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Galactose branching modulates the action of cellulase on seed storage xyloglucans

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Abstract

Xyloglucan is a cell wall polysaccharide that has a main β-D-(1 → 4)-glucan backbone branched at regular intervals with α(1 → 6) linked D-xylopyranosyl or β-D-galactopyranosyl(1 → 2)D-xylopyranosyl residues. These residues compromise almost all the structure of xyloglucan when it occurs as a storage polymer in seeds. This polymer is susceptible to the action of endo-β(1,4)glucanase (cellulase) at the non-branched glucosyl residues and this enzyme has been widely used in the study of the structure of xyloglucan. Storage xyloglucans from *Copaifera langsdorffii* and *Hymenaea courbaril* were hydrolysed with cellulase and the equilibrium reached after 24 h was analysed by gel filtration and high performance anion exchange chromatography. The equilibrium contained limit digest oligosaccharides (LDOs) and dimers and trimers of these LDOs that, despite their susceptibility to the enzyme, were not hydrolysed. Our data suggest that the side-chains of the polysaccharides can modulate the recognition of the fragments of xyloglucan by the enzyme: (1) the presence of a β-galactosidase in the system avoided the accumulation of these dimmers and trimmers, (2) less-branched LDOs are attacked preferentially by the enzyme, (3) polymers with different fine structures are hydrolysed at a different rates by the same enzyme. Considering that the branching pattern of the polysaccharide seems to have direct influence on the interaction of the enzyme with the substrate, we suggest that the structure of the polysaccharide would code at least part of the information required for its own metabolism.

Keywords: Xyloglucan; *Hymenaea courbaril* L.; Storage; Polysaccharides; Cell wall; Endo β-glucanase

1. Introduction

Seed xyloglucans have a main β-D-(1 → 4)-glucan backbone branched with α (1 → 6)-linked D-xylopyranosyl or β-D-galactopyranosyl(1 → 2)-D-xylopyranosyl residues. Except for the absence of terminal fucosyl units α-L-(1 → 2)-linked to the β-D-galactosyl groups, there is a remarkable similarity between seed reserve xyloglucan and structural xyloglucan from primary walls of dicotyledoneous vegetative tissues (Hayashi, 1989).

Using microbial cellulase, Kooiman (1961) found that tamarind seed xyloglucan is composed of the heptasaccharide Glc_4Xyl_3, with variation in the substitution with galactose residues. Following this finding, many authors have successfully used cellulase limit digest oligosaccharides (LDOs) as a diagnostic of the presence of xyloglucan in plant tissues. The experimental approach is to subject the soluble polysaccharide to the prolonged action of fungal endo-β-glucanases (cellulases) and perform analysis of the LDOs by TLC and/or HPAEC–PAD.

On the basis of the data produced until 1992, an unambiguous nomenclature for the structural blocks of xyloglucan has been proposed. This nomenclature takes the backbone as a central point. Unsubstituted glucose is assigned G; glucose residues branched with xylose are assigned X and if galactose is branching xylose, the trisaccharide is assigned L (Fry et al., 1993).

Usually, treatment of xyloglucan with different microbial cellulases (e.g. *Trichoderma* spp or *Penicilium* spp) gives similar results (Vincken, Beldman, Nissen, & Voragen, 1996). Another class of enzymes which is able to attack the main chain of xyloglucan is the XET—xyloglucan

Abbreviations: HPAEC–PAD, high performance anion exchange chromatography–pulsed amperometric detection.

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endo-transglycosilase (Farkas, Sulová, Stratilova, Hanna, & Mclachlan, 1992). Fanuti, Gidley, and Reid (1993) performed a comparative study using *Trichoderma* cellulase and nasturtium XET and showed that their pattern of action on xyloglucan is similar. Both produce a mixture of LDOs composed of XXXG, XLXG, XXLG and XLLG.

Vincken, York, Beldman, and Voragen (1997a) reviewed the literature of usage of LDOs to characterise xyloglucan and suggested that these polymers can be classified into two groups, the Solanaceae type, which is based on XXGG blocks, and the others, which are based on XXXG blocks.

A comparative study of the fine structure of seed storage XGs (*Tamarindus indica*, *Tropaeolum majus* and *Copaifera langsdorffii*) has shown that they present a similar structural pattern of LDOs, all being composed almost entirely of the Glc_4 subunits XXXG, XLXG, XXLG and XLLG (Buckeridge, Rocha, Reid, & Dietrich, 1992). Buckeridge, Crombie, Mendes, Reid, Gidley, and Vieira (1997) reported that the XG from seeds of *Hymenaea courbaril* displays unique structural features. Instead of being based on XXXG only, approximately 50% of the *H. courbaril* XG is composed of a family of oligosaccharides based on XXXXG.

In the present work, we performed a study of the action of cellulase from *Trichoderma* on XXXXG-based xyloglucan from *Hymenaea courbaril* as compared with other storage XXXG type polymers. We observed that galactose modulates the action of *Trichoderma* cellulase on the storage xyloglucans from *Copaifera langsdorffii*, *Tamarindus indica* and *Hymenaea courbaril*. This modulation appears to be solely by structural constriction due to galactose distribution in the polymer.

2. Materials and methods

2.1. Plant material and polysaccharide extraction

Seeds of *Hymenaea courbaril* L. were provided by the Seed Department of the Institute of Botany at São Paulo (Brazil) and those of *Copaifera langsdorffii* were harvested at the São Paulo Botanical Garden. The polysaccharides were extracted from cotyledon powders with water (1% w/v) at 80 °C for 8 h with constant stirring. After filtration, 3 volumes of ethanol were added to the aqueous extracts, kept overnight at 5 °C and centrifuged (12,000*g* for 15 min at 5 °C). The pellet was washed with acetone, dried at room temperature and, after re-suspension in water, freeze-dried.

2.2. Treatment of xyloglucan of Hymenaea courbaril with NaOH

The water soluble xyloglucan from *H. coubaril* was incubated with 100 mM NaOH supplemented with 0.01% $NaBH_4$ for 30 min at room temperature. The solution was neutralised with acetic acid and deionised with cationic and anionic Dowex resins. After freeze- drying, the polymer was digest with cellulase applied onto a Biogel P-6 column as described below.

2.3. Analysis by gel filtration

Five millilitres of a 0.5% (w/v) solution of xyloglucan in Na acetate buffer (50 mM pH 5.0) were digested with 200 μl of a 1:100 dilution of *Trichoderma viride* cellulase (Megazyme, 500 U ml^{-1} in 3.2 M ammonium sulfate, pH 7.0) for 24 h at 30 °C. The solution of oligosaccharides produced was loaded onto a Biogel P-6 (BioRad) column (1.5 × 170 cm) and eluted with a 0.05% solution of NaN_3. Fractions of 1 ml were collected and carbohydrates were quantitatively analysed by phenol-sulfuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.4. Monosaccharide analysis

Pooled fractions had their total sugar determined as above and a volume corresponding to 1 mg was freeze-dried and hydrolysed to their monossacharide constituents using 72% H_2SO_4–4% H_2SO_4 (Saeman, Buhl, & Harris, 1945). The monosaccharide solution was neutralised with 60 μl of a 50% (w/w) solution of NaOH (Fischer Scientific) to a pH between 6 and 8 and the monosaccharides were analysed on a Carbopak PA1 column (Dionex DX-500 system) using water as eluent (1 ml min^{-1}) for 50 min with a post-column with 500 mM NaOH (1 ml min^{-1}).

2.5. Analysis of xyloglucan oligosaccharides

Xyloglucan oligosaccharides were analysed by HPAEC–PAD on a Dionex system DX-500 using a CarboPak PA100 column and detected by pulsed amperometric detection (PAD). The samples were eluted with a gradient of sodium acetate (from 35 to 75 mM) in sodium hydroxide (88 mM) and with a flow of 0.9 ml/min^{-1}(gradient A). High molecular weight fragments were analysed on the same system with an acetate gradient from 50 to 330 mM of Na acetate in NaOH 88 mM for 50 min (0.9 ml min^{-1})(gradient B).

2.6. Purification of β-galactosidase

The β-galactosidase purification was adapted to an ÄKTA purifier system (Pharmacia) from Edwards, Bowman, Dea, and Reid (1998).

2.7. Digestion with cellulase and β-galactosidase

For the production of high molecular weight fragments of xyloglucan, solutions of storage xyloglucans (0.1 or 1.0%, w/v) were prepared on 50 mM Na acetate buffer pH 5.0. Two hundred microlitres of the polysaccharide solution were incubated with 50 μl of cellulase (1.0 U ml^{-1}). For assays with lower concentration of cellulase, the solution of

cellulase used was 0.5 U ml^{-1}. For incubations with β-galactosidase, the same incubation system was used, with the addition of 20 μl of *Tropaeolum majus* β-galactosidase.

3. Results

The digestion of *H. courbaril* storage xyloglucan with *Trichoderma* cellulase at low enzyme concentration rendered a mixture of oligosaccharides and low molecular mass polymers that appear as distinct peaks under chromatography on Biogel P-6 (Fig. 1). This chromatographic pattern was the same for digestion for 24 and 72 h (data not shown) indicating that hydrolysis had stopped after 24 h.

With the aim of checking the hypothesis that acetylation might have been a reason for resistance to further hydrolysis, *H. courbaril* XG was treated with NaOH. This hypothesis was ruled out on the basis that no change in the chromatographic pattern shown in Fig. 1(a) was observed after treatment with 100 mM NaOH for 1 h prior to cellulase incubation (Fig. 1(b)). This would have been expected if

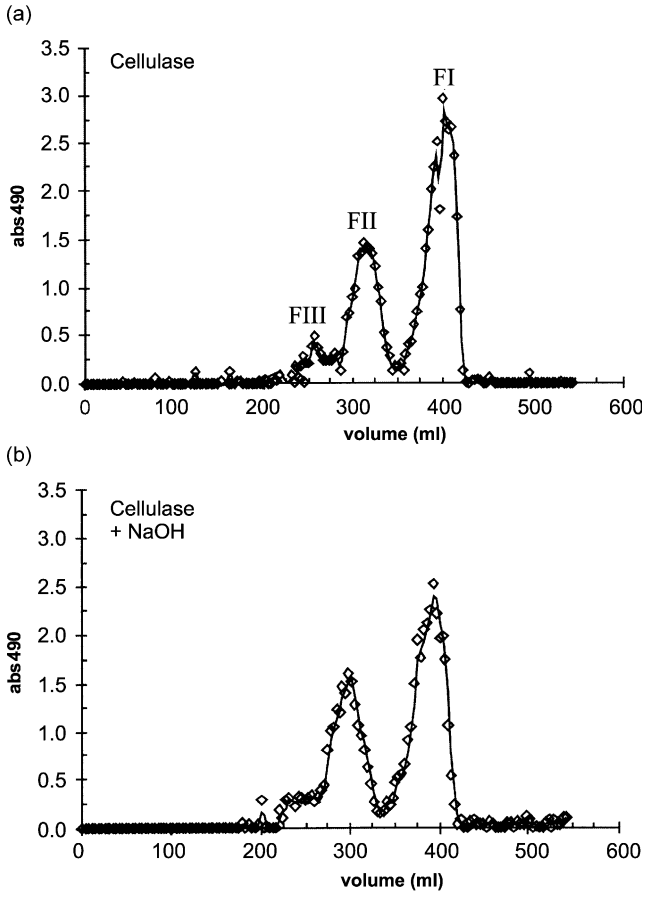

Fig. 1. Gel chromatography on Biogel P-6 of exhaustive digestion with cellulase from *Trichoderma* sp. (Megazyme, Australia) under suboptimal enzyme concentration. (a) Pattern of oligosaccharides obtained with the native polysaccharide; (b) polysaccharide was treated with 100 mM NaOH for 1 h prior to dialysis and enzyme assay.

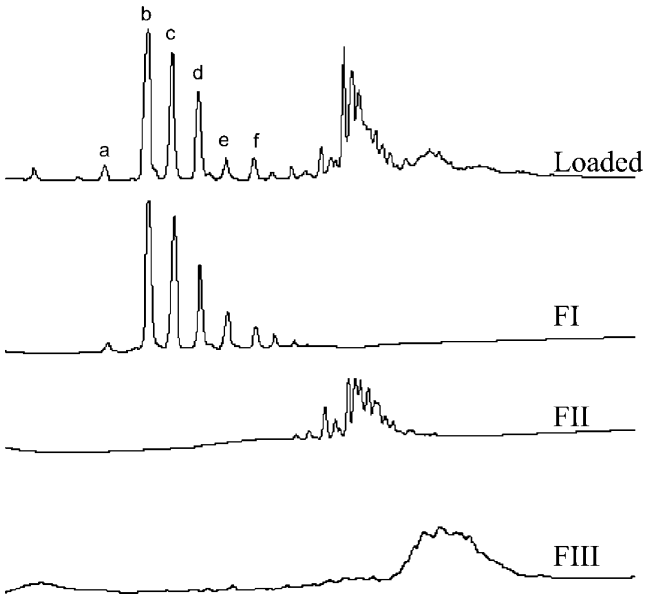

Fig. 2. Analysis by high performance anion exchange chromatography (gradient A) of the three fractions of LDOs from *Hymenaea courbaril* isolated by Biogel P-6 (FI, FII and FIII). The top chromatogram corresponds to the mixture of oligosaccharides loaded onto the column. The peaks in the LDOs fraction are: a = XXXG, b = XXLG, c = XLLG + XXXXG, d = XXXLG, e = XLXXG, f = XXLXG. The identification of peaks d, e and f will be published elsewhere.

acetyl substitutions were present, since they are hydrolysed at high pH.

HPAEC analysis of the different P-6 fractions (FI, FII and FIII) revealed that they are composed of a mixture of Glc_4Xyl_3 and Glc_5Xyl_4 LDOs (Fig. 2). Furthermore, monosaccharide analyses of the pools of LDOs obtained from BioGel P-6 showed an enrichment in galactose in the higher molecular weight fractions (Table 1). Trace amounts of arabinose were found in the mixture of LDOs from *Hymenaea* but its occurrence did not reach the necessary frequency to explain the resistance to cellulase attack.

Both, increase in enzyme concentration or decrease in substrate concentration improved hydrolysis efficiency (Fig. 3). However, the change in enzyme concentration had a much greater effect since doubling the enzyme concentration led to disappearance of fragments larger than two LDOs (compare Fig. 3(a) and (b) with (c) and (d)). The proportion of LDOs in the equilibrium reached after 24 h with lower cellulase concentration did not reflect

Table 1
Monosaccharide composition (ratios) of the pooled fractions from Biogel P-6 (cellulase limit digest oligosaccharides) from storage xyloglucan of *Hymenaea courbaril*

Fractions	Glucose	Xylose	Galactose
FI	5.0	3.0	1.0
FII	5.0	3.1	1.6
FIII	5.0	2.9	2.9

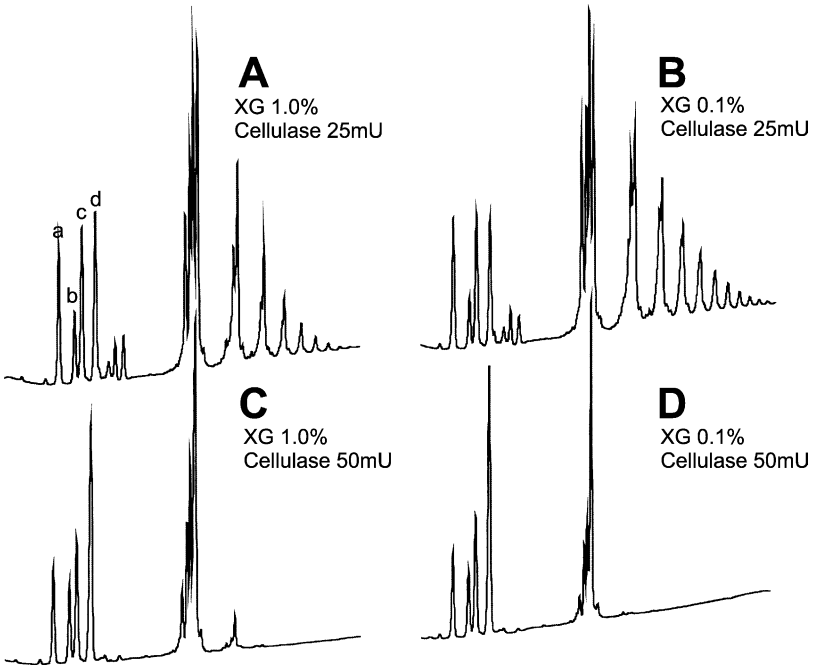

Fig. 3. Effect of enzyme and substrate concentration on the rate of hydrolysis of xyloglucan. Two concentrations of *Copaifera langsdorffii* xyloglucan (1.0 and 0.1%) were hydrolysed with 50 and 25 milliunits (mU) of cellulase and analysed by HPAEC-PAD (gradient B). The oligosaccharides in the LDO mixture are: a = XXXG, b = XLXG, c = XXLG and d = XLLG.

the proportion of oligosaccharides in the polymer. Fragments containing less 'XL…' motives (galactose adjacent to the non-reducing end) appear to have been the last to be released from the polymer (compare the proportions of LDOs in Fig. 3 (a) and (d)).

The fact that the reduction of substrate concentration led to an increase in hydrolysis raised the question whether the accumulation of lower molecular mass polymers might somehow be related to an inhibition effect. The oligosaccharides XXXG and XLLG were isolated from a mixture of LDOs from *T. indica* and *C. langsdorffii*, respectively and their addition at an initial concentration of 0.5% (w/v) to the reaction mixture did not change the pattern of reaction products at all (results not shown). As the addition of FII or FIII did not interfere with the reaction either, our results ruled out the hypothesis of inhibition by the products.

The isolated fractions from Biogel P-6 were further incubated (24 and 48 h) with *Trichoderma* cellulase (Fig. 4). Fractions FII and FIII were hydrolysed to produce the oligomers FI. This strongly suggests that FII and FIII are formed by multiples of FI oligosaccharides. We propose that FII are dimers whereas FIII are trimers of the XXXG/XXXXG families of oligosaccharides.

In order to test the hypothesis that galactose residues might be preventing further hydrolysis of the polymers, the storage xyloglucans from *H. courbaril* and *Tamarindus indica* were treated simultaneously with *Trichoderma* cellulase and purified nasturtium β-galactosidase (Nβ-gal) (Fig. 5). When native xyloglucan polymers are treated with Nβ-gal alone, small amounts of galactose were released from the non-reducing terminals of the polymers (Alcântara et al., 1999), but no oligosaccharides could be detected (Fig. 5). However, the prolonged action of a combination of

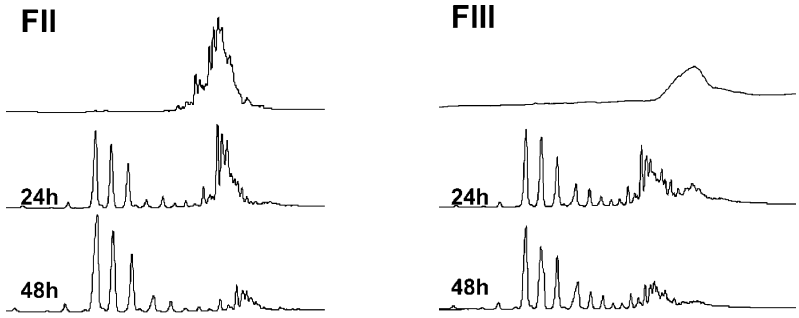

Fig. 4. Hydrolysis of the fragments of storage xyloglucan from *Hymenaea courbaril* (fractions FII and FIII from Biogel P-6) with *Trichoderma* cellulase. Both fragments are susceptible to hydrolysis with the enzyme, producing the FI oligosaccharides (gradient A).

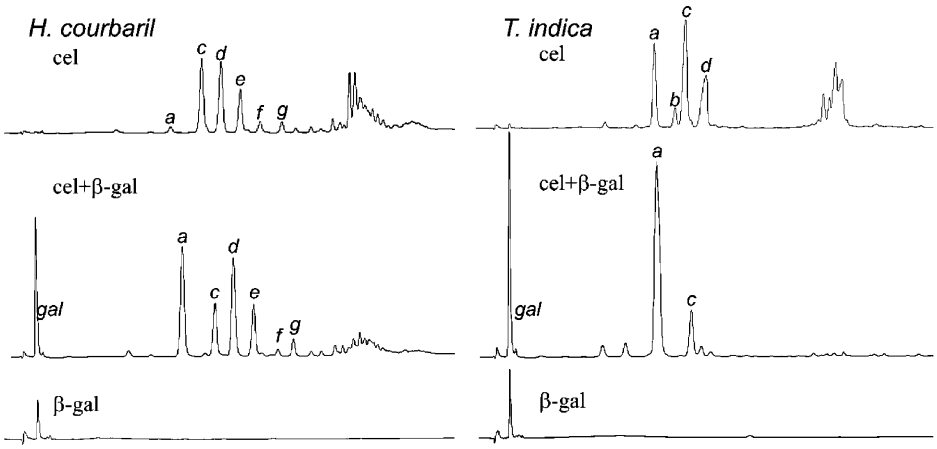

Fig. 5. Hydrolysis of *H. courbaril* and *T. indica* storage xyloglucans with cellulase (cel), cellulase plus nasturtium β-galactosidase (cel + β + gal) and nasturtium β-galactosidase alone (β-gal) for 24 h. The arrow points to the free galactose released, a = XXXG, b = XLXG, c = XXLG, d = XLLG + XXXXG (in *T. indica*, this peak contains only XLLG), e = XXXLG, f = XLXXG, g = XXLXG, gal = free galatose (gradient A was used in the analysis).

Trichoderma cellulase and Nβ-gal with xyloglucan polymers from *H. courbaril* and *T.indica* completely hydrolysed FII to FI. At the same time as the β-1,4 linkages were hydrolysed, the oligosaccharides produced were consistently degalactosylated. This can be deduced from the presence of high proportions of XXXG and XXXXG in *H. courbaril* and XXXG in *T. indica* together with the production of high amounts of galactose from both LDOs (Fig. 5). These experiments suggest that the presence of galactose at certain positions modulates cellulase action.

Fig. 6 shows the patterns of hydrolysis of xyloglucans from *H. courbaril* and *C. langsdorffii* with *Trichoderma* cellulase at half of the usual concentration (25 mU). In these chromatograms, it can be clearly seen that even at early steps of hydrolysis (1 h), fractions containing 2, 3, 4, 5 and higher blocks are produced. The reaction proceeded until it reached an equilibrium and it is noticeable that speed of reaction strongly decreased as smaller fragments are produced.

Whereas in *H. courbaril* the equilibrium was reached after 3 h of reaction, for *C. langsdorffii* it was observed only after 9 h, indicating that *H. courbaril* xyloglucan is more labile than the one from *C. langsdorffii*.

4. Discussion

In this work we confirm and extend the hypothesis that galactose modulates cellulase activity on xyloglucans by structural constriction. Other authors have suggested this modulation (Fanuti et., al 1993; Vincken, York, Beldman, & Voragen, 1997b) and with the use of storage xyloglucans,

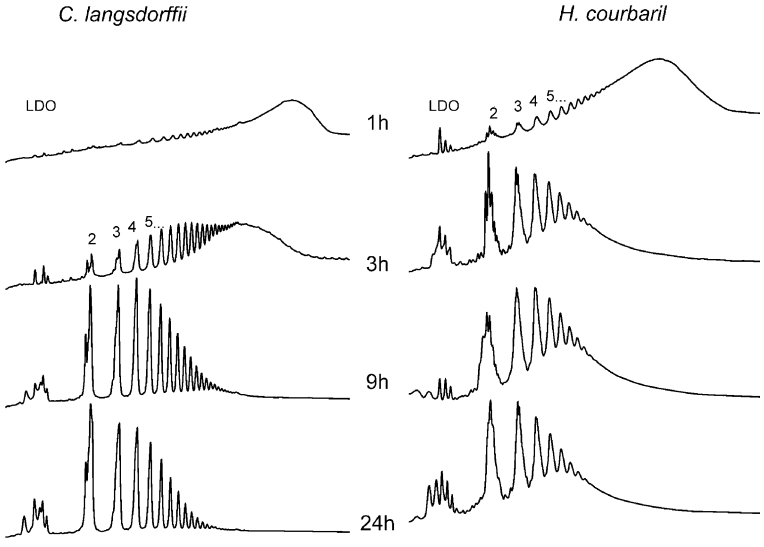

Fig. 6. Time course of hydrolysis of storage xyloglucans from *H. courbaril* and *Copaifera langsdorffii* from 1 to 24 h. The numbers above the peaks indicate the number of oligosaccharides in the fragment. Gradient B. LDO = cellulase limit digest oligosaccharides.

we obtained direct evidence for that by using a purified β-galactosidase from nasturtium cotyledons. Because *Hymenaea courbaril* XG was used as substrate, it was possible to evaluate the influence of galactose residues further ahead towards the reducing end of the chain, this due to the presence of XXXXG-based polymer in *Hymenaea courbaril* xyloglucan (Buckeridge et al., 1997).

The dimers and trimers of LDOs (FII and FIII) were susceptible to the attack of cellulase. This indicates that the accumulation of these fragments is not due to their intrinsic resistance to hydrolysis and that the reaction reaches an equilibrium even when sites of hydrolysis are still available. The presence of β-galactosidase in the assay alters the equilibrium reached. When the galactoses are removed from the fragments of xyloglucan as they are produced, the hydrolysis proceeds almost without accumulation of FII and FIII (Fig. 5). This also points to the importance of the galactosyl side-chains in the modulation of the attack of the enzyme on the substrate. Apparently, the accumulation of galactosylated oligosaccharides hinders the total hydrolysis of cellulase and the enzyme is not sensitive to the accumulation of free galactose. This highlights the importance of the interaction of the cellulase with the exo-hydrolases that occur in biological processes like fungal disassembling of the wall during pathogenesis and storage xyloglucan disassembling after seed germination. Although the exo-hydrolyses do not participate directly on the cellulase hydrolysis reaction, it seems to be capable of modulating the rate of reaction by altering the affinity of cellulase by the xyloglucan fragments. This supports the hypothesis that the activity of the exo-hydrolases might be an important control point of the xyloglucan disassembling process (Crombie, Chengappa, Hellyer, & Reid, 1998; Tine, Cortelazzo, & Bukeridge, 2000).

The proportion of LDOs obtained in the beginning of hydrolysis of xyloglucan from *Copaifera langsdorffii* was very different from the proportion of LDOs in the total polymer (compare the proportion of XXXG and XLLG in Fig. 3(a) and (d)). The mixture of LDOs was richer in XXXG in the beginning of the hydrolysis, although this is not one of the major oligosaccharides in the total polysaccharide composition. This difference in proportion suggests that the attack of the enzyme on the polymer is not random, and the less-branched regions are hydrolysed preferentially (XXXG). This preferential attack of the enzyme upon certain oligosaccharides releases fragments that are progressively poorer in these preferential sites of hydrolysis. Therefore, as hydrolysis proceed, the structure of the substrate changes continuously with the frequency of these high-affinity sites decreasing and the fragments produced become less susceptible to the enzyme attack. As a consequence, there appear to exist sites of action that, under low enzyme concentration, are attacked so slowly that are seen as being resistant to enzyme action towards the end of reaction.

The time course of hydrolysis of xyloglucans with different structures showed differences in the interaction between cellulase and xyloglucan. Under the conditions used, the reaction between cellulase and xyloglucan of *H. courbaril* reaches the equilibrium in less than three hours, with little alteration in the average molecular weight of the fragments in the next 21 h. The polysaccharide from *Copaifera langsdorffii*, on the other hand, reaches this point only between 3 and 9 h. The storage xyloglucan from *H. courbaril* differs from the one of *C. langsdorffii* in at least two points: (i) it has a series of five glucose-oligosaccharides, and (ii) has less oligosaccharides with the 'XL…' motif in its structure. The presence of XXXXG-based oligosaccharides alters the distribution of the points susceptible to the attack of cellulase along the polymer. The fifth glucosyl residue in the structure of the oligosaccharide inverts the position of the next oligosaccharide and, therefore, the position of the next attack of the cellulase. This means that XXXG-based polymers are asymmetric (considering a flat conformation), with all the points susceptible to cellulase in the same side of the polymer. In the presence of XXXXG-based oligosaccharides, however, the inversion of the chain generates a more symmetric polymer, with points of hydrolysis on both sides of the chain. This could make the polymer of *H. courbaril* susceptible to the enzyme even if complexed with other polymers. The differences in amount of 'XL…' motifs may also be an important element in the kinetics of hydrolysis of the polymer, since the presence of this galactose adjacent to the non-reducing end seems to play an important role in the interaction of the enzyme with the substrate.

Fanuti et al. (1991) performed a study of the subsite recognition of nasturtium XET. They found that the positions of the galactose residues on each LDO appear to modulate differentially the attack of the enzyme, studied the substrate specificity of endo-glucanases with a focus on the xyloglucanase activity. They found that galactose and fucose interfere with the action of the enzymes, usually by decreasing reaction speed. Together with our results, these data indicate that the interaction of the endo-glucanases (either from plants or from fungi) and xyloglucan is dependent on the branching pattern, which is a feature of the polysaccharide. This means that the polymer is capable to modulate the action of these enzymes upon itself and at least part of the information required for xyloglucan degradation is present in the polymer itself, i.e. on the branching pattern of the polymer produced during its biosynthesis.

Our results suggest that the fine structure of xyloglucans, especially regarding galactose positioning, seems to determine the speed with which cellulase attacks different points at the molecule. Although in the present work we did not perform experiments with XETs, Fanuti et al. (1993) found similar effects of galactose on cellulase and XET hydrolyses of tamarind xyloglucan. In the present work, we used *Copaifera*'s xyloglucan instead, but recent results from our laboratory show that the fine structure of these two xyloglucans (the proportion of oligosaccharides) is almost

identical (C.O. Silva, D.U. Lima and M.S. Buckeridge, unpublished).

The results presented above have interesting implications for the biological roles of storage xyloglucans. First, for a seed storage system where the proportion cellulose/hemicellulose is altered in relation to the primary wall, xyloglucan molecules have to maintain self-interactivity and galactose is thought to hinder it (Levy, York, Struike-Prill, Meyer, & Staehelin, 1991). On the other hand, positioning of the galactosyl residues are important to give higher solubility to the molecules, i.e. as higher the galactose content, the higher the water solubility (Buckeridge, Santos, & Tiné, 2000) so that a polymer with very high galactose proportions would be promptly soluble but according to our results would also be hydrolysed very slowly. The second implication regards the attack of a fungi to xyloglucan containing seeds. The existence of branching points that modulate degradation by cellulase, such as the one studied in the present work, poses the problem of increase of complexity to the micro-organism evolutionary performance and denotes important aspects of the co-evolution of legumes, that store xyloglucan, and fungi capable to attack such seeds.

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