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Note

A new family of oligosaccharides from the xyloglucan of *Hymenaea courbaril* L. (Leguminosae) cotyledons

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Abstract

The xyloglucan from cotyledons of *Hymenaea courbaril* was hydrolysed with *endo*-(1,4)-β-D-glucanase (cellulase) and analysed by TLC and HPAEC. The limit digest was different from those obtained from xyloglucans of *Tamarindus indica* and *Copaifera langsdorffii*. On treatment with nasturtium β-galactosidase, two main oligosaccharides were detected by TLC and HPAEC. Using a process of enzymatic sequencing involving alternate treatments with a pure xyloglucan oligosaccharide-specific α-xylosidase, and a pure β-glucosidase, both from nasturtium, their structures were deduced to be XXXG and a new oligosaccharide XXXXG. These structures were confirmed by ^{1}H NMR. The relative proportions of XXXG and XXXXG indicate that approximately half of the subunits in *Hymenaea* xyloglucan are based on the new oligosaccharides. In the native polymer the XXXXG subunits are likely to carry galactosyl substituents in varying proportions, since cellulase hydrolysates contained many bands which were converted to XXXXG on hydrolysis with nasturtium β-galactosidase. Although no comparative studies on the physico–chemical properties of *Hymenaea courbaril* xyloglucan have yet been performed, our results indicate that this polymer is less interactive with iodine when compared with *T. indica* and *C. langsdorffii* xyloglucans, suggesting that changes in conformation may occur due to the presence of XXXXG. © 1997 Elsevier Science Ltd.

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1. Introduction

Seed xyloglucans have a cellulose-like β-(1,4)-glucan backbone to which single-unit α-(1,6)-D-Xyl*p* substituents are attached. Some Xyl residues are further substituted at O-2 by β-D-Gal*p* residues [1]. In tamarind seed xyloglucan, which has been very extensively studied, the pattern of Xyl-substitution is remarkably regular, virtually the whole molecule being composed of repetitive units of Glc_4–Xyl_3 with variable Gal-substitution [2]. Recent work has shown that the xyloglucans of *Tropaeolum majus* and *Copaifera langsdorffii* are similar in structure to tamarind xyloglucan in that they are also composed almost entirely of the Glc_4 subunits XXXG, XLXG, XXLG, and XLLG [2,3]. However, these subunits are combined in different proportions to give a fine structure that varies according to the species and even to the populations of the same species [3]. Reid and coworkers have isolated four of the main enzymes responsible for xyloglucan degradation in *Tropaeolum majus*: a xyloglucan-specific *endo*-(1,4)-β-D-glucanase or xyloglucan *endo*-transglycosylase [4–6], a β-galactosidase with high specificity towards xyloglucan [7], a xyloglucan-specific oligosaccharide-specific α-xylosidase or oligoxyloglucan *exo*-xylohydrolase [8], and a β-glucosidase (Crombie et al., manuscript in preparation).

Kooiman [9] was the first to suggest the presence of xyloglucan in seeds of *Hymenaea*, on the basis of positive staining with iodine, which has been proved to be restricted to the wall thickenings in the cotyledons (Tiné, Buckeridge, and Cortelazzo, unpublished). Buckeridge and Dietrich [10] confirmed that *Hymenaea* seeds contain xyloglucan by monosaccharide analysis. Lima et al. [11] analysed the xyloglucan of *Hymenaea courbaril* by methylation and found the type of glycosidic linkages that should be expected for a 'normal' xyloglucan molecule. We now report that the xyloglucan of seeds of *Hymenaea courbaril* show unique structural features based on a different oligosaccharide block composition. A new family of xyloglucan oligosaccharides was observed and the structure of one of these oligosaccharides was examined. The molecular and biochemical implications of the discovery of this new xyloglucan family of oligosaccharides are discussed.

2. Results and discussion

Monosaccharide composition and fine - structure analyses of Hymenaea courbaril *xyloglucan using* endo-*(1,4)-β-glucanase ('cellulase') and β-galactosidase.*—Analysis of the monosaccharide composition of xyloglucan from seeds of *Hymenaea courbaril* by acid hydrolysis and HPAEC gave a ratio Glc–Xyl–Gal (4:3.4:1.2) having a slightly higher proportion of Xyl and less Gal compared to *Copaifera langsdorffii* (4:3.0:1.8) [10]. Deviation from the 4:3 Glc–Xyl ratio would not be expected if the molecule were based exclusively on XXXG, XLXG, XXLG, and XLLG subunits. Buckeridge et al. [3] demonstrated that hydrolysis with pure cellulase can magnify fine-structural details of seed xyloglucan molecules compared with acid hydrolysis. With fungal cellulase, *Tamarindus indica* and *Copaifera langsdorffii* produced a mixture of XXXG, XLXG, XXLG, and XLLG in different proportions (Fig. 1A and B), whereas the mixture produced from the xyloglucan from *Hymenaea courbaril* had additional higher molecular weight XGOs (Fig. 1C). The pattern obtained after exhaustive hydrolysis of the polysaccharide (72 h) did not change after repeated additions of fresh enzyme. Under the conditions used the

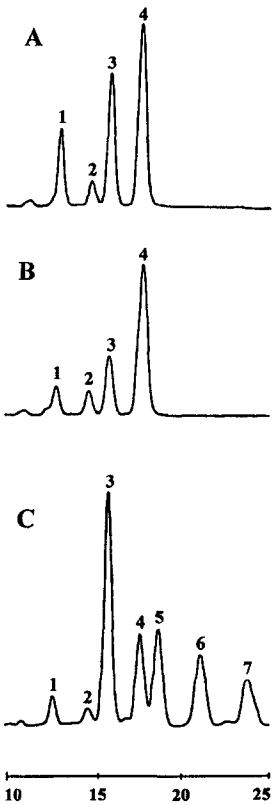

Fig. 1. HPAEC/PAD analysis of the cellulase limit digest oligosaccharides from xyloglucans from: *Tamarindus indica* (A), *Copaifera langsdorffii* (B), and *Hymenaea courbaril* (C). 1 = XXXG; 2 = XLXG; 3 = XXLG; 4 = XLLG; 5–7 = unknown oligosaccharides.

percentage of hydrolysis achieved was 68% of the dry weight of the polysaccharide.

On treatment with nasturtium β-galactosidase [4], both *Tamarindus indica* and *Copaifera langsdorffii* xyloglucan oligosaccharides had their Gal branches completely hydrolysed to mainly XXXG and free Gal (Fig. 2, results for *Copaifera langsdorffii* are shown), whereas the xyloglucan oligosaccharides from *Hymenaea courbaril* were hydrolysed to a mixture of XXXG and an unknown oligosaccharide. The HPAEC pattern did not change after addition of fresh enzyme. Although on TLC analysis the unknown oligosaccharide had the same R_f as XLXG and XXLG (Fig. 2A), HPAEC analysis showed that it is in fact a different XGO, since the retention time of the unknown XGO (peak 5 in Fig. 2B) does not correspond to those of XLXG (peak 2) or XXLG (peak 3).

Monosaccharide sequencing using α-xylosidase and β-glucosidase.—The following steps are thought to be involved in sequencing the oligosaccharide XXXG if pure α-xylosidase and β-glucosidase are used:

$$\text{first cycle: XXXG} \xrightarrow[\alpha\text{-xylosidase}]{} \text{GXXG} \xrightarrow[\beta\text{-glucosidase}]{} \text{XXG}$$

$$\text{second cycle: XXG} \xrightarrow[\alpha\text{-xylosidase}]{} \text{GXG} \xrightarrow[\beta\text{-glucosidase}]{} \text{XG}$$

$$\text{third cycle: XG} \xrightarrow[\alpha\text{-xylosidase}]{} \text{GG} \xrightarrow[\beta\text{-glucosidase}]{} \text{G}$$

As shown above, it would take three cycles of hydrolysis to produce free Glc and Xyl from an XXXG molecule. Thus, based on the number of cycles necessary to hydrolyse a given XGO to free Glc and Xyl, with examination of the transient products, it should be possible to determine the length of the main chain of an XGO.

To test the hypothesis that the unknown XGO from *Hymenaea courbaril* is an XXXXG, the results

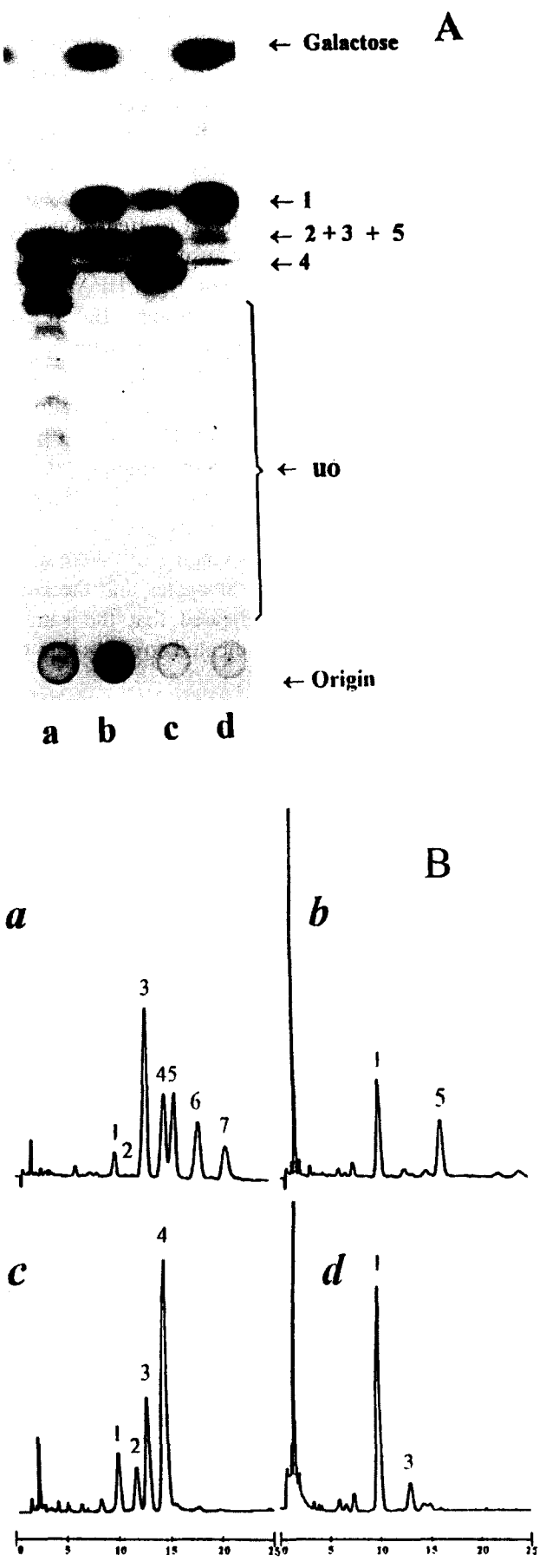

Fig. 2. TLC (A) and HPEAC/PAD (B) analyses of the cellulase (*a*, *c*) and cellulase/β-galactosidase (*b*, *d*) limit digest oligosaccharides from *Hymenaea courbaril* (*a*, *b*) and *Copaifera langsdorffii* (*c*, *d*). 1 = XXXG; 2 = XLXG; 3 = XXLG; 4 = XLLG; 5 = XXXXG; 6, 7, and uo = unknown oligosaccharides.

of HPAEC analysis were used to calculate the proportions of the main oligosaccharides at each cycle of hydrolysis (Table 1). The XGOs obtained by sequential hydrolysis with the xyloglucan degrading enzymes were identified using XGOs obtained by parallel degradation of nasturtium. Table 1 shows that after four cycles of hydrolysis with the *exo*-enzymes, almost all the XGOs were reduced to free monosaccharides (Glc and Xyl). These results were obtained by quantitative analysis using data from HPAEC and confirmed by TLC analysis (not shown). Before hydrolysis with the *exo*-enzymes, the dominant XGOs were XXXG and the unknown XGO. The first cycle of hydrolysis produced a mixture in which the dominant XGOs were XXG (27%) and XXXG (22%), the unknown XGO having almost completely disappeared. After the second and third cycles, respectively, XXG and XG were the dominant XGOs, and after the fourth cycle the monosaccharides were dominant. Thus, both the number of cycles and the composition of intermediates indicated that the sample contained both XXXG and indicated strongly that the unknown XGO was XXXXG.

The two main bands obtained by cellulase/β-galactosidase hydrolyses were isolated from preparative TLC plates and subjected to analysis by ^{1}H NMR. (Table 2). One oligosaccharide was confirmed to be XXXG and the other was consistent with an XXXXG structure, confirming the sequencing results. Thus, the xyloglucan in the seeds of *Hymenaea courbaril* is composed only to the extent of about 50% of the normal xyloglucan oligosaccharide subunits XXXG, XLXG, XXLG, and XLLG. The remaining 50% of subunits belong to a new class based on the structure XXXXG and comprising XXXXG itself plus its β-galactosyl substituted derivatives. Subunits of this type have not been reported either in seed xyloglucans or in the fucosylated xyloglucans from plant primary cell walls.

Preliminary studies of the interaction of xyloglucans from *T. indica*, *C. langsdorffii*, and *H. courbaril* with iodine show that the latter polysaccharide is comparatively less interactive with iodine under the conditions used (Mendes and Buckeridge, unpublished). It is likely that the unique structural features of *H. courbaril* xyloglucan implies conformational changes that may result in differences in the molecular properties of this xyloglucan such as water solubility and self-interactivity and interactivity with cellulose.

Table 1
Percentual distribution of the xyloglucan oligosaccharides during sequencing of the cellulase/β-galactosidase limit digest oligomers after each cycle of hydrolysis with α-xylosidase/β-glucosidase from germinating cotyledons of nasturtium. Data for calculation obtained from HPAE chromatograms

Mono/oligosaccharides	Percentage distribution				
	Cycles of hydrolysis				
	Cellu./β-gal [a]	1st	2nd	3rd	4th
Gal + Glc + Xyl	18.79	34.2	52.6	72.1	86.7
XG	0	11.3	18.8	16.5	6.7
XXG	2.3	27.1	19.8	17.0	2.5
XXXG	44.3	22.5	7.7	2.4	2.3
XXXXG	34.6	4.9	1.1	1.2	1.8

[a] Cellu. = *Trichoderma viride* cellulase; β-gal = nasturtium β-galactosidase.

Table 2
Analysis by ^{1}H NMR of anomeric signals in cellulase/β-galactosidase limit digests of *Hymenaea courbaril* xyloglucan

Chemical shift (ppm)	Assignment [a]	Relative integrals	
		Peak 1 [b]	Peak 5 [b]
4.961	'internal' 1-linked α-Xyl	2.0	3.0
4.944	'terminal' 1-linked α-Xyl	1.0	1.0
5.245	reducing 4-linked α-Glc	1.0	1.0
4.676	reducing 4-linked β-Glc	1.0	1.0
4.54–4.60	1,4-linked β-Glc	3.0	4.0

[a] From York et al. [2] and Fanutti et al. [8].
[b] From Fig. 2B, *b*.

3. Experimental

Seeds of *Hymenaea courbaril* L. (Leguminosae-Caesalpinioideae) were obtained from trees grown at the gardens of the Instituto de Botânica at São Paulo.

Xyloglucan extraction and preparation for enzyme hydrolysis.—Seeds of *Hymenaea courbaril* were decoated and the cotyledons were homogenised in distilled water. Sodium hydroxide (with sodium borohydrate) was added to a final concn of 1 M. After alkali extraction for 1 h at 90 °C, the homogenate was filtered through a nylon sieve and the filtrate was precipitated at 5 °C with 3 vols of 10:1 EtOH–CH_3COOH (v/v). The precipitate was washed sequentially with EtOH and acetone, resolubilised in distilled water and freeze dried.

For enzymatic hydrolysis, a 1% soln was prepared by homogenisation in distilled water at 90 °C, fol-

lowed by centrifugation (10,000 × *g*, 30 min, 5 °C). The supernatant was dialysed against 50 mM McIlvaine buffer pH 4.5.

Acid hydrolysis and sugar determinations.—The monosaccharide composition of the polysaccharides was determined after acid hydrolysis of samples in 72% (w/w) H_2SO_4 at 30 °C for 30 min. followed by 3% H_2SO_4 for 1 h at 121 °C in an autoclave. The low molecular weight carbohydrates were quantified colorimetrically as total [12] and reducing [13].

Enzymatic hydrolysis and monosaccharide sequencing of Hymenaea *xyloglucan oligosaccharides.*—Xyloglucan from *Hymenaea courbaril* was hydrolysed with *Trichoderma viride* 'cellulase' [*endo*-(1 → 4)-β-glucanase from Megazyme, Australia] for 24 h at 30 °C, pH 5.0, to produce the limit digest oligosaccharides. The XGOs obtained were incubated with nasturtium β-galactosidase for 36 h at 30 °C, pH 5.0. TLC analysis showed that under these conditions exhaustive hydrolysis was obtained, producing a mixture of XGOs composed of Glc and Xyl plus free Gal. XGOs left after hydrolysis with cellulase and β-galactosidase were subjected to sequential hydrolysis with pure α-xylosidase and β-glucosidase from cotyledons of nasturtium. Each enzyme was incubated with Gal free XGOs for periods of 16 h at 30 °C, pH 5.0. The sequential incubation with α-xylosidase [5] and β-glucosidase (Crombie et al., manuscript in preparation), each followed by boiling, was defined as 1 cycle of hydrolysis. This produced free Xyl and Glc from the non-reducing end of each XGO. The number of cycles necessary to produce a mixture of free monosaccharides, in conjunction with TLC and HPAEC analysis, was used to calculate the chain length of the unknown XGO.

Analysis of mono- and oligo-saccharides by TLC and HPAEC.—The products of xyloglucan and oligosaccharide enzymatic degradation were analysed by TLC on foil-backed silica-gel layers 0.2 mm in thickness (E. Merck DC-Alufolien, Kieselgel silica-gel 60 plates), using triple development in the solvent system 5:2:3 propan-1-ol–nitromethane–water (v/v). For monosaccharide analysis the solvent system 7:1:2 *n*-propanol–EtOH–water (v/v/v) was used [8]. Carbohydrates were detected by spraying plates with 5% H_2SO_4 in EtOH and heating at 100 °C for 5 min. HPAEC was performed according to Fanutti [8] in an ion-exchange Dionex system (Dionex Series 4000) using a HPLC-AS6 column (Carbopac PA1) column and an amperometric pulse detector. For monosaccharide analysis, samples were applied into a Carbopac PA1 anion-exchange column (250 × 4 mm) which was eluted isocratically with a 20 mM NaOH soln for 2 min, followed by water for 28 min at a flow rate of 1 mL/min. For oligosaccharide analysis the column was eluted with a gradient of NaOAc (50–100 mM) in NaOH (100 mM). Sugars were determined quantitatively using a pulsed amperometric detector. Detector responses were determined using the appropriate standards and molar response factors for standard monosaccharides were determined daily.

NMR Spectroscopy.—^{1}H NMR spectra were recorded on a model AMX 400 spectrometer (Bruker Analytical Messtechnik, Rheinstetten, Germany) operating at 400 MHz. Samples were dissolved in D_2O (1–2 mg/mL) and analysed at 85–90 °C. To ensure full signal responses for accurate integration, a 10-s recycle time was used between successive 90° pulses. Samples were lyophilised from D_2O prior to analysis to reduce interference from residual protiated solvent. Chemical shifts were referenced to external Et_4Si.

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