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LIQUID-CHROMATOGRAPHIC ANALYSIS OF THE DEPOLYMERIZATION OF (1→4)-β-D-MANNURONAN BY AN EXTRACELLULAR ALGINATE LYASE FROM A MARINE BACTERIUM

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ABSTRACT

The extracellular alginate lyase activity from a fermentative marine bacterium isolated from actively growing tissues of *Sargassum fluitans* has been purified and studied with respect to substrate specificity and mechanism. The enzyme endolytically depolymerizes (1→4)-β-D-mannuronan derived from alginate to oligomeric products possessing 4,5-unsaturated, nonreducing termini. Reversed-phase liquid chromatography has established that early in the reaction the tri-, tetra-, and pentameric oligomers are the predominant species. The pentamer and larger products that at first accumulate in the reaction are later degraded to smaller products. The trimer is the major product late in the reaction, at which time the dimer and tetramer are also present in significant amounts. By incubating purified oligomers with enzyme, the trimer is shown to be completely refractory to further depolymerization and therefore represents a limit product of the reaction catalyzed by this enzyme. The tetramer is slowly converted into trimer and monomer, whereas the pentamer is readily converted into trimer and dimer.

INTRODUCTION

Enzymes capable of degrading alginate are almost exclusively lyases, which catalyze depolymerization by eliminative cleavage of the (1→4)-glycosidic bonds, leaving 4,5-unsaturation at the newly generated, nonreducing terminal groups[1]. Such enzymes, generally referred to as alginate or alginolyases (EC 4.2.2.3), have been shown to be produced by marine and terrestrial bacteria[2–7], fungi[8], and marine invertebrates[9–11]. A lyase activity capable of degrading alginate has also been found in the brown algal species *Laminaria digitata*[12]. The arrangement of the constituent uronic acids of alginate, β-D-mannopyranosiduronic acid and α-L-gulopyranosiduronic acid residues in homopolymeric, poly(ManA) and poly(GulA), and heteropolymeric block-regions[13,14], confers unique physical properties to alginate[15,16]. These regions are recognized differentially by alginate lyase enzymes, which show preferences for either purified poly(ManA) or poly(GulA).

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Alginate lyases have been sought as specific probes to study the structure of soluble alginates[17–19] and polymers contributing to the structure of the cell walls of the brown algae[20], and have recently been evaluated for the production of protoplasts from *Sargassum* species[21,22]. Information about the substrate specificities and the modes of substrate cleavage by alginate lyases is valuable in defining their catalytic properties, and therefore their potential uses. Previous studies on the mechanisms of alginate depolymerization have included viscometric analyses[5,11,23], and the characterization of enzyme-generated products utilizing a number of methods including gel filtration[6,24], ion-exchange column chromatography[1,25], paper chromatography[1,5,25,26], and paper[17,26] and gel electrophoresis[7,20].

We have previously described the isolation and characterization of alginate lyase-producing oxidative and fermentative marine bacteria associated with actively growing tissues of marine brown algae, genus *Sargassum*[2,22]. One of the facultative anaerobes, isolate A, was shown to produce extracellular lyase activity that was specific for poly(ManA) versus poly(GulA) and endolytic in its action on alginate. The intracellular activities of this bacterium depolymerized both poly(ManA) and poly(GulA) and, in comparison with the extracellular preparation, showed a greater ratio of bond cleavage to increase in fluidity with native alginate, suggestive of exolytic as well as endolytic mechanisms. Here we describe a method, based upon l.c. separation of small oligomers, which has provided a kinetic evaluation of the depolymerization of poly(ManA) and has defined the limit products generated by the poly(ManA)-specific, extracellular enzyme.

EXPERIMENTAL

Materials. — Chemicals were analytical grade except as indicated. Acetonitrile (Fisher Scientific) and tetrabutylammonium hydroxide (Fisher Scientific and Sigma Chemical Co.) were HPLC grade. Commercially available, electrophoresis-grade reagents were used for electrophoretic analyses. All aqueous solutions were prepared with water which was deionized and distilled from glass.

Preparation of substrates. — Sodium alginate was purchased from Fisher Scientific Company as a purified grade originally isolated from *Macrocystis*. Poly(GulA) and poly(ManA) were obtained from HCl-hydrolyzed alginate following the methods developed by Haug *et al.*[14]. These preparations were further fractionated on Sephadex G-50 with 0.5M NaCl as eluent, and selected fractions were analyzed for reducing termini[27] and total carbohydrate[28,29] to obtain substrates of uniform size. Both ^{1}H- and ^{13}C-n.m.r.[30,31] analyses were carried out to assess the purity of substrates. Poly(ManA) preparations contained 10–15% guluronate, and poly(GulA) contained <5% mannuronate.

Enzyme assays. — Alginate lyase activity was quantified by spectrophotometric determination at 548 nm of the chromophore formed upon reaction of thiobarbituric acid with periodate-treated products[1,32], a specific measure of unsaturated, nonreducing termini of oligomeric products and the unsaturated monomer, 4-deoxy-L-*erythro*-5-hexosulosuronic acid. Substrate mixtures contained

either 0.1% alginate, poly(GulA), or poly(ManA), and 0.05M KCl, buffered with 0.03M sodium phosphate at the desired pH. Enzyme was mixed with 9 volumes of substrate to start the reactions, and reactions were terminated after 10 min by addition of aqueous periodic acid. One enzyme unit is defined as the amount of activity that catalyzes the formation of 1 nmol of nonreducing termini and/or monomer at pH 7.5 in one min at 22°. Protein was routinely estimated by absorbance at 280 nm. For more-quantitative measurements, the assay of Bradford[33] was utilized with bovine serum albumin as a standard protein.

Enzyme isolation. — The bacterium used in this study was obtained from healthy, apical tissue of *Sargassum fluitans*, and initially identified as an organism that secretes alginate-degrading activity, based on the appearance of extensive clearing zones surrounding colonies grown on solid alginate medium. The organism used for the work described here has been designated isolate A (complete designation SFFB080483A), and has been described[2]. Biochemical and morphological properties of this bacterium suggest its assignment to the genus *Photobacterium*, although its DNA has a G/C ratio of 0.454, somewhat greater than that of other species currently included in this genus. The organism has been maintained by monthly transfer on solid alginate medium[2].

For enzyme isolations, the organism was grown in 0.1% liquid alginate medium[2] with rapid gyrotory shaking at room temperature. In all subsequent purification steps, the extracellular and intracellular preparations were kept at ~4°. Bacterial cells were harvested at late exponential phase by centrifugation at 10,000*g* for 10 min, washed twice with water by resuspension and centrifugation, frozen in liquid nitrogen, and stored at −70°. The spent medium was concentrated, and dialyzed against distilled, deionized water by tangential-flow filtration, using a Millipore Pellicon cassette system with a polysulfone membrane, which allowed retention of proteins larger than 10,000 mol. wt.

For extracellular-enzyme preparations, the alginate lyase activity was precipitated together with remaining alginate products by dropwise addition of 10% polyethyleneimine to concentrated medium while stirring on ice. Prior to use, the polyethyleneimine (50% aqueous solution, Sigma Chemical Co.) was diluted with water, titrated to pH 7.5 with 12M HCl, and centrifuged at 10,000*g* for 10 min to remove insoluble particles. The relative volume of polyethyleneimine necessary for maximal precipitation of enzyme was found to be critical, and was determined for each batch of enzyme. Typically 1 mL of 10% polyethyleneimine would yield maximal precipitation of enzyme from 125 mL of concentrated medium derived from 10 μL of spent medium. The precipitate was collected by centrifugation at 10,000*g* for 15 min and resuspended in distilled, deionized water using a Potter–Elvehjem homogenizer driven by a variable-speed motor. The resulting suspension was centrifuged and the pellet homogenized in 0.25M NaCl, 0.1M sodium phosphate at pH 7.5, to elute the enzyme. The suspension was centrifuged for 2 h at 150,000*g*, and the supernatant solution was subjected to gel-permeation chromatography on Sephacryl S-200 (2.5 × 133 cm) with 0.1M sodium phosphate at pH 7.0. To obtain

protein concentration sufficient for preparative digestions and for quantification with the Bradford assay, fractions were concentrated by ultrafiltration using an Amicon cell with a YM 10 filter (10,000 mol. wt. cutoff).

Electrophoresis. — The method for native gel-electrophoresis was described by Shuster[34], and used the discontinuous buffer-system of Davis[35]. The running gel had a final concentration of 7.5% acrylamide and 0.2% bisacrylamide, was buffered at pH 8.9 with 0.38M Tris–HCl, and was polymerized with 0.07% ammonium peroxysulfate and 0.058% *N,N,N′,N′*-tetramethylethylenediamine. The stacking gel was composed of 2.5% acrylamide, 0.5% bisacrylamide, 0.062M pH 6.8 Tris–HCl, and was polymerized with 0.058% *N,N,N′,N′*-tetramethyleneethylenediamine and 0.01mM riboflavin phosphate, using a fluorescent light to activate the polymerization process. The running buffer was composed of 0.3% Tris base, 1.44% glycine at pH 8.9. Vertical slab-gels were 0.15 cm thick and were subjected to electrophoresis at 30 mA per gel until the bromophenol tracking-dye had reached the end of the gel. The gels were cut into 0.5-cm slices, which were incubated at room temperature with 200 μL of sodium alginate pH 7.5 substrate mixture. Following the incubation, 100 μL of the solutions were withdrawn and assayed for products of the lyase reaction.

Analysis of alginate lyase-generated products. — Analytical chromatographic separation of products was accomplished by ion-paired, reversed-phase l.c. using a system that is a modification of that developed by Voragen *et al.*[36] to fractionate pectate products. The column was a C_{18} μBondapak 8MB 10 μm column housed in a Z-Module radial-compression system (Waters). A Rainin 0.5-μm stainless-steel filter and a Waters RCSS Guard-PaK C_{18} prefilter cartridge were positioned between the column and the injector. The column was run isocratically at room temperature with 10% acetonitrile–10mM tetrabutylammonium hydroxide–0.1M sodium phosphate buffered at pH 6.5. Unsaturated oligomers were detected by monitoring u.v. absorbance of the effluent from the column at 230 nm with a Gilson Holochrome variable-wavelength detector equipped with a 1.0-cm flow cuvette. A waters Tri-Mod system was used for programming a 6000A pump, integration of peak areas, and for automated injection of samples onto the column. A Waters U6K injector was used for manual injection of samples.

Preparative fractionation of lyase products was performed by gel filtration with a column (2.5 × 133 cm) of Biogel P-2 eluted with 0.1M NH_4HCO_3, collecting 5.8 mL per tube. For a successful isolation of the products, 110 mg of poly(ManA) was dissolved in a 1.6-mL solution of the extracellular enzyme (900 units/mL) buffered at pH 7.0 with 0.1M sodium phosphate, and incubated for 12 h at room temperature. Under these conditions, the digestion was not complete at the time the mixture was applied to the P-2 column, allowing some of the larger oligomeric mannuronans to be obtained. Absorbance at 230 nm was determined for each tube.

Contents of tubes comprising each peak from the P-2 column were pooled, lyophilized, and stored at −20° over anhydrous calcium sulfate. The lyophilized products were dissolved in 0.1M sodium phosphate buffer, pH 7.0, and were

analyzed for unsaturated, nonreducing termini by measuring thiobarbituric acid-reactive material generated by periodate oxidation[1,32], with 3-deoxy-D-*manno*-octulosonic acid (KDO, Sigma Chemical Co.) as the standard. For preparation of the KDO standard, the compound was desiccated overnight *in vacuo*. The chromophore generated by the reaction of thiobarbituric acid with the 3-formylpyruvate formed by periodic acid oxidation of the KDO was quantified spectrophotometrically according to Preiss and Ashwell[1] and according to Koseki *et al.*[37], and indicated that the sample was 90 and 85% pure, respectively. Total uronic acid content was measured by the method of Blumenkrantz and Asboe-Hansen[38] using D-mannurono-3,6-lactone (Sigma Chemical Co.) as the standard. Based upon the expected extinction at 520 nm for the chromophore from D-mannurono-3,6-lactone, the desiccated standard was 84% pure. Absorbance at 232 nm was measured in a 1.00-cm cuvette after diluting samples 200-fold with 0.01M HCl.

Samples of lyophilized fractions from the P-2 column effluent were sent to Triangle Laboratories, Inc., Research Triangle Park, North Carolina for fast-atom-bombardment mass spectrometry (f.a.b.–m.s.) under the direction of Ronald Hass. Analyses were performed on a VG 7070H mass spectrometer with a VG11-250 data system. The acceleration voltage was 3 kV for the trimer analysis and 2 kV for the other samples. An Iontech saddle-field ion-source was used with xenon as the bombarding species. The gun was operated at 7 keV with a discharge current of ~1.5mA. The samples were analyzed after dissolving in water, and applying 1–2 μL of the solution to 1-thioglycerol on the probe. The mass spectrometer was scanned at 5 s per decade of mass from 1200–100, at a mass resolution of 1000.

RESULTS AND DISCUSSION

Chromatographic and electrophoretic behavior of the extracellular activity. — The extracellular preparation from a fermentative marine bacterium, designated isolate A, was shown previously to be highly active on poly(ManA) and native alginate, but inactive or possessing only trace activity with poly(GulA)[2,22]. The preparation was endolytic with alginate as substrate, as shown by comparing the rate of bond cleavage with the increase in the reciprocal of specific viscosity, namely, specific fluidity. For purification and characterization of extracellular poly(ManA) lyases, the concentrated preparation was first treated with 10% polyethyleneimine to remove the partially degraded alginate that remained after dialysis of the medium. This procedure is effective in reducing the viscosity, allowing a greater quantity of enzyme to be applied to the gel-filtration column. When subjected to gel filtration on Sephacryl S-200, a single peak of lyase activity eluted at 0.67 column-bed volumes. The column allowed complete removal of remaining products derived from alginate, which appeared as thiobarbituric acid-reactive material eluting at 0.94 column-bed volumes, and separation of some of the contaminating proteins from the enzyme. The pooled enzyme-fractions represented recovery of 82% of the alginate-degrading activity loaded onto the

column. At this stage of purification, the specific activity of the pooled enzyme fraction was typically 7000 units per mg of protein with alginate as the substrate and the ratio of activities on poly(GulA) versus poly(ManA) was 0.14. Based upon denaturing polyacrylamide gel-electrophoresis (SDS-PAGE with 5 proteins as molecular-weight standards) and gel-filtration liquid chromatography (TSK 4000-SW column with 7 proteins as molecular-weight standards), the enzyme was shown to consist of a single polypeptide having an apparent mol. wt. of 29,000 (unpublished results).

Samples from individual tubes containing the extracellular alginate-lyase fraction that eluted from the Sephacryl S-200 column were subjected to native polyacrylamide gel-electrophoresis followed by detection of activity. The individual fractions comprising the peak of alginate lyase eluting from the S-200 column showed the same single-activity component, which migrated as a homogeneous band (data not shown), indicating that the extracellular fraction contained a single enzymic activity.

L.c. analysis of poly(ManA) depolymerization. — When the extracellular enzyme (14 units) from the S-200 column was incubated at room temperature with 5 mg of poly(ManA) in 0.5 mL of 0.1M sodium phosphate buffered at pH 7.0, unsaturated oligomers were produced that could be fractionated by l.c. Profiles generated after 15 min and 4 h of depolymerization are shown in Figs. 1a and 1b, respectively. At least six peaks were detected by absorbance at 230 nm, and five of these were sufficiently distinct to be integrated by the data-analyzing system. Individual oligomers, detected as A_{230} peaks (Fig. 1b), were designated numerically from 1–6 in the order of their elution with retention times (in min) of 5.87, 7.54, 10.00, 13.87, 19.83, and 29.29, respectively. When the depolymerization was monitored over a 30-h period (Fig. 2), several features of interest were noted. Component 1, and to a lesser extent component 2, exhibit initial lags in their rates of accumulation. Components 4 and 5 increased until the digestion had continued for 6 and 4 h, respectively, and thereafter decreased. Components 1, 2, and 3 never showed declines, although the rates of accumulation of 2 and 3 decreased at ~6 h, and the rate of accumulation of 1 began to decrease gradually at 6 to 10 h. The delays in appearance of the two smaller products, namely, 1 and 2, indicated that these compounds were generated to a significant extent from products that accrued from initial depolymerization reactions. The decrease in the concentration of the larger compounds, 4 and 5, after initial accumulation, suggested that these must be subject to depolymerization by the enzyme, and that their relative rates of formation and degradation determine the levels at any give time. The unsaturated monomer is not detected by absorbance at 230 nm because of tautomerization to the α-keto acid form[1]. Detection at 205 nm revealed a minor component that eluted prior to 1 (Fig. 1), and analysis by the thiobarbituric acid method of fractions collected from a reversed-phase separation of a preparative poly(ManA) digest also showed a minor component of reactive material eluting prior to component 1 (data not shown). This material, presumed to be the monomer, constituted <4% of the

products, including dimers to pentamers produced by the depolymerization of poly(ManA) catalyzed by this enzyme.

Purification and characterization of the reaction products. — In order to obtain sufficient quantities of the oligomeric products for characterization, 110 mg of poly(ManA) was digested with extracellular enzyme for 12 h and the products were resolved by chromatography on Biogel P-2 eluted with 0.1M NH_4HCO_3. Four components, measured by absorbance at 230 nm, were resolved from one another. These were eluted at column volumes of 0.46, 0.50, 0.56, and 0.64, and are designated as fractions 1, 2, 3, and 4, respectively. A small fraction, ~9% of the total absorbance at 230 nm, eluted between the void volume of the column and the leading edge of the peak designated as fraction 1. Chromatography in ammonium formate led to a similar profile; however, the lyophilization of these fractions resulted in discoloration of some of the fractions, and subsequent l.c. analysis demonstrated significant degradation. Products obtained after elution with NH_4HCO_3 were, after lyophilization, fluffy and white, although they were quite hygroscopic.

Samples of the peak tubes from the P-2 NH_4HCO_3 column were analyzed by l.c. (data not shown), which identified the contents of tubes 52, 57, 63, and 72 as the oligomers comprising peaks 4, 3, 2, and 1, respectively of the l.c. profiles (Fig. 1). Absorbance spectra in the u.v. range for samples diluted in 0.01M HCl showed maxima at ~232 nm (profiles not shown), typical of products generated by alginate lyases.

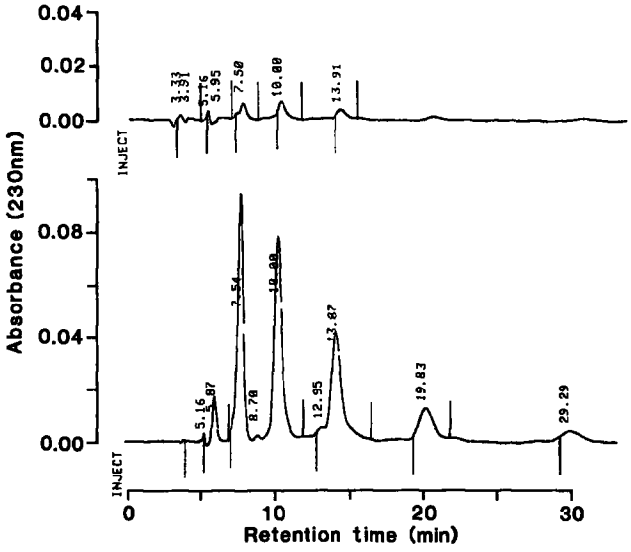

Fig. 1. Liquid-chromatographic analysis of products generated by digestion of poly(ManA) by extracellular lyase. The unsaturated oligomers produced from poly(ManA) by the extracellular enzyme were resolved by l.c. as described in the text. Sample volumes of 10 μL were delivered with automatic injection (Waters, WISP) and eluted isocratically at 1.0 mL/min. Profiles of the products that had accumulated after 15 min (a) and 4 h (b) are shown.

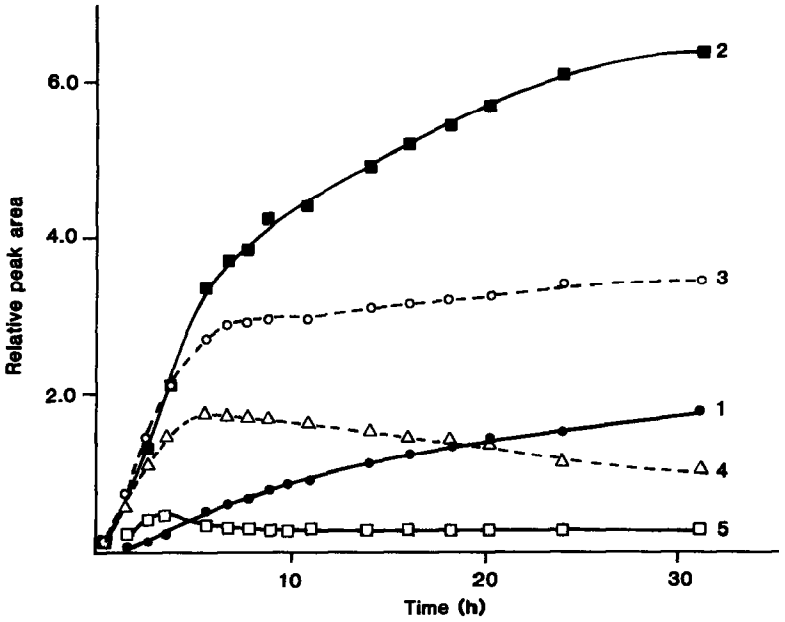

Fig. 2. Kinetic analysis of poly(ManA) depolymerization by extracellular alginate lyase. The depolymerization reaction described in Fig. 3 was sampled periodically over 30 h, and samples subjected to l.c. as described in the text. The peak areas of the major products are plotted against the times at which the reaction was sampled. The individual products are numbered according to their order of elution from the column: 1, fastest moving, representing a dimer, through 5, which represents a hexamer. The dimer peak integrates as 1.1×10^5 or 0.11×10^6 area units per nmol.

The pooled, lyophilized fractions from the P-2 column were further analyzed to establish the molecular sizes of the unsaturated products. Comparisons of the uronic acid content with content of nonreducing residues (Table I) allowed an estimation of the degree of polymerization (d.p.) of each product. The d.p. values estimated from the ratio of total uronic acid to nonreducing termini show a trend consistent with assigning the unsaturated oligomers in fractions 1–4 as the pentamer, tetramer, trimer, and dimer, respectively.

Based upon the concentration of the nonreducing, unsaturated terminal groups estimated by the thiobarbituric acid assay of the periodate-treated products, and the absorbance values at 232 nm of products in 0.01M HCl, an estimation of the molar absorptivities for the dimer to pentamer series, presumed to contain a single unsaturated residue in each molecule, ranged from 5,160 (Fr. 1) to 5,420 (Fr. 3) $M^{-1}cm^{-1}$. The concentrations of each component, based upon gravimetric preparations of lyophilized samples and calculated molecular weights of each as the ammonium salt, were calculated on the assumption that fractions 1, 2, 3, and 4 constituted the pentamer, tetramer, trimer, and dimer, respectively. These values, as divisors for the A_{232} values listed in Table I, led to calculated molar absorptivities that ranged from 3350 (Fr. 2 as the tetramer) to 3,890 (Fr. 1 as the pentamer). The

TABLE I

ANALYSIS OF UNSATURATED OLIGOMERS FROM LYOPHILIZED P-2 FRACTIONS[a]

Fraction no.	*Abs.*[b] *(232 nm)*	*Unsat.*[c] *termini (mM)*	*Uronic*[d] *acids (mM)*	*D.p. uronic acids/ unsat. termini*
1	39.2	7.59	40.5	5.3
2	42.0	7.94	37.5	4.7
3	64.0	11.8	36.3	3.1
4	84.6	16.2	45.6	2.8

[a]Lyophilized products from the Biogel P-2-fractionated, preparative poly(ManA) digest were obtained by methods described in the text and were dissolved in 0.1M sodium phosphate buffer, pH 7.0, to a final concentration by weight of 10 mg/mL. [b]Solutions were analyzed for absorbance at 232 nm in a 1.00-cm quartz cuvette, after diluting 200-fold with 0.01M HCl. [c]Unsaturated, nonreducing-terminal groups were determined by the thiobarbituric acid assay of periodate-treated products, with KDO as the standard. Values were adjusted to correct for the apparent 90% purity of the standard. [d]Uronic acid residues were determined by using D-mannurono-6,3-lactone as a standard. Values were adjusted to correct for the apparent 84% purity of our standard compound, based on the expected yield of chromophore given by Blumenkrantz and Asboe-Hansen[38].

individual fractions, in particular the putative dimer, were sufficiently unstable when heated to preclude high-temperature desiccation, and these lower molar absorptivities, in comparison with those determined from the concentrations determined by the thiobarbituric acid assay, may reflect the presence of water not removed by the lyophilization process.

Samples of unsaturated dimeric and tetrameric products obtained from digestion of bacterial alginate with a poly(ManA) lyase from a *Pseudomonas aeruginosa* isolate[25] were graciously provided by Dr. Alfred Linker, and shown to possess the same l.c. mobilities as our dimer and tetramer, respectively; molar absorptivities at 232 nm in 0.01M HCl of 6,400 and 5,500$M^{-1}cm^{-1}$ were obtained for this dimer and tetramer, respectively (data not shown).

Further evidence of the d.p. of the major products was obtained by f.a.b.–m.s. (Table II) of the lyophilized fractions. Spectra from each of the products contained major ions that correspond to within 1 mass unit of the calculated M + NH_4 and the M + NH_4 + H_2O ions. The trimer product showed an additional ion that represents M + NH_4 + thioglycerol, and the spectrum of the dimer showed two major ions (373, 391) which could not be explained, based on the expected structure of the dimer. The dimer sample was the only one that was not white, and we assume that these ions resulted from some decomposition of the dimer during transit or in handling prior to f.a.b.–m.s.

Although f.a.b.–m.s. established the molecular masses of the products, the contamination of poly(ManA) preparations by 10–15% of guluronate residues allows that some or all of the unsaturated oligomers may contain some guluronate. Studies by ^{13}C- and ^{1}H-n.m.r. spectroscopy to determine the content of guluronate and its localization in the unsaturated products are planned.

Activity of the extracellular enzyme on unsaturated, oligomeric products. —

TABLE II

ANALYSIS OF UNSATURATED OLIGOMERS BY F.A.B. MASS SPECTROMETRY[a]

Product	*Observed ions*			
	$M + NH_4$	$M + NH_4 + H_2O$	$M + NH_4 +$ *thioglycerol*	*Unidentified*
Pentamer	898	916		
Tetramer	722	740		
Trimer	546	564	654	
Dimer	370	388		373, 391

[a]Samples of lyophilized fractions from the P-2 column were analyzed by f.a.b.–m.s. according to methods described in the text.

Although the l.c. kinetic analysis of poly(ManA) digestion yields valuable information about the reaction, the complex nature of the process, wherein several products compete for binding to the enzyme and some products are degraded as they accumulate, does not allow detailed consideration of the activity of the enzyme on individual molecular species. To test the capability of the extracellular enzyme to further degrade products that accumulate during depolymerization of poly(ManA), the lyophilized trimer, tetramer, and pentamer purified by P-2 column chromatography were individually incubated with extracellular enzyme and the reactions were sampled at 5 min and 5 h and subjected to l.c. The conditions and the resulting profiles are shown in Fig. 3. Profiles generated from poly(ManA) digestion (a1 and a2) are included for comparison, as the retention times for the products had changed over several months of column use since the profiles shown in Fig. 1 were obtained. It is clear that the trimer is not subject to depolymerization by the enzyme, as profiles b1 and b2 are identical. The tetramer (c1 and c2) is not a good substrate for the enzyme, as predicted from the kinetic analysis (Fig. 2); however, a small amount of trimer was generated from the tetramer over the 5-h period. An equal amount of monomer should also have been produced, although it would not be detected by absorbance at 230 nm, as already noted. The pentamer was readily degraded by the enzyme, which converted almost 50% of the initial quantity into equal amounts of dimer and trimer, but produced little or no tetramer in 5 h of incubation.

Few other bacterial poly(ManA) lyases have been characterized to an extent that would allow a detailed comparison with the present enzyme. Doubet and Quatrano demonstrated that a cell-bound enzyme from a marine bacterium could degrade poly(ManA) by an apparent exolytic mechanism[20]. Davidson *et al.* described an endolytic poly(ManA) lyase which was induced by phage infection of *Azotobacter vinelandii*[26]. This enzyme seems to be quite similar to the one here studied in that it generates a series of unsaturated products ranging from dimers through pentamers, although neither the relative levels of the products nor the limit products were determined. Kashiwabara *et al.* measured poly(ManA) (SM)-degrading

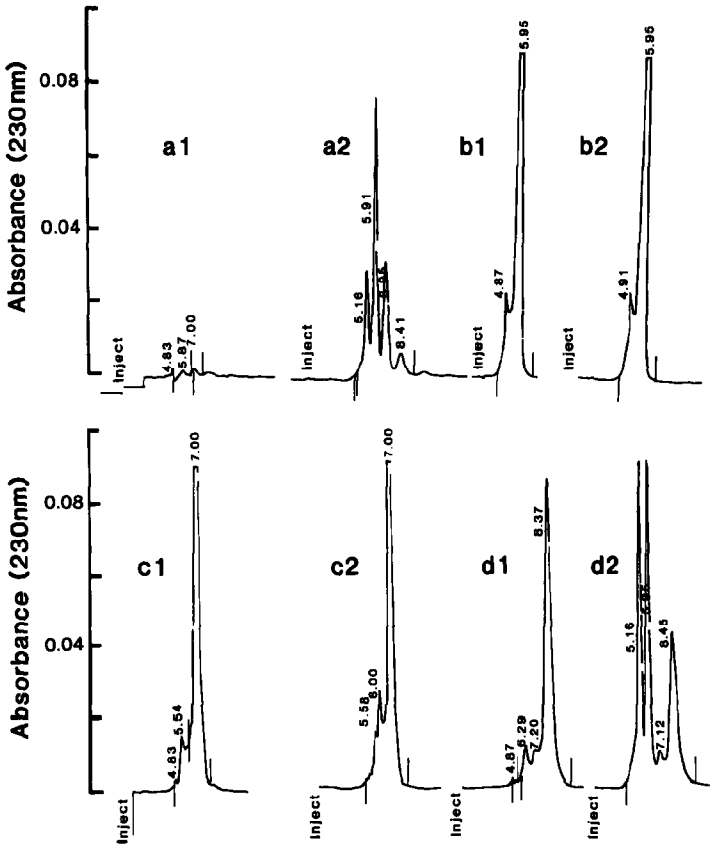

Fig. 3. Activity of the extracellular enzyme on purified trimer, tetramer, and pentamer products. The lyophilized products obtained after P-2 column chromatography were dissolved in 0.1-M sodium phosphate at pH 7.0 such that the final concentration in the mixtures was 5 mg/mL, and enzyme was added to a final concentration of 100 units per mL to start the reactions. The mixtures were incubated at room temperature and 5 μL samples withdrawn at 5 min and 5 h, and subjected to reversed-phase l.c. The chromatographic profiles generated from poly(ManA), trimer, tetramer, and pentamer are designated a, b, c, and d, respectively, and numbers 1 and 2 indicate sampling times of 5 min and 5 h.

activities in crude extracts of two marine pseudomonads[5]. The activities were weak in relation to the endogenous poly(GulA) lyase activities, and although the products were not well characterized, an unsaturated trimer was shown to be the major one. Linker and Evans examined an intracellular poly(ManA) lyase from a *Pseudomonas aeruginosa* isolate that generated unsaturated oligomers[25] ranging from dimers through pentamers. This enzyme was apparently incapable of producing monomer, and cleaved the unsaturated tetramer to form dimeric products. Although the major products generated by the enzyme studied here are similar to those produced by the *P. aeruginosa* enzyme, the catalytic mechanisms of the two enzymes clearly differ, as shown by the l.c. analysis of the conversion of unsaturated tetramer to trimer.

The approach utilizing reversed-phase ion-pairing l.c. to evaluate the mechanisms of the lytic depolymerization of alginate is now being applied to alginate lyases from other marine bacteria having different substrate specificities. This method should also prove useful for the study of lyases acting on other glycuronans. Detection of products based on refractive index or absorbance of u.v. at shorter wavelengths (for instance, 205 nm) should extend the applicability of the method to hydrolytic systems as well.

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