Purification and Properties of an Alginate Lyase from a Marine Bacterium

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An unidentified pseudomonad isolated by enrichment procedures from decomposing seaweed was grown in defined medium containing sodium alginate as the sole carbon source. The alginate lyase recovered from disrupted bacterial cells was purified by a procedure of $(\text{NH}_4)_2\text{SO}_4$ precipitation, gel filtration and ion-exchange chromatography. From sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis experiments a mol.wt. of about 50000 was determined. The enzyme was active against both algal and bacterial alginate preparations. Kinetic studies together with analysis of the unsaturated oligouronide products of alginate lyase action indicated the enzyme was specific for guluronic acid-containing regions of the macromolecular substrate. The specificity of the enzyme can be used to give information about the primary composition of alginate samples.

Alginic acid is a co-polymer of D-mannuronic acid and L-guluronic acid, in which the uronic acids are arranged in a block fashion in the polymer chain, with blocks of one type of monosaccharide residue being separated by segments in which the two residues alternate (Haug & Larsen, 1965). X-ray-diffraction studies (Atkins *et al.*, 1970) on samples of polymannuronic acid and polyguluronic acid have indicated that D-mannuronic acid residues are β -linked in the C1 conformation and L-guluronic acid residues are α -linked in the 1C conformation.

Alginates have found widespread application in pharmaceutical and food industries owing to their ability to form viscous solutions at relatively low concentrations and to form gels with Ca^{2+} . These physical properties are strongly influenced by the ratio and arrangement of the mannuronic acid and guluronic acid residues in the polymer. Despite the economic importance of alginic acid (see Whistler, 1973) and the structural studies that have been performed on the polymer, the routine analysis of alginates remains notoriously difficult. Added requirements for such analyses have come from the recognition of alginates as bacterial exopolysaccharides from *Azotobacter vinelandii* (Gorin & Spencer, 1966) and certain strains of *Pseudomonas aeruginosa* (Linker & Jones, 1964), which may thus provide an alternative to the traditional algal sources. Both bacterial polysaccharides are partially O-acetylated.

Only a limited amount of work has been reported on alginolytic enzymes. Preiss & Ashwell (1962) described the enzymic formation of unsaturated

oligouronides from alginic acid. That alginate lyases might show certain specificities for linkages with the polysaccharide was proposed by Nakada & Sweeny (1967). Two alginate-degrading enzymes, characterized as alginate lyases (EC 4.2.2.3) were separated from the hepatopancreas of abalone (*Haliotis rufescens* and *Haliotis corrugata*).

The alginases, designated I and II, exhibited different properties. Alginase I caused a rapid fall in the viscosity of alginate solutions and appeared to be specific for β -1,4-glycosidic linkages as indicated by the high affinity that the enzyme exhibited towards mannuronic acid-rich substrates. Alginase II showed an increased affinity for linkages involving guluronic acid and was thought to be an exo-lyase.

The aim of the present study was to investigate the properties of a bacterial alginate lyase and its possible role in analysis of alginates of algal and bacterial origin.

Materials and Methods

Bacterium

The alginase-producing bacterial strain used in this study was isolated from decomposing seaweed by an enrichment procedure and was provisionally identified as a pseudomonad by the criteria described by Cowan & Steel (1961).

Conditions of cultivation

The unidentified pseudomonad was grown in 15-litre batch fermenters (L. H. Engineering Ltd., Stoke

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Poges, Bucks., U.K.) in a medium containing (g/litre) sodium alginate, 2.0; K_2HPO_4 , 7.0; KH_2PO_4 , 3.0; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; NaCl, 23.5. After incubation at 22°C for 18h, bacteria were harvested by centrifugation at 10000g for 20min. The yield of bacteria was 1g/litre of wet cells.

Purification of alginate lyase

All procedures were carried out at 4°C.

Step 1: preparation of crude extract. The bacteria were suspended in cold 10mM-Mops [3-(*N*-morpholino)propanesulphonic acid]/NaOH buffer, pH7.5, and disrupted by passage once through a pre-cooled French pressure cell (American Instrument Co., Silver Spring, MD, U.S.A.) at 20684 KPa. Cell debris was removed by centrifugation at 10000g for 20min. The supernatant fluid was retained and dialysed overnight against a large volume of the Mops/NaOH buffer, pH7.5.

Step 2: $(NH_4)_2SO_4$ precipitation. Solid $(NH_4)_2SO_4$ was added to the crude enzyme solution to obtain 40% saturation. The resultant precipitate was removed by centrifugation at 10000g for 20min and discarded. Further $(NH_4)_2SO_4$ was added to obtain 80% saturation and the suspension was again centrifuged at 10000g for 20min. The precipitate was dissolved in 10mM-Mops/NaOH buffer, pH7.5, and dialysed overnight against a large volume of the same buffer (the supernatant fluid contained no appreciable enzyme activity and was discarded).

Step 3: batch ion-exchange chromatography. The material obtained from step 2 was mixed with DEAE-cellulose (DE-32; Whatman Biochemicals Ltd., Maidstone, Kent, U.K.). Alginase activity was recovered by the stepwise elution of the suspension with a NaCl gradient (0.05–0.5M) in 0.05M-Tris/HCl buffer (pH7.8), elution of enzymic activity occurring at 0.25M.

Step 4: gel filtration. The sample obtained from step 3 was divided into four equal portions and each was fractionated in a column (51cm×2cm) of Sephadex G-100, with 10mM-Mops/NaOH buffer, pH7.5, as eluent. Fractions showing alginase activity were pooled and further fractionated by DEAE-cellulose ion-exchange chromatography.

Step 5: column ion-exchange chromatography. A DEAE-cellulose (DE-32) column (55cm×3cm) was equilibrated with 0.05M-Tris/HCl buffer, pH7.8. After application of the enzyme, the column was eluted by application of a linear 0–0.5M-NaCl gradient in 0.05M-Tris/HCl buffer, pH7.8. Fractions (5ml) were collected and those showing alginase activity were pooled and retained.

Step 6: CM-Sephadex chromatography. A CM-Sephadex (C-50) column (40cm×1.6cm) was equilibrated with 0.05M-sodium phosphate buffer, pH7.8. The pooled material from step 5 was applied to the

column, which was eluted with a linear gradient in the manner described above. The fractions showing alginase activity were pooled and dialysed against 10mM-Mops/NaOH buffer, pH7.5. The enzyme solution was stored at –20°C.

Measurement of enzymic activity

The amount of unsaturated material produced by lyase action was measured by the periodate/thiobarbituric acid procedure of Weissbach & Hurwitz (1958). A unit of enzyme activity was defined as the amount of enzyme required to liberate the equivalent of 1 μ mol of β -formylpyruvic acid/min; 0.01 μ mol of β -formylpyruvic acid produces an E_{549} of 0.290 in the thiobarbituric acid test (Preiss & Ashwell, 1962).

Analytical procedures

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Total carbohydrate was determined by the phenol/ H_2SO_4 procedure of Dubois *et al.* (1956).

Preparation and analysis of substrates

Algal alginates were obtained from various commercial sources. Bacterial alginates were prepared from sucrose-grown *A. vinelandii* in batch culture under conditions described by Imrie (1973). Alginates were partially purified by the procedure of Bucke (1974). Mannuronic acid/guluronic acid ratios were determined by total acid hydrolysis of the polymer by the procedure of Haug (1964) with modification by C. J. Lawson & K. E. Rudland (unpublished work), involving high-voltage paper electrophoresis, followed by staining with lead acetate and densitometric measurement.

Homopolymeric substrates (polymannuronic acid and polyguluronic acid blocks) were prepared by partial acid hydrolysis of alginic acid by the procedure of Penman & Sanderson (1972). The authenticity of the preparations was confirmed by proton-magnetic-resonance spectroscopy.

Paper chromatography and electrophoresis

Comparative paper chromatography was carried out by descending irrigation of Whatman no. 1 paper. Whatman 3 MM paper was used for preparative work. The following solvent systems were used; A, ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.); B, ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.) and C, butan-1-ol/acetic acid/water (50:12:25, by vol.). Paper electrophoresis was performed on Whatman no. 1 paper in pyridinium acetate buffer (pH5.3) by using a current of 80–

100mA at 3.5kV for 1.5h on Locarte (London, S.W.7, U.K.) equipment with a cooled plate area of 75cm×20cm. Sugars and oligosaccharides were detected with alkaline AgNO₃ (Trevelyan *et al.*, 1950) or with periodate/sodium thiobarbiturate (Warren, 1960).

Column chromatography

Oligouronides released from sodium alginate by enzymic action were fractionated on a column (38cm×1.2cm) of Sephadex G-15 with 0.16M-pyridinium acetate buffer (pH5.3) as eluent.

Disc-gel electrophoresis

Polyacrylamide-gel electrophoresis of proteins was performed by the method of Weber & Osborn (1969). Standards were purchased from Boehringer, Lewes, Sussex, U.K.

K_m and V_{max} determinations

K_m and V_{max} constants were calculated by the method of Lineweaver & Burk (1934).

Enzymic hydrolysis of algal and bacterial alginates

Alginates (50mg) were dissolved in 5ml of 10mM-Mops/NaOH buffer, pH7.5, and sufficient alginate lyase was added to give maximum hydrolysis in 20h at 30°C. Microbial growth was inhibited by the addition of toluene. Hydrolysates were examined by paper chromatography (solvents A, B and C), and individual unsaturated oligouronides accumulated by preparative paper chromatography (solvent B). Oligouronides, shown to be chromatographically pure in solvents A and B, were hydrolysed by heating

with 2M-HCl for 2h at 100°C. The solutions were evaporated to dryness, repeatedly redissolved and re-evaporated before examination of the hydrolysates by paper chromatography (solvent C) and high-voltage electrophoresis.

Results

Enzyme purification

The purification procedure gave reliable purification, yielding material of a high specific activity (Table 1). The elution profiles of the fractionation of the enzyme solution on Sephadex G-100, DEAE-cellulose and CM-Sephadex are shown in Figs. 1, 2 and 3 respectively. The purified enzyme retained activity for up to 12 months when stored at -20°C.

General properties of enzyme

The enzyme synthesized by the marine pseudomonad released from alginate solutions products that gave a positive thiobarbituric acid test and absorbed strongly in the u.v. region (with maxima at 232nm), indicating that the enzyme was of the lyase type (EC 4.2.2.3) described by Preiss & Ashwell (1962). The enzyme was active in the pH range 6.0-9.0 and showed maximum activity at pH7.5. The optimum temperature for enzymic activity was 30°C.

Molecular weight

Studies on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that the mol.wt. of the alginate lyase was about 50000. This value was obtained by comparing the electrophoretic mobility of the enzyme (0.29) with those of standard marker proteins; bovine serum albumin (0.21), catalase

Table 1. *Purification of alginate lyase (EC 4.2.2.3)*

The enzyme was isolated from broken cells of the marine pseudomonad (15g wet wt.) and the activity of each fraction measured by the thiobarbituric acid assay of the products released under standard conditions described in the Materials and Methods section. The units are defined as the amount of enzyme required to liberate the equivalent of 1 μ mol of β -formylpyruvic acid/min under the standard conditions of assay. Details of the purification step are given in the text.

Step	Description	Vol. (ml)	Protein (mg/ml)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
1	Crude extract	250	5.5	3.2	800	0.6	100	1
2	(NH ₄) ₂ SO ₄ precipitation	150	0.62	4.3	640	7.0	80	11.7
3	Batch DEAE-cellulose purification	100	0.41	3.8	380	9.2	47	15.3
4	G-100 Sephadex-gel filtration	50	0.11	3.8	352	33.0	44	55.0
5	Purification on DEAE-cellulose column	30	0.075	4.1	184	55.0	23	92.0
6	Purification on CM-Sephadex column	24	0.05	3.3	80	73.0	10	120.0

(0.255), ovalbumin (0.34), lactate dehydrogenase (0.42), trypsin (0.61) and lysozyme (0.79). The mol.wts. of the standards are 68000, 60000, 43000, 36000, 23300 and 14300 respectively.

Specificity of bacterial enzyme

When incubated with a series of alginate samples of different mannuronic acid/guluronic acid ratios, the enzyme released more thiobarbituric acid-positive

material from algal substrates with higher guluronic acid content (Table 2). The rate of release of thiobarbituric acid-positive material from homopolymeric substrates (prepared from algal alginate), is shown in Fig. 4. The Michaelis constant determined for the enzyme varied according to the nature of the alginate sample used as substrate. However, when the constant was corrected to accommodate the variable amount of guluronic acid within the polyuronide, a reproducible value was obtained (Table 3).

Fig. 1. Purification of alginate lyase from bacterial isolate SA1

Gel filtration on Sephadex G-100 was used to purify the enzyme (step 4 in the purification procedure outlined in the Materials and Methods section). Mops buffer (10mM, pH7.5) was used as eluent. The protein content (Δ , E_{280}) and enzyme activity ($\bullet$, E_{549}) of the fractions were measured.

Enzyme products from alginate

The unsaturated oligouronides released from alginate solutions by the action of the alginate lyase were examined by paper chromatography and gel filtration. Fractionation of the products in a calibrated column of Sephadex G-15 indicated that the fastest moving compound (on paper) was a disaccharide. The mobility, on paper, of the products relative to the mobility of guluronic acid is shown in Table 4. The acid hydrolysate of each oligouronide was examined by paper chromatography and high-voltage electrophoresis. After staining with alkaline AgNO_3 , the approximate ratio of mannuronic acid to guluronic acid present in each oligosaccharide was determined (Table 4).

Fig. 2. Anion-exchange chromatography in the purification of alginate lyase

The enzyme, dissolved in 0.05M-Tris/HCl buffer (pH7.8), was applied to a column of DEAE-cellulose as described in the Materials and Methods section (purification step 5). A linear elution gradient of NaCl in buffer (—) was applied and samples were collected and assayed for protein content (Δ , E_{280}) and for enzyme activity ($\bullet$, E_{549}).

Fig. 3. Cation-exchange chromatography of alginate lyase

The material with enzyme activity eluted from the DEAE-cellulose column was equilibrated with phosphate buffer (0.05M, pH7.8) and eluted from a CM-cellulose column with a linear gradient of NaCl (—) in buffer. Δ , Protein (E_{280}); $\bullet$, enzymic activity (E_{549}).

Table 2. *Rate of release of thiobarbituric acid-positive material from a series of alginate samples of different composition*

Algal and bacterial alginates of different mannuronic acid/guluronic acid ratios were incubated with purified alginate lyase at 30°C for 1 h in 10mM-Mops/NaOH buffer (pH 7.5). The amount of unsaturated uronic acid liberated in each incubation mixture was determined by the thiobarbituric acid test described in the Materials and Methods section.

Alginate	Mannuronic acid (%)	Thiobarbituric acid assay (E_{549})
I*	28	2.10
II†	33	1.83
III†	42	0.50
IV*	48	1.60
V†	55	0.96
VI†	67	0.96
VII†	73	0.76

* Algal alginate.

† Bacterial alginate.

Table 3. *Kinetics of bacterial alginase action*

K_m values for the bacterial enzyme were determined as described in the Materials and Methods section for the alginate samples of differing mannuronic acid/guluronic acid ratios. The values shown in column 3 reflect the quantity of available substrate (i.e. guluronic acid) within each alginate sample. The kinetic constants were obtained from double-reciprocal plots.

Alginate	Mannuronic acid content (%)	K_m (mg/ml)	K_m (guluronic acid, mM)
I	28	1500	6.13
VIII	48	1850	5.51
IV	48	2460	7.27
V	55	2180	5.57
VII	73	4400	6.74

Discussion

From the kinetic studies and from analysis of oligouronide fragments, the alginate-degrading enzyme elaborated by the marine pseudomonad appears to show specificity towards guluronic acid-containing linkages within the alginate macromolecule; and as

Fig. 4. *Rate of release of thiobarbituric acid-positive material from homopolymeric substrates by the guluronate lyase*

Homopolymeric substrates were prepared from alginate as described in the Materials and Methods section. These were dissolved in 10mM-Mops/NaOH buffer (pH 7.5) and digested with purified enzyme at 30°C. Samples were removed at various time-intervals for assay of the lyase products by the thiobarbituric acid test. Δ, polyguluronic acid; ▲, polymannuronic acid.

Table 4. *Mobility and composition of oligouronide products of alginate decomposition by bacterial alginate lyase activity*

The oligosaccharide products from both algal and bacterial alginate samples were isolated and purified as described in the Materials and Methods section. Their composition was determined by hydrolysis with 2M-HCl, followed by chromatography in solvent C. —, Uronic acid not detected; +, trace amounts of uronic acid; +++, very strong spot equidistant with uronic acid standard. n.d., Not determined.

Oligouronide (unsaturated)	R_{GuA} in solvent			Component sugars			
	A	B	C	ManA*	GuA*	ManA†	GuA†
Dimer	0.63	0.94	0.92	—	+++	—	+++
Trimer	0.32	0.64	0.72	—	+++	—	+++
Tetramer	0.27	0.46	0.53	+	+++	—	+++
Pentamer	0.12	0.21	0.43	+	+++	+	+++
Hexamer	0.05	0.09	0.20	n.d.	n.d.	n.d.	n.d.

* Algal alginate.

† Bacterial alginate.

Fig. 5. Use of bacterial alginate (guluronate) lyase in the determination of guluronic acid/mannuronic acid ratios of alginic acid preparations

The K_m values were obtained by using alginate preparations of different mannuronic acid content, by incubating samples at 30°C in 10mM-Mops/NaOH buffer (pH7.5) with purified enzyme. Enzyme activity was measured as release of thiobarbituric acid-positive material. Plot of the reciprocal K_m value against mannuronic acid content of the alginate sample then shows a linear relationship, thus providing a means of defining the primary structure of any alginate sample.

such may be described as a poly-(1,4- α -L-guluronate) lyase (EC 4.2.2.3).

This specificity makes it possible to estimate the ratio of mannuronic acid/guluronic acid within the polymer (Fig. 5). Although not an absolute procedure, the amount of 'real' substrate (as defined below) within any alginate molecule may thus be conveniently estimated from kinetic studies.

The failure to detect mannuronic acid in acid hydrolysates of the smaller oligouronide products of lyase activity may indicate that the enzyme cleaves one or both the uronic acid sequences shown below, where G = L-guluronic acid, M = D-mannuronic acid and Δ = 4-deoxy-5-oxo-uronic acid.

The disaccharide 4-deoxy-5-oxo-uronosylguluronic acid could therefore have arisen from either polyguluronic acid blocks or alternating sequences of the polysaccharide. Since, however, both mannuronic acid and guluronic acid on transesterification give rise to 4-deoxy-5-oxo-uronic acid, it is not possible to state the exact origin of this disaccharide. Guluronic acid-containing oligouronides larger than disaccharides probably arose from homopolymeric guluronic acid sequences.

The relationship of the enzyme described here to previously reported enzymes with alginates as substrates is difficult to determine as, apart from the

studies of Nakada & Sweeny (1967) few authors have characterized the substrate adequately. Although Preiss & Ashwell (1962) noted the lyase nature of the enzyme studied, and the formation of oligouronides, neither the mannuronic acid/guluronic acid ratio of the substrate nor the exact nature of the products was determined. Enzymes apparently differing in their specificities towards substrates of high and low molecular weight have been described, and one such enzyme was thought to be specific for short-chain polyguluronides (Kashiwabara *et al.*, 1969). The product of this enzyme, also from a pseudomonad, was an unsaturated triuronide. Most other reports have indicated enzymes with specificity towards the mannuronic acid portion of the molecule. Such enzymes have been found in molluscs (*Littorina* sp., Elyakova & Favorov, 1974), and bacteriophage for an *Azotobacter vinelandii* strain (Davidson *et al.*, 1976).

The existence of an enzyme complementary to those polymannurono-lyases is consistent with the understood structure of alginic acid, since both specific enzymes would be necessary for the complete degradation of the polysaccharide.

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