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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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Received May 23, 1986; Revised Manuscript Received August 25, 1986

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)].

† These studies were supported by the Gas Research Institute and the Institute of Food and Agricultural Sciences, University of Florida, as CRIS Project MCS-02183-BI. This publication represents Journal No. 7304 of the University of Florida Institute of Food and Agricultural Sciences Experiment Station. The material is part of the thesis presented by T.R. to the Department of Microbiology and Cell Science, University of Florida, in partial fulfillment of the degree of Doctor of Philosophy.
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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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Table I: Extent of Degradation of Purified Block Regions of Alginate, Sodium Alginates, and Propylene Glycol Modified Alginates by Poly(ManA) Lyase

substrate[a]	F_M[b]	F_{MM}[c]	F_{MMM}[d]	extent of depolymerization, $F_{\Delta X}$[e]	F_{MM}[f]/F_{MM}-[poly(ManA)]	F_{MMM}[g]/F_{MMM}-[poly(ManA)]	$F_{\Delta X}$[h]/$F_{\Delta X}$-[poly(ManA)]
poly(ManA)	0.89	0.81	0.61	0.16	1.00	1.00	1.00
poly(ManA,GulA)	0.61	0.36	0.12	0.027	0.44	0.20	0.17
poly(GulA)	0.11	0.043	0.025	0.006	0.053	0.04	0.04
sodium alginate							
1	0.67	0.58		0.093	0.71		0.58
2	0.66	0.53		0.086	0.65		0.54
3	0.38	0.31		0.042	0.38		0.26
PG-alginate							
50	0.14			0.016			0.10
85	0.27			0.010			0.06

[a] Oligomeric block regions were prepared from partial acid hydrolysates as described under Materials and Methods. Sodium alginate 1 was alginate which was originally purified from *Macrocystis* (Fisher Scientific Co.); sodium alginates 2 and 3 were purified from *Macrocystis* (KGHV) and *Laminaria* (Manugel "DMD"), respectively, and were generously provided by Kelco. Propylene glycol alginates, PG-alginates 50 and 85, were Kelcoloid LVF and Kelcoloid S, respectively, and were modified at 50% and 85% of their carboxylate moieties, as determined by Kelco. [b] The mole fraction of mannuronic acid, as determined by ^{1}H NMR. [c] The frequency of occurrence of the mannuronic acid diad in the substrates, as determined by ^{1}H NMR. [d] The frequency of occurrence of the mannuronic acid triad sequence in block polymers, based upon ^{13}C NMR data of Grasdalen et al. (1981). [e] The fraction of the total uronic acid residues which were converted to unsaturated termini in 25 h of exposure to enzyme, as determined by the TBA assay. [f] The frequency of mannuronic acid diad of the substrates normalized with respect to that found for poly(ManA). [g] The frequency of mannuronic acid triad normalized with respect to that of poly(ManA). [h] The extent of depolymerization normalized as the ratio of that quantity of unsaturated termini generated from a given substrate at 25 h vs. the amount of unsaturated termini generated from poly(ManA) in 25 h, as measured by the TBA assay.

The present paper provides information about the specific type of bond which is cleaved by the purified enzyme and the minimum region of polymer necessary for substrate recognition. The major products of alginate depolymerization are identified by HPLC, and the effects of products on the reaction rate are examined.

Materials and Methods

Chemicals and Reagents. Chemicals were analytical or HPLC grade, except as indicated. Deuterium oxide was 99.996% isotopically pure (Sigma Chemical Co., St. Louis, MO). Water for all aqueous solutions was deionized and glass distilled.

Substrates. Sodium alginates were obtained from Fisher Scientific Co. (Springfield, NJ) and Kelco (San Diego CA), as purified grades originally obtained from *Macrocystis* (S-211, Fisher; KGHV, Kelco) and *Laminaria* (Manugel "DMD", Kelco), and alginates with carboxyl moieties which are esterified with propylene glycol were kindly provided by Kelco. Homopolymeric block regions of alginate, poly(ManA), and poly(GulA), and blocks with a high percentage of alternating sequence, poly(ManA,GulA), were prepared from HCl-hydrolyzed alginate by using methods developed by Haug et al. (1967) and fractionated by gel filtration chromatography on Sephadex G-50 with 0.5 M NaCl as eluent. The degree of polymerization, DP, of each fraction was determined by comparison of total carbohydrate (Dubois et al., 1956; Haug et al., 1962) to reducing termini (Nelson, 1944). The DP values of fractions chosen for these studies were 16–20, 22 (average), and 16–40 for poly(ManA), poly(GulA), and Poly(ManA,GulA), respectively. The uronic acid compositions and the frequencies of mannuronic acid diads for the substrates were determined by the use of ^{1}H NMR with a Nicolet NT-300 instrument operating in the Fourier-transform mode [see Penman & Sanderson (1972) and Grasdalen et al. (1979)]. The analyses were kindly provided by Sandra Bonetti and Dr. John E. Gander in the Department of Microbiology and Cell Science at the University of Florida.

Enzyme Purification and Assay. The methods and materials for purifying poly(ManA) lyase were described in the preceding paper (Romeo & Preston, 1986b), including the measurement of activity with the thiobarbituric acid assay, which quantifies unsaturated nonreducing residues and/or monomer which are generated by the eliminative depolymerization of substrate (Preiss & Ashwell, 1962).

Analysis of Products from the Depolymerization of Alginate by Poly(ManA) Lyase. A reaction mixture containing 5 mg/mL alginate (from *Macrocystis*, purchased from Fisher Scientific Co.) and 200 units of poly(ManA) lyase in 0.1 M sodium phosphate buffer at pH 7.0 was incubated at room temperature and sampled periodically over 15 h. Aliquots of 10 μL were subjected to ion-paring reversed-phase HPLC as previously described (Romeo & Preston, 1986a).

Determination of K_m and Measurement of Inhibition by Limit Products. The conjugated π electron system of the unsaturated oligomeric products absorbs UV light with a maximum at 232 nm (Preiss & Ashwell, 1962). Inhibition and K_m data were obtained by continuously monitoring the absorbance at 232 nm of the depolymerization reactions in 1.00-cm quartz cuvettes, using a Gilford Model 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) with a Honeywell chart recorder (Ft. Washington, PA). Apparent initial velocities were maintained for sufficient periods of time (at least 1 min) to allow their direct graphical interpretation.

Results

Extents of Depolymerization of Alginates, Block Regions of Alginate, and Propylene Glycol Alginates by Poly(ManA) Lyase. The time course of the depolymerization of several potential substrates by the enzyme with the formation of oligomeric uronides containing unsaturated nonreducing terminal residues is shown in Figure 1. At the substrate concentrations chosen for the reactions, 0.20 mg/mL, the initial rates were indicative of the final extent to which each substrate was depolymerized. At 25 h, the reactions had almost ceased, yet the addition of a small quantity of substrate to three of the reactions demonstrated that the enzyme had remained active (Figure 1). The substrates were therefore assumed to be degraded to a practical limit.

Table I quantitatively compares the extent to which each substrate was depolymerized at 25 h. Poly(ManA) allowed the greatest number of unsaturated terminal residues to be formed and hence the greatest amount of depolymerization;

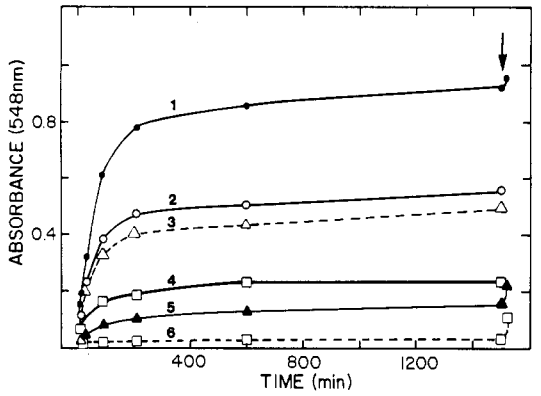


FIGURE 1: Extent of depolymerization of alginates and purified oligomeric block regions of alginate by poly(ManA) lyase. Reaction mixtures containing final concentrations of 0.20 mg/mL substrates, 0.05 M Tris-HCl at pH 7.8, and 0.3 M NaCl were incubated with poly(ManA) lyase in 1.5-mL capped polypropylene vials. Samples (100 μL) were withdrawn at the indicated times, and the extent of depolymerization was determined by the TBA assay (1.475 mL final assay volume), measuring the absorbance at 548 nm. Curves are numbered to identify substrates: (1) poly(ManA), 89% ManA; (2) sodium alginate (Fisher, *Macrocystis*), 67% ManA; (3) sodium alginate (Kelco, KGHV, *Macrocystis*), 66% ManA; (4) sodium alginate (Kelco, Manugel DMD, *Lamanaria*), 38% ManA; (5) poly(ManA,GulA), 61% ManA; (6) poly(GulA), 11% ManA. At 25 h, 20 μg of poly(ManA) was added to the reactions (indicated by an arrow) which contained poly(ManA), poly(ManA,GulA), and poly(GulA), curves 1, 5, and 6, respectively, and the reactions were allowed to continue for 10 min before sampling and assaying.

native alginates allowed from 26% to 58% of the number of cleavage reactions observed for poly(ManA), and the alternating polymer and poly(GulA) allowed still less, 17% and 4%, respectively. This suggests that the ManA–ManA bond is cleaved more readily than any other which may exist in these substrates. For poly(ManA), the fraction of uronic acid residues converted to unsaturated terminal residues at 25 h, $F_{\Delta X}$, was 0.16, or a mole fraction of 1 out of 6.3 residues.

To allow interpretation of the values describing the extent of depolymerization of poly(ManA) and other substrates in terms of the specific kind of site which is recognized by the enzyme, parameters which express quantitative estimates of the content of mannuronic acid, and of its arrangement in the polymers, are given. The fraction of each substrate which is composed of mannuronic acid, F_M, and the frequency of occurrence of the mannuronic acid diad, F_{MM}, were determined by ^{1}H NMR, and the triad frequency, F_{MMM}, was taken from previously published ^{13}C NMR data for purified block polymers derived from alginate (Grasdalen et al., 1981).

An average DP of 3.4 may be estimated for the dimeric through hexameric products which are present late in the reaction with poly(ManA), on the basis of the HPLC quantitation described (Romeo & Preston, 1986a). Since the fraction of the residues in poly(ManA) which were converted to unsaturated termini at 25 h was 0.16, an estimation of the fraction of the total uronic acids converted to low DP products is 3.4 × 0.16 or 0.54. The mannuronic acid triad frequency of the poly(ManA), 0.61, agrees fairly well with this, but the diad frequency is considerably higher, 0.81.

Then when the mannuronic acid diad and triad frequencies of other block polymers are normalized relative to those of poly(ManA), and the fractions of residues converted to unsaturated residues are similarly normalized, a direct quantitative relationship is observed for the extent of depolymerization relative to mannuronic acid triad frequency. Although mannuronic acid triad frequencies were not available for the alginates, the diad frequencies, as for the isolated block

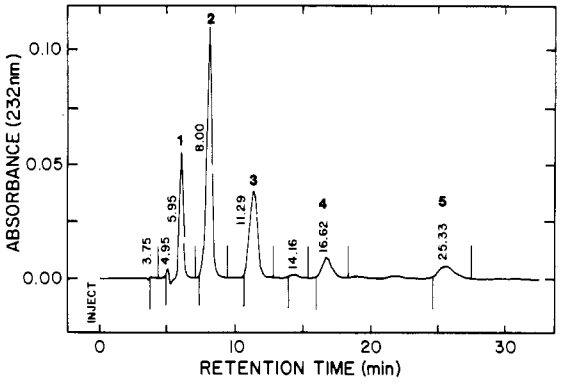


FIGURE 2: HPLC analysis of products generated by depolymerization of alginate by poly(ManA) lyase. The unsaturated oligomeric products which had accumulated after 15.1 h of depolymerization of alginate (5 mg/mL) by the poly(ManA) lyase (200 units/mL) were fractionated by reversed-phase ion-pairing HPLC. The major peaks are numbered in the order of their elution from the column; peaks 1 through 5 represent the unsaturated dimer through hexamer, respectively, based on comparison of their retention times with those of the products from poly(ManA) depolymerization (see Figure 3).

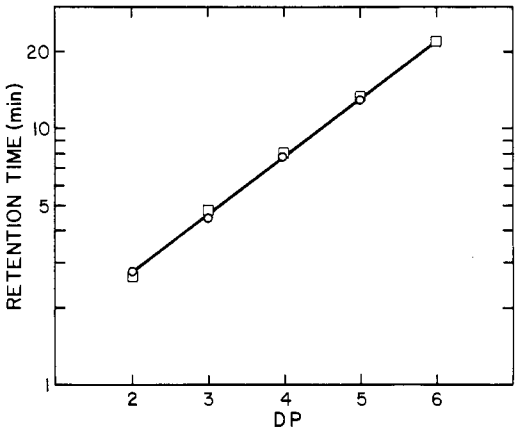


FIGURE 3: Relationship of the DP values of unsaturated uronic acid oligomers with their retention times after reversed-phase HPLC. The retention times of unsaturated dimeric through pentameric products which were generated from the depolymerization of poly(ManA) by the poly(ManA) lyase were corrected for the dead volume of the chromatography system and plotted on semilog paper against their DP values [previously established (Romeo & Preston, 1986a), shown as circles] along with the retention times and inferred DP values of the analogous products from alginate depolymerization (squares).

polymers, overestimated the amount of potential substrate.

The esterification of the carboxyl moieties of alginate with propylene glycol appears to completely block enzyme activity; alginates which were 50% and 85% esterified were depolymerized to 10% and 6%, respectively, of the extent measured for poly(ManA) (Table I).

Products of Alginate Depolymerization. A reaction mixture which contained 200 units/mL poly(ManA) lyase and 5.0 mg/mL sodium alginate (*Macrocystis* alginate from Fisher Scientific Co.) in 0.1 M sodium phosphate buffered at pH 7 was sampled periodically, and 10-μL aliquots were subjected to reversed-phase HPLC. The elution profile of the major products generated after 15.1 h at 25 °C is shown in Figure 2. The products generated from alginate were indistinguishable from those generated from poly(ManA) on the basis of the HPLC analysis. A linear relationship exists between the size (DP) of the products generated from alginate and poly(ManA) vs. the log of their retention times on HPLC, as shown in Figure 3. The DP values of the dimer through pentamer generated from poly(ManA) by the poly(ManA) lyase were previously established (Romeo & Preston, 1986a);

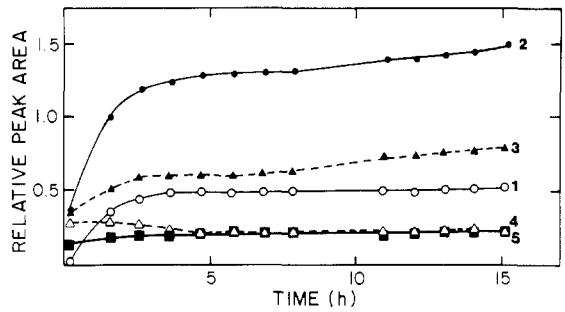


FIGURE 4: Kinetic evaluation of the unsaturated uronides generated from alginate by the poly(ManA) lyase. The reaction described in the legend of Figure 2 was monitored over 15.1 h by periodically sampling the reaction and subjecting the products to reversed-phase HPLC. The curves are numbered according to the individual products which they represent (see Figure 2). The unsaturated dimer product from poly(ManA) has a peak area of 0.11 unit/nmol (Romeo & Preston, 1986a).

Table II: Comparison of Relative Levels of Unsaturated Products Generated by Poly(ManA) Lyase from Alginate and Poly(ManA)

	products[c]				
substrate	dimer	trimer	tetramer	pentamer	hexamer
alginate[a]	0.35	1.00	0.49	0.24	0.16
poly(ManA)[b]	0.28	1.00	0.54	0.16	0.05

[a] The reaction mixture contained final concentrations of 5 mg/mL sodium alginate (Fisher) and 200 units/mL poly(ManA) lyase, buffered with 0.1 M sodium phosphate at pH 7.0. [b] This reaction mixture contained 10 mg/mL poly(ManA) and 50 units/mL poly(ManA) lyase. [c] The peak areas for products which had accumulated after 1.91 and 31 h from alginate and poly(ManA), respectively, which represent similar extents of depolymerization, were calculated as fractions of the trimer peak area for each reaction.

the other DP values were inferred from the logarithmic relationship presented in Figure 3.

When the levels of products present in the alginate depolymerization reaction were measured at various times by HPLC, the pattern of product accumulation and disappearance (Figure 4) was similar to that observed for poly(ManA) (Romeo & Preston, 1986a). The ratios of the concentrations of products changed as the reaction progressed. The concentrations of dimer and trimer, relative to other products, increased beyond the first time of sampling, approximately 0.1 h, and the absolute level of the pentamer decreased slightly from that time. The concentrations of the dimer and trimer continued to increase from approximately 7 to 15.1 h (the final time of sampling), while concentrations of other products remained constant. The levels of products at similar extents of reaction for alginate and poly(ManA) are shown in Table II, at which time the [trimer] > [tetramer] > [dimer] > [pentamer] > [hexamer]. The two reactions differed in that the relative levels of the larger unsaturated oligomers (pentamer and hexamer) present late in the reactions were somewhat higher when alginate was the substrate.

Effect of Reaction Products on Enzyme Activity. The K_m of the reaction with poly(ManA) was determined to be 1.6 mM, with respect to uronic acid residues, from a Lineweaver–Burke plot (data not shown). Two products which were previously shown to accumulate in, and were isolated from, the reaction with poly(ManA) (Romeo & Preston, 1986a) showed only slight inhibition of the depolymerization of poly(ManA) (Table III), even at relative uronic acid residue concentrations of 5.7- and 7.6-fold of the trimer and tetramer, respectively, over poly(ManA). The addition of poly(GulA) also did not inhibit the poly(ManA) depolymerization but allowed a slight increase in the initial rate of the reaction.

Table III: Effects of Unsaturated Mannuronic Acid Trimer and Tetramer and Poly(GulA) on the Initial Rate of Poly(ManA) Depolymerization by Poly(ManA) Lyase[a]

[poly-(ManA)][b] (mM)	[trimer][c] (mM)			[tetramer] (mM)	[poly(GulA)] (mM)
	0.02	0.4	0.4[d]	0.4	5.0
0.21		91	95	91	120
1.7	96	83		68	

[a] Values are reported as percentages of the apparent initial velocities measured in the absence of added trimer, tetramer, or poly(GulA), as determined by measuring increasing absorbance of the solutions at 232 nm. [b] The concentrations of poly(ManA) and poly(GulA) are reported as uronic acid concentrations (millimolar). [c] Concentrations of trimer and tetramer were determined on the basis of A_{232} and therefore represent those of the given oligomer. [d] Data for this reaction were obtained by incubation of the enzyme with trimer for 20 min before addition of poly(ManA); all other reactions were started upon addition of the enzyme.

DISCUSSION

Previously, a crude preparation of extracellular alginate lyase from a facultative marine bacterium (designated isolate A, or SFFB080483 A, with a tentative assignment to the genus *Photobacterium*) which was isolated from actively growing *Sargassum fluitans* was shown to preferentially depolymerize poly(ManA) relative to alginate and poly(GulA) as measured in 10-min assays (Preston et al., 1985). The single endolytic alginate lyase activity from this preparation was purified [see Romeo & Preston (1986b)] and has now been examined with respect to its activity on various alginate polymers.

Alginate and poly(ManA) are depolymerized to low DP products with identical retention times in the HPLC system employed and include dimeric through hexameric forms. The identities of the products from alginate are inferred by comparison of their mobilities with those of products from poly(ManA) depolymerization. The latter have been characterized by a number of criteria, including fast atom bombardment mass spectrometry (Romeo & Preston, 1986a). The linear relationship observed for the log of the retention times of the unsaturated uronides in ion-paring reversed-phase HPLC with their DP values is a feature of the method which should allow facile determination of the size (DP values) of uronides which belong to a homologous series containing two or more known members.

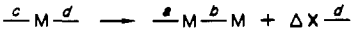
In addition to the low DP unsaturated uronic acid oligomers from alginate, the enzyme must also release larger fragments of alginate which should contain poly(GulA) and poly(ManA,GulA) block regions of alginate. However, these are not detected by the HPLC system due to their strong interaction with the column. The larger products should make up a fairly small mole fraction of the reaction products, based on the sizes of the poly(GulA) and poly(ManA,GulA) regions of the polymer, although on a weight basis they are significant.

The enzyme is apparently unable to act upon alginate which has been modified by formation of propylene glycol esters. This suggests a role for the carboxyl moieties of alginate in substrate recognition, as the esterification of carboxyl moieties of 1–4-linked uronic acid polymers actually increases their susceptibility to base-catalyzed elimination (Kiss, 1974). The effect on activity appears to be similar to that observed for a poly(ManA) lyase from a marine mollusc, which had 40% as much activity on PG-alginate as with native alginate (Muramatsu et al., 1977), although these results cannot be directly compared, since the level of esterification of the alginate was not reported.

The kinetics of the accumulation of the major low DP products (dimer–tetramer) generated from alginate are similar

to those of the products from poly(ManA) depolymerization (Romeo & Preston, 1986a); i.e., the relative abundance of the analogous oligomers is similar at comparable extents of substrate depolymerization. However, compared to the depolymerization of poly(ManA), depolymerization of alginate led to the accumulation of a relatively greater concentration of pentamer and hexamer late in the reaction. This may be due to the presence of guluronic acid in these products. Guluronate residues in a pentamer or hexamer may make them refractory to further depolymerization by the enzyme; guluronate should be present to a greater extent in products from alginate depolymerization than those from poly(ManA). Support for this possibility comes from the previous observation that the unsaturated pentamer which was obtained from partially depolymerized poly(ManA) is a good substrate for the enzyme (Romeo & Preston, 1986a). The limit products should not contain unsaturated pentamer unless it contains some guluronate.

In considering the specificity of the enzyme active site, the observations that (a) the tetramer is slowly converted to a trimer and a monomer, but not to dimer (Romeo & Preston, 1986a), (b) the unsaturated trimer and dimer molecules represent true limit products derived from either native alginate or poly(ManA), and (c) the frequency of the mannuronic acid triad is correlated with the extent to which a particular substrate is degraded suggest a cleavage reaction which requires the interaction of the enzyme with at least three sequential mannuronic acid residues, and a location for the reaction between the second and third residues of this site of interaction or bond *c* as shown:



Cleavage of bond *b* appears to be ruled out based upon the formation of trimer but not dimer from the tetramer. The data do not rule out the possibility that bond *a* is broken. However, as the configuration of the unsaturated terminal residue of the tetramer is sufficiently different from that of a mannuronic acid residue, or guluronic acid residues which might share bond *a*, in a polymer this is not expected to occur. Subsite affinity studies of the kind which have been carried out for amylase enzymes (Suganuma et al., 1978; MacGregor & MacGregor, 1985; Sano et al., 1985) would help clarify these points.

Neither the trimer nor the tetramer is an effective inhibitor of poly(ManA) depolymerization. Poly(GulA) was not inhibitory either but instead caused a slight increase in the initial velocity of the reaction, presumably due to contaminating mannuronic acid sequences. On the other hand, Boyd and Turvey (1977) found that a polyguluronate lyase from *Klebsiella aerogenes* was competitively inhibited by a trimer which did not contain a terminal unsaturated residue. One of the advantages of the lyase mechanism may be that the new nonreducing termini which are generated limit further interaction with the enzyme, thus yielding products which have much reduced affinities for the endolytic enzyme which generated them. Transglycosylation and condensation reactions, which occur under high substrate concentrations for hydrolytic enzymes, such as amylases (Suganuma et al., 1978), would then be avoided. In the reaction of the poly(ManA) lyase with unsaturated products (Romeo & Preston, 1986a), we did not observe higher oligouronides to be formed, e.g., dimer and trimer, but no hexamers were formed when the enzyme was incubated with the unsaturated pentamer. In effect then, the lyase mechanism may allow the depolymerization reactions to proceed at rates independent of the concentrations of the products generated by the reaction.

The K_m of the poly(ManA) lyase for its preferred substrate is relatively high, 1.6 mM. The K_m of a guluronate lyase from another marine bacterium is also high, 5.51 mM for guluronic acid residues (Davidson et al., 1976), relative to alginate lyases from a marine mollusc, 0.19 mM (Elyakova & Favorov, 1974), and from *Klebsiella*, 0.11 mM (Boyd & Turvey, 1977). The high K_m of the poly(ManA) lyase for substrate and the apparent lack of product inhibition may help it to avoid substrate and product inhibition during activity on algal cell walls.

The enzyme activity examined in this study is the major extracellular alginate-degrading activity from a bacterium which probably derives much of its carbon and energy from alginate in its native environment, the surface of *Sargassum* algae. The organism also produces intracellular or cell-bound activities capable of degrading both poly(ManA) and poly(GulA) and which are more exolytic in their overall mechanisms (Romeo et al., 1986). Unlike the extracellular enzyme, these are able to produce a considerable amount of apparent monomer product from poly(ManA) (Romeo, 1986). This organism, which produces a single extracellular poly(ManA)-specific lyase, may therefore obtain partially degraded poly(GulA) and/or poly(ManA,GulA) regions from the action of the extracellular enzymes of other bacteria which share the same unique microenvironment, and which have been shown to produce extracellular enzymes active on poly(GulA) (Preston et al., 1985; Romeo et al., 1986).

ACKNOWLEDGMENTS

We greatly appreciate the NMR analyses provided by Sandra Bonetti and John E. Gander, Department of Microbiology and Cell Science, University of Florida, and the critical review of the manuscript by Dr. Gander. We are grateful for the suggestions of L. O. Ingram for plotting HPLC data. We thank Donna Huseman for preparing the figures and Martina Champion for typing the manuscript.

Registry No. Poly(ManA), 105280-81-7; poly(GulA), 105280-79-3; poly(ManA,GulA), 105280-80-6; poly(ManA) lyase, 86922-62-5; kelcoloid LVF, 9005-37-2; alginic acid, 9005-32-7; alginate lyase, 9024-15-1.

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Purification and Characterization of High Ca^{2+}-Requiring Neutral Proteases from Porcine and Bovine Brains

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Received May 12, 1986; Revised Manuscript Received August 20, 1986

ABSTRACT: Porcine and bovine brain high Ca^{2+}-requiring neutral proteases were purified to homogeneity by the same isolation procedures, and their properties were compared. A high degree of similarity existed between the two proteases. The purification procedures included ion-exchange chromatography on DEAE-cellulose, hydrophobic chromatography on phenyl-Sepharose CL-4B, second DEAE-cellulose chromatography, second phenyl-Sepharose CL-4B chromatography, and gel filtration on Ultrogel AcA 34. Both purified enzymes were composed of M_r 75 000 and 29 000 subunits, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Both enzymes required 250 μM Ca^{2+} for half-maximal activity and 700 μM Ca^{2+} for maximal activity. Sr^{2+} and Ba^{2+}, but not Mg^{2+} or Mn^{2+}, also activated both enzymes but not as effectively as Ca^{2+}. Both enzymes displayed maximum activity at pH 7.5–8.0. Leupeptin, antipain, and *trans*-epoxysuccinyl-L-leucylagmatine inhibited both enzymes. Neurofilament triplet proteins and microtubule-associated proteins were extensively hydrolyzed by both proteases, but tubulin and actin were not hydrolyzed. The amino acid compositions of the two proteases were very similar. Antisera against bovine brain protease cross-reacted with porcine brain protease when examined by immunoelectrotransfer blot techniques.

Calcium-activated neutral proteases (CANPs)[1] have been identified in many tissues of various species (Murachi et al., 1981; Ishiura, 1981). Recently, two forms of CANP which differ in their Ca^{2+} requirement, CANPI requiring low Ca^{2+} and CANPII requiring high Ca^{2+}, have been recognized in several tissues (Mellgren, 1980; Dayton et al., 1981; DeMartino, 1981; Kubota & Suzuki, 1982; Croall & DeMartino, 1983; Inomata et al., 1983; Malik et al., 1983; Otsuka & Tanaka, 1983; Yoshimura et al., 1983). However, the physiological significance of the existence of such a dual enzyme system is not known, and the relationship between the two forms of CANP is still not clear. It is suggested that CANPI, which requires micromolar levels of Ca^{2+}, is the physiologically active form of CANP. However, CANPI is a minor form in most tissues (Mellgren, 1980; Dayton et al., 1981; DeMartino, 1981; Kishimoto et al., 1981; Kubota & Suzuki, 1982; Croall & DeMartino, 1983; Inomata et al., 1983; Murachi, 1983; Otsuka & Tanaka, 1983; Yoshimura et al., 1983). At present, it remains unknown which form, CANPI or CANPII, plays a more important role in the cell. It seems reasonable that the major form of CANP must play an important role in the cell. There are also discrepancies as to the subunit composition of CANPIIs. Some investigators have suggested that proteases are heterodimers composed of M_r 70 000–80 000 and 18 000–30 000 subunits (Dayton et al., 1976; Suzuki et al., 1979; Truglia & Stracher, 1981; Tsuji & Imahori, 1981; Hataway et al., 1982; Kubota & Suzuki, 1982; Mellgren et al., 1982; Wheelock, 1982; Inomata et al., 1983; Otsuka & Tanaka, 1983; Yoshida et al., 1983; Yoshimura et

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[1] Abbreviations: CANP, calcium-activated neutral protease; CANPI, low Ca^{2+}-requiring protease; CANPII, high Ca^{2+}-requiring protease; SDS, sodium dodecyl sulfate; MAPs, microtubule-associated proteins; E-64, *trans*-epoxysuccinyl-L-leucylagmatine, a thiol protease inhibitor; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-8396$01.50/0