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Kinetic analysis of pectate lyases by high-performance liquid chromatography*

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ABSTRACT

Ion-pair reverse-phase high-performance liquid chromatography has been used to resolve and quantify unsaturated oligosaccharides generated by pectate lyases. Increasing resolution is obtained with increasing size of oligomer, and there is a proportional relationship between the molecular weight of a given oligomer and the logarithm of the retention time. The rates of formation of individual unsaturated oligogalacturonic acids ranging in degree of polymerization from 2 to 11 and in concentrations from 0.02 to 1.6mM have been measured as products of endolytic eliminative D-galacturonan depolymerases. This system has provided a convenient and sensitive method to assay the kinetic properties of pectate lyases (EC 4.2.2.2) secreted by *Erwinia chrysanthemi*, and has been used to clearly distinguish different activities based upon their respective mechanisms and the formation of individual limit products.

INTRODUCTION

Glycuronan ("polyuronide") lyases are enzymes that cleave glycosiduronosyl bonds through an eliminase reaction. They are important in the depolymerization of such plant glycuronans as alginate[1,2] and pectate[3,4], and animal glycosaminoglycans, for example hyaluronate[5], heparin[6], and chondroitin sulfate[7,8]. The products formed by these enzymes contain a 4,5-unsaturated residue in the newly generated nonreducing termini and may be monitored by absorbance at 230–235 nm when the alkenic bond is preserved, as it is in products with a d.p. of 2 or more. Spectrophotometric methods may therefore be conveniently applied to the kinetic evaluation of these enzymes, and along with viscometric methods, have provided information on the relative activities and mechanisms of the depolymerization process.

A more rigorous characterization of this type of enzyme and its mechanism may be achieved with the quantitation of the individual products formed. Methods involving paper chromatography and electrophoresis have been employed to determine the pattern of products formed by different lyases involved in the depolymerization of pectate, and have been useful in identifying the limit products formed for a given enzyme-catalyzed reaction[9,10]. These methods require the detection of the individual

*Abbreviations: f.a.b.-m.s. fast-atom bombardment mass spectrometry; PL, pectate lyase; d.p., degree of polymerization; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of 0.2% sodium dodecyl sulfate.

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products on the solid support with the aid of a chromogenic reagent, such as silver ion reduction to detect reducing termini, or pH indicators to detect carboxyl groups, and are in general semiquantitative, with sensitivity limits down to 10 nmol.

A number of h.p.l.c. systems have been examined for their ability to quantify the depolymerization products of polysaccharides[11,12]. Ion-pairing reagents that confer hydrophobic properties on ionized compounds have allowed the use of reverse-phase chromatographic systems, with their high partitioning capabilities, for the separation of charged molecules. Reverse-phase ion-pair h.p.l.c. has been applied to the analysis of products generated in the enzyme-mediated depolymerization of both pectate[12,13] and hyaluronate[14]. A similar system was developed for the kinetic assay of mannuronan and guluronan lyases secreted by alginate-degrading bacteria[15,16]. The advantage of this system was demonstrated with the analysis of endolytic mannuronan-specific lyase, where the resolution (as retention time) was proportional as a logarithmic function to the molecular weight of the product formed[17]. In the work presented here, this approach has been extended to the kinetic assay of pectate lyases secreted by *Erwinia chrysanthemi*, with the resolution and quantitative detection of unsaturated oligogalacturonic acids ranging in degree of polymerization from 2–11. Applications of this methodology to the analyses of pectate lyases secreted by different species of bacteria have been presented[18,19].

EXPERIMENTAL

Organism and reagents. — *Erwinia chrysanthemi* strain P860281 was provided by Dr. T. Shubert from the Division of Plant Industry, Florida Division of Agricultural and Consumer Services, Gainesville, Florida. For enzyme production, cultures containing 100 mL of minimal salts medium[20] and 0.5% pectate (Burger Enterprises) as carbon source were incubated in 2.8 L Fernbach flasks with shaking (140 r.p.m., New Brunswick G10 gyrotory shaker) at room temperature. Galacturonan (Pfaltz and Bauer) for enzyme substrate was prepared in solutions (1% in 0.1M NaOAc, pH 4.2) by dialysis on an Amicon concentration/dialysis unit equipped with a PM 10 membrane filter to remove components having molecular weights < 10 000. Acetonitrile was purchased as h.p.l.c. grade. All other chemicals were reagent grade or a biochemical grade of the highest commercially available purity.

Enzyme preparations. — The extracellular pectate lyases (EC 4.2.2.2) were prepared as a mixture from concentrated media from late exponential-phase cultures of *Erwinia chrysanthemi* strain P860201. This PL mixture, which was concentrated as a 60–90% saturated $(NH_4)_2SO_4$ fraction, was dialyzed against Buffer A (0.05M Tris-HCl, pH 8.5; 0.2mM $CaCl_2$) and used to prepare unsaturated oligogalacturonic acid standards ranging in d.p. from 2–6. Individual activities were prepared by chromatofocusing to give four major fractions eluting at pH values of 8.6, 8.3, 6.0, and <6, and designated A, B, C, and D, respectively. Fraction B showed a single protein band of molecular weight 37 200 with SDS-PAGE and two activity bands of pI 7.5 and 7.9 by thin-layer isoelectric focusing. Fraction C contained a single protein component of

molecular weight 40 200 by SDS-PAGE and a single activity band by analytical isoelectric focusing with a pI of 4.8. The detailed characterization of these activities will be presented elsewhere.

Lyase activities were measured spectrophotometrically[4] at 235 nm with the production of unsaturated nonreducing termini based upon a molar absorptivity of 4600. It should be noted that, when this reaction results in the formation of an unsaturated monomer, tautomerism results in the conversion of this product into the keto form with a shift in absorbance from 235 to 210 nm. The activities measured in this study were monitored only through their formation of unsaturated products having a d.p. of 2 or more. Mixtures containing 0.1% PGA in Buffer A were incubated at room temperature (23–25°). One unit of activity is defined as that which catalyzes the formation of one nmol of unsaturated residue per min under these conditions.

Preparation of oligogalacturonic acid standards. — The production of several mg of unsaturated oligogalacturonic acids ranging in d.p. from 2 to 6 was conveniently achieved upon incubating 4 units of the PL mixture in 200 mL of Buffer A containing 0.1% dialyzed D-galacturonan in an Amicon model 400 concentration/dialysis unit fitted with a PM 10 membrane. Products with molecular weights of 10 000 or less were collected as effluent in 5-min fractions at an initial flow rate of 0.5mL per min. Fractions (2.5 mL) having significant absorbance at 235 nm were pooled and lyophilized. This concentrated dialyzate fraction was applied to a pre-equilibrated P2 column (Bio-Rad P-2 200–400 mesh, 2.5 cm × 145 cm) and saccharides were eluted with 0.1M NH_4HCO_3 at room temperature. Fractions of 5 mL were collected and assayed for absorbance at 235 nm and total carbohydrate[21]. Fractions comprising individual oligogalacturonic acid peaks were pooled and lyophilized. Molecular masses of each oligomer ranging in d.p. from 2 to 6 were established by f.a.b.-m.s.

Chromatographic analyses. — The procedures employed for the resolution and detection of unsaturated oligogalacturonic acids were similar to those used for the separation of unsaturated oligomers derived from the lyase-mediated depolymerization of alginate[14]. The column system (Waters/Millipore) included a C_{18} RCSS guard cartridge preceding a 8MB C_{18} 10μ column enclosed in a Z module compression unit, and was developed isocratically at room temperature with 10% MeCN:0.1M sodium phosphate buffer, pH 6.5:0.01M Bu_4NOH. The solvent was delivered at a constant flow-rate with a 6000A pump. Compounds were detected by continuous analysis of absorbance at 235 nm with a Holochrome photodiode spectrophotometer (Gilson Medical Electronics) equipped with an 8-μL flow cell with a 1.00-cm light path. An h.p.l.c. system (Waters/Millipore) including a 721 controller, 710B sample injector, and a 730 data module was used to program runs for timed automatic sample-injection and the plotting and integration of detector signals. Mixtures were filtered through Millex GV 0.22μ filters (Waters/Millipore) immediately after the addition of enzyme. The filtered mixture was then split for the simultaneous kinetic analysis by h.p.l.c. and continuous recording spectrophotometric analysis with a Gilford model 2400 recording spectrophotometer.

RESULTS

Resolution of pectate lyase-generated products by h.p.l.c. — Unsaturated oligomeric products ranging in d.p. from 2 to 6 were resolved in quantity by column chromatography on Bio-Gel P-2 with 0.1M NH_4HCO_3 as the eluant: these oligomers served as standards for the calibration of the h.p.l.c. column. The reverse-phase ion-pair chromatography of the standards on the C_{18} column allowed the resolution of each and provided retention times for the assignment of each in a mixture of products. Figure 1a identifies the resolution of the dimer, trimer, tetramer, and pentamer standards, with an increase in the resolution with the size of the oligomer. Each oligomer could be quantified in the range of 1.0–80 nmol in a given sample applied to the column (Fig. 1b). A plot of the logarithm of the retention time for these defined standards (d.p. 2–5) versus the molecular weight gave a straight line for the five standards (Fig. 3).

The h.p.l.c. analysis of products generated directly by the unpurified secreted pectate lyases, as well as by two purified enzymes, reveals the ability of this system to

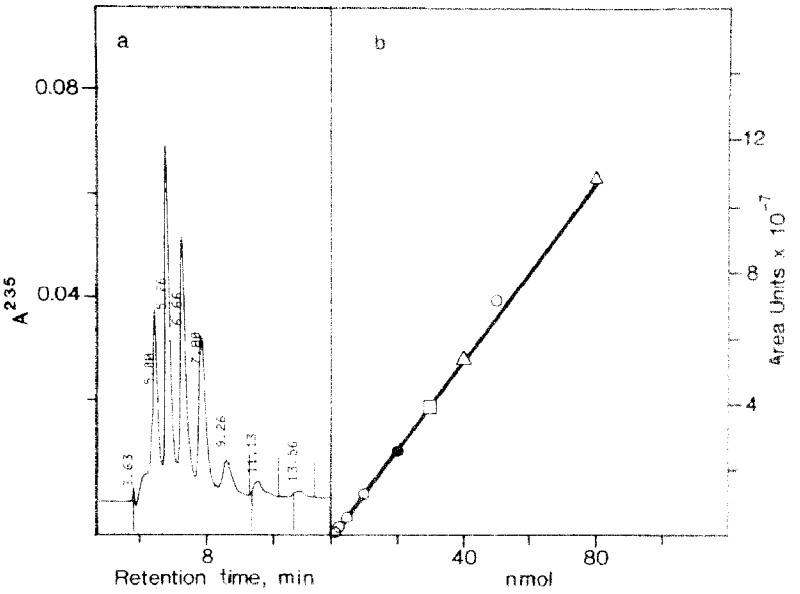

Fig. 1. Resolution and quantification of unsaturated oligomers by h.p.l.c.. Unsaturated oligomers ranging in d.p. from 2 to 5 were prepared with a mixture of *E. chrysanthemi* enzymes in an Amicon flow-cell, fractioned by P-2 gel filtration, and analyzed by f.a.b.-m.s. (a) A mixture containing 0.2mM each of dimer, trimer, tetramer, and pentamer was prepared and 0.025 mL injected and chromatographed on a C_{18} column with 10% CH_3CN:10mM Bu_4NOH: 0.1M sodium phosphate buffer, pH 6.5 at 1.0 mL/min. The effluent was analyzed by continuous flow at 235 nm. Based upon injection and analysis of individual oligomers, the dimer, trimer, tetramer, and pentamer eluted with retention times of 5.00, 5.76, 6.66, and 7.80 min, respectively. Small amounts of hexamer, heptamer, and octamer were present in the P-2 pentamer fraction and are presumed to be the components having the longer retention times. (b) Quantitation of unsaturated oligomers. Individual oligomers ranging in concentration from 0.02 to 1.6mM were injected in 0.05-mL volumes, eluted at 1 mL/min, and analyzed as before. Dimer, open circles; trimer, open triangles; tetramer, open square; pentamer, closed circle.

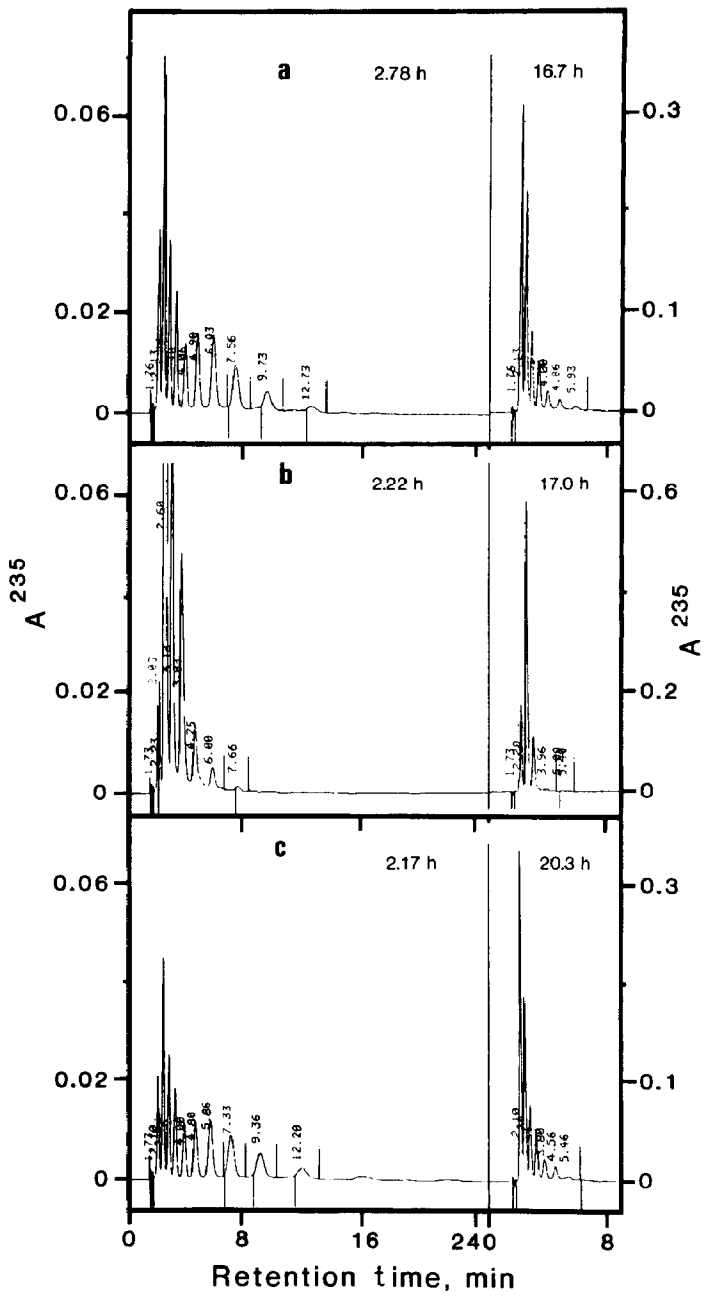

Fig. 2. H.p.l.c. profiles of early and limit reactions catalyzed by secreted pectate lyase activities. Mixtures contained 4 units of enzyme activity per mL and 0.1% D-galacturonan. Injections of 0.05 mL were made from reaction mixtures with a WISP automatic sample-injector and eluted at 2.4 mL/min with a run time of 30 min. (a) Combined enzymes in ammonium sulfate fraction. (b) Fraction B after chromatofocusing. (c) Fraction C after chromatofocusing.

resolve oligomers constituting an homologous series of unsaturated products generated by endolytic mechanisms (Fig. 2). At the same time, the ability of each enzyme to provide unique limit-products is demonstrated for comparison to the crude mixture of enzymes. The products found at the later times for the enzyme mixture (Fig. 2a, 16.7 h) and fraction C (Fig. 2c, 20.3 h) represent near limit-products and include dimer > trimer > tetramer as the major products with smaller amounts of larger oligomers. Products found at 17 h for fraction B (Fig. 2b) include trimer >> dimer > tetramer; these comprise true limits since the addition of more enzyme did not change their quantities, while the addition of more D-galacturonan as substrate gave rise to more product (data not shown). The exponential relationship of retention time to molecular weight for both the standards and the products generated after one h of incubation is evident in Fig. 3, and allows assignments of d.p. values of 2–13 to the products formed.

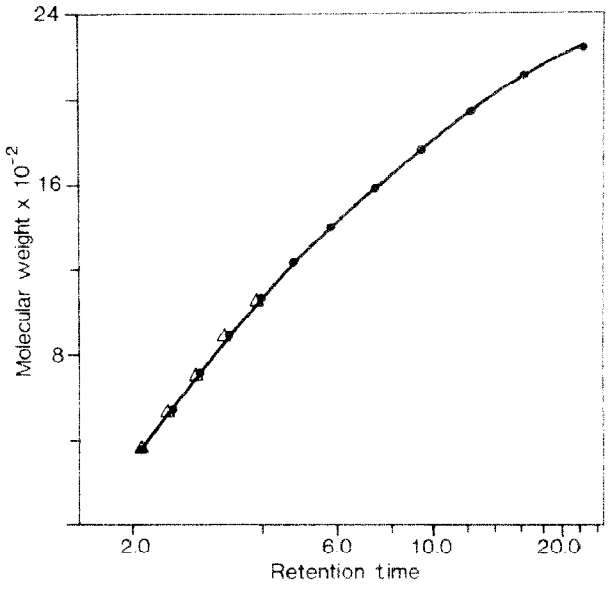

Fig. 3. Relationship of retention time and molecular weight of unsaturated oligomers separated by reverse-phase ion-pair h.p.l.c. The column was developed with a flow rate of 2.4 mL/min. Open triangles identify reference oligomers analyzed by f.a.b.-m.s. Closed circles identify oligomers generated as reaction product by incubation for 2.17 h with PL fraction C and resolved in the h.p.l.c. profile shown in Fig. 2c.

Kinetic comparisons of pectate lyases by h.p.l.c.. — With the WISP automatic sample injector set to inject a sample every 30 min and the flow rate set at 2.4 mL/min, a condition was provided to resolve and quantify the detectable products prior to a second injection. A series of injections could then be delivered over time to provide a kinetic analysis of the individual products formed during the reaction. Kinetic comparison of two purified activities is presented in Fig. 4. Fraction B (Fig. 4a) produced trimer at the greatest rate, with lesser rates of formation of tetramer, pentamer, hexamer, and eventually some dimer. The lag observed for the formation of dimer matched the point

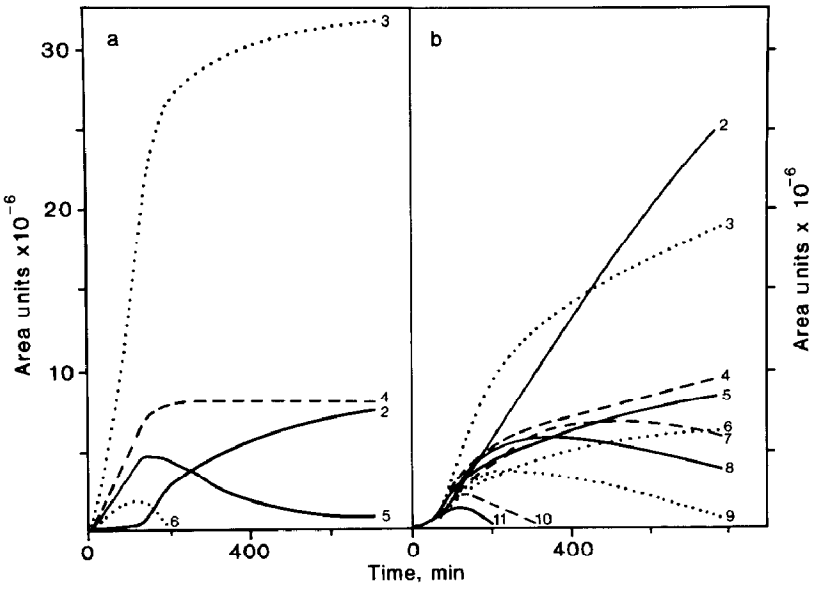

Fig. 4. Kinetic comparison of purified pectate lyases by h.p.l.c. Conditions for the assay were the same as those described for Fig. 2. Numbers beside each curve identify the d.p. value for each product. (a) Profile for fraction B. (b) Profile for fraction C.

at which the pentamer began to decline and indicates a relatively slow conversion of pentamer. The tetramer appears as an early limit-product that is not further depolymerized. Both the predominant product, trimer, as well as dimer, are formed as additional limit-products. The early generation of pentamer and hexamer, along with trimer and tetramer, indicate an endolytic process. The absence of production of larger oligomers as compared to fraction C (Fig. 4b) suggests that this process is not random. An initial rate of formation of trimer was estimated at 0.074 nmol per min; a maximum rate between 100 and 150 min of 0.148 nmol per min was observed. This increase of rate with time suggests that early endolytic processing provides substrate for subsequent exolytic processing, and may reflect the presence of more than one enzyme.

Fraction C (Fig. 4b) showed a much different pattern, with the early and rapid formation of trimer as well as other oligomers, which could be quantified up to a d.p. value of 11. The formation of dimer lagged behind that of the trimer, but proceeded at a constant rate, overtaking that of the trimer after 450 min. Oligomers having d.p. $\geqslant 7$ were all degraded during the later stages of the depolymerization process. The decrease in the rate of formation of trimer after ~ 200 min suggests that it may be preferentially formed from oligomers having d.p. values > 7, which also decrease with time. Alternatively, the trimer may be generated by an endolytic mechanism while the dimer is formed by a slower, exolytic mechanism.

DISCUSSION

As previously shown by Voragen *et al.* ref. 13, reverse-phase ion-pair h.p.l.c. with C_{18} provides an effective way to resolve oligomers generated as products of pectate lyase activities. Here we have shown that there is a nearly linear relationship between the molecular weight and the logarithm of the retention time, similar to that previously observed for the products generated from alginate by an alginate lyase[17]. The increased resolution of oligomers with increasing size provides a contrast to that observed for gel filtration, and offers a potential preparative method for the production of quantities of the larger oligomers.

Coupled with the WISP automatic sample-injection system, this h.p.l.c. system provides a convenient and powerful method for distinguishing pectate lyases on the basis of unique depolymerizing mechanisms. As many as five pectate lyases secreted by different strains of *Erwinia chrysanthemi* have been identified as unique gene products[22-24], four of which have been cloned and compared on the basis of DNA sequences[25]. Studies on these enzymes have classified them all as endolytic on D-galacturonan with some differences noted in the pattern of products resolved by paper chromatography[9,10]. The h.p.l.c. method described here clearly establishes that fractions B and C are different not only with respect to mechanism, but also in limit product composition. The action pattern of fraction B suggests a predominantly exolytic mechanism with the formation of trimer > tetramer > pentamer > hexamer. However, unsaturated oligogalacturonates with d.p. 7–10 could be detected in the very early stages of the reaction (15 min incubation) but could not be quantified due to their low concentrations. Pectate lyase PLb secreted by *Erwinia chrysanthemi* EC16 depolymerizes D-galacturonan with a similar action pattern[19] and was also active in increasing permeability and macerating potato tuber tissue[10]. It is therefore likely that this enzyme possesses endolytic properties necessary to attack the D-galacturonan within the rhamnogalacturonan complex of the plant cell wall, and may contain an endolytic as well as an exolytic type of mechanism indicated by the action pattern. The action pattern of fraction C indicates a random endolytic attack on the D-galacturonan with respect to the generation of trimer through undecamer. However, the rate of formation of dimer suggests an additional exolytic mechanism in addition to this one, whereby dimer is formed from products generated from the initial endolytic attack. This method should prove useful in comparing different pectate lyases with respect to mechanisms on different substrates, both natural and artificial, and allow a better understanding of the function of the different enzymes on the pathogenic processes associated with *Erwinia* infections. It may also assist in the identification of specific pectate lyases that are most effective as elicitors of phytoalexins[26].

The automated h.p.l.c. system has been important in the analysis of eliminases involved in the depolymerization of galacturonan and alginate. This system should also find itself applicable to the kinetic analysis of eliminases acting on glycosaminoglycans, and has been so used[14,27], although without the application of the automatic sample-injector. It should be noted that the presence of enzyme in the injected sample did not affect the elution of products, nor did it significantly affect the life of the C_{18} column.

With occasional replacement of the RCSS precolumn cartridge, the 8MB C_{18} cartridge was used to analyze more than a thousand samples with negligible change in retention times. By using a lower wavelength, such as 210 nm, to monitor carboxyl groups, or using a refractive-index detector, it should be feasible to follow the depolymerization of glycuronans catalyzed by hydrolases as well.

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