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Purification and Structural Properties of an Extracellular (1–4)-β-D-Mannuronan-Specific Alginate Lyase from a Marine Bacterium†

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ABSTRACT: The major extracellular alginate-degrading enzyme produced by a fermentative marine bacterium during growth on alginate as a sole carbon source has been isolated. This enzyme was purified 49-fold and was homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and high-performance liquid chromatography. The enzyme is a lyase which catalyzes depolymerization of the (1–4)-β-D-mannuronan block regions of alginate. It has an apparent native molecular mass of 29 kilodaltons, is composed of a single subunit, and has a significant fraction of potentially acidic amino acids, 14.9% Asx and 12.5% Glx. The determination of the partial amino-terminal sequence indicates the purified protein represents a single gene product. Isoelectric focusing of the purified protein yields several closely migrating forms which have p*I* values ranging from 4.2 to 5.0, suggesting posttranslational modification. The secondary structure is 74% α-helix by circular dichroism spectroscopy. The salt and pH requirements of the enzyme reflect the conditions of the marine environment in which it functions.

Alginate is a linear uronic acid polymer composed of β-D-mannuronic acid and α-L-guluronic acid in 1–4 linkages. Enzymes capable of depolymerizing alginate are almost exclusively eliminases, or lyases, and have been detected in and/or isolated from marine molluscs (Nakada & Sweeny, 1967; Muramatsu et al., 1977; Elyakova & Favorov, 1974) and from bacteria (Kashiwabara et al., 1969; Boyd & Turvey, 1977; Sutherland & Keen, 1981; Doubet & Quatrano, 1982; Hansen et al., 1984; Preston et al., 1985a; Romeo & Preston, 1986a). Previous studies utilizing these enzymes have examined the structure of alginate (Min et al., 1977; Boyd & Turvey, 1978), the composition of alginate containing cell walls of brown algae (Quatrano & Peterman, 1980), and the feasibility of generating protoplasts of brown algal species (Preston et al., 1985b; Romeo et al., 1986). The possibility that the alginate produced by *Pseudomonas aeruginosa* strains colonizing the lungs of cystic fibrosis patients is involved in the morbidity of that disease has recently led to the identification of alginate lyases in isolates of clinical origin (Linker & Evans, 1984; Dunne & Buckmire, 1985).

With few exceptions alginate lyase enzymes have been examined as impure mixtures of proteins, or even as preparations containing more than one activity, disallowing firm conclusions to be drawn about their substrate specificities, mechanisms, and structural properties. The result is that the only investigations on the structures of these enzymes, with the exception

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of molecular weight determinations, have been carried out on two isozymes from the mid-gut gland of the wreath shell *Turbo cornutus* (Muramatsu & Egawa, 1982; Muramatsu et al., 1984).

We previously reported the isolation of an alginate lyase capable of depolymerizing (1–4)-β-D-mannuronan [poly(ManA)],[1] derived from alginate, and an analysis of the products of this enzymatic reaction (Romeo & Preston, 1986a). The present paper describes the purification of this enzyme to homogeneity and some of the structural features of the purified enzyme. The subject of the following paper (Romeo & Preston, 1986b) is the mode of action of this enzyme, including studies on its substrate specificity, and products from its degradation of alginate.

Materials and Methods

Reagents. Chemicals were analytical grade except as indicated. Commercially available electrophoresis-grade reagents were used for SDS–polyacrylamide gel electrophoresis. Reagents and chemicals for amino acid analysis and N-terminal sequencing were commercially available ultrapure grades. Water for all aqueous solutions was deionized and glass distilled.

Sodium alginate was purchased as a purified grade (Fisher Scientific Co.) originally obtained from *Macrocystis*. The content of mannuronic acid was determined to be 67% by ^{1}H NMR, using methods established by Penman and Sanderson (1972) and Grasdalen et al. (1979). The poly(ManA) was prepared from HCl-hydrolyzed alginate according to Haug et al. (1967) and fractionated by size through Sephadex G-50 with 0.5 M NaCl as eluent. The fraction used for these studies was shown to contain 89% ManA by ^{1}H NMR. Comparison of total carbohydrate (Dubois et al., 1956) to reducing termini (Nelson, 1944) indicated that the range for the degree of polymerization was 16–22.

Enzyme Assays. The poly(ManA) lyase activity was quantified by spectrophotometric determination of the chromophore generated upon reaction of thiobarbituric acid (TBA) with periodate-treated products (Preiss & Ashwell, 1962; Weissbach & Hurwitz, 1959). The following conditions have been used for the enzyme reactions, unless otherwise noted: pH 7.5, 0.03 M sodium hydrogen phosphate, 0.05 M KCl, 0.10% sodium alginate, incubated for 10 min at room temperature, 22 °C. One unit of activity generates 1 nmol of unsaturated termini and/or unsaturated monomer in 1 min.

The quantity of protein present in fractions at various stages of purification was determined by the Coomassie blue binding assay of Bradford (1976), using bovine serum albumin as the standard. When a more accurate estimate of the protein concentration of the purified poly(ManA) lyase was needed, spectrophotometric analysis at 205 and 280 nm was used (Scopes, 1974). This method, unlike that of Bradford [see Tal et al. (1985) and Compton & Jones (1985)], has relatively little variation of response to proteins of differing chemical constitution.

Purification of Poly(ManA) Lyase. The poly(ManA) lyase was purified from a bacterium originally isolated from healthy tissues of *Sargassum fluitans*; the bacterium grew on alginate as the sole carbon source and secreted significant alginate lyase activity. The properties of this fermentative marine bacterium, designated as isolate A, or SFFB 080483 A, have been described (Preston et al., 1985a; Romeo et al., 1986), and isolates have been deposited with the American Type Culture Collection. The metabolic properties of this isolate, including the accumulation of poly(β-hydroxybutyrate) granules, would allow its assignment to the genus *Photobacterium*, while the GC mol % (45.4; Preston et al., 1985a) is 1.4 mol % higher than the upper value currently inclusive of the range typifying members of this genus. The purification steps were carried out at 4 °C, except for chromatography in the HPLC systems, which was at room temperature. For enzyme isolations, the organism was grown to late exponential phase in 0.1% liquid alginate medium (Preston et al., 1985a), with rapid gyrotory shaking at 22 °C. Bacterial cells were separated from the medium by centrifugation (10000*g*, 10 min), and the medium was concentrated and dialyzed against water by tangential flow filtration using a Millipore Pellicon cassette system with a polysulfone membrane (PTCG) which allowed retention of proteins larger than 10 kDa.

The enzyme was precipitated along with remaining products of alginate degradation by dropwise addition of 10% poly(ethylenimine) (PEI; purchased from Sigma Chemical Co., St. Louis, MO; titrated to pH 7.5 with concentrated HCl, diluted with water, and centrifuged at 10000*g* for 10 min to remove insoluble particles) to the concentrated medium. The relative volume of PEI needed for maximal precipitation of alginate lyase activity was determined by titrating soluble alginate lyase activity. The PEI precipitate was collected by centrifugation (10 min at 10000*g*) and washed by resuspension in water with a Potter–Elvehjem homogenizer driven by a variable-speed motor. The suspension was centrifuged and enzyme activity eluted by homogenization in 0.25 M NaCl and 0.1 M sodium hydrogen phosphate at pH 7.5. Insoluble material was removed by centrifugation at 150000*g* for 2.5 h and the supernatant solution subjected to gel filtration chromatography.

A preparation which was derived from 43 L of growth medium (yielding 71.3 g of wet cells) was fractionated on a column of Sephadex G-75 superfine grade (5 × 79 cm) at 4 °C with 0.1 M sodium phosphate buffered at pH 7.0. The recovered activity was concentrated by pressure filtration in an Amicon stirred cell (Amicon Corp., Lexington, MA) with a YM 10 membrane.

The concentrated activity was applied in three separate runs to a Mono Q HR 5/5 anion-exchange column (5 × 50 mm, Pharmacia, Inc., Piscataway, NJ) and eluted at room temperature with a gradient of NaCl (0–1.0 M) buffered at pH 7.0 with 0.01 M sodium hydrogen phosphate at a flow rate of 0.5 mL/min. The chromatography system included an LKB Ultrachrome GTi HPLC system (2152 controller, 2150 pump, 2154-002 injector; LKB-Produkter AB, Bromma, Sweden). A Gilson Holochrome variable-wavelength detector fitted with a 1.00-cm cell (Gilson Medical Electronics, Inc., Middleton, WI) was used to measure absorbance at 280 nm, which was recorded with a Linear 800 Versagraph (Linear Instruments Corp., Irvine, CA). Fractions of 0.5 mL were collected and assayed for alginate lyase activity.

Enzymatically active fractions from the three Mono Q column runs were concentrated and desalted by using the Amicon cell, and again applied to the Mono Q column and eluted as above. Activity was recovered, and concentrated, and subjected to gel filtration HPLC using an UltroPac TSK-G4000 SW column (7.5 × 600 mm, LKB) run at room temperature with a buffer of 0.1 M sodium hydrogen phos-

[1] Abbreviations: poly(ManA), (1–4)-β-D-mannuronan; poly(GulA), (1–4)-α-L-guluronan; TBA, 2-thiobarbituric acid; PEI, poly(ethylenimine); HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); CD, circular dichroism; PTH, phenylthiohydantoin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

phate, pH 7.0, containing 0.1 M NaCl, at a flow rate of 0.2 mL/min. The column was fitted to the HPLC system described above.

Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed (Laemmli, 1970) by using single-dimension 1.5-mm-thick slab gels of 9.73% acrylamide/0.27% bis(acrylamide), pH 8.8, for the running gel and 3.85% acrylamide/0.11% bis(acrylamide), pH 6.8, for the stacking gel. The conditions for the analyses are described in detail in the Hoefer Scientific Instruments catalog (Hoefer Scientific Instruments, San Francisco, CA).

Isoelectric focusing gels were purchased as 1.0-mm-thick prepared gels, Ampholine PAG plates, pH 3.5–9.5 (LKB), and were run by using an LKB Multiphore system according to instructions provided with the gels. The isoelectric point of the enzyme was determined by comparison with standard proteins. Enzyme activity in the gel was determined by sectioning the gel into 0.5-cm slices which were incubated overnight with 200 μL of alginate under standard conditions. Samples (100 μL) of the solutions were withdrawn and assayed for unsaturated products. Proteins present in both SDS and isoelectric focusing gels were fixed with acetic acid/ethanol/water (1:5:4) and were visualized by staining at 65 °C for 30 min with Coomassie brilliant blue R-250, 0.46 g/400 mL of destain solution (acetic acid/ethanol/water, 1:2.5:6.5), and destaining of the gels with several changes of destain solution.

Circular Dichroism Spectroscopy. Analyses were performed by using a Jasco J500C spectropolarimeter. The scan speed was 20 nm/min at a sensitivity of 1 mdeg/cm using a spectral bandwidth of 1 nm and a time constant of 2 s. The samples were contained in a 0.1-cm path-length cuvette. Data processing was accomplished with an Oki IF 800 Model 30 computer to provide scan averaging and molar ellipticity values.

Amino Acid Composition. Purified alginate lyase was dialyzed against water and concentrated by using a Centricon-10 unit (Amicon), lyophilized, and hydrolyzed in 6 N HCl under N_2 in sealed tubes for 24 h at 110 °C. The amino acids were resolved and quantified by using a Beckman 6300 amino acid analyzer with a Nelson analytical data acquisition system. The amino acid analyses were carried out by B. Parten and B. Dunn in the Department of Biochemistry and Molecular Biology at the University of Florida.

The content of cystine plus cysteine was obtained after hydrolysis in the presence of dimethyl sulfoxide, which converts these amino acids to cysteic acid (Spencer & Wold, 1969). Tryptophan content was estimated after hydrolysis in the presence of 4% thioglycolic acid (Matsubara & Sasaki, 1969).

Serine and threonine are known to be degraded slightly under the conditions of hydrolysis, and their levels were estimated by extrapolation to 0 h of hydrolysis from their levels at 24 and 48 h of hydrolysis (Hirs et al., 1954).

N-Terminal Amino Acid Sequence. An Applied Biosystems Model 470A gas phase protein sequencer was used for automated Edman degradation. The program (02RPTH) ran 30 cycles with 2 nmol of protein. The repetitive yield was 94%, and the initial yield was 70–80% with a myoglobin standard. The PTH–amino acids were identified and quantified by using reversed-phase HPLC with a Waters Model Trimod system including a 721 programable system controller, 730 data module, and a WISP 710B automatic injector, using a Waters Model 440 absorbance detector to monitor absorbance at 254 nm. A Novapak C-18 column (3.9 × 150 mm) was eluted with a gradient of methanol, 10–90% in 0.025% acetic acid, to resolve the PTH–amino acids. These analyses were also made by B. Parten and B. Dunn in the Department of Biochemistry and Molecular Biology.

Table I: Purification of Poly(ManA) Lyase

	fraction	total[a] act. (units)	total[b] protein (μg)	sp act. (units/μg)	yield (%)	x-fold purification[c]
(1)	crude	17000			100	
(2)	PEI eluate	9000	9300	0.97	53	1.0
(3)	Sephadex G-75	7300	2700	2.7	43	2.8
(4)	Mono Q-1	6700	260	26	39	27
(5)	Mono Q-2	5100	110	46	30	47
(6)	TSK	4100	86	48	24	49

[a] Based on the TBA assay for unsaturated nonreducing termini, generated as described under Materials and Methods. Total activity is that recovered from 43 L of concentrated, dialyzed growth medium. [b] Assayed according to Bradford (1976) with bovine serum albumin as the standard protein. [c] Calculated relative to the PEI eluate. The high level of acidic carbohydrate in the crude extract prevented assay of its protein content.

RESULTS

Purification of Poly(ManA) Lyase. Table I summarizes the purification procedures. The first step in the purification of the enzyme from the crude extract, precipitation with PEI followed by elution of enzyme activity with 0.1 M sodium hydrogen phosphate containing 0.25 M NaCl, separates a large amount of acidic carbohydrate from the enzyme activity, decreases the viscosity of the precipitation, and allows a greater amount of activity to be fractionated on the Sephadex column.

Chromatography on Sephadex G-75 separated alginate lyase activity (a single peak at 0.5 column volume) from alginate degradation products which eluted near the total bed volume as compounds which were oxidized by periodate to yield TBA-reactive products, and from some of the larger contaminating proteins which eluted in the void volume of the column (profile not shown). The enzyme activity was completely dependent upon the addition of exogenous substrate after this step.

Anion-exchange HPLC using a Mono Q column afforded a purification of 27-fold. Less than 1 h was required for recovery of the enzyme. The conditions shown in Figure 1a allowed optimum separation of enzyme activity, which eluted at approximately 0.4 M NaCl, from proteins with different charge properties. The procedure was repeated once with slight (1.8-fold) improvement in specific activity (Figure 1b).

A final step in the purification was gel filtration HPLC using a Biosil TSK-G4000 SW column (Figure 2, panel a). Although this procedure yielded only 1.04-fold purification (Table I) of the enzyme for the preparation considered here, for other batches of enzyme the purification was as high as 1.2-fold, and the step has been used routinely in the purification sequence. Samples containing up to 250 μg of protein from the Mono Q column have been successfully purified with the TSK column, and the method has allowed the detection of minor contaminants which were not apparent in the SDS–polyacrylamide gel analyses (data not shown). The poly(ManA) lyase was judged to be pure when enzyme activity eluted as a single homogeneous peak of UV-absorbing material (280 nm) on gel filtration HPLC, a single band was observed on SDS gel electrophoresis (see Figure 2, panel b), and a specific activity of approximately 48 units/μg (as calculated in Table I) was obtained.

The absorbance spectrum of the purified enzyme shows a maximum at 280 nm, a minimum at 250 nm (the ratio of absorbance at 280 to 250 nm is 2.6), and no measurable absorbance in the visible range (data not shown). The absor-

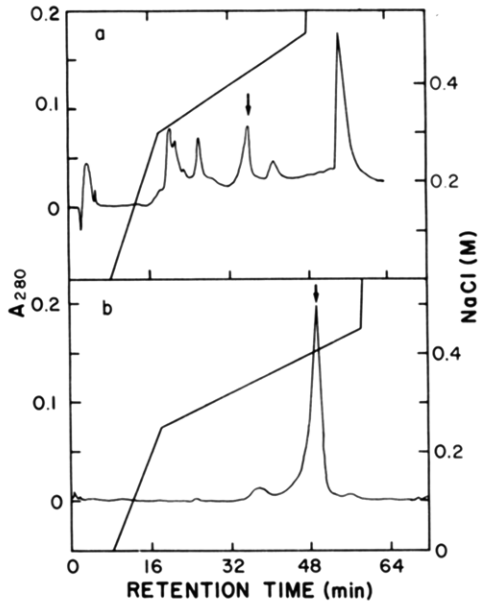

FIGURE 1: Anion-exchange HPLC of poly(ManA) lyase. A preparation containing 730 units of activity and 270 μg of protein from the Sephadex G-75 column was fractionated with a Mono Q column using a gradient of NaCl (up to 1.0 M, top portion of gradient not shown) to elute the activity (panel a). The single peak which possessed alginate lyase activity is indicated by an arrow. Activity peaks from three column runs were pooled, concentrated, and desalted, and this combined preparation was subjected again to anion-exchange chromatography with the Mono Q column (panel b).

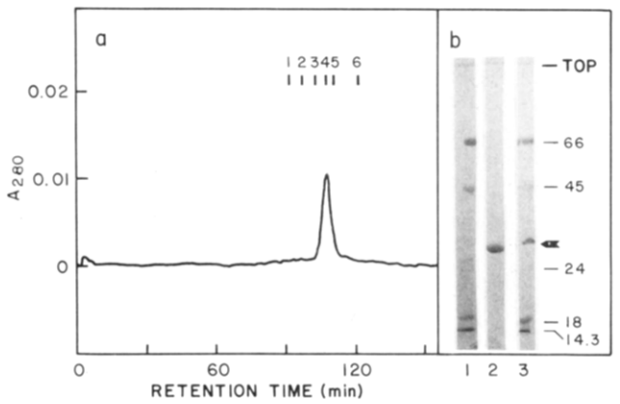

FIGURE 2: Gel filtration HPLC and SDS–polyacrylamide gel electrophoresis of poly(ManA) lyase. A sample of lyase activity (10 μg of protein) from the second pass through the Mono Q column was analyzed by gel filtration HPLC on a TSK4000 column as described under Materials and Methods (panel a). The elution of standard proteins shown for comparison is as follows: (1) α-amylase, 200 kDa; (2) bovine serum albumin, 66 kDa; (3) egg albumin, 45 kDa, and dimeric β-lactoglobulin, 37 kDa; (4) carbonic anhydrase, 29 kDa; (5) trypsinogen, 24 kDa; (6) lysozyme, 14.3 kDa. Activity from the second pass through the Mono Q column was denatured in the presence of SDS and 2-mercaptoethanol and analyzed in the presence and absence of standard proteins (panel b). Lane 1 contains the following standard proteins, 4.2 μg of each: bovine serum albumin, 66 kDa; egg albumin, 45 kDa; trypsinogen, 24 kDa; β-lactoglobulin B, 18.4 kDa; lysozyme, 14.3 kDa. Lane 2 contains 10 μg of poly(ManA) lyase, and lane 3 contains lyase plus standards.

bance at 280 nm of a 1 mg/mL solution of enzyme at pH 7 was 1.6 when the protein concentration was calculated by the method of Scopes (1974). The dye binding assay of Bradford (1976), using bovine serum albumin as a standard protein, underestimated the protein concentration of the purified enzyme relative to the method of Scopes by approximately 50%.

Molecular Mass of the Active Enzyme and Its Single Subunit. The molecular mass of the native enzyme was estimated by gel filtration HPLC (Figure 2, panel a) to be 29 kDa by using a plot of log M_r vs. retention time. Polyacrylamide gel electrophoresis in the presence of SDS showed a single band estimated as 29 kDa (Figure 2, panel b) from a plot of log M_r vs. mobility, indicating that the active enzyme is composed of a single polypeptide chain.

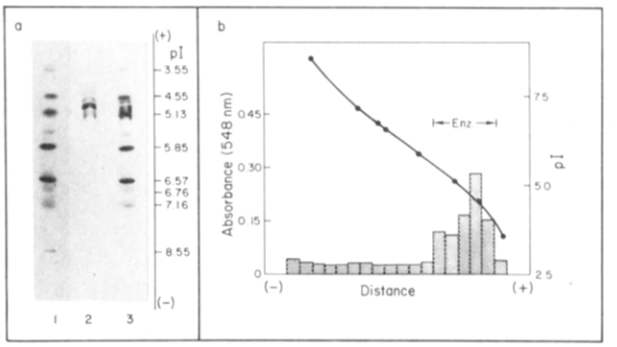

FIGURE 3: Isoelectric focusing (IEF) of poly(ManA) lyase. (Panel a) Horizontal IEF polyacrylamide gel electrophoresis was used to analyze the poly(ManA) lyase as described under Materials and Methods. Lane 1, standard proteins (Sigma isoelectric focusing markers): *Aspergillus oryzae* amyloglucosidase, p*I* = 3.55; soybean trypsin inhibitor, p*I* = 4.55; β-lactoglobulin A, p*I* = 5.13; bovine erythrocyte carbonic anhydrase, p*I* = 5.8; human erythrocyte carbonic anhydrase, p*I* = 6.57; horse heart myoglobin, p*I* = 6.76 and 7.16; rabbit muscle L-lactic acid dehydrogenase (M_4), p*I* = 8.55; lane 2, poly(ManA) lyase purified to apparent homogeneity through HPLC Mono Q and TSK steps (4 μg); lane 3, purified poly(ManA) lyase plus standard proteins. (Panel b) A lane containing poly(ManA) lyase was sliced, and enzyme activity was detected as described under Materials and Methods. The relative positions of standard proteins are shown as closed circles.

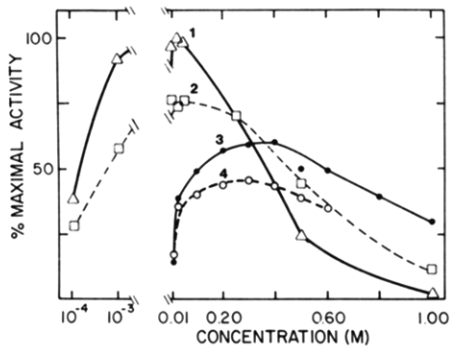

FIGURE 4: Effect of cation concentration on enzyme activity. The enzyme was assayed in reaction mixtures containing 0.1% poly(ManA), 5 mM Tris-HCl at pH 7.8, and the indicated salt concentrations. Curves are numbered to indicate salts which were present: (1) $CaCl_2$; (2) $MgCl_2$; (3) NaCl; (4) KCl.

Isoelectric pH of Poly(ManA) Lyase. Isoelectric focusing showed that the purified enzyme migrated as several closely spaced bands of protein with p*I* values from 4.2 to 5.0 (Figure 3a). Analysis of several batches of purified enzyme showed similar focusing patterns, and the pattern was visible, although somewhat obscured by other bands, in preparations of enzyme which were purified only through Sephadex G-75 chromatography (profiles not shown). Enzyme activity was detected over a similar pH range (Figure 3b).

Optimum pH. Sodium phosphate (for 11 pH values from 5.6 to 7.6) and Tris-HCl (for 10 pH values from 7.3 to 9.15) buffers were utilized and yielded a reasonably continuous pH profile which identified pH 7.8 as optimal with alginate as the substrate. Less than 10% of the maximum activity was observed below pH 5.8 or above 9.1.

Cation and Salt Requirements. When poly(ManA) was used as the substrate for the lyase, enzyme activity was found to require significant levels of salts for maximal activity (Figure

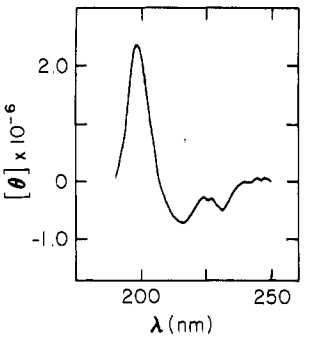

FIGURE 5: Circular dichroism spectroscopy of poly(ManA) lyase. The CD spectrum of the purified enzyme at a concentration of 104 μg/mL in 0.01 M sodium hydrogen phosphate buffer was obtained (scanned 10 times and averaged). The molar ellipticity from 190 to 250 is shown. No apparent changes in the CD spectrum of the protein were observed when the ionic strength was increased by addition of NaCl up to 1.0 M (data not shown).

4). Optimal activities were obtained at approximately 0.05 M with divalent cations and between 0.3 and 0.4 M with monovalent cations. The ratios of maximal activities obtained for chloride salts were 1:0.76:0.60:0.46 for Ca:Mg:Na:K.

CD Spectroscopy. The CD spectrum of the enzyme at pH 7.0 in 0.10 M sodium hydrogen phosphate is shown in Figure 5. In the presence of NaCl (0.05–1.0 M), the spectrum was not observably altered. The helical content of the protein was calculated to be 74% based upon the molar ellipticity at 208 nm (Greenfield & Fasman, 1969).

Amino Acid Composition. The amino acid composition was determined after the hydrolysis of the protein in 6 N HCl for 24 h, with the exceptions noted for tryptophan, serine and threonine, and cysteine (Table II). The content of potentially acidic residues is high: Asx (aspartic acid plus asparagine), 37 residues or 14.9%; Glx (glutamic acid plus glutamine), 31 residues or 12.5%. Serine is also abundant with 20 residues. The contents of Cys (cysteine and/or half-cystine), methionine, and histidine are low; four residues of each are present.

Amino-Terminal Sequence. Thirty cycles of Edman degradation allowed 19 of the 30 amino-terminal residues of the protein to be unambiguously assigned. The first six cycles established a continuous and constant amino-terminal sequence containing Asp-Ser-Ala-Pro-Tyr-Asp, with no ambiguous residues. The HPLC profiles of PTH derivatives at cycles 9 and 17 showed no assignable residues, possibly indicating the presence of cysteine, threonine, or arginine, which are not readily detected. Asp and Lys were assigned to positions 15 and 18, respectively.

Table II: Amino Acid Composition of Poly(ManA) Lyase

amino acid	no. of residues/ molecule[a]	integer
Asx	37.18	37
Thr[b]	12.99	13
Ser[b]	19.99	20
Glx	31.14	31
Pro	6.73	7
Gly	11.51	12
Ala	16.16	16
Val	16.12	16
Met	3.50	4
Ile	13.94	14
Leu	14.87	15
Tyr	16.57	17
Phe	7.25	7
His	3.97	4
Lys	11.32	11
Arg	9.36	9
Cys[c]	4.43	4
Trp[d]	12.38	12

[a] Based upon an estimated molecular mass of 29 kDa. [b] The contents of serine and threonine were corrected for slight decomposition by extrapolation to 0 h of hydrolysis using values obtained at 24 and 48 h (Hirs et al., 1954). [c] Total cysteine plus cystine was estimated by conversion to cysteic acid by hydrolysis in the presence of dimethyl sulfoxide (Spencer & Wold, 1969). The value was corrected for an approximate yield of 67.5%. [d] Tryptophan was estimated after hydrolysis in the presence of 4% thioglycolic acid (Matsubara & Sasaki, 1969). The value was corrected for 83% recovery.

Discussion

This work represents the first time that an enzyme which specifically depolymerizes poly(ManA) has been purified to homogeneity from a bacterial source. Previously, such enzymes have been purified from invertebrates (Muramatsu et al., 1977; Elyakova et al., 1974) and have been examined in partially purified states from marine (Doubet & Quatrano, 1984) and terrestrial bacteria (Sutherland & Keen, 1981; Hansen et al., 1984; Dunne & Buckmire, 1985) and from *Azotobacter vinelandii* after bacteriophage infection (Davidson et al., 1977). The method involves concentration of the spent growth medium of the bacterium by tangential flow ultrafiltration and precipitation of the enzyme activity and acidic polymers with poly(ethylenimine) followed by elution of the enzyme with buffered NaCl. A procedure involving precipitation with poly(ethylenimine) has been utilized routinely for removing acidic polymers in the purification of RNA polymerase (Jendrisak & Burgess, 1975). Further fractionation of the preparation through a single gel filtration step with Sephadex G-75 and two kinds of HPLC, anion-exchange chromatography using a Mono HR 5/5 column and gel filtration on an Ultropac G4000 SW column, yield enzyme which is purified to homogeneity. The percentage yields for the chromatography steps were quite good (from 77% to 91% for individual steps). The use of HPLC allows the procedure to be accomplished relatively rapidly (less than 1 h to elute the enzyme from the Mono Q column and less than 2 h for the TSK column). The total amount of enzyme protein obtained from the medium was 4.1 μg/L [assayed by the method of Scopes (1974)]. This protein yield is comparable to that obtained for an intracellular poly(GulA)-degrading enzyme from a marine bacterium, 8 μg/L of medium providing the harvested cells (Davidson et al., 1976). The overall purification of 49-fold starting with the PEI eluate indicates that the enzyme constitutes no more than 2% of the protein secreted. The fold purification of the other bacterial extracellular alginate lyase which has been purified, a poly(GulA) lyase, was 5.7-fold (Doubet & Quatrano, 1984).

The pH optimum (7.8) and salt optimum (300–400 mM with NaCl and 10–100 mM with $CaCl_2$) of the enzyme make it well suited to its marine environment, where it is presumed to depolymerize alginate present in the cell walls of *Sargassum* algae. The products which are generated may provide a pool of oligomeric uronides which is available to bacterial epiphytes for further depolymerization by intracellular or cell-bound lyases (Romeo et al., 1986), for conversion to the monomer 4-deoxy-L-erythro-5-hexoseulose uronic acid (Preiss & Ashwell, 1962).

The mass of the native enzyme and that after reduction and SDS treatment indicate that the enzyme is composed of a single polypeptide with a molecular mass of 29 kDa. Analyses of other alginate lyases from bacteria have indicated molecular weights, estimated by SDS–polyacrylamide gel electrophoresis, of 50 (Davidson et al., 1976), 40 (Hansen et al., 1984), and 35 and 100 kDa (Doubet & Quatrano, 1984). Only in the case of the alginate lyase secreted by *Bacillus circulans*

(Hansen et al., 1984) was the molecular weight of the native enzyme evaluated with the suggestion that this enzyme is active as a single polypeptide subunit with a molecular mass of 40 kDa.

The gel pattern obtained for the native enzyme after isoelectric focusing showed that the enzyme is heterogeneous in its charge properties. All of the observed forms focus in a relatively narrow region of the anodic side of the gel, pH 4.2–5.0. The acidic nature of the enzyme is also indicated by its interaction with the Mono Q column. This property may be expected on the basis of the amino acid composition which is high in Asx and Glx, 14.9% and 12.5%, respectively, on a molar basis, and low in basic residues, 4.5% and 3.8% for Lys and Arg, respectively. The amino acid composition of the enzyme differs considerably from that of two poly(ManA) lyase isoenzymes from a marine mollusc, *Turbo cornutus* (Muramatsu & Egawa, 1982); acidic residues (Asx and Glx) and Ala, Val, Ile, and Tyr are higher in the bacterial enzyme by 30–150% on a residue per molecule basis; Gly, Phe, and His are 70–200% higher in the mollusc enzymes; levels of other residues are similar.

The low isoelectric pH range of this enzyme makes it quite different in net charge from the poly(ManA) lyases of *T. cornutus* (Muramatsu et al., 1984), which have p*I* values of 7.5 and 7.7, but similar to that of an endolytic poly(GulA) lyase present in growth media of a marine bacterium, which was described as having a high net negative charge at pH 8.5 (Doubet & Quatrano, 1984). These workers suggested that their enzyme may be a glycoprotein. The acidic charge of the extracellular bacterial enzymes is not unexpected for proteins which must function in a saline environment (Lanyi, 1974).

Although the specific chemical nature of the charge heterogeneity of the poly(ManA) lyase is not established, the isoelectric focusing pattern of the purified enzyme suggests that the protein is subject to posttranslational modification by one or more of the enzymatic or spontaneous reactions which are known to alter the net charge of proteins (Wold, 1981). The N-terminal sequence analysis indicates that this region of the protein does not account for the observed heterogeneity and suggests that the enzyme is a single gene product. The method does not rule out the possibility that glutamine or asparagine of this region is partially deamidated or that gaps in the sequence may contain modified amino acids. The single sharp band which is consistently observed for the purified enzyme on SDS gel electrophoresis tends to rule out proteolysis. It is relevant that enzyme activity is detected as a peak which spans several 0.5-cm slices of the isoelectric focusing gel (see Figure 3b), suggesting that some or all of the observed protein bands are enzymatically active and are not due to gross structural changes which destroy activity.

The CD spectrum of poly(ManA) lyase indicates that the predominant structure (74%) is helix. This is quite different from the results obtained for the two poly(ManA) lyase isozymes from *T. cornutus* (Muramatsu et al., 1984), which were predominantly β structure. The functional significance of these observations is not immediately obvious, although it is clear that poly(ManA) lyases from bacteria and invertebrate sources differ significantly in their primary and secondary structures.

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Depolymerization of Alginate by an Extracellular Alginate Lyase from a Marine Bacterium: Substrate Specificity and Accumulation of Reaction Products†

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ABSTRACT: The endolytic depolymerization of alginate by the major extracellular alginate lyase from a marine bacterium has been examined. The reaction proceeds more extensively with polymers which contain a preponderance of (1–4)-β-D-mannuronic acid over (1–4)-α-L-guluronic acid. The extent of depolymerization of propylene glycol esters of alginate is inversely proportional to the extent of esterification. For isolated homopolymeric and heteropolymeric block regions of alginate, the extent of depolymerization is closely correlated with the frequency of occurrence of mannuronic acid triad, F_{MMM}, and not with the diad frequency, F_{MM}, or simply the fraction of mannuronic acid residues present. The major products which accumulate from alginate are indistinguishable from those from (1–4)-β-D-mannuronan by high-performance liquid chromatography analysis and include dimeric through hexameric uronides possessing Δ-4,5, unsaturated nonreducing terminal residues. The results indicate that only bonds between adjacent mannuronic acid residues are severed and that at least three such residues must occur in sequence. Kinetic analyses suggest that reaction products, including the unsaturated trimer and tetramer as well as purified (1–4)-α-L-guluronan, effect little or no product inhibition on this enzyme.

Alginate lyase enzymes which have been isolated and examined show preferences for either (1–4)-β-D-mannuronan, [poly(ManA)][1] or (1–4)-α-L-guluronan [poly(GulA)] blocks of the polymer. Alginate lyases from a varieity of molluscs generally show specificity for poly(ManA), and some of these enzymes have been isolated and characterized (Nakada & Sweeny, 1967; Favorov et al., 1979, Muramatsu, 1984). Bacteria have been isolated which produce enzyme activities with a demonstrated preference for poly(ManA) (Dunne & Buckmire, 1985; Linker & Evans, 1984; Sutherland & Keen; 1981; Hansen et al., 1984) or poly(GulA) (Kashiwabara et al., 1969; Davidson et al., 1976; Boyd & Turvey, 1977) or activities recognizing both of these regions of alginate (Doubet & Quatrano 1982; Preston et al., 1985; Romeo et al., 1986). Depolymerization of the other region of alginate which consists primarily of alternating sequences of mannuronic and guluronic acid, poly(ManA,GulA), can apparently be effected by some enzymes which are specific for poly(GulA) over poly(ManA) (Boyd & Turvey, 1978; Min et al., 1977).

We previously reported on the characterization of the extracellular alginate lyase activity from a fermentative marine bacterium associated with actively growing *Sargassum* (Romeo & Preston, 1986a). The activity (in 10-min assays) was greater when poly(ManA) rather than poly(GulA) or alginate was used as a substrate, and HPLC was used to kinetically monitor products which accumulate during the endolytic depolymerization of poly(ManA). The unequivocal identity of the specific bond(s) cleaved by the enzyme was not established. The enzyme recently has been purified to homogeneity, and some of its structural features have been reported [see Romeo & Preston (1986b)].

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[1] Abbreviations: poly(ManA), (1–4)-β-D-mannuronan; poly(GulA), (1–4)-α-L-guluronan; poly(ManA,GulA), alternating polymer; PG-alginate, propylene glycol alginate; DP, degree of polymerization; HPLC, high-performance liquid chromatography; TBA, 2-thiobarbituric acid; Δ, an unsaturated double bond; ΔX, an unsaturated terminal uronic acid residue; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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