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Kinetic comparisons of trimer-generating pectate and alginate lyases by reversed-phase ion-pair liquid chromatography*

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ABSTRACT

Reversed-phase ion-pair liquid chromatography has been applied to the kinetic analysis of trimer-generating pectate lyases secreted by *Clostridium populeti*, *Lachnospira multiparus*, and two different strains of *Erwinia chrysanthemi*. A trimer-generating alginate lyase secreted by a marine bacterium was also analyzed and compared. A common feature of these enzymes is the initial formation of trimer, tetramer, pentamer, and larger oligouronates. Dimer can be formed from the later eliminative cleavage of pentamer and larger oligouronates. A general depolymerizing process involving an endolytic, followed by an exolytic mechanism, has been ascribed to these enzymes, with subtle differences noted with respect to the quantities of products formed with time.

INTRODUCTION

Bacteria which depolymerize pectate and alginate polymers associated with cell walls of land plants and algae generally secrete specific lyases which catalyze the cleavage of the glycosidic bonds through a β-elimination reaction. Plant pathogens belonging to the genus *Erwinia* initiate their phytopathogenic response *via* the secretion of pectate lyases which macerate the plant tissues to provide a soluble environment for their growth. In *Erwinia chrysanthemi* EC-16, four extracellular pectate lyases with isoelectric points ranging from 5.2 to 9.8 have been identified, each as a unique gene product[1]. Collectively, these enzymes provide three different depolymerization processes as determined by the kinetics of individual oligogalacturonates formed, and result in the rapid formation of dimers, trimers, and tetramers which may be assimilated and catabolized by the bacterium. These enzymes comprise a battery of endolytic and exolytic mechanisms that appear to be responsible for the strong macerating activity and the consequent pathogenic response[2].

In contrast to the mixture of lyases produced by pathogenic *Erwinia* sp., sapro-

* Abbreviations: PGA, polygalacturonate; TBAH, tetrabutylammonium hydroxide; Δ, unsaturated bond; PL, pectate lyase; AL, alginate lyase; d.p., degree of polymerization; h.p.l.c., high-performance liquid chromatography.

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phytic or commensal species, *i.e.*, *Clostridium populeti* and *Lachnospira multiparus*, secrete acidic pectate lyases with a more limited potential, forming trimer as the predominant product for subsequent uptake and catabolism[2]. Pectate lyase activity from enzymes secreted by *L. multiparus* catalyzes a depolymerization process similar to that of two of the pectate lyases secreted by *E. chrysanthemi* EC-16 as well as a single analogous activity secreted by another strain of *E. chrysanthemi*[2,3]. This same type of trimer-generating process was observed for the depolymerization of polymannuronate by a D-mannuronan-specific alginate lyase secreted by a marine bacterium associated with the brown algal species, *Sargassum fluitans*[4,5].

Here we present a comparative examination of the depolymerization processes catalyzed by these pectate and alginate lyases, using reversed-phase ion-pair high-performance liquid chromatography to detect and quantify individual oligouronates as a function of time.

EXPERIMENTAL

Bacterial cultures. — *Erwinia chrysanthemi* strain P86021 (here designated as EC-PI) was isolated from *Anthurium* species and identified by T. Shubert (Division of Plant Industries, University of Florida). Cultures were grown with shaking on a minimal salts medium containing 0.5% polypectate[3]. The gene coding for PLb from *Erwinia chrysanthemi* EC-16 was obtained on plasmid pPEL344[1] and used to transform *E. coli* HB 101[6]. Enzyme PLb was then expressed in *E. coli* cultured with shaking to stationary phase on Luria broth containing ampicillin[7]. *Clostridium populeti*, isolated as a pectinolytic and cellulolytic culture from a wood-based (poplar) methane digestor, was grown as standing cultures to stationary phase under CO_2:N_2 (2:8) on a defined medium containing 0.5% polypectate[8,9]. *Lachnospira multiparus* strain D15d was grown to stationary phase as for *C. populeti*. The gene from the Alg A *Sargassum* isolate coding for the D-mannuronan-specific alginate lyase was expressed in *E. coli* TC-4 following transformation with pAL-A3[5,10]. Transformed cultures were grown to late exponential phase on Luria broth containing ampicillin.

Enzyme and substrate preparations. — Extracellular pectate lyases (EC 4.2.2.2) were purified from media which had been partially dialyzed against appropriate assay buffer (see below) and concentrated on a Model CH2PRS Amicon Diaflo hollow-fiber concentration/dialysis unit fitted with an Amicon H1P10-43 filter. Further concentration to 50 mL was completed with an Amicon Model 8400 ultra-filtration cell equipped with a PM10 membrane.

The PL from EC-PI was purified from the concentrated medium by precipitation with ammonium sulfate (between 60 and 90% saturation) and chromatofocusing. The activity fraction which eluted at pH 8.3 after chromatofocusing (designated CF-B) showed an activity band following thin-layer isoelectric focusing (i.e.f.) with a pI of 8.65 and a single protein band corresponding to 37.2 kD following SDS-PAGE[3]. The PL from *C. populeti* was partially purified from concentrated medium following anion exchange (DEAE-Spectragel M) and gel filtration (Sephacryl-300), and gave a single

activity band after thin-layer i.e.f. with a pI of 4.8. The PL from *L. multiparus* was partially purified by anion-exchange chromatography and gave two adjacent activity bands after thin-layer i.e.f. with pI values of 4.7 and 4.8. The PL from *E. chrysanthemi* EC-16 and the AL (EC 4.2.2.3) from the *Sargassum* associated bacterium AlgA isolate were each expressed as single gene products in *E. coli* and purified from the periplasmic fractions[11].

Polygalacturonate (Pfaltz and Bauer) for pectate lyase substrate was prepared by the concentration dialysis method described previously[3]. D-Mannuronan was prepared from *Macrocystis* alginate (Fisher Scientific Co., purified grade) to give preparations containing 10–15% D-guluronate, as previously described[4].

Chromatographic analysis. — Reversed-phase ion-pair h.p.l.c. was carried out as described in the preceding paper[3]. Kinetic analyses were accomplished with the programmed injection of a fixed volume (0.100 mL or 0.050 mL) of reaction mixture using a Waters WISP automatic sample injector. For PL assays, reaction mixtures contained 0.1% PGA in 50mM Tris-HCl (pH 8.2) and 0.2mM $CaCl_2$. AL assay reaction mixtures contained 0.1% D-mannuronan in 30 mM sodium phosphate buffer, 50 mM KCl, pH 7.6. Reactions were initiated with the addition of enzyme to give a 4.0-mL reaction mixture, filtered through Millex GV 0.22 μ filters (Waters/Millipore), and split to provide one sample for h.p.l.c. analysis and a separate sample for continuous spectrophotometric analysis. Chromatographic analyses were made at room temperature. Unsaturated oligogalacturonate standards ranging from d.p. 2–6 were prepared and used to standardize the column for PL analyses as described in the preceding paper[3]. Unsaturated oligomannuronate standards ranging from d.p. 2–5 were prepared and used to standardize the column for AL analysis as previously described[4]. Continuous h.p.l.c. kinetic profiles were obtained by fitting data points to curves using a Slide-Write-Plus program (Advanced Graphics Software, Inc., Sunnyvale, (CA) run on an IBM PS/2 50Z desk-top computer.

Spectrophotometric assay. — Both PL and AL activities were measured on the basis of unsaturated residues formed per minute at room temperature by detection on a continuously recording spectrophotometer (Gilford Model 2400). A molar absorptivity value of 4600M^{-1} cm^{-1} at A_{235} was used to determine the rate of double-bond formation[12]. A unit of enzyme activity is defined as 1 nmole of unsaturated residue formed per min at 25°.

RESULTS

Comparison of products formed early and late in the depolymerization process. — The h.p.l.c. system resolved unsaturated oligogalacturonates ranging from dimer to decamer within 15 min at a flow rate of 1.0 mL/min. Fig. 1 shows the elution profiles for these products formed in 35 min (0.6 h) and 50 h for the selected PLs. Standards of unsaturated di-, tri-, and tetra-oligogalacturonates provided retention times of 4.80, 5.33, and 5.96 min, respectively, for the chromatographic conditions employed here. The assignment of size to a given A_{235} peak was made on the basis of the relationship

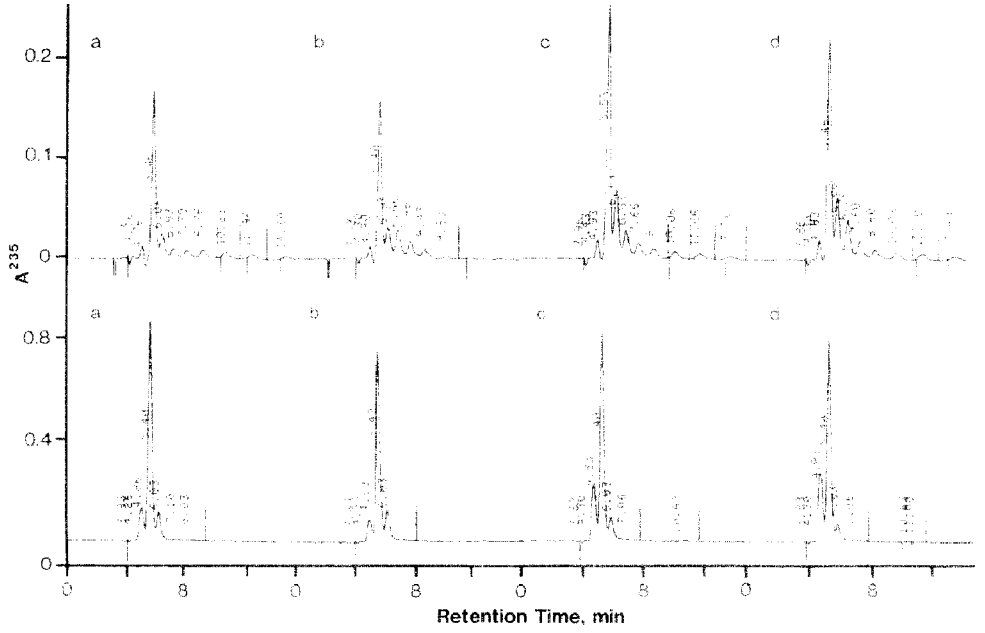


Fig. 1. H.p.l.c. profiles of early (0.6-h) and near limit (50-h) reactions catalyzed by different PLs. Each reaction was initiated with the addition of PL activity to 13 units/mL. Eighteen × 0.05mL samples were injected at 17.3-min intervals and subjected to reversed-phase ion-pair h.p.l.c. at a flow rate of 1 mL/min. Later injections were made at specific times. The upper series of profiles represent 0.6-h incubations, and the lower series of profiles represent 50-h incubations. (a) PL from *E. multiparus*; (b) PL from *C. populeti*; (c) PL-b from cloned pPEL344 from *E. chrysanthemi* EC-16, expressed in *E. coli*; (d) PL-CFB from *E. chrysanthemi* PI.

observed between the logarithm of the retention time and the molecular weight[3]. In the earliest time of analysis, *e.g.*, a 17-min reaction time, very small amounts of oligomers corresponding to undecamer and larger were detected, but these were present below the level which could be quantified (not shown). By the end of the 35-min reaction time, these products had completely disappeared, leaving the decamer (retention times ranging from 13.7 to 14.2 min with different analyses) as the largest detectable oligomer. Nearly equal molar equivalents of oligouronates ranging from hexamer to decamer observed for 0.6-h reactions indicate a random endolytic process, at least for the PLs from *E. multiparus* (a), *E. chrysanthemi* EC-16 (c), and *E. chrysanthemi* PI (d). Each of the PLs examined, however, produces trimer in the largest quantity and very small amounts of dimer in the early portions of the reaction. After 50 h of incubation, the larger oligomers have disappeared, with the accumulation of trimer and lesser amounts of dimer and tetramer. For both the 0.6-h and the 50-h times presented here, the depolymerization processes for the two *E. chrysanthemi* enzymes appear essentially identical. The depolymerization processes depicted for *E. multiparus* and *C. populeti* are similar but not identical with those for the two *E. chrysanthemi* strains, possibly due to different rates of depolymerization of the larger oligomers generated during the early portions of the reaction.

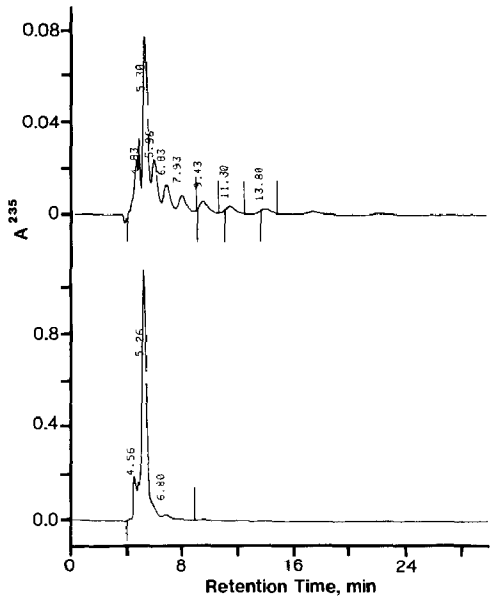
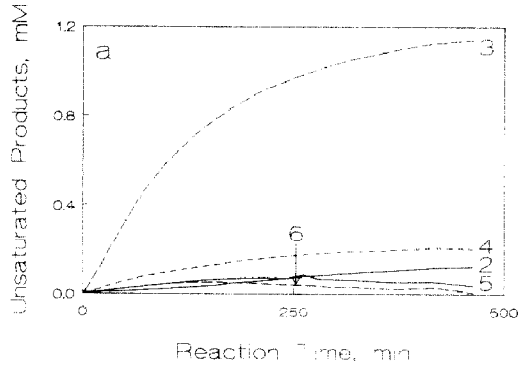


Fig. 2. H.p.l.c. profile of early (0.6-h) and near limit (24-h) reactions catalyzed by D-mannuronan-specific AL from AlgA marine bacterium. Enzyme was expressed in *E. coli* transformed with pAL-A3 plasmid and obtained from the periplasmic fraction. The reaction was initiated with the addition of 4.25 units/mL of AL. The kinetic analysis was performed as for Fig. 1, with the injection of 0.1-mL samples every 33 to 34 min. The upper panel is a profile of a 0.6-h reaction. The lower panel is a profile of a 24-h reaction.

Depolymerization of the D-mannuronan for 0.6 h by the pAL-A3 alginate lyase provided a profile (upper panel, Fig. 2) as was found following 0.6 h of reaction catalyzed by the PLs (Fig. 1). Standards of unsaturated trimer, tetramer, and pentamer D-mannuronans eluted with retention times of 5.26, 5.93, and 6.80 min, respectively. The relationship between logarithm retention time (ordinate) and molecular weight (abscissa) provided a nearly linear relationship with a slope slightly greater than that found for the analogous relationship for the unsaturated D-galacturonans (data not shown). Consequently, the retention time projected for the D-mannuronan nonamer was nearly the same as that projected for the retention time of the decamer of the D-galacturonan. The rate of formation for trimer is the greatest followed in series from tetramer to nonamer, with lower amounts of the higher molecular weight oligomers which could not be quantified. After 24-h incubation the pAL-A3 AL had converted all of the products to trimer, dimer, small amounts of pentamer (retention time of 6.80 min), and possibly some tetramer appearing as a trailing shoulder to the trimer peak.

Continuous kinetic profiles for the different enzymes are given in Fig. 3. Each of the PLs as well as the pAL-A3 AL show depolymerization profiles with the following common features: (*i*) trimer is the predominant product throughout the course of the reaction; (*ii*) tetramer is formed early in the reaction, but at a rate less than a third that of the trimer; (*iii*) oligouronates with d.p. ≥ 5 are formed early in the reaction and then disappear; (*iv*) the formation of dimer occurs at a rate that nearly matches the disappear-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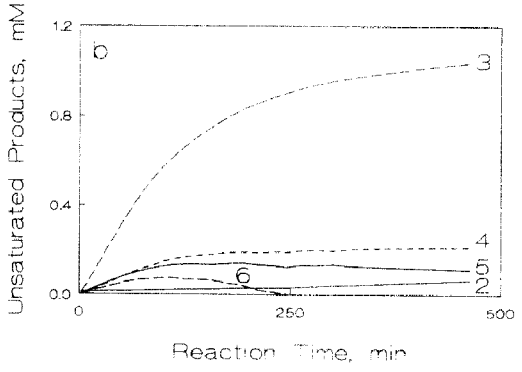
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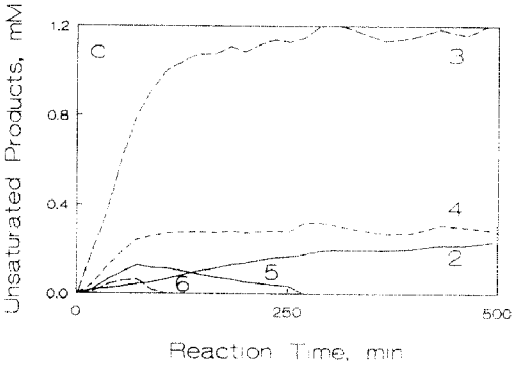
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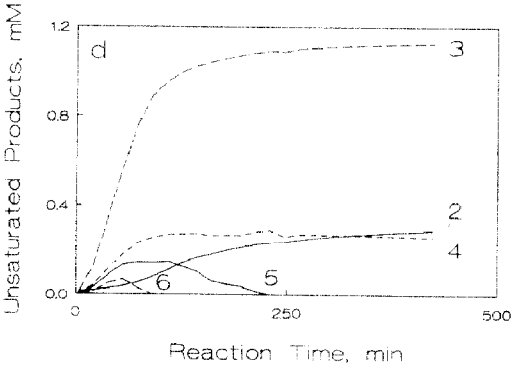
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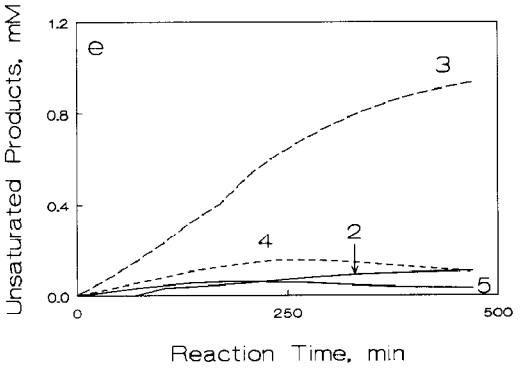

Fig. 3. Kinetic comparisons of trimer-generating pectate lyases and D-mannuronan specific AL. Conditions for the analyses are described in the legend to Fig. 1. Points taken at 15- or 30-min intervals were used to develop the curves using computer software described in the text. A number beside each curve identifies the d.p. value for each product: (a) PL from *L. multiparus*, (b) PL from *C. populeti*, (c) PLb from cloned pPEL344 from *E. chrysanthemi* EC-16, expressed in *E. coli*, (d) PL CFB from *E. chrysanthemi* PI, (e) AL from cloned pAL-A3 from marine bacterium AlgA, expressed in *E. coli*.

ance of pentamer. Each of the enzymes evaluated here show a common depolymerization process, with the rate of formation of trimer > tetramer > pentamer > hexamer, and the formation of dimer occurring through the depolymerization of pentamer. In all of the PLs assayed in the present study, the tetramer accumulates as an apparent limit product over the 500-min course of the reaction. The AL profile (Fig. 3e) shows an early formation of tetramer which later disappears, presumably through its conversion to smaller oligomers.

To identify limit products formed by the different PLs, the reactions were incubated at room temperature and assayed at 24 and 50 h. The relative activity after incubation was determined by adding fresh substrate (0.1% PGA) to a five-fold dilution of the reaction mixture and following the formation of total unsaturated residues by continuous recording spectrophotometry at 235 nm. As shown in Table I, each enzyme preparation showed diminished activity in the 50-h reaction mixture (V_{50}), ranging from 52 to 69% of the initial activity at the start of the reaction (V_0). Nevertheless, the activity remaining after 50 h was substantial and sufficient to form products representing true limits of the reaction. By 50 h most (82 to 92%) of the PGA substrate could be accounted for in the unsaturated products detected in the h.p.l.c. profile. Individual unsaturated oligogalacturonate products were measured by h.p.l.c. at 24 and 50 h, and reflect an approach to the limit products formed by each enzyme. Both dimer and trimer appear to be limit products. For the PL reactions from the two *E. chrysanthemi* strains and *L. multiparus*, the tetramer was slowly depolymerized with time. The PLs from the two *E. chrysanthemi* strains show an increase in dimer with a nearly concomitant decrease in tetramer, suggesting an ability of these enzymes to slowly convert tetramer to dimer.

TABLE I

Unsaturated oligogalacturonates accumulated during pectate lyase-catalyzed approach to limit products

PL source	V_0[b]	V_{50}[b]	*Percent conversion*[c]	*Unsaturated oligomers, d.p. 2–6, mM*[a]									
				24 h					50 h				
				2	*3*	*4*	*5*	*6*	*2*	*3*	*4*	*5*	*6*
L.m.	13.0	6.8	89	0.19	1.14	0.26	–	0.03	0.23	1.23	0.21		0.01
C.p.	13.0	6.8	82	0.10	1.05	0.23	0.10		0.16	1.12	0.25	–	–
EC-16	12.5	8.2	92	0.29	1.11	0.27	0.01	–	0.43	1.20	0.18		
EC-PI	11.8	8.2	92	0.36	1.09	0.22	–	–	0.51	1.18	0.14	0.02	

[a] Unsaturated oligogalacturonates with d.p. from 2–6 were quantified as area units from a 0.05-mL injected sample, which were converted to nmoles by comparison with a trimer standard and corrected to concentration in the reaction mixture. [b] Initial velocities as nmol unsaturated product min^{-1} mL^{-1} of enzyme solution were determined on the recording spectrophotometer at the start of the reaction (V_0) at the same time the h.p.l.c. analyses were initiated. V_{50} values were apparent initial velocities determined with 0.2 dilutions of the reaction mixtures (incubated for 50 h and analyzed by h.p.l.c.) which were reinitiated with the addition of fresh PGA to 0.1% concentration. [c] Percent conversion for the 50-h reaction of initial PGA substrate was estimated from the total weight of the individual oligogalacturonates quantified by h.p.l.c.

DISCUSSION

The reversed-phase ion-pair h.p.l.c. system has been useful for resolving and quantifying individual unsaturated oligogalacturonates generated by the eliminase reaction[13,14]. In the system used here, a consistent relationship may be obtained between the logarithm of the retention time and the molecular weight of the oligouronates, thus providing a convenient way of assigning a size identity to a depolymerization product using a few standards to calibrate this relationship[3]. The use of an automatic sample injector with an h.p.l.c. delivery system and a u.v. detector to follow absorption at 235 nm has been applied to the kinetic comparisons of both alginate and pectate lyases[3,4]. Of the different ALs and PLs analyzed by this technique, enzymes with different depolymerization mechanisms have been distinguished, ranging from randomly endolytic to predominantly exolytic. The predominantly exolytic reaction is typified by an L-guluronan-specific AL secreted by a marine bacterium[13], while the randomly endolytic mechanism is typified by one or more of the enzymes secreted by the plant pathogen, *E. chrysanthemi*[2,3].

A D-mannuronan-specific AL from a marine bacterium and one or more PL secreted by *E. chrysanthemi* have been distinguished from the exolytic and randomly endolytic enzymes in that they generate large amounts of trimer with the transient formation of pentamers and larger oligomers[2,4]. Here we have examined the kinetic profiles of these enzymes, cloned and expressed in *E. coli*, and compared them to the PLs secreted by the phytopathogenic (*E. chrysanthemi*) and saprophytic (*C. populeti* and *L. multiparus*) bacteria. Our results indicate that each of these organisms secretes at least one enzyme that is trimer generating with a potential endolytic/exolytic mechanism.

It could be argued that these enzymes are strictly endolytic with a processive mechanism that favors the sequential release of trimer once the enzyme has bound and initiated an endolytic glycosidic cleavage. However, such an argument is not supported by the observations reported here. For the *E. chrysanthemi* PLs examined here, there is an increase in the rate of trimer formation with time, suggesting that initial endolytic cleavage in fact provides substrate to which enzyme binds to continue the depolymerization through an exolytic type of reaction. The transient formation of oligouronates with d.p. ≥ 5, the early and continued formation of trimer and tetramer, and the absence of dimer formation in the initial stage of the reaction, would best be explained by two separate processes. The first would be the endolytic attack on the polymer, a requisite reaction when that polymer is included in a complex polysaccharide like rhamnogalacturonan or alginate. The second reaction would involve an exolytic mechanism with the enzyme binding to the nonreducing terminus of substrate and cleaving most frequently at the third, less frequently at the fourth, and rarely at the second glycosidic bond. Most of the dimer which appears somewhat later could be derived from the cleavage of trimer from unsaturated pentamer formed in the early portion of the depolymerization process.

Additional studies with defined oligouronates, with or without modification at the nonreducing termini, are needed to provide definitive data discerning the require-

ments for enzyme binding. A biological justification for the proposed endolytic/exolytic mechanism would allow that oligomers released from the endolytic cleavage of complex polymers would be further degraded by an exolytic mechanism to generate trimer, tetramer, and dimer as products which could then be assimilated and catabolized to provide carbon and energy for growth.

The common depolymerization processes ascribed to the AL and PLs examined here show subtle if not dramatic differences with respect to the mix of products formed at a given time. While the enzymes may catalyze the same basic reactions, and even share a common active site, they may have different affinities for different oligomers derived from the depolymerization of polyuronate substrates. The structural gene for the *E. chrysanthemi* EC-16 PLb enzyme has been cloned and sequenced and provides a reference for comparison with the other genes coding for the other enzymes. The structural gene for the D-mannuronan-specific AL has also been cloned and will be sequenced for comparison. Through these approaches, it should be possible to define the relationship of specific protein structure and a depolymerization mechanism which is common to several different bacteria.

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