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Degradation of apple fruit xyloglucan by endoglucanase

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A purified, alkali-extractable apple fruit xyloglucan (APfxg) was treated by endoglucanase (endoIV) from *Trichoderma viride*. The degradation products were fractionated by size-exclusion chromatography on BioGel P-2; the pentamer to dodecamer fractions were further fractionated by semi-preparative high-performance anion-exchange chromatography. The purified oligosaccharides were characterized by monosaccharide analysis, mass spectrometry and degradation with Driselase. Based on these data, tentative structures were proposed for most products. Apple xyloglucan is composed of a diversity of repeating units. Next to the major oligosaccharides XXXG, an octamer, XXFG and XLFG (Renard, C.M.G.C., Lomax, J.A. & Boon, J.J. (1992). *Carbohydr. Res.*, **232**, 303–320), XG, XXG, FG and two new fucose-containing oligosaccharide building blocks (hexasaccharide and dodecasaccharide) were found. Larger xyloglucan fragments were obtained by partial degradation of APfxg by endoIV, and treatment of apple cell wall material by endoI. These fragments were predominantly composed of fucose-containing oligosaccharides. Our results show that both endoIV and endoI are hindered by fucosylated side chains. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Xyloglucans are extensively branched polysaccharides which occur in many primary cell walls of plants. Side chains of α-D-Xyl*p*-(1→6)-, β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)- or α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)- are attached to ca 75% of the β-(1→4)-D-Glc*p* residues of the backbone (Hayashi, 1989; York *et al.*, 1990; Hisamatsu *et al.*, 1991). This consistency in chemical configuration led Fry *et al.* (1993) to define a one-letter code for each different type of side chain substitution which can unambiguously describe a xyloglucan structure. It should further be mentioned that the galactosyl residues can be *O*-acetylated (Kiefer *et al.*, 1989).

A common repeating structure, found in xyloglucans of various origin, is that of a hexadecasaccharide (Fig. 1A). Treatment of xyloglucan with a crude cellulase preparation derived from *Trichoderma viride* does usually not accumulate XXFGXXXG; endoglucanase, an important constituent of these preparations, can cleave this fragment at the reducing side of an internal unsubstituted glucosyl residue to yield a nonasaccharide (XXFG) and a heptasaccharide (XXXG). Kiefer *et al.* (1990) demonstrated that endoglucanase action was blocked when there was an additional substitution of the glucan backbone with α-L-Ara*f*-(1→2)- as is indicated in Fig. 1B. Removal of this residue by mild acid hydrolysis and subsequent endoglucanase treatment gave the expected XXFG and XXXG. Hisamatsu *et al.* (1992) identified a whole group of these endoglucanase-resistant oligosaccharides. Next to an α-L-Ara*f*-(1→2)-side chain, β-D-Xyl*p*-(1→2)- and α-L-Ara*f*-(1→3)-β-D-Xyl*p*-(1→2)-side chains were also found; all extra side chains were attached to the glucan backbone as indicated in Fig. 1B. In addition, they showed that a structure containing two adjacent fucosylated side chains (as in XFFG; Fig. 1C) could only be degraded with a large overdose of endoglucanase. Apparently, fucosyl residues play a role in the digestibility of xyloglucans which was further substantiated by York *et al.* (1995).

The cellulase complex of *Trichoderma viride* contains several distinct endoglucanases (Beldman *et al.*, 1985), which differ in their activity toward apple fruit xyloglucan; endoIV was shown to be a much better xyloglucanase than endoI (Vincken *et al.*, 1994). Further, larger xyloglucan fragments (degree of polymerization, DP > 15) accumulated upon treatment of apple cell wall material (WUS) by endoI compared to those obtained by endoIV. Renard *et al.* (1992) showed that apple fruit xyloglucan is predominantly built from XXXG, XXFG

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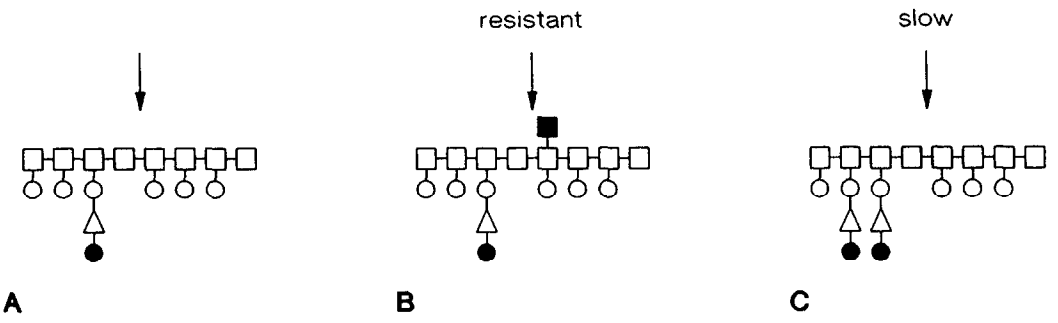

Fig. 1. Schematic structures of xyloglucan composed of two building units. A, common, easily degradable, fragment (XXFGXXXG); B, endoglucanase-resistant fragment (XXFGAXXG); C, fragment (XFFGXXXG) which is partially resistant to cleavage by endoglucanase. Symbols: □, β-D-Glc*p*-(1→4)-; ○, α-D-Xyl*p*-(1→6)-; Δ, β-D-Gal*p*-(1→2)-; ●, α-L-Fuc*p*-(1→2)-; ■, α-L-Ara*f*-(1→2)-. Sites of (possible) endoglucanase attack are indicated by arrows.

and XLFG units; smaller oligosaccharides (DP approximately 6) were also reported but these were not further characterized. The objective of the present study is to characterize the oligosaccharides, including the small ones, obtained by treatment of apple fruit xyloglucan with endoIV. Larger fragments, obtained by treatment of WUS by endoI, were also purified and their oligosaccharide composition was determined by degradation with endoIV. The apparent endoI-resistancy of these xyloglucan fragments is discussed.

MATERIALS AND METHODS

Materials

Substrates

Apple (*Malus malus* L., Rosaceae, var. Golden Delicious) cell wall material (WUS) was obtained after extensive extraction of apple pulp with water as described previously (Vincken *et al.*, 1994). Xyloglucans were extracted from WUS by strong alkali (4 N KOH) and depectinized on a DEAE-Sepharose CL-6B column. This purified xyloglucan (further referred to as APfxg) contained small amounts of (1→4)-Man and (1→4)-Xyl suggesting a slight contamination with mannan and (arabino)xylan (Vincken *et al.*, 1994). A mixture of cellodextrins (DP 1–6) was obtained from Merck (Darmstadt, Germany).

Enzymes

Two endoglucanases (endoI and endoIV) [E.C.3.2.1.4] and CBH [E.C.3.2.1.91], previously referred to as exoIII, were purified from a commercial enzyme preparation from *Trichoderma viride* (Maxazyme Cl, Gist-Brocades, Delft, The Netherlands) as described by Beldman *et al.* (1985). A Driselase preparation (Sigma, St Louis, MO, USA) was desalted on a Fast Desalting Column HR 10/10 (Pharmacia, Uppsala, Sweden) and used for 'sequencing' of xyloglucan oligosaccharides without further purification.

Preparation of xyloglucan fragments

Nomenclature

Two individual purification procedures of xyloglucan fragments were carried out. The first procedure used APfxg as starting material, which was degraded by endoIV. The digest was fractionated by BioGel P-2 chromatography and semi-preparative HPAEC (see later). Fractions obtained by this procedure are summarized in Fig. 2A. Larger xyloglucan fragments were obtained by the second fractionation procedure in which WUS was degraded by a combination of endoI plus CBH. The resulting fragments were fractionated by BioGel P-4 chromatography (see later). Roman numerals were used to refer to these fractions as is indicated in Fig. 2B.

Preparation of oligosaccharides from APfxg using endoIV

APfxg (40 mg) was treated with endoIV (40 μg of protein) in 20 ml of a 50 mM sodium acetate buffer, pH 5 at 40°C. After 24 h a similar amount of fresh enzyme was added. The enzyme was inactivated (10 min, 100 °C) after a total incubation time of 72 h. The digest was concentrated under reduced pressure, freeze-dried, dissolved in 2 ml of distilled water and applied onto a column (100 × 2.6 cm, i.d.) of BioGel P-2 (200–400 mesh, Bio-Rad, Richmond, CA, USA) at 60°C and eluted with distilled water (18 ml/h). Fractions (2.0 ml) were assayed for total neutral sugar content. Appropriate fractions were pooled; digits ***3–20*** were used to refer to these pools. The column was calibrated using a mixture of glucose, cellobiose, raffinose, stachyose and Dextran T150 (Pharmacia).

The BioGel P-2 fractions ***5–12*** were fractionated by semi-preparative high-performance anion-exchange chromatography (HPAEC) using a Dionex Bio-LC GPM-II quaternary gradient module equipped with a Dionex CarboPac PA-1 column (250 × 9 mm, Sunnyvale, CA, USA). Samples (5 × 200 μl) were injected using a SP8780 autosampler (Spectra Physics, San José,

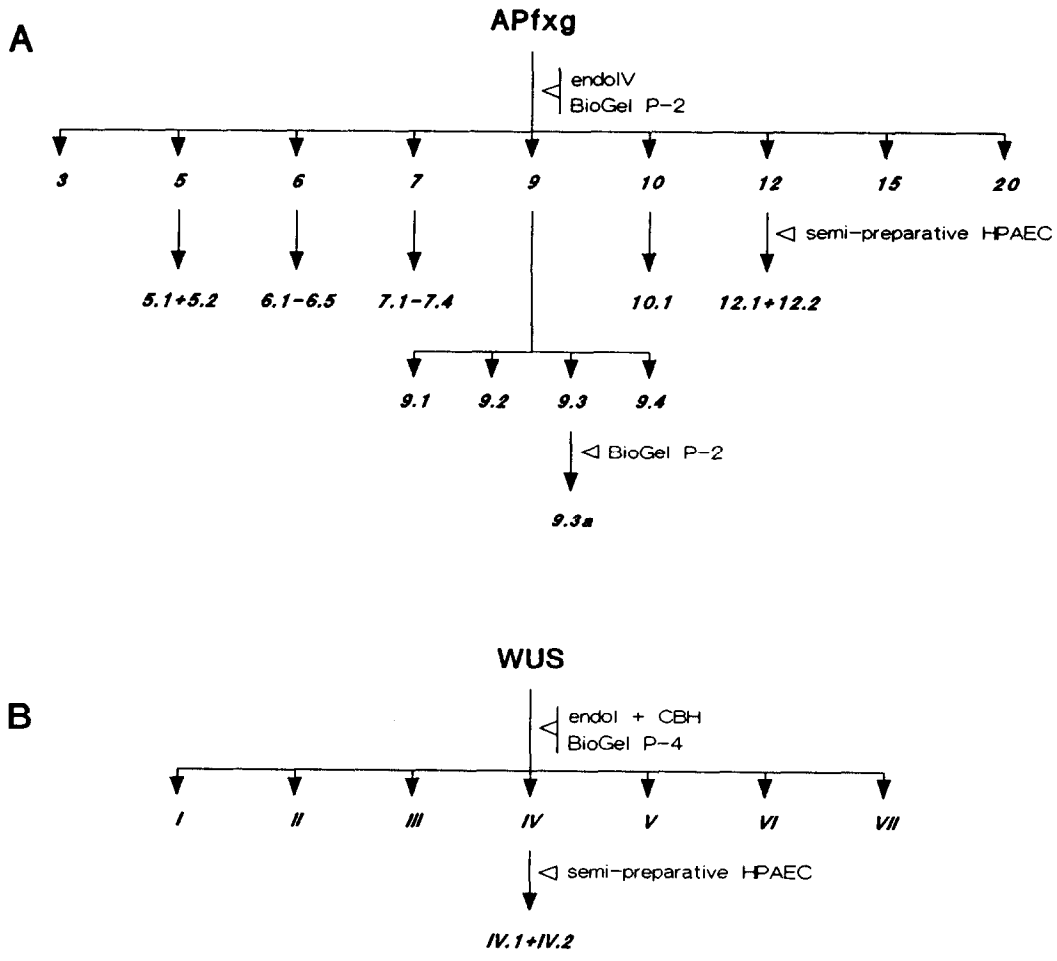

Fig. 2. Purification scheme of xyloglucan fragments obtained after degradation of apple xyloglucan (APfxg) by endoIV (A), and released from apple cell wall material (WUS) by endoI plus CBH (B).

CA, USA) equipped with a Tefzel rotor seal in a 7010 Rheodyne injector valve. The fractions were eluted (5 ml/min) at 20°C with 100 mM NaOH containing different concentrations of sodium acetate. The NaOAc gradients were adapted for the individual BioGel P-2 pools: *5*, 0–20 min, 40 mM NaOAc (isocratic); *6*, 0–30 min, 50 mM NaOAc (isocratic); *7* and *10*, 0–35 min, 60 mM NaOAc (isocratic); *9*, 0–35 min, linear gradient of 60–70 mM NaOAc; *12*, 0–30 min, 80 mM NaOAc (isocratic). After each run, the column was washed for 5 min by elution with 100 mM NaOH containing 1 M 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 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 with 1 M acetic acid and the appropriate fractions (ca 1 ml) were combined, desalted using columns (30 × 80 mm) of Dowex 50W X8 (H^+) and AG3 X4A (OH^-) resins (Bio-Rad) in series (eluent: distilled water), and concentrated under reduced pressure. The oligosaccharides were analyzed for sugar composition by mass spectrometry.

Preparation of fragments from WUS using endoI

WUS (200 mg), suspended in 10 ml of a 50 mM succinate buffer at pH 5, was treated with endoI (30 μg) and CBH (100 μg) for 48 h. After centrifugation (20 min, 50,000 *g*), the water-solubles were freeze-dried, dissolved in 2 ml of distilled water and applied onto a column (100 × 2.6 cm, i.d.) of BioGel P-4 (100–200 mesh, Bio-Rad) at 60°C and eluted with distilled water (18 mL/h). Appropriate fractions were combined according to their neutral sugar and uronic acid content. BioGel P-4 pools are referred to by roman numerals *I–VII*. Fraction *IV* was further fractionated on HPAEC using 110 mM NaOAc (isocratic) for 20 min. Appropriate fractions were pooled, dialyzed against distilled water, concentrated under reduced pressure and dried under a stream of air. All fractions were analyzed for sugar composition. The BioGel P-4 column was calibrated as described for BioGel P-2.

Chemical and enzymic treatment of oligosaccharides

Reduction
Oligosaccharides of ***IV.2*** were labeled at their reducing terminus by treating ca 100 μg of this fraction with 200 μL 1.5 N ammonia containing 75 mg/ml $NaBH_4$ for 1 h at 30°C. The reduced oligosaccharides were dialyzed against distilled water and dried under a stream of air.

Mild acid hydrolysis
Fucose was removed from ***9.2***, ***10.1***, and ***IV.2*** by treating ca 50 μg of these oligosaccharides with 1 ml of 50 mM TFA for 1 h at 100°C. The resulting defucosylated oligosaccharides were evaporated to dryness to remove TFA, and used for enzymic degradation studies without further purification. Removal of fucosyl residues from polymeric xyloglucan was done using a different procedure. APfxg (40 mg) was treated with 8 ml of 25 mM TFA for 90 h at 60°C. The defucosylated xyloglucan (referred to as APxg) was dialyzed extensively against distilled water and freeze-dried.

'Sequencing' of oligosaccharides with Driselase
Oligosaccharides ***7.4***, ***9.2***, and ***10.1*** (ca 50 μg) were treated with Driselase (ca 1 μg of protein) in 200 μl of a 50 mM acetate buffer pH 5 (40°C). Release of Fuc and a Glc–Xyl disaccharide were monitored by analyzing aliquots of 10 μl by HPAEC.

Further degradation of larger fragments by endoglucanase
Approximately 50 μg of ***20***, ***15***, ***III***, ***IV.1***, ***IV.2***, and reduced ***IV.2*** were incubated (24 h, 40 °C) with endoIV (1 μg of protein) in 100 μl of a 50 mM acetate buffer at pH 5. Degradation products were analyzed by HPAEC. In a similar manner, ***IV.2*** and defucosylated ***IV.2*** were treated with endoI (1 or 20 μg of protein).

Analytical methods

Uronic acid content
Uronic acids were estimated colorimetrically with an automated *m*-hydroxydiphenyl test (Thibault, 1979) using concentrated sulphuric acid containing 0.0125 M $Na_2B_4O_7$ for hydrolysis.

Total neutral sugar content
The total neutral sugar content was determined colorimetrically with an automated orcinol/sulphuric acid assay (Tollier & Robin, 1979). Glucose was used as a standard.

Neutral sugar composition
BioGel P-2 and P-4 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 (Vincken *et al.*, 1994). Oligosaccharides, obtained after fractionation on HPAEC, were hydrolyzed as above and the sugar composition was determined by HPAEC, as described by De Ruiter *et al.* (1992).

Protein content
Protein content of enzyme preparations was determined according to Sedmak and Grossberg (1977). BSA 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, CA, USA), equipped with a 20 kV conversion dynode and a Finnigan MAT electrospray interface as described previously (Tinke *et al.*, 1993).

Analysis of xyloglucan oligosaccharides
Xyloglucan oligosaccharides and their degradation products were analyzed on a CarboPac PA-1 column (250 × 4 mm, Dionex) combined with PAD analysis (as described above), eluted (1 ml/min) with the following sodium acetate gradient in 100 mM NaOH: 0–5 min, linear gradient of 0–50 mM NaOAc; 5–30 min, linear gradient of 50–80 mM NaOAc; 30–35 min, linear gradient of 80–130 mM NaOAc; 35–50 min, linear gradient of 130–180 mM NaOAc. After each analysis, the column was washed for 5 min with 1 M NaOAc in 100 mM NaOH, and equilibrated in 100 mM NaOH for 15 min. Some samples were analyzed on CarboPac PA-100 which enabled a slightly better separation with the following sodium acetate gradient in 100 mM NaOH: 0–5 min, linear gradient of 0–30 mM NaOAc; 5–45 min, linear gradient of 30–80 mM NaOAc; 45–55 min, linear gradient of 80–200 mM NaOAc. After each analysis the column was washed and conditioned as described for the CarboPac PA-1 column.

RESULTS

Characterization of xyloglucan oligosaccharides obtained by endoIV

A purified apple xyloglucan was degraded with endoIV, and the resulting digest was subsequently fractionated by BioGel P-2 chromatography as shown in Fig. 3. The fraction numbers ***3–20*** correspond to the DP of the oligosaccharides, which was extrapolated from the elution of standards on BioGel P-2. This DP was confirmed by mass spectrometry of the oligosaccharides (see later).

Fractions ***5–12*** were further fractionated by HPAEC (Fig. 4) with an optimized gradient for each pool. In this study, the focus is on the main oligosaccharides of

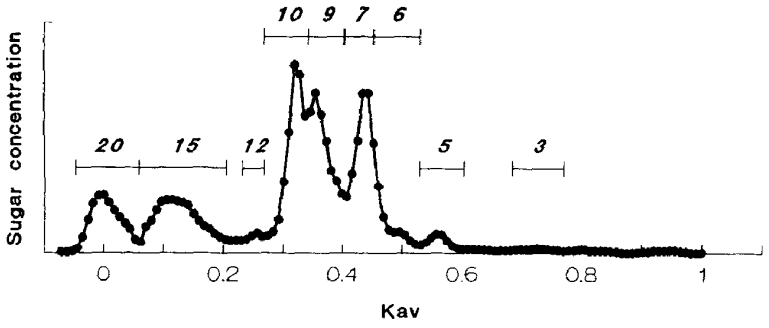

Fig. 3. Elution pattern of an apple xyloglucan digest on BioGel P-2.

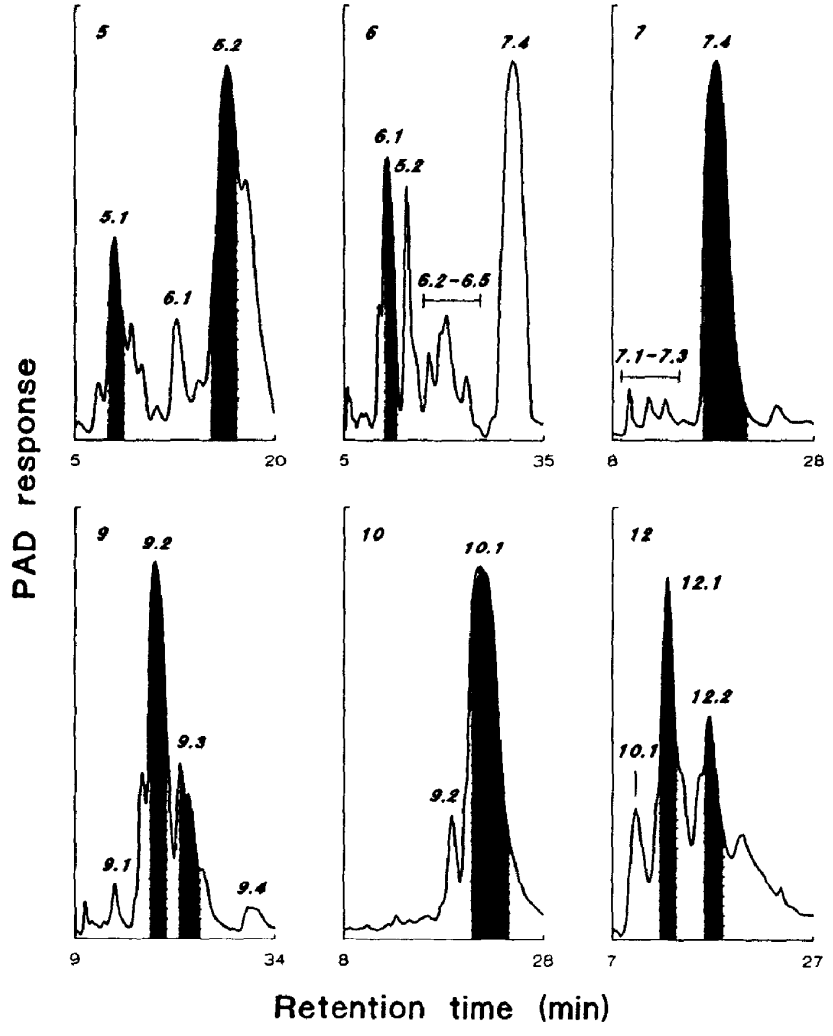

Fig. 4. Elution profiles on semi-preparative HPAEC (CarboPac PA-1) of BioGel P-2 pools *5*, *6*, *7*, *9*, *10*, and *12*. Products in shaded peaks were further characterized.

apple xyloglucan (indicated by the shaded areas), but small amounts of other products were also present. Fraction numbers are indicated in Fig. 4. HPAEC analysis of *5–12*, using the same gradient for all pools, showed that *5.2*, *6.1*, *7.4*, *9.2* and *10.1* were present in more than one BioGel P-2 fraction. Comparison of retention times obtained for *6.2–6.5* and *7.1–7.3* suggested that these fractions might represent similar oligosaccharides. Figure 4 indicated that *9.3* contained more than one component, and therefore this fraction was rechromatographed on a BioGel P-2 column. After removal of the main product of pool *9* (*9.2*) by HPAEC, a good separation of *9.3a* and a fraction similar to *10.1* was obtained by BioGel P-2 chromatography (data not shown). The purity of all oligosaccharides was confirmed by HPAEC and electrospray mass spectro-

Table 1. Analysis of oligosaccharides obtained after degradation of alkali-extracted xyloglucan by endoIV and subsequent fractionation by BioGel P-2 chromatography and HPAEC

BioGel P-2 fractions		
	20	***15***
Glc (mol %)	25	44
Xyl (mol %)	45	32
Gal (mol %)	16	14
Fuc (mol%)	4	10
Ara (mol%)	10	0

HPAEC sub-fractions of *3* to *12*										
	3	***5.1***	***5.2***	***6.1***	***7.4***	***9.2***	***9.3a***	***10.1***	***12.1***	***12.2***
Glc (mol%)	59	40	56	49	54	43	48	38	48	48
Xyl (mol %)	41	26	40	18	43	35	38	31	21	24
Gal (mol %)	0	16	2	16	1	11	12	19	16	14
Fuc (mol %)	0	18	2	17	2	11	2	12	15	14
Molecular weight[a]	nd[b]	782	768	944	1062	1370	1224	1532	1870	nd
$H_xP_yD_z$[c]	nd	$H_3P_1D_1$	H_3P_2	$H_4P_1D_1$	H_4P_3	$H_5P_3D_1$	H_5P_3	$H_6P_3D_1$	$H_8P_2D_2$	nd
Structure[d]	XG	FG	XXG	[FG]G	XXXG	XXFG	XLXG	XLFG	?	?

[a]Determined by electrospray mass spectrometry.
[b]Not determined.
[c]Number of hexoses (H), pentoses (P) and deoxyhexoses (D), *x*, *y* and *z*, respectively, which correspond to the molecular weight.
[d]Tentative structure based on sugar composition, molecular weight, defucosylation experiments and Driselase degradation patterns. Nomenclature according to Fry *et al.* (1993).

metry (>90%); only fractions ***12.1*** and ***12.2*** contained ca 20% of contaminants (data not shown). Sugar composition and molecular weight of all purified compounds are shown in Table 1. Although Man was present in the starting material, this glycosyl residue was not recovered in any of the fractions. Monosaccharide composition of ***15*** and ***20*** showed that xyloglucan fragments are a major constituent of these fractions. The presence of these large xyloglucan fragments indicated that the degradation of xyloglucan by endoIV might be incomplete, which will be discussed later. The high xylose and arabinose content of ***20*** suggested that this pool contained an arabinoxylan contamination. Although HPAEC analysis of ***15*** and ***20*** showed that these pools contained many compounds, no further fractionation was attempted.

Fucose residues of ***9.2*** and ***10.1*** can be removed by treatment with mild acid. The release of fucose was analyzed by HPAEC; only minute amounts of galactose were released under these conditions (data not shown). Although the molecular weight of the compounds was reduced, their retention times upon HPAEC increased. Fucose removal from oligosaccharides is therefore easily recognized by HPAEC. Similar observations were made by McDougall and Fry (1991).

In order to obtain additional structural information, the most abundant oligosaccharide units in apple xyloglucan, ***7.4***, ***9.2***, and ***10.1***, were treated with a crude Driselase preparation. Apart from α-fucosidase and β-galactosidase activity, this enzyme preparation has been shown to contain a 'xyloglucosidase', an enzyme being able to release α-D-Xyl*p*-(1→6)-β-D-Glc (isoprimeverose) from the non-reducing terminus of xyloglucan oligosaccharides (Hisamatsu *et al.*, 1991; McDougall & Fry, 1991). The substrate specificity of a similar enzyme, derived from a preparation of *Aspergillus oryzae*, was described in detail by Kato *et al.* (1985). This 'xyloglucosidase' had a higher activity toward XXXG (50 times) than toward cellotetraose, and was unable to by-pass Gal- and Fuc-containing side chains. The degradation of fractions ***7.4***, ***9.2***, and ***10.1*** was monitored by taking samples at certain time intervals and analyzing these samples by HPAEC. A representative elution profile after partial degradation of the individual oligosaccharides is shown in Fig. 5.

Considering sugar composition, molecular weight (Table 1) and the mode of action of endoglucanase, it is concluded that ***3***, ***5.2***, and ***7.4*** correspond to XG, XXG and XXXG, respectively. Treatment of XXXG (***7.4***) with Driselase yielded XXG and isoprimeverose at the early stages of degradation. The release of isoprimeverose confirmed that the 'X' element was on the non-reducing side of the molecule. At later stages, XXG was further degraded to XG and isoprimeverose. The retention time of isoprimeverose was used as a reference for the incubations of ***9.2*** and ***10.1***.

Treatment of ***9.2*** with Driselase gave an unknown intermediate product and isoprimeverose at the early stages of degradation. The intermediate product was further degraded to ***5.1*** and more isoprimeverose. These results demonstrate that there are two adjacent 'X'; elements at the non-reducing side of the oligosaccharide. This observation, the sugar composition, the molecular weight and the known mode of action of endoglucanase indicated that ***9.2*** and ***5.1*** correspond to XXFG and FG, respectively. Some other products are

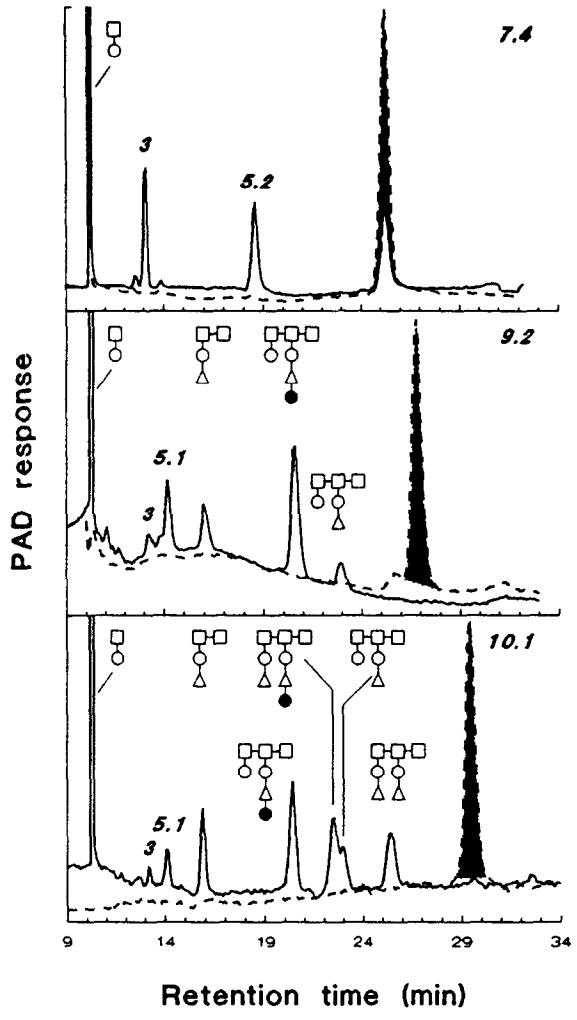

Fig. 5. Elution patterns on HPAEC (CarboPac PA-1) of oligosaccharides *7.4*, *9.2*, and *10.1* after partial degradation with a crude Driselase preparation. Shaded peaks and dashed line (—) indicate the original material; solid line (——) shows a representative pattern during the course of degradation. Probable structures of degradation products are indicated. Symbols as in Fig. 1.

formed as well. These are probably a result of α-fucosidase and β-galactosidase activity in Driselase. Tentative structures (LG, XFG, and XLG) for these intermediate products are indicated in Fig. 5.

Isoprimeverose was released from *10.1* as an initial product, which showed that this disaccharide is at the non-reducing terminus. FG appeared much later during the course of degradation, which demonstrated that the penultimate glucosyl residue at the reducing side of the molecule carried the fucosylated sidechain. These observations and the results of Table 1 show that *10.1* corresponds to XLFG. XLFG can be degraded according to one of the following routes: XLFG → LFG → XFG → FG → LG, or XLFG → XLLG → LLG → XLG → LG, or XLFG → LFG → LLG → XLG → LG. Tentative structures for all intermediates are proposed in Fig. 5.

Characteristics of the purified oligosaccharides are summarized in Table 1. Based on these characteristics and the degradation studies, tentative structures were proposed. The experiments were not decisive on the structure of *6.1*, *9.3a*, and *12.1*. In principle, there are three possible structures for octamer *9.3a*: XXLG, XLXG, and LXXG. Comparison of retention times of XXLG (defucosylated XXFG) and *9.3a* upon HPAEC showed that these oligosaccharides were different. Thus, *9.3a* does not correspond to XXLG. The existence of LXXG has never been reported before; thus, XLXG seems most probable for *9.3a*. *6.1* and *12.1* did not fit in any of the Driselase degradation patterns. The mode of action of endoglucanase implies that both oligosaccharides have an unsubstituted glucosyl residue on the reducing terminus; however, the position of the trisaccharide side chain(s) is unknown. *6.1* will be further referred to as [FG]G (brackets indicate that the F and G elements are interchangable).

The elution profile of apple xyloglucan, which is completely degraded to its oligosaccharide building units by endoIV, is shown in Fig. 6. Products are indicated according to Table 1; the elution times of cellodextrins are indicated for comparison. It should be noted that a CarboPac PA-100 column was used instead of a PA-1 column. Oligosaccharides have a tendency to elute at lower sodium acetate concentrations on CarboPac PA-100 due to a smaller particle size and lower capacity of this packing material. However, an advantage of this material was that it allowed some separation of XLXG and XLFG. The presence of Glc and cellobiose suggested that apple fruit xyloglucan contained a small amount of contiguous β-(1→4)-Glc residues. The asterisk indicates a group of unknown compounds, corresponding to *6.2–6.5* and *7.1–7.3* (see Fig. 4). Degradation of XXFG (*9.2*) and XLFG (*10.1*) by Driselase indicates that XFG, LFG, XLG, and LLG elute at similar retention times (range 23–29 min).

Purification of xyloglucan fragments obtained by endoI plus CBH

In order to obtain larger xyloglucan fragments, apple fruit cell wall material (WUS) was degraded by a combination of endoI and CBH. Solubilized products were fractionated by BioGel P-4 chromatography into seven pools as shown in Fig. 7. Monosaccharide composition of these pools is given in Table 2. Fraction *VII* contained predominantly cellobiose. The Glc:Xyl ratio suggested that xyloglucan fragments were present in *I* to *VI*; fraction *I* contained mainly pectic material, *V* and *VI* were contaminated with Man. HPAEC analysis showed that XXXG was the major component of *VI*. Fractions *III* and *IV* eluted at retention times which are indicated by '§' in Fig. 6. Fraction *IV* was further fractionated by semi-preparative HPAEC (data not shown), which resulted in two pools, *IV.1* (ca 30% of the material) and *IV.2* (ca 70% of the material).

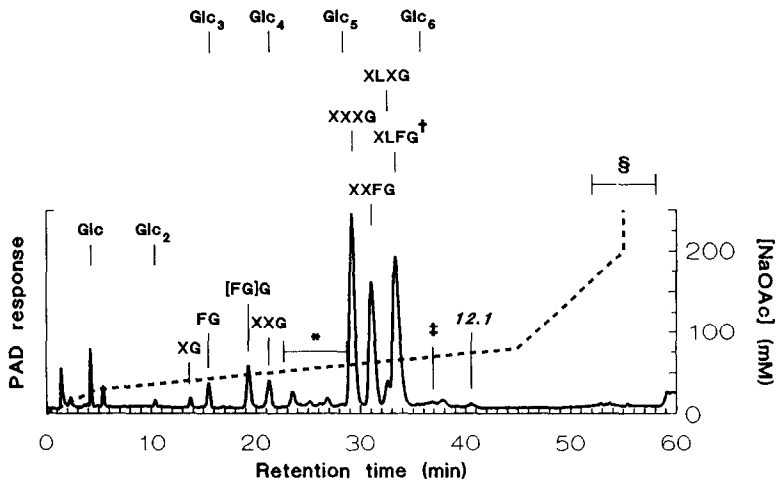

Fig. 6. Elution profile of apple xyloglucan, which is completely degraded by endoIV, on HPAEC (CarboPac PA-100). The profile with a solid line (—) indicates PAD response; the profile with a dashed line (----) shows the sodium acetate gradient. Products are indicated in the chromatogram by letter-codes or, in case of unknowns, by fraction number. *, degradation of XXFG (*9.2*) and XLFG (*10.1*) by Driselase suggests that XFG, LFG, XLG, and LLG elute in this region. †, after defocosylation of XXFG, the resulting oligosaccharide coelutes with XLFG. ‡, elution of product obtained after defucosylation of XLFG. §, indicates the retention times where fragments of two repeating xyloglucan oligosaccharides (such as *15*, *20*, *III*, and *IV*) elute. Retention times of cellodextrins are indicated by Glc to Glc_6.

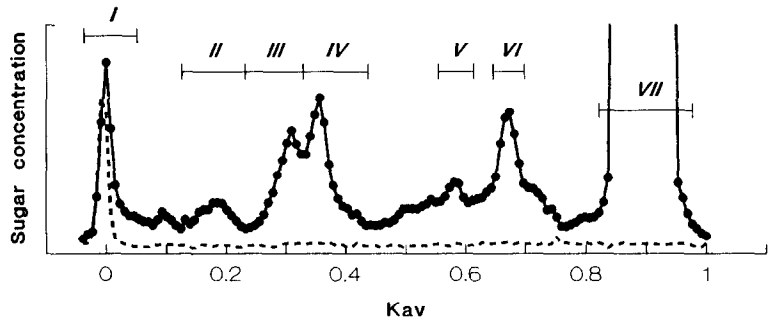

Fig. 7. Elution profile on BioGel P-4 of the compounds solubilized from apple WUS after treatment with a combination of endoI and CBH. Neutral sugar response (—) and uronic acid response (----). Roman numerals are used to indicate BioGel P-4 pools.

Further degradation of larger xyloglucan fragments

Both fractionation procedures described above have provided a number of fractions, containing fragments which consisted of more than one oligosaccharide building unit (*20*, *15*, *III*, *IV.1*, and *IV.2*). To investigate if these fragments were resistant to endoglucanase, they were treated with a large dose of endoIV and analyzed by HPAEC. All fractions could be degraded to oligosaccharides, identical to those described in Table 1; only in the case of fraction *20* was this degradation was incomplete. Time course studies demonstrated that no intermediary products were formed (data not shown), which shows that all larger fragments were built up of two oligosaccharide subunits. Table 3 summarizes these oligosaccharide subunits. The diversity of subunits shows that each original fraction contains more than one compound. The main component of *20* is probably a dimer of XLFG. *III* and *IV.2* are composed of two oligosaccharides having four glucosyl residues in the backbone (such as XXXG, XXFG, and XLFG). *15* and *IV.1* contain considerable amounts of smaller subunits (such as XG, FG, XXG, and [FG]G). The monosaccharide composition of the different fractions (Tables 1 and 2) is in agreement with this subunit composition. It should be noted that XXXG, the

Table 2. Neutral sugar composition (mol%) of BioGel P-4 fractions obtained after degradation of apple-WUS by the combined action of endoI plus CBH

	I	*II*	*III*	*IV*	*V*	*VI*	*VII*
Glc	19	43	44	47	49	54	96
Xyl	11	30	31	34	27	34	1
Gal	15	14	16	12	10	3	0
Fuc	1	8	7	5	4	1	0
Ara	49	3	1	1	1	1	0
Man	1	2	1	1	9	7	3
Rha	4	0	0	0	0	0	0

Table 3. Summary of subunits formed after degradation of some larger xyloglucan fragments by endoIV. Quantification of subunits was based on peak areas using HPAEC (CarboPac PA-100 column). Symbols: −, not detected; ±, traces; +, present in small amounts; + +, most important subunit

	20[a]	*15*[a]	*III*[b]	*IV.1*[b]	*IV.2*[b]	*IV.2*[b,c]
DP<7[d]	±	+	−	+ +	−	−
XXXG	±	+	±	±	+ +	−
XXFG	±	+	+ +	+ +	+	+ +
XLFG[e]	+ +	+ +	+ +	+ +	+	+ +

[a]Pools from BioGel P-2 fractionation containing larger xyloglucan fragments.
[b]Selected pools from BioGel P-4 fractionation.
[c]Fragment was reduced prior to treatment with endoIV.
[d]Contains the smaller xyloglucan oligosaccharides: predominantly FG and [FG]G, but also XG and XXG.
[e]May contain small amounts of XLXG which cannot be recovered as a separate peak when the XLFG:XLXG ratio is high.

major oligosaccharide in apple fruit xyloglucan, is not an important constituent of any of the above mentioned fractions (except ***IV.2***). Fucosylated oligosaccharides, however, were very abundant subunits of these fractions. From these experiments it was concluded that fucosyl-containing structures were poorly degradable, but that none of the fragments was endoglucanase-resistant.

Fraction ***IV.2*** was further studied because this fraction contained similar amounts of non-fucosylated (XXXG) and fucosylated (XXFG and XLFG) repeating units (Table 3). It seems unlikely that ***IV.2*** contains structures like XLFGXLFG, XLFGXXFG, or XXFGXXFG because these fragments would have eluted in fraction ***III*** (see also Table 3). Therefore, it is expected that the fragments of ***IV.2*** are composed of XXXG and either XXFG or XLFG. To investigate which subunit was at the reducing end, ***IV.2*** was labeled by reduction, and subsequently degraded by endoIV. Oligosaccharides XXFG and XLFG were almost completely recovered, but no XXXG was found. This showed that XXXG is positioned predominantly at the reducing terminus, and that ***IV.2*** is composed of XXFGXXXG and XLFGXXXG. Apparently, cleavages like XXXG ↓ XXFG are preferred over those like XXFG ↓ XXXG in the degradation of apple xyloglucan.

Activity of endoglucanase towards defucosylated apple xyloglucan

Treatment of APfxg by mild acid specifically removes fucosyl residues from the xyloglucan molecules (results not shown); this material is further referred to as APxg. The activity of endoI towards APxg was three times higher compared to that towards APfxg (data not shown). Defucosylation also enhanced endoIV activity, but to a much smaller extent (data not shown). It can be concluded that the action of endoI is more hindered by fucose than that of endoIV.

The endoI-resistancy of XLFGXXXG (***IV.2***) was tested by treating this fraction with endoI (a similar amount of protein was used compared to endoIV). When the endoI dose was increased 20 times, this fraction was partially degraded. Removal of fucose by mild acid had a positive effect on the ability of endoI to degrade this fragment. In this case, the material was completely degraded to XXXG, XXLG, and XLLG by the lower dose of endoI. These results indicated that fucose-containing side chains of xyloglucans strongly hinder endoI.

DISCUSSION

In a previous paper (Vincken *et al.*, 1994), it has been demonstrated that some endoglucanases (endoIV) display a much higher activity toward xyloglucans than others (endoI). This study investigates the products released by both enzymes in more detail. For this purpose, a purified, alkali-extracted apple fruit xyloglucan was degraded by endoIV. The resulting digest could be fractionated satisfactorily by combining BioGel P-2 chromatography and HPAEC. Although the latter purification step is the most distinctive of the two, prefractionation by BioGel P-2 chromatography is necessary due, with respect to molecular mass, to a rather unpredictable elution behavior of fucosylated compounds upon HPAEC. For instance, a fucose-containing decasaccharide coeluted (on CarboPac PA-1) with an octasaccharide lacking this residue.

Based on the BioGel P-2 elution pattern and Table 1, it can be calculated that XXXG, XLXG, XXFG, and XLFG form ca 80% of the building units of apple xyloglucan in a ratio of 4:2:3:4. Different responses of monosaccharides with the neutral sugar assay and the incomplete degradation of ***15*** and ***20*** were accounted for. Further, it was assumed that the PAD response factors of the compounds within one BioGel P-2 pool were equal. These results are in agreement with those reported by Renard *et al.* (1992).

An important part of apple xyloglucan is composed of other, mainly smaller, oligosaccharides (ca 20% in total). Our study confirms that XXG is part of apple xyloglucan, but no evidence was found for the presence of a hexasaccharide composed of 4 glucosyl and 2 xylosyl residues, as reported by Renard *et al.* (1992). This component might be among the unidentified oligosaccharides ***6.2–6.5*** or ***7.1–7.3***. Typically, our data suggest a diversity in fucosylated compounds: FG and two new structures, [FG]G and ***12.1***. Both unknown compounds contain a relatively high percentage of Glc. Since the degradation of xyloglucan was not complete,

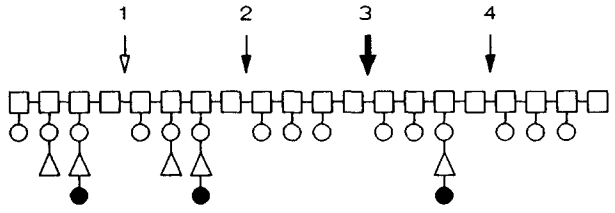

Fig. 8. Schematic representation of the degradation by endoglucanases of an imaginary fragment consisting of five repeating xyloglucan oligosaccharides (XLFG–XLFG–XXXG–XXFG–XXXG). Cleavage of linkage '3' is preferred over '2' and '4'. Linkage '1' is least preferred. Symbols as in Fig. 1.

these might be intermediary products. A detailed characterization (^{1}H-NMR, MSMS) of these structures will be reported in a separate paper.

Our results do not suggest resistancy of certain xyloglucan fragments (DP > 15; Vincken *et al.*, 1994) to endoI. Accumulation of these fragments is merely due to the low specificity of endoI toward xyloglucan. Considering the accumulation of e.g. XLFGXLFG (***20***, poorly degradable by endoIV) and the facilitated cleavage of XLFGXXXG (***IV.2***) by endoI after removal of fucose, our results suggest that fucosylated side chains hamper the action of both endoglucanases. Similar observations have been made by Hisamatsu *et al.* (1992) and York *et al.* (1995) who showed that similar fragments from sycamore were partially resistant against a mixture of endoglucanases.

Levy *et al.* (1991) indicated by modeling that the fucosylated side chain of xyloglucan plays an important role in stabilizing certain molecular conformations. In solution, xyloglucan adopts a twisted conformation and the trisaccharide side chain folds toward the reducing end. As a result of this the linkages indicated with '1', '2' and '4' (Fig. 8) might become poorly accessible to endoglucanases. Linkage '3' is much easier cleaved which explains that fragments like XXFGXXXG (***IV.2***) can accumulate. The presence of XLFGXLFG (***20***) or XLFGXXFG (***III***) shows that both endoglucanases are able to split linkage '2'. Apparently, this cleavage site is preferred over '1', because XXFG or XXLG hardly accumulate upon treatment of WUS by endoI. Possibly, galactose residues (as in XLFG) play a role in the relatively slow cleavage of linkage '1'.

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