Real-Time Monitoring of Enzymatic Hydrolysis of Galactomannans

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Received 18 October 2000;
accepted 9 March 2001

Abstract: Enzymatic hydrolysis was monitored in real-time using time dependent static light scattering (TDSLS) for a variety of galactomannans from native Brazilian flora. α-Galactosidase, which strips only the (1–6)α-D-galactose side groups, and β-mannanase, which hydrolyses only the (1–4)β-D-mannan main chain into oligosaccharides were investigated separately and in combination. The time-dependent signatures matched those describing side-chain stripping for galactosidase, whereas those resulting from the action of mannanase followed the signature typical of random backbone cleavage. Use of both enzymes together required that the TDSLS theory of polymer degradation be extended to the case where random backbone cleavage sites appear as side chains are stripped by the first enzyme. Whereas galactosidase allowed mannanase to access more backbone cleavage sites as time passes, leading to a higher degree of hydrolysis, there was no increase in rate constants. The distribution of random fragments in the case of mannanase digestion alone followed reasonably well the predictions for random cleavage of a single-strand polymer with a restricted number of cleavage sites. The fragment distributions were evaluated by size exclusion chromatography. © 2001 John Wiley & Sons, Inc. Biopolymers 59: 226–242, 2001

Keywords: enzymatic hydrolysis; real-time monitoring; galactomannans; time dependent static light scattering; oligosaccharides; random backbone cleavage; size exclusion chromatography

INTRODUCTION

Galactomannans

Galactomannans (GM) are the energy-reserve polysaccharides mainly found in the endosperm of seeds from the Leguminosae family. These polysaccharides consist of a main chain of (1–4) linked β-D-mannopyranosyl residues, most of which are substituted at O-6 with single-unit α-D-galactopyranosyl side-chain residues. The ratio of mannose to galactose (M/G) depends on the plant source and the method of extraction and can range from 1.0 to 5.0.1,2

GM is rich in cis-OH groups that allow aggregation from chain to chain via hydrogen bonds, so that hydration becomes more complicated if interchain cross-linking can take place. Commercial sources derive from the seeds of Cyamopsis tetragonolobus (guar; M/G 1.5), Ceratonia siliqua (carob, locust bean; M/G 3.5), and Caesalpinia spinosa (tara; M/G 3).3 The main field of GM application is their use as thickening or gelling agents (only when associated with other polysaccharides) in feed and food industries.

Hydrolysis of GM in endosperms of germinating seeds requires the presence of at least three enzymes4:
The galactose-depleted GM has been used as a product to improve the interaction between other polysaccharides as xanthan, agarose, and carrageenan. Results obtained by some authors\textsuperscript{10–14} showed that this system increases the degree of interaction, forming a firm, rubbery gel. Enzymatic degradation of high molecular weight GM are being developed for application properties in food processing, paper products, textile printing, sizing and oil gas industry.\textsuperscript{3,15–18}

The structural and rheological characterization of GM from\textit{ Mimosa scabrella}, known as bractatinga, family Mimosaceae, a native tree of Brazil, was previously carried out.\textsuperscript{19,20} Chemical analysis and \textsuperscript{13}C NMR confirmed a highly substituted GM (M/G 1.1) with a linear backbone of (1–4) linked \textit{d}-mannopyranosyl units, to which were bonded single (1–6) linked \textit{\alpha}-\textit{d}-galactopyranosyl side-chain residues. The analysis of oligosaccharides obtained by mild acid hydrolysis suggest that galactosyl groups along the main chain are randomly distributed.\textsuperscript{19}

Rheological properties of GM solutions from the seeds of \textit{M. scabrella} showed that it is a random coil polymer with a critical concentration overlap $c^* = 2.6/\eta_0$. Typical values of $\eta_0$ range from 700 to 1000 mL/g. The average persistence length $L_p$, which characterizes the local stiffness of the \textit{d}-mannan chain in which all the mannose units are 6-substituted by galactosyl groups, is in the range of 95 Å.\textsuperscript{21}

This polysaccharide has been used in a system interacting with xanthan,\textsuperscript{22} processed in a pilot scale,\textsuperscript{23} and has potential application in food\textsuperscript{24,25} and cosmetic industries as a substitute for guar gum. GM from \textit{M. scabrella} (M/G 1.1) was used in a system interacting with xanthan, and a lower temperature of gel formation was found ($T_g = 20^\circ\mathrm{C}$), in comparison with less substituted GM. In this case only disordered xanthan chains can interact with GM and the interaction does not depend strongly on M/G.

The first use of light scattering on a degrading polymer to make structural deductions about polymer structure was first reported by Thomas,\textsuperscript{26} who found early evidence of the double-strand nature of the DNA molecule, by manually withdrawing aliquots of a DNA solution undergoing hydrolysis. The use of time-dependent static light scattering (TDSLs) to continuously follow degradation reactions of polymers was introduced over a decade ago.\textsuperscript{27} Since then, a number of theoretical and experimental works have appeared. Theory and application to random scission of single-strand polymers was detailed in several publications.\textsuperscript{27–31} It was shown that the technique could
be used to rapidly and accurately determine Michaelis–Menten–Henri parameters to investigate the enhanced degradability of copolymers to determine sidechain branching and multiple modes of degradation, and to make other structural and mechanistic determinations (multiple strands). A more general formalism for deriving TDSLS signatures of degrading polymers was also introduced.

The current work makes use of the TDSLS technique to monitor enzymatic hydrolysis of Mimosa scabrella, Moldenhawera floribunda, and Melanoxylon brauna, three sources of GM from Brazilian flora, each of which has a different degree of galactopyranosyl side-chain substitution. The aim is to determine kinetic parameters for GM, to investigate enzymatic hydrolysis, to make deductions concerning the mechanism (e.g., random, endwise, weighted toward central segments, etc.) are all contained in the model whether singly or multiply stranded) and the cleavage still intact. The details of the polymer structure (e.g., whether undergoing a degradation reaction, then the excess light scattering behavior throughout the reaction.

If side chains are stripped from the backbone in a first-order reaction of rate \( \alpha \), such that the decrease in the mass (or number of monomers) of the polymer is given by

\[
M(t) = M_p + M_s \exp(-\alpha t) \quad (5)
\]

then the remaining concentration of material in the backbone is

\[
c(t) = c_0 \frac{M(t)}{M(0)} \quad (6)
\]

where \( M_p \) is the amount of mass in the backbone and \( M_s,0 \) is the initial amount of mass in the side groups. Then, substituting into Eq. (1), the time-dependent scattering signature of a sidechain stripping reaction is

\[
P(q, t) = \frac{2}{N} \sum_{i=2}^{N} \sum_{j=1}^{i-1} W(r, i, j) \langle \exp(-i q \cdot r) \rangle \quad (4)
\]

where \( N \) is the number of cleavable units in a polymer, \( r_{ij} \) is the vector connecting cleavable units \( i \) and \( j \), and \( W(r, i, j) \) is the probability that after an average of \( r \) cuts on a polymer the segment between \( i \) and \( j \) is still intact. The details of the polymer structure (e.g., whether singly or multiply stranded) and the cleavage mechanism (e.g., random, endwise, weighted toward central segments, etc.) are all contained in the model for \( W(r, i, j) \), and the double average \( \langle \langle \rangle \rangle \) is taken over all spatial orientations and conformations of the polymer. Several forms for \( P(q, r) \) have been published and investigated experimentally.
\[ \frac{Kc_0}{I(q, r)} = \frac{1 + u(t)/3}{M_{10}(f_p + (1 - f_p)\exp(-\alpha t))^2 + 2A_2(t)c_0} \]

where \( M_{10} \) is the total initial polymer mass (\( = M_p + M_{s,0} \), where \( M_{s,0} \) is the initial side-chain mass), \( f_p \) is the initial fraction of mass in the backbone and

\[ u(t) = q^2\langle S^2 \rangle_c(t) \]

where \( \langle S^2 \rangle_c(t) \) is the mean square \( z \)-average radius of gyration. It is assumed that the stripped side chains themselves scatter insignificantly compared to the remaining backbone. The above form will hold for stripping side chains from any polymer conformation, as long as \( u < 1 \). For the case of stripping from an ideal random coil (which GM resemble), the numerator \( 1 + u/3 \) can be replaced by \( u/2 \) for the case where \( u > 3 \).

The \( 2A_2(t)c_0 \) term in Eq. (7) deserves special attention. In the case of backbone side-group stripping by Gase, a large change in \( A_2 \) can arise. Since

\[ A_2(t) = \frac{\beta(t)N_h}{2M(t)^{3/2}} \]

where \( \beta \) is the excluded volume between polymers, then

\[ 2A_2(t)c_0 = \frac{2A_2\rho c_0M_{s,0}^5\beta(t)}{M(t)^{3/2}c_0} \]

\[ A_2,0 \] is the second virial coefficient measured for undegraded GM, \( \beta_0 \) is the excluded volume of the undegraded polymer, and \( M(t) \) is given by Eq. (5).

Here it is assumed, with experimental justification shown below, that the excluded volume does not change significantly during side-chain stripping, because \( \langle S^2 \rangle^{1/2} \), which controls \( \beta \), decreases by only about 6%. This form for \( 2A_2(t)c_0 \) with \( M(t) \) from Eq. (5) is used below both for the Gase and combined Mase/Gase fits, since in both cases the only significant change to this term comes from the stripping of side chains from the GM. With the approximation that \( \beta(t) \approx \beta_0 \) [Eq. (7)] becomes

\[ \frac{Kc_0}{I(q, t)} = \frac{[1 + u(t)/3 + 2A_2,0c_0M_{s,0}]}{M_{10}(f_p + (1 - f_p)\exp(-\alpha t))^2 + 2A_2(t)c_0} \]

In other words, when the change in \( \langle S^2 \rangle_z \) is small during a reaction, the side-chain stripping rate \( \alpha \) and the fraction of mass in the backbone \( f_p \) can be computed without any explicit knowledge of \( M_{s,0} \), \( A_2,0 \), or \( \langle S^2 \rangle_{z,0} \). Even \( K \) and \( c_0 \) need not be known, since it will suffice to measure simply the relative solvent subtracted scattering level instead of the absolutely calibrated \( I(q, t) \). The \( f_p \) can be obtained, if the predicted plateau of \( Kc/I(q, t) \) is reached during the experiment from the ratio of the initial to final values

\[ f_p = \frac{Kc_0I(q, 0)}{Kc_0I(q, t_{final})} \]

so that no fitting is required for \( f_p \). [Again, relative excess scattering could be substituted for \( I(q, t) \) in Eq. (12), and \( K \) and \( c_0 \) omitted.] The decay rate \( \alpha \) can then be obtained by a single parameter fit to \( Kc_0I(q, t) \) according to Eq. (11), where all the constants on the right-hand side are simply the measured value of \( Kc_0I(q, 0) \). This could even be obtained from a straight line fit to the initial slope of \( Kc/I(q, t) \).

Furthermore, within the approximation that \( u(t)/3 \) varies little in time, it suffices to measure \( I(q, t) \) at any angle (experimental proof is given below). This considerably simplifies the amount of experimental work and auxiliary information needed.

Of course, if the auxiliary parameters are known, then extrapolation to \( q = 0 \) will directly yield the time course of the diminishing backbone mass \( M(t) \), according to

\[ M_n(t) = \sqrt{\frac{[M_{s,0}(1 + 2A_2,0c_0M_{s,0})]}{Kc_0I(0, t)}} \]

**Random Cleavage of the Mannose Backbone by Mase**

Mannanase cleaves the GM backbone randomly at a restricted number of sites. If GM resembles a random coil, then this will lead to a qualitatively very different time-dependent scattering signature from the sidechain stripping reaction. In this case, a broad distribution of fragments is produced and the number of unhydrolyzed initial chains decreases as \( \exp(-r) \), where \( r \) is the average number of initial cuts per polymer.

For random cleavage of a random coil, the scattering will follow the form of Eq. (1) expressed in terms of \( r \)

\[ \frac{Kc_0}{I(q, r)} = \frac{c_0}{MC_0(M)P(q, r)\rho^M} + 2A_2c_0 \]

where \( c_0 \) is the initial polymer concentration, and \( C_0(M) \) is the initial polymer mass distribution. This
functional form does not require explicit knowledge of \( C(M, r) \) (i.e., how the fragment distribution evolves with \( r \)). The function \( P(q, r) \) in Eq. (14) is given by a Debye scattering function
\[
D(u) = \frac{2}{u^2} (e^{-u} - 1 + u) \tag{15}
\]
where the usual argument \( u = q^2(S^2)_r \) is shifted by the average number of cuts \( r \) on the polymer, \( 26 \) so that
\[
u(t) = q^2(S^2)_r(t = 0) + r(t) \tag{16}\]
The Debye function has two important limiting cases, which give the following simplifications:
\[
\frac{1}{D(q, r)} = 1 + u/3, \quad u < 1 \tag{17a}
\]
which applies at sufficiently low angles, and/or small number of average cuts \( r \), whereas
\[
\frac{1}{D(q, r)} = \frac{u}{2}, \quad u > 3 \tag{17b}
\]
Since TDSLS data are collected in time, the connection between \( r \) and \( t \) must be established. Again, for an enzyme randomly attacking a polymer with \( n(t) \) cleavable sites, the rate equation for the number of cuts should be pseudo-first order, and proportional to the number of remaining cleavable sites \( n_0 - r \), where \( n_0 \) is the initial number of cleavable sites;
\[
dr \frac{dt}{dt} = k(n_0 - r) \tag{18}\]
where the rate constant \( k = k'[E] \), with \( k' \) the effective second-order rate constant and \([E]\) the enzyme concentration, assumed constant, unless some deactivation process occurs.

This gives a time dependence for \( r(t) \),
\[
r(t) = n_0[1 - \exp(-kt)] \tag{19}\]
or using \( R_0 \), the average number of cleavable sites per g/mole for a polymer of initial mass \( M \),
\[
R_0 = \frac{n_0}{M} \tag{20}\]
\( r(t) \) becomes
\[
r(t) = MR_0\left[1 - \exp(-kt)\right] \tag{21}\]
This can be substituted directly into Eq. (16) to obtain \( u(t) \), which then specifies \( D(u) \) in Eq. (15), which becomes the function \( P(q, r) \) in Eq. (14) above, to give
\[
\frac{Kc_0}{I(q, t)} = \frac{c_0}{\int_0^\infty MC_0(M)D(u(t))dM} + 2A_c c_0 \tag{22}\]

The limiting cases of Eq. (17b) have especially interesting consequences for Eq. (22). At \( u > 3 \), the result is independent of the initial polydispersity \( C_0(M) \) for random coils:
\[
\frac{Kc_0}{I(q, t)} = \frac{1}{2M_{n,0}} + \frac{\gamma q^2}{2} + \frac{R_0(1 - e^{-kt})}{2} + 2A_c c_0 \tag{23}\]
This result was derived in Ref. 27, except that their use of a linearly increasing number of cuts with time (\( \beta t \) in the original reference) has been replaced here with \( r(t)/t \) from Eq. (21). The other terms have their original definitions; \( M_{n,0} \) is the initial number average polymer mass, and \( \gamma \) is the factor relating ideal random coil \( \langle S^2 \rangle \) to mass
\[
\langle S^2 \rangle = \gamma M \tag{24}\]

For all \( u \), there is no angular dependence to the progress of the reaction with respect to \( r \), and hence time, so that it suffices to make light scattering measurements at a single angle. It is noted that only \( \langle S^2 \rangle_0(t = 0) \) figures in the above equations, so that the latter assertion is rigorously true. For the side-chain stripping case, the angular independence is true when \( \langle S^2 \rangle(t) \) varies little during the stripping reaction.

**Combined Backbone Degradation and Side-Chain Stripping**

Now, if Gase and Mase act together, then the simplest model is the following one: The Mase can immediately begin to randomly cleave the GM at unsubstituted sites (or minimum sequences of unsubstituted sites, discussed below) on the GM, \( n_0 \). Simultaneously, the Gase strips side chains and opens more cleavage sites accessible to the Mase. This leads to a time dependence for the number of cleavable sites of the form
\[ n(t) = n_0 - r + (N - n_0)[1 - \exp(-\alpha' t)], r \leq N \] (25)

where \( \alpha' \) will be smaller than \( \alpha \) in Eq. (5) if more than one galactose must be stripped in a sequence to open a cleavable site. \( N \) is the total number of cleavable sites on a GM, where \( N - n_0 \) are opened up by the Gase stripping. This leads to a rate equation for the number of cleavages

\[
\frac{dr}{dt} + kr = kn_0 + k(N - n_0)[1 - \exp(-\alpha' t)]
\] (26)

whose solution is

\[
r(t) = N + kn_0 \left[ \frac{(1 - (N/n_0)e^{-\alpha t})}{k - \alpha'} + \frac{((\alpha')/(kNn_0 - 1))e^{-kt}}{k - \alpha'} \right]
\] (27)

where, again, \( r(t) = R(t)M \). It can be shown that \( r(t) \), according to Eq. (27), recovers the proper expressions for all limiting cases—e.g., for \( \alpha' = 0 \) or \( \alpha' \to \infty \) holds, the case of no Gase.

The form of \( Kc_0/I(q, t) \) follows from Eq. (1) by recognizing that the product \( MC(M) \) in the integral diminishes as \([ f_p + (1 - f_p)e^{-\alpha t}]^2 MC_0(M) \), where \( C_0(M) \) is the initial mass distribution of the polymers. The time dependence due to side-chain stripping hence factors out of the integral, and the original result for random degradation of a random polymer still applies. The term involving \( A_2 \) in Eq. (1), however, is shifting in time. The decrease due to stripping is the same as outlined in the above section on side-chain stripping, whereas for the backbone degradation part \( A_2 \) changes negligibly.

Combined with the original equation for random degradation of a polymer, the combined stripping leads to a new TDSLS signature of the form

\[
Kc_0 = \frac{1}{[ f_p + (1 - f_p)e^{-\alpha t}]^2} \times \left[ \frac{c_0}{M_{c_0}(M)P(q, r)dM} + 2A_{2,0}c_0 \right]
\] (28)

Analysis carried out at high \( u \) allows the limit of Eq. (17b) to be used, so that in fitting the results the following expression will be used:

\[
Kc_0 = \frac{1}{[ f_p + (1 - f_p)e^{-\alpha t}]^2} \times \left[ \frac{1}{2M_{c,0}} + \frac{\gamma q^2}{2} + \frac{R(t)}{2} + 2A_{2,0}c_0 \right]
\] (29)

where \( r(t) \) is now given by Eq. (27), and again, \( R(t) = r(t)/M \). Since \( f_p \) and \( \alpha \) are known from the analysis of the sidechain stripping data with Gase alone, and \( k \) and \( n_0 \) are known from the random degradation by Mase, the only unknown parameters in the expression involving the two enzymes are \( N/M \), the total number of cleavable sites per g/moles of initial polymer mass, and \( \alpha' \).

### MATERIALS AND METHODS

Seeds of *Mimosa scabrella* Benthan (Ms), *Molendawer floribunda* (Mf), and *Melanoxylon brauna* (Mb) were obtained from Embrapa-Empresa Brasileira de Pesquisa Agropecuaria, and Instituto Ambiental do Paraná, Brazil. Milled seeds were boiled in water for 3 min in a microwave oven and submitted to water extraction at 25°C for 4 h. The supernatants were centrifuged twice at 20,000 g for 1 h and filtered successively through 8 and 3 μm nitrate cellulose membranes. The filtrates were precipitated with one volume of ethanol and afterward washed on a gradient of ethanol (70–100%). GM were collected and dried under vacuum at 30°C.

The GM were hydrolysed with 1N trifluoroacetic acid (5 h, 100°C), reduced with sodium borohydride and acetylated with pyridine: acetic anhydride (1:1 v/v) overnight at room temperature. The resulting alditol acetates were analyzed by gas-liquid chromatography (GLC) using a model 5890 SII HP Gas Chromatograph at 220°C (free induction decay and injector temperature, 250°C) with DB-225 capillary column (0.25 mm id x 30 m). The carrier gas was nitrogen.

\(^{13}\)C NMR spectroscopy was performed, after samples were sonicated (Branson B2, 150 W apparatus), with a Bruker AC-300 spectrometer at 75 MHz in the Fourier-transform mode, with complete proton-decoupling at 80°C, using D\(_2\)O as solvent.

\( \alpha \)-\( \beta \)-Galactosidase from guar seed and \( \beta \)-\( \beta \)-mannanase from *Aspergillus niger* were obtained from Megazyme (ammonium sulphate form). These enzymes are furnished already highly purified, and the percentage of \( \beta \)-mannanase and \( \beta \)-mannosidase contaminants is less than 0.0001% for the \( \alpha \)-galactosidase (guar), and \( \beta \)-mannosidase and \( \alpha \)-galactosidase is less than 0.001% for mannanase. The enzyme solutions were dissolved in 0.2 mol/L acetate buffer and filtered in 0.22 μm Millex-GV sterile nitrate cellulose filters before GM hydrolysis.

The enzyme units are defined by the manufacturer as follows: one unit-U (Kat) of \( \beta \)-mannanase activity is the amount of enzyme that will release 1 mol of mannose reducing-sugar equivalent from soluble \( \beta \)-mannan at pH 4.5 and 40°C in 1 s. One unit-U (Kat) of \( \alpha \)-\( \beta \)-galactosidase is the amount of enzyme that will release 1 mol of \( p \)-nitrophenol from \( p \)-nitrophenyl \( \alpha \)-\( \beta \)-galactopyranoside at pH 4.5 and 40°C in 1 s.

Protein contents were determined by a modified Bradford method. GM (5 mL; 1 mg/mL) in 0.2 mol/L acetate
buffer, filtered in 0.22 μm Millex-GV sterile nitrate cellulose filters, were treated by guar α-β-galactosidase (0.36 U/mL galactomannan; pH 4.5) and A. niger β-β-mannanase (0.6 U/mL galactomannan; pH 4.5) at 25°C. Enzymatic hydrolysis was monitored continuously by TDSLS. SEC analysis was performed on starting and end products.

A Wyatt Dawn-DSP light scattering photometer was used for the TDSLS measurements, using scintillation vials in “batch mode.” Custom software was written by one of the authors to control the instrument and analyze data. Typically, the light scattering at 18 angles was sampled every 1 s. For all hydrolysis experiments a GM concentration of 1 mg/mL was used. All experiments were carried out at 25°C.

SEC was carried out on 0.5 mg/mL sample solutions using a multidetection equipment consisting of an HP 1100 isocratic pump, an ANSPEC ECR-7522 differential refractometer, a Shimadzu SPV-10AV UV/visible flow spectrophotometer, a Wyatt Dawn-F scattering photometer operating in flow mode, and a homebuilt single capillary viscometer based on a Validyne P55D pressure transducer. Two Shodex columns (OH-pakSB 804 and 806) were connected in series and coupled to the detectors. A 0.1 mol/L NH₄NO₃ solution, in which NaN₃ (0.2 mg/mL) was added as a preservative, was the eluent. SEC data were collected and analyzed by software written by one of the authors. The system has been previously described.

RESULTS

TDSLS Results on Enzymatic Hydrolysis of GM

Figure 1 shows the time dependence of the raw scattered light intensity (in volts) at θ = 90° when 1 mg/mL Ms is hydrolyzed by galactosidase at a concentration of 0.36 U/mL. The curve shows an initially concave downwards curvature, followed by concave upwards behavior. The inflection point is at about 10,200 s. This type of inflection is characteristic of a single-stranded chain from which side chains are being randomly stripped. It was shown that the condition for an inflection point is that the fraction of material in the backbone \( f_p \), be less than \( \frac{2}{3} \). The smaller \( f_p \) the stronger the initial downwards curvature. This condition is clearly met since by GLC, methylation analysis, and NMR analysis, \( f_p < 0.52 \).

Figure 1 inset shows the raw scattered intensity at \( \theta = 90° \) for 1 mg/mL Ms hydrolyzed by Mase at 0.6 U/mL, and also by a mixture of Mase (0.6 units/mL) and Gase at 0.36 U/mL. The hydrolysis is rapid at this enzyme concentration, and few points are recorded.
during the initial precipitous fall. Under pure Mase digestion the scattering reaches a plateau in about 100 s. This is interpreted as cleavage of the Ms backbone at a finite number of exposed sites—i.e., at sites with enough sequential mannopyranosyl residues to permit digestion by Mase. There is considerable evidence in the literature, as well as from these data, that more than a single unsubstituted site is necessary for Mase to cleave. In contrast, when Gase and Mase are mixed, no plateau is reached, since Gase continuously opens new attack sites for Mase by stripping off galactopyranosyl side groups.

The reciprocal representation in terms of $Kc/I(t)$ provides a more revealing depiction of the hydrolysis processes. Figure 2 shows this quantity at $\theta = 90^\circ$ for Gase hydrolysis of Ms (M/G 1.1), Mb (M/G 2.6), and Mf (M/G 1.6). In each experiment the concentration of Gase was 0.36 U/mL. The curve for Ms shows the strongest initial upwards curvature, consistent with it having the lowest percentage of mass in the backbone. Mf shows only little upward curvature, and arrives at its plateau within the time frame of the experiment. Mb, which has the least side-group mass, shows virtually no upward curvature and arrives at its plateau more quickly than the other samples. The subsequent plunge of $Kc/I$ shows the aggregation of the completely galactose-stripped Mb backbones. This is consistent with the fact that it is the galactose substitution that confers water solubility to the galactomannans. The other samples also showed aggregation and precipitation well after the experiments were completed.

Tayal et al. studied enzymatic degradation of guar (M/G 1.6) using Gamanase (mixture of endo-\beta-mannanase and \(\alpha\)-galactosidase). They showed in their Figure 3 samples of $1/M_w$ vs time of guar solutions, and described it as a linear relation. Actually, the data appear to have the type of initial upward curvature and inflection as seen in Figure 2 in the present work for Ms and Mf.

Fits to the TDSLs [Eq. (11)] are also shown in Figure 2. The only fitting parameter was the rate constant $\alpha$, since $f_p$ was determined according to Eq. (12). The exception was for Ms, for which $f_p$ was used as a fitting parameter, since the final plateau was not reached during the experiment. Table I summarizes rates and other parameters of each galactomannan.

The aggregation phenomenon mentioned for Mb can also be seen in the angular dependence of the
TDSLS signal. The Figure 2 inset shows samples of $Kc/I$ vs $\sin^2(\theta/2)$ (which is proportional to $q^2$) curves at different time points during the hydrolysis of Mb by Gase. At the outset of the experiment $Kc/I$ is linear with a low $Kc/I(0)$ intercept. As galactopyranosyl side-chain stripping proceeds $Kc/I(q)$ remains straight, but the intercept increases, indicating the loss of mass. At the end of the experiment, corresponding to the precipitous drop in Figure 2, $Kc/I$ is low, showing that the mass of the aggregates is large compared to the size of the original, unhydrolyzed Mb.

The TDSLS models summarized above can now be further applied quantitatively to the other hydrolysis processes. Mase digestion corresponds to random degradation of a linear polymer at a finite number of randomly distributed cleavage points. Figure 3 shows hydrolysis behavior at $[\text{Mase}] = 0.6 \text{ U/mL}$ enzyme dissolution for Ms, Mf, and Mb. The most striking features is the height of the plateau, which is linearly related to the number of available cleavage sites on the original polymers. The plateaus clearly follow the increasing order Ms:Mf:Mb, and are used to determine the values of $R_0$ given in Table I, by using eq 23 in the plateau region.

\[
\frac{R_0}{2} = \frac{Kc}{I(q, t)} \left|_{t=\text{plateau}} - \frac{Kc}{I(q, 0)} \right. 
\]

Also shown in Figure 3 are fits to the random scission of a linear polymer with two parameters; $k$, the rate constant, defined in Eq. (18), and a second parameter that is simply the sum of the first, second, and fourth constant terms on the right side of Eq. (23).

Table I shows the rate constants from these fits, determined for the various samples at different Mase concentrations. There is fairly good agreement among the various samples for the rate constant at each Mase concentration, which suggests that there is no large difference in the random substitution of galactose with varying degree of substitution. The number of cleavable sites per $10^6$ g/mole of initial polymer mass for each GM is also shown in Table I.

Figure 4 shows $Kc/I$ for two different hydrolyses of Ms, each using combined Mase and Gase enzymes. One figure has $[\text{Gase}] = 0.36 \text{ U/mL}$, and $[\text{Mase}] = 0.6 \text{ U/mL}$, whereas the other has $[\text{Gase}] = 0.225 \text{ U/mL}$ and $[\text{Mase}] = 0.38 \text{ U/mL}$. In both cases the pH was 4.5. The fitted curves shown are according to Eq. (29) with $R(t)$ as in Eq. (27), where the values of $f_p$ and $k$ are those determined from the previous Gase and Mase fits.
The slope of the nearly straight lines reached in the two curves in Figure 4 can be analyzed as the limit of Eq. (27) for \(1/\alpha \gg t \gg 1/k\), and \(k \gg \alpha'\). Equation (27) becomes

\[
\lim_{1/\alpha' \gg t \gg k} r(t) = N + n_0 \left[ \left( 1 - \frac{N}{n_0} \right)(1 - \alpha' t + \ldots) \right] \tag{31}
\]

The \(t = 0\) intercept to this line is related to \(n_0\) from Eq. (29) by

\[
n_0 = 2M \left( \frac{Kc}{I(t = 0)} \right)_{\text{above approximation}} - \left( \frac{Kc}{I(t = 0)} \right)_{\text{experimental}} \tag{32}
\]

The intercepts from all four Mase/Gase experimental curves are close (only two experiments are shown), and yield \(n_0 = 14 \pm 3\%\), which is within error bars of the value found by Mase digestion alone (Table I). The slopes are equal to \(\alpha' N/2\) by Eqs. (29) and (31). Unfortunately, the individual values of \(N\) and \(\alpha'\) cannot be found from this value alone. Nonetheless, the slopes scale approximately as the concentration of Gase.

The Figure 4 inset shows combined Mase/Gase hydrolysis of \(\text{Mf}\), where \([\text{Gase}] = 0.09\ \text{U/mL}\), and \([\text{Mase}] = 0.15\ \text{U/mL}\). Because there are significantly more Mase cleavage sites initially exposed (ten times more than in Ms), and because of the short time scale of the experiment, the second phase due to the first exponential in Eq. (27) is not apparent, as it was for Ms. The form of the Figure 4 inset is expected from Eq. (27) when \(n_0\) is on the order of \(N\), and \(\alpha' \ll k\). In this case, the second exponential term in Eq. (27) dominates in bringing \(Kc/I\) to the final plateau. The same occurred for \(\text{Mb}\).

An advantage of TDSLS is that kinetic determinations can be made without extrapolation to zero angle. It hence suffices to follow the hydrolysis kinetics at a single scattering angle in order to obtain kinetic and structural information. For Mase digestion of Ms (0.06 U/mL of enzyme) the average value over all 18 angles is \(k \approx 0.00395 \pm 0.0035\), with no angular dependence. This means, for example, that even a device such as a spectrofluorimeter (right angle detection with excitation and observation wavelengths set equal) could be used for these types of experiments.

Extrapolation to 0 angle, and correction for \(A_2\) as described above, allows both the mass and mean square radius of gyration to be followed. Figure 5a

### Table I Properties of Galactomannans, and Rate Constants and Other Parameters Deduced from Kinetic Data

<table>
<thead>
<tr>
<th></th>
<th>Ms</th>
<th>Mf</th>
<th>Mb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield % (w/w)</td>
<td>30</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>M/G ratio (GLC analysis)</td>
<td>1.1</td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>(dn/dc)</td>
<td>0.115</td>
<td>0.141</td>
<td>0.119</td>
</tr>
<tr>
<td>(M_w) (g/mole)(^a)</td>
<td>1,510,000</td>
<td>1,180,000</td>
<td>1,460,000</td>
</tr>
<tr>
<td>(\langle S^2 \rangle) (Å)(^a)</td>
<td>1050</td>
<td>1140</td>
<td>1230</td>
</tr>
<tr>
<td>(A_2) (cm(^3) mol/g(^2))(^a)</td>
<td>(6.5 \times 10^{-4})</td>
<td>(8.8 \times 10^{-4})</td>
<td>(6.8 \times 10^{-4})</td>
</tr>
<tr>
<td>(10^6R_0): Mase cleavable sites per 10(^6) g/mole of initial mass [see Eq. (20)]</td>
<td>13.7 ± 1.3</td>
<td>140 ± 23</td>
<td>245 ± 43</td>
</tr>
<tr>
<td>Backbone cleavage rate constant [Eq. (18)] (k) (s(^{-1})) at [Mase]</td>
<td>0.06 (U/mL)</td>
<td>0.0038</td>
<td>0.0301</td>
</tr>
<tr>
<td></td>
<td>0.15 (U/mL)</td>
<td>0.0076</td>
<td>0.0091</td>
</tr>
<tr>
<td></td>
<td>0.38 (U/mL)</td>
<td>0.0283</td>
<td>0.0295</td>
</tr>
<tr>
<td></td>
<td>0.60 (U/mL)</td>
<td>0.0357</td>
<td>0.0378</td>
</tr>
<tr>
<td>Galactosidase side-chain stripping rate constant (\alpha) (s(^{-1})) at [Gase]</td>
<td>0.36 U/mL</td>
<td>4.03 \times 10^{-5}</td>
<td>4.07 \times 10^{-5}</td>
</tr>
<tr>
<td>(f_p) by TDSLs of galactosidase digestion, by Eq. (12)</td>
<td>0.458(^b)</td>
<td>0.602</td>
<td>0.683</td>
</tr>
<tr>
<td>(f_p) by (^1^3)C NMR (f_p) by methylation</td>
<td>0.521</td>
<td>0.590</td>
<td>0.754</td>
</tr>
<tr>
<td>(f_p) by GLC</td>
<td>0.523</td>
<td>xx</td>
<td>xx</td>
</tr>
<tr>
<td>(q) by TDSLs and Eq. (33)</td>
<td>0.190(^c)</td>
<td>0.339</td>
<td>0.535</td>
</tr>
<tr>
<td>Minimum number of consecutive mannose sites for Mase cleavage, (u_{min})</td>
<td>2.6 ± 0.6</td>
<td>2.7 ± 0.3</td>
<td>3.4 ± 0.7</td>
</tr>
</tbody>
</table>

\(^a\)By Zimm plot determination.

\(^b\)No plateau reached, value from kinetic fit to Eq. (11).

\(^c\)From GLC data.

The intercept to this line is related to \(n_0\) from Eq. (29) by

\[
n_0 = 2M \left[ \left( \frac{Kc}{I(t = 0)} \right)_{\text{above approximation}} \right] - \left( \frac{Kc}{I(t = 0)} \right)_{\text{experimental}} \tag{32}
\]

The intercepts from all four Mase/Gase experimental curves are close (only two experiments are shown), and yield \(n_0 = 14 \pm 3\%\), which is within error bars of the value found by Mase digestion alone (Table I). The slopes are equal to \(\alpha' N/2\) by Eqs. (29) and (31). Unfortunately, the individual values of \(N\) and \(\alpha'\) cannot be found from this value alone. Nonetheless, the slopes scale approximately as the concentration of Gase.
shows $M_w$ and $\langle S^2 \rangle_2^{1/2}$ vs time for Gase hydrolysis of Mf. (0.36 U/mL of enzyme). $\langle S^2 \rangle_2^{1/2}$ diminishes very little, from about 900 to 840 Å, justifying the use of $b(t) \approx b_0$ going from Eq. (10) to Eq. (11) in the discussion of $A_2$ corrections above. $M_w$, on the other hand, decreases from $1.18 \times 10^6$ g/mole to $6.8 \times 10^5$ during degradation.

Figure 5b shows $M_w$ and $\langle S^2 \rangle_2^{1/2}$ vs time for Gase hydrolysis of Mb (0.36 U/mL of enzyme). A somewhat larger change in $\langle S^2 \rangle_2^{1/2}$ is observed, from 810 to 680 Å, but a smaller percentage change in $M_w$, from 9.2 to $6.2 \times 10^5$, reflecting the fact that Mb has less galactose substitution than Ms. At later times the onset of the aggregation phenomenon is seen as both $M_w$ and $\langle S^2 \rangle_2^{1/2}$ begin to rise abruptly.

The Figure 5b inset shows $M_w$ and $\langle S^2 \rangle_2^{1/2}$ vs $t$ for Mase (0.15 U/mL of enzyme) hydrolysis of Ms. Both of these parameters plunge significantly ($M_w$ final is about 6 times less than the initial value), and $\langle S^2 \rangle_2^{1/2}$ falls to less than half its original value.

**SEC Results Compared to TDSLS**

Figure 6a shows raw SEC chromatograms of Ms, before enzymatic hydrolysis, after separate hydrolyses by Gase and Mase, and after combined Gase/Mase hydrolysis. Under Gase digestion, the RI trace shows that the essential shape of the unhydrolyzed peak does not change qualitatively, but moves to higher elution volumes with lower scattering signal—i.e., to lower masses. This is consistent with side-chain stripping, leaving unsubstituted GM backbones at roughly half the initial mass, but with no significant change in the polydispersity. The light scattering trace closely follows the RI behavior in this case. (As demonstrated above, there is no appreciable change in the hydrodynamic volume of GM via Gase hydrolysis of side chains. It is recalled that, especially in aqueous SEC, size exclusion is often not the main mode of polymer separation. The reduced amplitude of the scattering for Gase hydrolyzed Ms in figure 6a attests to the mass reduction more so, a priori, than a shift to higher elution volume).

The action of Mase in Figure 6a is quite distinct from that of Gase. The unhydrolyzed peak becomes a broad distribution, as seen in the RI data for Mase. This corresponds to a finite number of random scissions of the initial backbone. Unlike the Gase case, the light scattering data now bear no resemblance to the RI trace. The only residual light scattering is at

![Figure 4](image_url)
FIGURE 5  (a) $M_{w, ap}$ and $(S^2)^{1/2}$ of Mf (1 mg/mL) as a function of degradation time hydrolysis experiments with Gase (0.36 U/mL enzyme; pH 4.5, at 25°C). (b) $M_{w, ap}$ and $(S^2)^{1/2}$ of Mb (1 mg/mL) as a function of degradation time hydrolysis experiments with Gase (0.36 U/mL enzyme; pH 4.5; at 25°C). Inset shows $M_{w, ap}$ and $(S^2)^{1/2}$ of Ms (1 mg/mL) as a function of degradation time hydrolysis experiments with Mase (0.15 U/mL enzyme; pH 4.5; at 25°C).
FIGURE 6  (a) Raw SEC chromatograms of Ms (0.5 mg/mL), before enzymatic hydrolysis, after separate enzymatic hydrolysis by Mase (0.6 U/mL enzyme; pH 4.5, 1000 s) and Gase (0.36 U/mL enzyme; pH 4.5, 20 h), and after combined hydrolysis by Mase/Gase (pH 4.5, 1000 s), at 25°C. (b) Raw SEC chromatograms of Ms, Mf, and Mb (all at 0.5 mg/mL), before and after enzymatic hydrolysis by Mase (0.6 U/mL enzyme; pH 4.5, 1000 s), at 25°C.
high elution volume, where there are large fragments of the original GM left, but the rest of the fragments, which constitute the majority of the RI signal, give no appreciable scattering, since they are so small.

The combined action of Gase and Mase has the expected effect of producing small fragments that have no discernible light scattering signal but that give a large RI signal shifted to very high elution volume, with a discernible, broad peak. Notably, the elution volume for the final fragments from Gase/Mase combined digestion is lower (i.e., higher mass) than those from pure Mase digestion in Mf and Mb, discussed below.

Figure 6b focuses more closely on the Mase hydrolysis of each GM. Whereas Mase produced a broad fragment distribution, Mf shows a very well-defined peak of small fragments, before being absorbed into the large RI rise at about 19.5 mL elution volume, which corresponds to the solvent mismatch between the acetate buffer used for GM digestion and the 0.1M NH₄NO₃ SEC eluent. This symmetric peak for the fragments is what is expected for random degradation of a random polymer. Likewise, the small trace of fragments disappearing into the solvent peak for Mb show the beginnings of a well-defined distribution. Again, the effects of a much larger number of Mase cleavable sites \( n_0 \), for Mf and Mb, is reflected in the fact that the fragments produced are so markedly smaller than for Ms.

Figure 7 shows an example of the resulting absolute mass distributions, as determined by the SEC light scattering and RI detectors. The distributions are for both the unhydrolyzed and galactosidase hydrolysed Mb. The main effect is to shift the central peak to lower mass, and largely retain the initial shape, as expected for side-chain stripping. The stripped galactopyranosyl monomers do not show up in the distribution.

**DISCUSSION**

The TDSLS experiments allow quantitative rate constants to be determined and structural analysis to be made. The most immediate result is that all the data fit well by using random models: The signature of Gase side-group stripping is that of an enzyme randomly stripping sidechains. The signature of Mase backbone degradation is that of an enzyme randomly attacking randomly distributed cleavage sites. The behavior of the two enzymes together is the result of random side-chain stripping at randomly distributed sites, and random backbone degradation at an increasing number of sites.
A striking feature of the Mase data is that the number of initially cleavable sites \( n_0 \) is much smaller than the number of unsubstituted sites. This strongly suggests that Mase cannot cleave a single unsubstituted site surrounded by galactose substituted sites, but rather requires a minimum number of sequential unsubstituted sites \( u_{\text{min}} \).

McCleary et al.\(^8\) showed that lucerne galactomannan (high level of substitution M:G 1.1) can be hydrolyzed on the subsites with unsubstituted mannose residues adjacent substituted mannosyl residues. The degree of hydrolysis by \( \beta\)-d-mannanase was 5% for guar galactomannan (M:G 1.6) and 22% for tara (M:G 2.6), showing that the specific subsites present in lower man:gal ratio galactomannans can improve the hydrolysis by \( \beta\)-d-mannanase. These results are in agreement with this work.

As suggested by the data, we assume that the galactose substitutions are essentially random. Let \( N_g \) and \( N_m \) be the initial number per g/mole of initial polymer, of galactose substitution sites, and number of mannose monomers on a GM chain, respectively. Then, the fraction of unsubstituted sites \( q \) is given by

\[
q = \frac{(N_m - N_g)/N_m = (2f_p - 1)/f_p}{(33a)}
\]

and the number of unsubstituted sites per g/mole is

\[
N_u = (2f_p - 1)/m \tag{33b}
\]

where \( m \) is the monomer mass (162 g/mole), and \( N_m \) is

\[
N_m = f_p/m \tag{34}
\]

\( N_m \) is the same as the total number of sites along the side chain, since mannose forms the backbone.

Now, if we begin with an unsubstituted site and take a step along the chain, the probability of finding an unsubstituted site is \( q \), and of finding a substituted site is \( 1 - q \). That is, the probability of finding a sequence of unsubstituted sites just one site long is \( (1 - q) \). Continuing the trial where an unsubstituted site was found, the probability on the next step of finding a substituted site is \( q(1 - q) \). This is the probability of finding a sequence of two unsubstituted sites. In general, the probability of finding \( u \) sequential unsubstituted sites, followed by a substituted site, is

\[
p(u, q) = (1 - q)q^{u-1} \tag{35}
\]

This can also be interpreted as the fraction of all unsubstituted sequences that contain \( u \) consecutive unsubstituted sites. This is a standard geometric sequence that describes random distributions of comonomers in the closely related case of random copolymers.\(^4\) The sum of probabilities from \( u = 0 \) to infinity is normalized (i.e., unity), since

\[
\sum_{u=1}^{\infty} q^{u-1} = \sum_{u=0}^{\infty} q^u = \frac{1}{1 - q} \tag{36}
\]

The quantity \( 1/(1 - q) \) is also the number average sequence length number \( L_n \), defined as

\[
L_n = \frac{\text{Total no. of unsubstituted sites}}{\text{No. of sequences of unsubstituted sites}}
\]

\[
(1 - q) \sum_{u=1}^{\infty} uq^{u-1} = \frac{1}{1 - q} \tag{37}
\]

The total number of sequences \( L_t \) per g/mole is hence

\[
L_t = N_f/L_n \tag{38}
\]

The fraction of sequences that have at least \( u_{\text{min}} \) sequential unsubstituted sites before a substituted site is encountered is

\[
p(u \geq u_{\text{min}}, q) = (1 - q) \sum_{u=u_{\text{min}}}^{\infty} q^{u-1} = q^{u_{\text{min}}-1} \tag{39}
\]

Now \( R_0 \) is the number of initially cleavable sequences per g/mole of initial polymer, as determined, for example, from the TDSLS measurements presented above. The fraction of initially cleavable sequences \( R_0/L_t \) is then

\[
\frac{R_0}{L_t} = q^{u_{\text{min}}-1} \tag{40}
\]

Hence \( u_{\text{min}} \) is given by

\[
u_{\text{min}} = \frac{\log(R_0/L_t)}{\log(q)} + 1 \tag{41}
\]

\( L_t \) is determined from Eqs. (33b), (37), and (38) above. Table I shows the values of \( u_{\text{min}} \) computed from the experimental values for \( L_t \), \( R_0 \), and \( q \). The error bars were obtained by computing the range of \( u_{\text{min}} \) values obtained when the error bars on \( R_0 \) and \( q \)
are taken into account. All $\mu_{\text{min}}$ values are within error bars for the three GM, and show that at least 3 units are needed for hydrolysis. Though this number seems small, it radically reduces the number of cleavable sites on a chain, since the number of sequences with a given number of units decreases geometrically with the number of units.

This sequentiality result is consistent with the report of McCleary and Matheson, who analyzed the action patterns and substrate binding requirements of $\beta$-$d$-mannanase with mannosacharides and galactomannans. They demonstrated that a chain of at least four mannoses is required for a significant hydrolysis rate; with five subsites (pentaose), the initial rate of hydrolysis is as high as with the hexaose, suggesting five significant binding subsites, which is in agreement with our results.

**Summary**

TDSLS has been used to follow the enzymatic hydrolysis of GM with various degrees of galactose substitution. An enzyme (Gase) has been used that only strips the galactose substitution sites, and a second enzyme (Mase), which only cleaves the backbone at sites with a sufficient number of consecutive mannose units. The enzymes have been used together, which leads to the highest degree of hydrolysis in the case of the most highly galactose substituted, Ms. The action of the two enzymes separately, and the two enzymes together, generate unique TDSLS signatures, which are analyzed within the framework of previous TDSLS theory, and an extension to the simultaneous enzyme case developed here. This type of analysis rapidly yields the rate constants for each enzyme, indicates that the mechanism in each case is random scission of randomly distributed substrates, and allows estimation of the amount of material in the form of sidechains. These values are in good agreement with both those of SEC and the other methods for sidechain content determination listed in Table I. The method has also demonstrated that there is a minimum sequence of unsubstituted mannose units needed for hydrolysis by mannanase to occur.

The actual data gathering is simple and automatic, and it is hoped the method will prove to be of general utility for studying kinetics and structure of polysaccharides and other natural products, and complement other techniques.

This work was supported by CNPq—Conselho de Desenvolvimento Científico e Tecnológico do Brazil and PRONEX—Carboídritos. Support from U.S. National Science Foundation CTS-9877206, and Louisiana BoR RD-B-11 are gratefully acknowledged.

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