residues. Galactosyl residues are more equally distributed over both xylosyl residues; [XL]G (**6.1**) and [LX]G (**6.2**), as well as G[XL]G (**7.1**) and G[LX]G (**7.2**) plus [LX]GG (**7.3**), are present in similar amounts.

The mol wt of **6.3** and **8.2** corresponds to $Hexose_3Pentose_3$ and $Hexose_5Pentose_3$, respectively. The absence of arabinosyl residues in these oligosaccharides (Table 3), suggests that Xyl accounted for all three pentoses. The possibility that **8.2** corresponds to XLXG can be excluded because exoI removed Glc from this molecule. Thus, the data indicate that sidechains containing two xylosyl residues are present, which is in accordance with the small percentage of 1,4-linked Xyl found in the methylation analysis of POsxg. Ryden and Selvendran [19] provided some evidence for the presence of 1,4-linked Xyl in the sidechains of xyloglucan derived from *Phaseolus coccineus*. Possibly, **6.3** and **8.2** contain similar structures in their sidechains.

Mode of action of endoV and endoIV.—The release of oligosaccharides from POsxg by endoIV or endoV was monitored in time by HPAEC analysis. An example of a typical degradation pattern of POsxg is shown in Fig. 3 for both enzymes. No cellobiose or cellotriose was detected in any of the digests. Products eluting between 5 and 15 min only appeared upon incubation with endoV; they probably originate from the galactomannan contamination in POsxg. The numerals in Fig. 3 correspond to those in the last column of Table 4. It can be seen that galactosylated oligosaccharides elute much faster upon HPAEC than arabinosylated oligosaccharides [compare, for instance, [XL]G (**6.1**) and [XS]G (**6.5**) or GLLGG (**9.1**) and GSSGG (**9.4**)]. Further, oligosaccharides are retarded upon HPAEC when they contain an extra unsubstituted glucosyl residue; the effect is most pronounced when this residue is present at the reducing terminus [compare, [LX]G (**6.2**), G[LX]G (**7.2**), and [LX]GG (**7.3**) or [XS]G (**6.5**), G[XS]G (**7.4**), and [XS]GG (**7.6**)].

The degradation of POsxg by endoV, as shown in Fig. 3, was not yet complete. Peaks indicated with 'dimers' disappeared during the course of degradation, while the relative amounts of products 1 to 19 remained similar (data not shown). The absence of 'dimers' in the dashed line (---) of Fig. 3 suggested that the degradation of POsxg by endoIV was complete. Different products (peaks 16–19) than with endoV were accumulated. Further, only a small amount of Glc was released by endoIV. This amount increased, however, at prolonged incubation times with a concomitant shift in retention time of

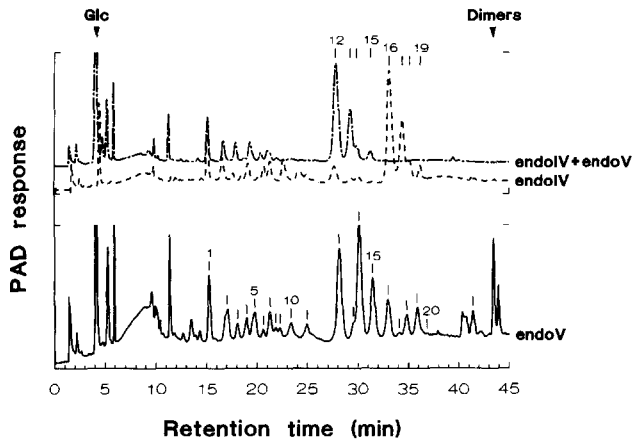

Fig. 3. Elution profiles on HPAEC of a POsxg digest by endoV (————), endoIV (---), and endoIV after subsequent treatment with endoV (– · –). Peak numbers correspond to numerals and tentative structures in Table 4.

most other products. Upon treatment of the POsxg digest of endoIV by exoI, a few minor oligosaccharides (for instance peaks 5 and 19) disappeared and a small amount of Glc was released (data not shown). Apparently, only a small percentage of the oligosaccharides contained an unsubstituted glucosyl residue at the non-reducing terminus; thus, only very small amounts of G[..]G-type of oligosaccharides are formed by endoIV. Incubation of the same digest with endoV resulted in the release of Glc and a shift of the main products of endoIV towards smaller retention times (peaks 12–15; Fig. 3, – · –). The retention time of peak 13 is similar to that of the product of G[LS]G **(8.4)** after exoI treatment; peak 13 probably represents [LS]G.

4. Discussion

The present study investigates the branching pattern of potato xyloglucan. Characterization of oligosaccharides obtained by endoglucanase treatment shows that all building units have two adjacent glucosyl residues bearing sidechains (Tables 3 and 4). An important question was how these clusters of sidechains were distributed along the glucan backbone. This distribution can be deduced from the action pattern of endoIV on POsxg. EndoIV mainly releases [..]GG-type of oligosaccharides and little Glc from POsxg. Prolonged incubation with endoIV showed that these oligosaccharides were further degraded to [..]G-type of products and Glc. Therefore, the relatively small amounts of [..]G and Glc in Fig. 3 (solid line) probably originate from 'trimming' of slightly larger oligosaccharides ([..]GG). It is concluded that blocks of two adjacent unbranched glucosyl residues are characteristic for potato xyloglucan. Thus, [..]GG is the major repeating unit of POsxg, which is illustrated by Fig. 4. It might be argued that methylation analysis of POsxg indicates a higher proportion of 1,4-linked Glc than is shown in Fig. 4. However, the monosaccharide composition of the BioGel P-2 void

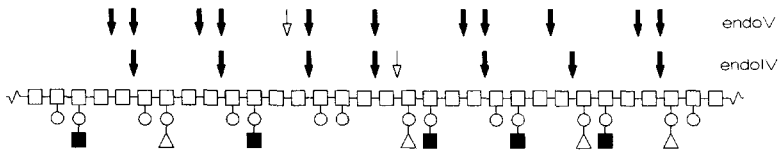

Fig. 4. Tentative structure of POsxg based on methylation analysis, data in Table 4 and degradation pattern of POsxg by endoIV. The location of arabinosyl and galactosyl residues is in accordance with Table 4; it should be realized that the exact position of these residues still needs to be determined. Building blocks with two arabinosyl or two galactosyl residues also occur; these were not indicated because they are present in very small quantities. The different mode of action of endoIV and endoV towards POsxg is illustrated by arrows. Solid and open arrow heads indicate preferred and possible cleavage sites, respectively. Note that we do not have evidence for the release of hexasaccharide XXGG. Symbols: □, β-D-Glc *p*-(1 → 4)-; ○, α-D-Xyl *p*-(1 → 6)-; △, β-D-Gal *p*-(1 → 2)-; ■, α-L-Ara *f*-(1 → 2)-.

fraction of a digest, obtained after degradation of POsxg by endoIV, suggested that POsxg contained some residual starch. The data of methylation analysis were therefore not considered in the degree of branching of POsxg as indicated in Fig. 4, but only to demonstrate that galactose and arabinose were present as terminal residues. It should be noted that the proposed structure is not necessarily representative for all xyloglucan in potato. The 1 M KOH extract contained some xyloglucan as well (data not shown), but the polysaccharides of this fraction were not further investigated. Compared to other xyloglucans, the structure of POsxg is rather unique. It resembles barley [8] and rice xyloglucan [7] in having clusters of two adjacent branched glucosyl residues; however, the number of unsubstituted glucosyl residues between these clusters is two for POsxg instead of three as established for barley and rice (repeating units of [..]GG versus [..]GGG). The data for barley and rice xyloglucan leave room to speculate that their branching pattern is similar to that of potato. Further, POsxg differs from apple [4,5], sycamore, tamarind [20] and many other xyloglucans [1] in its degree of substitution ([..]GG versus X[..]G), although the backbone length of their repeating units is similar.

The release of different oligosaccharides by endoIV and endoV demonstrates that endoglucanases can differ in their mode of action towards xyloglucans. This has never been observed before because in most studies either crude cellulase mixtures or too heavily branched xyloglucans were used. EndoIV gives predominantly a [..]GG-type of oligosaccharides, even when both xylosyl residues of the oligosaccharide are substituted. EndoV seems to cleave more at random, but the action of this enzyme is influenced when both of two adjacent xylosylated glucosyl units are substituted with arabinose and/or galactose. In such a case, endoV releases oligosaccharides only as G[..]G, and not as [..]G or [..]GG (Table 4). Apparently, substitution of both xylosyl residue hinders endoV, and cleavage of a more accessible linkage (e.g. -G↓GLSGG-) is preferred over (e.g. -GG↓LSGG-). Based on this observation, it was expected that endoV would show a similar behavior in the case of GGLXGG (only the xylosyl residue towards the non-reducing end is substituted), i.e. -G↓GLXGG- is preferred over -GG↓LXGG-. However, this is contradicted by finding both [LX]G and [XL]G. The possible mode of action of endoIV and endoV is summarized in Fig. 4. The fact that endoIV releases similar amounts of XXG as endoV, and that no indications for the presence of GXXG or

XXGG were found, suggests that endoIV is also influenced by arabinosyl and galactosyl substitution (see open arrows in Fig. 4). Therefore, determination of the exact location of the galactosyl and arabinosyl residues is still necessary to further refine Fig. 4. The release of Glc and relatively large amounts of a [..]G-type of oligosaccharide by endoV shows that this enzyme is better in 'trimming' unsubstituted glucosyl residues from the reducing end of oligosaccharides ([..]G↓G) than endoIV; in case of the latter, Glc release is very slow. This might be related to a difference in the number of substrate binding sites of both enzymes.

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