Time-dependent viscometry study of endoglucanase action on xyloglucan: A real-time approach

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A R T I C L E   I N F O

A B S T R A C T

Hydrolysis of xyloglucan from *Tamarindus indica* and *Hymenaea courbaril* seeds with endoglucanase (EGII), which randomly breaks the $(1 \rightarrow 4)$-linked β-glycosidic bonds of the polymer chain, was monitored in real time using time-dependent viscometry analysis (TDV). For both samples there was a decrease in the intrinsic viscosity ($[\eta]$), viscosity average molar mass (M_v), radius de gyration (R_g) and persistence length (L_p) immediately after the addition of the enzyme. It was observed the formation of oligosaccharides and oligomers composed of ~2 units, up to 140 min. Galactose-containing side chains two positions away from the non-substituted glucose, modulated the action of EGII, and the complete hydrolysis of the XG oligomers occurred after 24 h. The results demonstrate for the first time the real-time degradation of xyloglucan as well the macromolecular and oligosaccharide composition during the EGII hydrolysis process.

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1. Introduction

Xyloglucan structures are composed of a 1,4-linked β-D-glucan main chain that is partially substituted with α-D-Xyl side chains at the O-6 atoms. Depending on the source, the side chains can be β-D-Gal-1,2-α-D-Xyl or α-L-Fuc-1,2-β-D-Gal-1,2-α-D-Xyl [1–5].

Xyloglucans are soluble in water, forming highly viscous solutions at low concentrations and gels with other polymers that control the structure and texture of commercial products [6]. Xyloglucans' unique rheological properties lend themselves to potential applications in the food, pharmaceutical and medical industries [7,8].

The structural, physical and biological properties of xyloglucan from seeds have been extensively studied [2,3,5,9–11]. The oligosaccharide composition of xyloglucan samples obtained from seeds of *Tamarindus indica* (Tamarind), *Copaifera langsdorffii* (Copaíba) *and Hymenaea courbaril* (Jatobá) have shown that even xyloglucan from different sources have some similar structural pattern of partially digested oligosaccharides, being composed of four xyloglucan oligomers, with tetra glucose, representing a glucan backbone (**GGGG**). The unsubstituted backbone of glucose is represented as **G**. The reducing end is not substituted so is always

labeled **G**. **X** refers to a single Xyl-sidechain, whereas L refers to a Gal-Xyl-sidechain, as presented in Fig. 1 for the oligosaccharides XXG, XXXG, XLXG, XXLG, XLLG, XXXXG, XXXLG, XLXXG and XXLXG.

However, differently from other sources of xyloglucan, those obtained from *H. courbaril* seeds have a unique feature in which a new series of oligomers were found. The **XXXXG** series correspond to ~50% of the oligosaccharide composition, with different positions of galactose in the sidechain [2,5,9,12,13].

The xyloglucan enzymatic hydrolysis reaction occurs by means of enzymes that act as catalysts to break the $(1 \rightarrow 4)$-linked β-glycosidic bonds. The oligomers obtained could be used to explain, for example, the interaction from xyloglucan with cellulose. Lima et al. [9] observed that short oligomers of storage xyloglucans are as efficiently adsorbed onto cellulose as fucosyl branched structural xyloglucans. Different oligomers, with different molar mass and different lateral chains, have different enthalpic interactions with cellulose. Additionally, it can be used to determine how enzymatic treatment influences the molar mass and chain conformation, and the first oligosaccharides released during the process [2,9,13].

In this article we provide more details on xyloglucan enzymatic degradation using a real-time technique, based on continuous analysis of the reactor, producing a stream through a detector train and allowing several polymer characteristics to be determined. The detector's signals offer extensive information on the evolution of xyloglucan properties during hydrolysis reactions, and allow observing the changes in polymer behavior during the reactions,
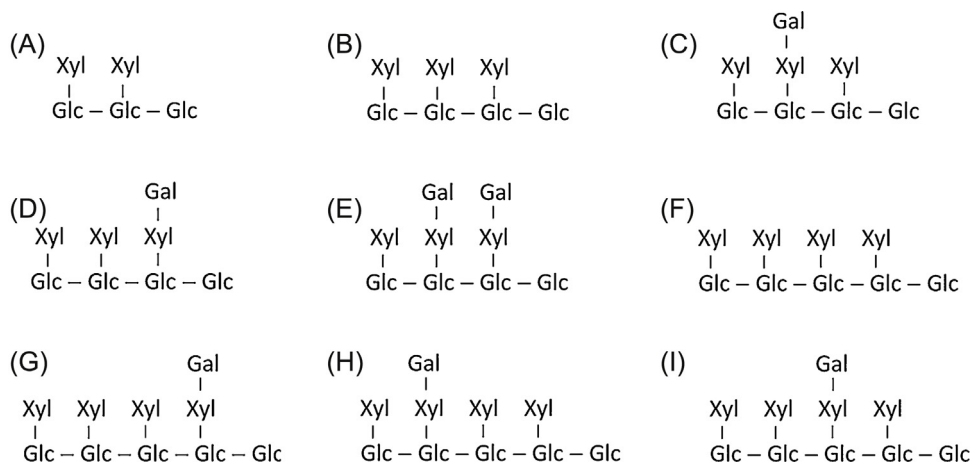
Fig. 1. Schematic representation of the unit structures of digested oligosaccharides from xyloglucan (A) XXG, (B) XXXG, (C) XLXG, (D) XXLG, (E) XLLG, (F) XXXXG, (G) XXXLG, (H) XLXXG and (I) XXLXG, denominated according to the nomenclature described by Fry [23], where G is the unsubstituted glucose, X is Glucose substituted by xylose and L is the xylose substituted by galactose.

elucidating how enzymes act in xyloglucans backbone. Complementary techniques were also used to observe the formation of digested oligosaccharides and allow comparisons between the two different types of xyloglucan (Tamarind and Jatobá).

Based on this, we applied real-time monitoring techniques to evaluate the hydrolysis from xyloglucan with endoglucanase II (EGII). Time dependent viscometry (TDV) is a powerful tool for this investigation, since it can be used to monitor changes in the viscosity on the time scale of the hydrolysis process, reducing the effects of aggregates that could influence the light scattering results. It yields comprehensive, model-independent information about polymer degradation [14]. The oligomers generated during the enzymatic process were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC), using small fraction of sample from the reactor, as well by high performance size exclusion chromatography (HPSEC) coupled to multidetectors.

2. Experimental

2.1. Material

Seeds of *T. indica* L. (XGT) were provided by a commercial source, Conceição de Almeida, Bahia state, Brazil. Seeds of *H. courbaril* L. (XGJ) were harvested from trees growing in the Foz do Chopin Forest Reserve, Paraná state, Brazil.

The enzyme cellulase, or *endo*-1,4-β-D-glucanase (EGII), from *Trichoderma longibrachiatum* was purchased from Megazyme (Bray, Co., Wicklow, Ireland) and was used without any purification.

2.2. Methods

2.2.1. Plant material and polysaccharide extraction

Xyloglucan was obtained by exhaustive aqueous extraction at 25 °C of pooled and milled seeds. A solution at $1.0\,g\,L^{-1}$, after 16 h of hydration, was centrifuged at $8000 \times g$ for 20 min at 40 °C and the supernatant was passed sequentially through cellulose acetate filters (Millipore, Merck KGaA, Darmstadt, Germany) with pore sizes of 3.0 and 0.8 μm. The filtered polymer was obtained after precipitation with two volumes of 96% ethanol (Copalcool, São Paulo, SP, Brazil) and washed with acetone (Synth, Diadema, SP, Brazil) and then dried under vacuum at 25 °C [14].

2.2.2. Characterization

2.2.2.1. Sample characterization by high-performance size exclusion chromatography. The native samples or enzymatically degraded ones, are characterized by high-performance size exclusion chromatography (HPSEC) using a Viscotek-HPSEC multidetector system (Malvern Instruments, Worcestershire, UK) equipped with a Shodex OHpak SB-806 HQ column (Showa Denko America, New York, NY, USA), connected in series and coupled to a differential refractometer (Viscoteck VE3580 RI detector), a viscometric detector and a laser light scattering detector (model 270 dual detector) with low angle 7° (LALLS) and right angle 90° (RALLS) lasers with λ 632.8 nm, at 30 °C using $0.1\,mol\,L^{-1}$ NaNO$_3$ (Vetec, Duque de Caxias, RJ, Brazil) with 0.02% (w/v) NaN$_3$ (Vetec, Duque de Caxias, RJ, Brazil) as eluent. Xyloglucan solutions ($1\,g\,L^{-1}$) were prepared in the HPSEC mobile-phase during 16 h and passed through a 0.22 μm cellulose acetate filter (Millipore, Merck KGaA, Darmstadt, Germany) before analysis. All data collected were analyzed using the OmniSEC 4.7 program (Dällikon, Switzerland) and the figures were created by Kaleidagraph version 4.1 (Reading, PA, USA).

2.2.2.2. Real time analysis of xyloglucans enzymatic hydrolysis. Xyloglucan solutions ($0.5\,g\,L^{-1}$) were prepared in ultrapure water (MilliQ system, Billerica, MA, USA) dissolved during 16 h with a magnetic stirrer; after which the solution was filtered sequentially through 0.8, 0.45 and 0.22 μm cellulose acetate filters (Millipore, Merck KGaA, Darmstadt, Germany) to remove insoluble compounds. The enzyme EGII was used mainly at $0.009\,U\,mL^{-1}$ (few results are also presented at 0.006 and $0.012\,U\,mL^{-1}$). The data, collected from a continuous flow rate at $0.5\,mL\,min^{-1}$, were used to measure the parameters: intrinsic viscosity ($[\eta]$); viscosity average molar mass (M_v), radius of gyration (R_g) from the intrinsic viscosity and reactor concentration ($g\,L^{-1}$), in real time.

Some small fractions were removed from the reactor, heat inactivated at 100 °C for 10 min, for further analysis of oligosaccharide composition and HPSEC analysis, at times 0, 10, and 140 min, to obtain comparative results with real time experiments (Section 2.2.2.1).

The real-time experiments were performed using time-dependent viscometry (TDV) configuration. TDV was applied to analyze the xyloglucans' enzymatic degradation using EGII, as follows: a custom-built system was used where XGT and XGJ samples were incubated in a reactor at 40 °C. The solutions were passed through the TDV device to obtain baseline signals and then EGII

was added. The system is sensible enough to determine the viscosity of small oligomers with 2 or 3 units of oligosaccharides. The analysis were carried out using a serial detection device consisting of a homebuilt single capillary viscometer based on a Validyne DP15 pressure transducer and Validyne CD12 carrier demodulator (Validyne Engineering Corp., Northridge, CA, USA), and a 2414 Waters differential refractometer (RI) (Milliford, MA, USA). Also, was used a multi-angle static laser light scattering module (DSP-F, Wyatt Technology). The data were collected using the Wyatt Technology ASTRA 4.0 (Santa Barbara, CA, USA) program. All samples were systematically studied, and it was possible to determine the $[\eta]$, M_v, L_p and R_g.

The viscometer was selected, instead of static laser light scattering, due to the presence of a small fraction of aggregates, formed during the real time experiments. Also, the smaller R_g after the enzymatic treatment, also suggest the use of viscometer to calculate the R_g by the Flory–Fox equation (Eq. (1)). The M_v was obtained by the Mark–Houwink–Sakurada equation (Eq. (2)), to reduce the effect of aggregates.

$$[\eta] = \frac{6^{3/2} * \phi * R_g^3}{M_v} \qquad (1)$$

$$[\eta] = K * M_v^\alpha \qquad (2)$$

where $[\eta]$ is the intrinsic viscosity (obtained from the reduced viscosity, calculated from the viscometer in $dL\,g^{-1}$), $\phi = 2.56.10^{23}\,mol^{-1}$ is the Flory–Fox constant, and R_g. The K and α of equation 2 are the constants of the xyloglucan in water, and K was 4.9×10^{-3} and $7.6 \times 10^{-4}\,dL\,g^{-1}$, α was 0.51 ± 0.04 and 0.65 ± 0.10, respectively to XGT and XGJ gum.

The effect of EGII on xyloglucan was analyzed via the persistence length (L_p) parameter. The calculation of L_p in real time, using equation (Eq. (3)), is given by the square of the radius of gyration (Flory–Fox R_g was used) and the contour length ($L = (M_w/m_0)b$, where m_0) is the monomeric molar mass and b is its length.

$$R_g^2 = \frac{L * L_p}{3} - L_p^2 + \frac{2 * L_p^3}{L} - 2 * \left(\frac{L_p^4}{L^2}\right) * \left[1 - \exp * \left(\frac{-L}{L_p}\right)\right] \qquad (3)$$

2.2.2.3. Analysis of the oligomers produced. The quantification of the digested oligosaccharides produced was performed by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC) analysis in a Thermo Scientific ICS-5000 system (Thermo Scientific Dionex, Sunnyvale, CA, USA), using a Carbopack PA-100 column (Thermo Scientific Dionex, Sunnyvale, CA, USA), ED gold electrode and an amperometric pulse detector (PAD). The eluent used was NaOH 88 mmol L^{-1} (Sigma–Aldrich, St. Louis, MO, USA) with a gradient of NaOAc 1 mol L^{-1} (Sigma–Aldrich, St. Louis, MO, USA) from 7 to 15%, flow rate of 0.9 mL min^{-1} at 30 °C. The data were treated with the Chromeleon 7 program (Thermo Scientific Dionex, Sunnyvale, CA, USA).

The identification of the oligosaccharides were realized using a secondary standard of xyloglucan oligosaccharides from Tamarind

and Jatobá, hydrolyzed with EGII for 48 h and identified according Freitas et al. [2].

3. Results

3.1. Endoglucanase hydrolysis of the xyloglucan

The XGT and XGJ were treated with EGII 0.009 U mL^{-1}, and the native and enzymatically treated products aliquots were collected from the reaction and analyzed by HPSEC, as shown in Table 1.

Both samples, before the enzymatic treatment, showed a homogeneous and unimodal elution profile by HPSEC according to LS and RI detector, with dispersity ($Đ = M_w/M_n$) of 1.8 for XGT and 1.4 for XGJ. The values of M_w for XGT and XGJ seeds, before hydrolysis (Table 1), were 3.80×10^5 and 7.9×10^5 g mol^{-1}, the $[\eta]$ values were 359 and 410 mL g^{-1}, while the R_g values were 50 and 56 nm.

HPSEC analysis of aliquots at 10 up to 140 min of enzymatic hydrolysis with EGII shows that M_w values of XGT decreased to 3365 g mol^{-1}. The same behavior was observed for XGJ, where M_w decreased to 2393 g mol^{-1}. The $[\eta]$ values decreased from 359 to 5.5 mL g^{-1} for XGT and from 410 to 1.9 mL g^{-1} and for XGJ. All the values of R_g presented in the Table 1 were determined using viscometer and the Flory–Fox equation by the HPSEC-viscometer detector (Eq. (1)), since it was impossible to determine such small R_g by laser light scattering. The R_g decreased to unit, for both XGT and XGJ. Obviously such small value of R_g is compatible with the scission, into oligomers and oligosaccharides, of the xyloglucan chain by the EGII.

From HPSEC, at 0 or 140 min of hydrolysis (**Supplementary data S1**), the xyloglucan was reduced to a mixture of oligomers, from approximately 1–2 units of oligosaccharides in the chain, as can be observed in Table 1, justifying the values of R_g. We assumed the presence of disaccharides up to 140 min, based in the average molar mass of the oligosaccharides.

Similar results were observed by the raw responses from the detectors in the TDV, at 0.009 U mL^{-1} of EGII, for XGT and XGJ, respectively (Fig. 2). The addition of the enzyme in the reactor caused a decrease of viscosity in both samples. The addition of the EGII did not interfere, by itself, in the viscometer or even light scattering signal.

The light scattering signal reduced during the real time reactions, but the molar mass calculated was much higher than the results obtained by viscometry or HPSEC. As the viscometer is much less sensible to presence of dense aggregates, and correlated results were obtained between the viscometer and HPSEC analysis. During the HPSEC analysis the aggregates were undone due to the heating of the samples, to inactivate the enzyme EGII, and also the re-filtration through a 0.22 μm filters, removing some few aggregates presents.

TDV experiments (Fig. 2) show that M_v was immediately reduced after the addition of the EGII. The residual mass in the polymer chain was almost the same of HPSEC, indicating that the information of the viscometer is giving real time information. The

Table 1
The xyloglucan fractions from Tamarind (XGT) and Jatobá (XGJ) seeds characterized after real time enzyme reaction 0.009 U mL^{-1}, measured by HPSEC.

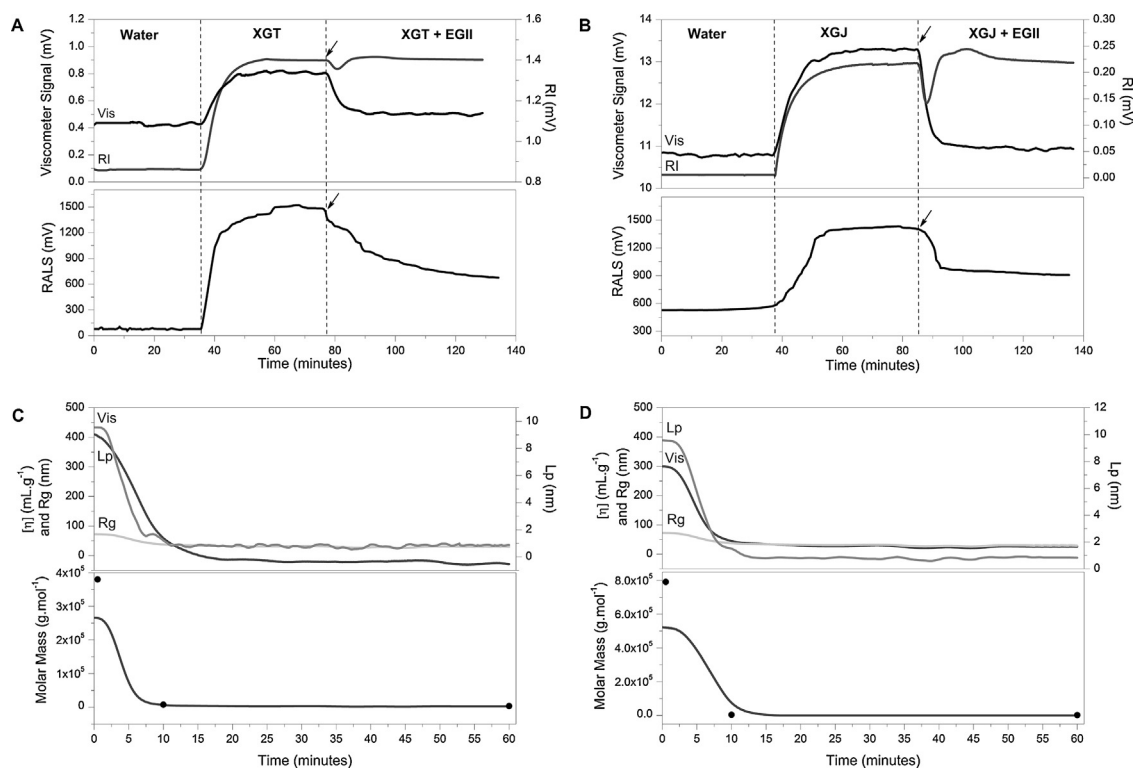| Sample	[EGII] U mL^{-1}	Time (min)	M_w (g mol^{-1})	M_w/M_n	$[\eta]$ (mL g^{-1})	R_g[a] (nm)
XGT	0.009	0	3.80×10^5	1.8	359.0	50
		10	7534	1.7	28.0	3
		140	3365	1.6	5.5	2
XGJ	0.009	0	7.90×10^5	1.4	410.0	56
		10	4562	1.4	21.0	2
		140	2393	1.3	1.9	1

[a] From Flory–Fox equation.

Fig. 2. Real time hydrolyses of xyloglucan from Tamarind (XGT) (A) and Jatobá (XGJ) (B) seeds at 0.5 g mL^{-1} of EGII 0.009 U mL^{-1} light scattering detection at 90° (RALS), viscometry and refractive index (RI). Macromolecular parameters obtained during hydrolysis of XGT (C) and XGJ (D) intrinsic viscosity [η], radius of gyration (R_g), persistence length (L_p) and viscosity average molar mass (M_v). The arrows indicate the moment the enzyme was added.

other parameters, [η], R_g and L_p also reduced with the addition of EGII.

The RI detector signals during the reaction, which are directly proportional to the solution's concentration, confirm that the polysaccharide concentration in the reactor remained constant (Fig. 2A and B).

Table 2 shows the oligosaccharide obtained from XGT and XGJ with EGII 0.009 U mL^{-1}, analyzed by HPAEC. The proportion of oligomers formed during the first minute of the reaction remained constant up to 140 min, when compared with the values of the oligomers formed in the total hydrolysis reaction of 24 h. This was observed for both xyloglucan samples, and the data of EGII hydrolysis with 0.006 and 0.012 U mL^{-1} are presented in the **Supplementary data (S2, S3 and S4)**.

The oligosaccharide chromatographic pattern was different for digestion for 24 h of XGT and XGJ (Fig. 3). So, the oligomers are completely degraded to the oligosaccharide in Fig. 1 after only a long incubation time (24 h). Total hydrolysis was carried out in order to compare the monosaccharides formed with aliquots collected during the reaction in real time. For this reaction, which lasted 1440 min. For the XGJ sample, there was formation of monosaccharides in the same proportions for all enzyme concentrations.

3.2. Discussion

Although the oligosaccharides of xyloglucan are relatively well characterized, important details of the action of the enzyme on the polymer chain remain poorly understood. A detailed analysis of the action mechanism of enzymes on xyloglucan in real time can provide new insights into the formation of oligomers during the hydrolysis reaction, as well information about the context in which it occurs. This knowledge from real-time analysis can help explain the reason for the formation of oligosaccharides with higher and lower susceptibility to enzymatic hydrolysis.

In TDV, since [η] and M_v reduced, these suggest the xyloglucan degradation into smaller chains, oligomers or oligosaccharides (Fig. 2). As expected, the product of the action of endoglucanase on polysaccharides, there is release of oligosaccharides with various degrees of polymerization. The catalytic activity can be followed by decreasing the viscosity of the medium, due to the decrease in average molar mass of the polymer. This action also depends on the degree of substitution of the main chain of xyloglucan and the presence of galactose side chains, which limit the interaction of the enzyme with its active site, thereby slowing the enzymatic degradation due to the formation of oligomers resistant to the EGII action [15,16].

Previous studies with EGII hydrolysis show that xyloglucan structures have similar patterns of oligosaccharides, mainly composed of oligosaccharides: XXXG, XLXG, XXLG, and XLLG. The first differences between the proportions of oligosaccharides from XGT and XGJ were previously identified by Buckeridge et al. [12], Freitas et al. [2] and Tiné et al. [5]. Similar results were found in aliquots withdrawn during the hydrolysis reaction in real time in HPAEC analysis, where we observed the sequence of formation of oligosaccharides for each xyloglucan sample hydrolyzed with EGII as a function of hydrolysis time (Table 2).

The proportion of oligosaccharides formed during the first minutes of the reaction remained constant up to 140 min, in contrast to the values of the oligosaccharides formed in the total hydrolysis reaction of 24 h. This was observed in both xyloglucan samples.

The standard chromatographic profile of oligosaccharide hydrolysis differed between 140 min and 24 h for XGT and XGJ. Initially are formed of between 1 and 2 units of oligosaccharides, which in turn are more resistant to enzymatic hydrolysis. Oligomers having in average 2 units of oligosaccharides are completely degraded to oligosaccharides with 1 unit (Fig. 1) mainly after 24 h of degradation. It can be seen that increasing the hydrolysis time increases the efficiency of the reaction.
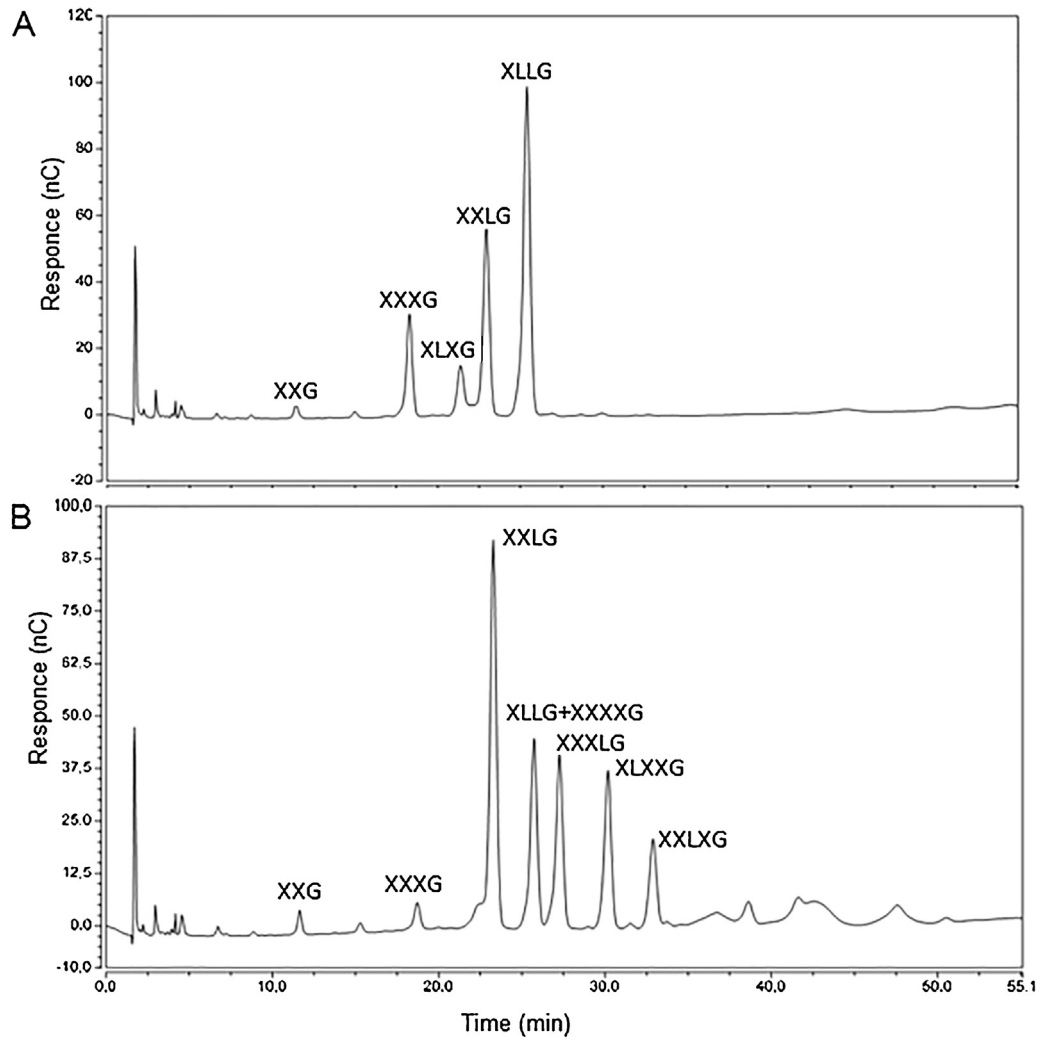
Fig. 3. Elution profile of oligosaccharides pattern obtained of XGT (A) and XGJ (B) by EGII 0.012 U mL^{-1} digestion during 24 h by HPAEC.

Our results are well correlated with Mkedder et al. [17], showing that xyloglucan treated with endoglucanase produced a constant viscosity a short incubation time with endoglucanase (12 min of reaction), and form an oligomer with the molar mass of 2 oligosaccharides units. The same authors also identify the presence of few aggregates, formed by the oligomers of xyloglucan.

The enzymatic activity of endoglucanase has optimal conditions for activity and is mostly above 40 °C and pH values between neutral and mildly acidic [18,19]. In pure water, the hydrolysis of cellulose occurs spontaneously, but more slowly [20]. Thus, it is possible that the degradation observed for the xyloglucan can also be influenced by the solvent and can possibly occur

Table 2
Oligosaccharides from Tamarind (XGT) and Jatobá (XGJ) xyloglucans obtained from partial digestion with EGII 0.009 U mL^{-1}, measured by HPAEC.

Time (min)	XXG	XXXG	XLXG	XXLG	XLLG + XXXXG[a]	XXXLG	XLXXG	XXLXG
XGT								
10	4.9	45.1	5.5	35.6	8.9	–	–	–
30	3.2	42.2	5.1	38.2	11.2	–	–	–
60	3.1	41.5	5.7	38.2	11.6	–	–	–
90	3.3	42.2	5.1	38.2	11.2	–	–	–
120	3.2	41.9	5.6	37.6	11.8	–	–	–
140	3.1	40.2	5.8	38.9	11.9	–	–	–
1440	1.7	14.4	8.9	23.1	51.8	–	–	–
XGJ								
10	0.3	3.8	–	31.8	1.9	42.8	17.4	1.9
30	3.0	3.0	–	27.5	5.7	41.5	17.1	2.2
60	2.7	2.7	–	25.5	1.4	49.8	16.1	1.9
90	3.0	3.3	–	30.6	2.4	37.4	18.8	4.4
120	3.1	3.3	–	30.7	2.2	37.4	18.7	4.6
140	3.3	3.5	–	31.5	2.4	39.7	17.4	2.1
1440	1.5	3.0	–	35.0	18.5	17.7	15.2	9.2

[a] XLLG + XXXXG in *T. indica*, this peak contains only XLLG.

more slowly, since the experiments shown were performed in water.

The proportion of oligosaccharides remained nearly constant from ~10 to 140 min of real-time analysis, with predominant formation of oligosaccharides with xylose and glucose in the chain. The oligosaccharides of galactose substitution formed more slowly. This is due to steric hindrance caused by the presence of galactose in vicinal positions on unsubstituted glucose, inhibiting the activity of the enzyme. But there was a change in the proportions of oligosaccharides in aliquots from the reactions within 24 h, with predominance of the oligosaccharide XLLG to xyloglucan from Tamarind and XXLG from Jatobá seeds.

The interesting result of this study is that the galactose located two units (XLXG, XLLG and XXLXG) from unsubstituted glucose, where the enzyme EGII can act, was more significant in inhibiting the formation of oligosaccharides than when the galactose was found in a neighboring position to the unsubstituted glucose, i.e., XXXLG or XXLG. Thus regardless of whether oligosaccharides are composed of tetra or penta glucose, galactose is decisive in the formation of oligomers resistant to enzymatic hydrolysis, mainly that located two units away from the point of hydrolysis of the reducing end of the oligosaccharide. Alcântara, Dietrich and Buckeridge [21] showed the activity of β-galactosidase on xyloglucan from *Copaifera langsdorffii* and suggested that the position of galactose in the backbone of the polysaccharide influences the enzyme's activity. Crombie, Chengappa, Hellyer and Reid [22] studied the performance of four hydrolases (β-galactosidase, endo-β-(1 → 4)-glucanase, α-xylosidase and β-glucosidase) on xyloglucan from *Tropaeolum majus*. They observed that the presence of galactose at the non-reducing end of the oligosaccharides (GLXG and GLLG) inhibits the activity of β-glucosidase in the formation of the oligosaccharides, but not GXLG and GXXG. So, the presence of galactose two positions away from the reducing end appears to be responsible for steric hindrance, and therefore is essential to mobilize xyloglucan for the enzyme.

4. Conclusion

Real time analysis was used to characterize the time-dependence enzymatic degradation of xyloglucan with EGII, with good correlations between the viscometer and HPSEC results. The light scattering detector during the real-time analysis was influenced by the presence of few aggregates, formed during the enzymatic reaction. We observed the formation of dimers, resistant to hydrolysis and that the presence of galactose-containing side chains two position away from the non-substituted glucose (local action EGII) has a stronger influence on the EGII formation of oligosaccharides, when compared with a galactose neighboring glucose, or even the absence of galactose.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2015.08.023.

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