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Isolation and characterization of an alginate lyase from *Klebsiella aerogenes*

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Abstract. The bacterium *Klebsiella aerogenes* (type 25) produced an inducible alginate lyase, whose major activity was located intracellularly during all growth phases. The enzyme was purified from the soluble fraction of sonicated cells by ammonium sulfate precipitation, anion- and cation-exchange chromatography and gel filtration. The apparent molecular weight of purified alginate lyase of 28,000 determined by gel filtration and of 31,600 determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the active enzyme was composed of a single polypeptide. The alginate lyase displayed a pH optimum around 7.0 and a temperature optimum around 37° C. The purified enzyme depolymerized alginate by a lyase reaction in an endo manner releasing products which reacted in the thiobarbituric acid assay and absorbed strongly in the ultraviolet region at 235 nm. The alginate lyase was specific for guluronic acid-rich alginate preparations. Propylene glycol esters of alginate and O-acetylated bacterial alginates were poorly degraded by the lyase compared with unmodified polysaccharide. The guluronate-specific lyase activity was applied in an enzymatic method to detect mannuronan C-5 epimerase in three different mucoid (alginate-synthesizing) strains of *Pseudomonas aeruginosa*. This enzyme which converts polymannuronate to alginate could not be demonstrated either extracellularly or intracellularly in all strains suggesting the absence of a polymannuronate-modifying enzyme in *P. aeruginosa*.

Key words: *Klebsiella aerogenes* – Alginate – Alginate lyase – Purification – Characterization – Mannuronan C-5 epimerase assay – Mucoid – *Pseudomonas aeruginosa*

Alginate is a (1-4)-linked copolymer of β-D-mannuronate and its C5 epimer α-L-guluronate (Haug and Larsen 1962). The monomer residues may be arranged in homopolymeric block structures of (1-4)-β-D-mannuronan [poly(ManA)] and (1-4)-α-L-guluronan [poly(GulA)] that are linked by heteropolymeric sequences of randomly distributed mannuronate and guluronate residues (Haug et al. 1967). In contrast to algal alginates from brown seaweeds (*Phaeophyceae*), bacterial alginates are extracellular polysaccharides in *Azotobacter vinelandii* (Gorin and Spencer 1966) and a number of *Pseudomonas* species (Evans and Linker 1973; Govan et al. 1981; Fett et al. 1986) and additionally contain O-acetyl groups on some mannuronate residues (Skjåk-Bræk et al. 1986). The degree of acetylation varies strain-dependent from 4%–57% (Skjåk-Bræk et al. 1986). In contrast to alginate from *A. vinelandii*, alginates from *Pseudomonas* species completely lack consecutive guluronate residues (Skjåk-Bræk et al. 1986). Alginates are enzymatically depolymerized by lyases (eliminases) which catalyze the cleavage of the glycosidic linkages by β-elimination with formation of an unsaturated bond between carbon atoms 4 and 5 of the uronic acid residue on the non-reducing end at the cleavage site. Alginate lyases differ in their preferences for poly(ManA) and poly(GulA) regions in the alginate molecule.

An extracellular poly(GulA)-specific alginate lyase of *Klebsiella aerogenes* has been described (Boyd and Turvey 1977). Crude preparations of this enzyme have been used for establishing a sensitive assay of an extracellular D-mannuronan C-5-epimerase, which catalyzes the final step of alginate biosynthesis in *A. vinelandii* (Currie and Turvey 1982). The progress of the epimerization reaction, i.e. conversion of mannuronate to guluronate residues, could be followed by assaying the increase in susceptibility of epimerized substrate to the action of poly(GulA)-specific lyase (Currie and Turvey 1982). The presence of a similar mannuronan C-5 epimerase activity in mucoid (alginate-synthesizing) strains of *Pseudomonas aeruginosa* is still controversial (Pigott et al. 1981; Singh et al. 1987).

The present study is concerned with the purification and partial characterization of the cell-bound alginate lyase of *K. aerogenes* and with preliminary attempts to identify mannuronan C-5 epimerase activity in mucoid strains of *P. aeruginosa* by use of the purified poly(GulA) lyase from *K. aerogenes*.

Materials and methods

Bacterial strains

Klebsiella aerogenes type 25 was kindly provided by F. Haugen, Trondheim, Norway. Mucoid strains *Pseudomonas*

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Abbreviations: poly(ManA), (1-4)-β-D-mannuronan; poly(GulA), (1-4)-α-L-guluronan; TBA, 2-thiobarbituric acid

aeruginosa 4335, 7293 and 5386b isolated from sputa of cystic fibrosis patients were kindly provided by N. J. Russell and P. Gacesa, Cardiff, Wales. Mucoid strain *P. aeruginosa* CF1/M1 was isolated from a cystic fibrosis patient at the Children's University Hospital, Essen, FRG.

Purification of alginate lyase

The nutrient broth alginate (NBA) growth medium used for the production of alginate lyase by *K. aerogenes* was composed of (g/l): nutrient broth (Oxoid), 13, and sodium alginate (Manucol LH), 10. A 50 ml portion of NBA medium was inoculated with single colonies of *K. aerogenes* and incubated at 37° C for 22 h on a rotary shaker (180 rev/min). 0.2 ml samples were added to 400 ml portions of NBA medium in 1-l flasks. After incubation at 37° C for 22 h (180 rev/min) the bacteria were collected by centrifugation (16,000 × *g*, 20 min) and resuspended in one eighth of the original volume of 50 mM phosphate buffer pH 7.0 containing 1 mM EDTA. Cells were disrupted by sonication (6 × 30 s, 90 W) under cooling on ice. Cell debris and envelopes were removed by centrifugation at 6,000 × *g* for 10 min followed by centrifugation at 40,000 × *g* for 60 min. Solid ammonium sulfate was added to the clear supernatant to 50% saturation. After 3 h at 4° C the precipitate was removed by centrifugation (15,000 × *g*, 20 min) and ammonium sulfate concentration was increased to 90% saturation. After standing overnight at 4° C precipitated material was collected by centrifugation (15,000 × *g*, 20 min) and resuspended in one tenth of the original volume of 50 mM phosphate buffer pH 7.0. The crude enzyme preparation was dialyzed against 50 mM Tris buffer pH 8.5. Up to 20 ml of sample were applied to a column (1.6 × 26 cm) of DEAE-Sepharose CL-6B. The column was eluted with 80 ml of 50 mM Tris buffer pH 8.5, 130 ml of a linear gradient of 0 – 0.5 M NaCl and 200 ml of 2 M NaCl in the same buffer at a flow rate of 20 ml/h. All fractions (5 ml) were assayed for protein (A_{280}) and alginate lyase activity as described below. Enzyme-containing fractions were pooled, dialyzed against 50 mM phosphate buffer pH 7.0 and applied to a column (1.6 × 26 cm) of CM-Sepharose CL-6B. The column was eluted with 80 ml of 50 mM phosphate buffer pH 7.0, 50 ml of 0.15 M NaCl and 200 ml of 2 M NaCl in the same buffer at a flow rate of 20 ml/h. All fractions (5 ml) were assayed for protein (A_{280}) and alginate lyase activity. Two milliliters of alginate lyase-containing peak fraction obtained by elution with 0.15 M NaCl from the cation exchanger were applied on a column (1.6 × 90 cm) of Sephacryl S-200 and eluted with 180 ml of 50 mM phosphate buffer pH 7.0 containing 0.3 M NaCl at a flow rate of 16.6 ml/h. The same column was calibrated under identical elution conditions using the following protein size standards (Bio-Rad): thyroglobulin (670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and cyanocobalamin (1,350).

Electrophoresis

Alginate lyase preparations of several steps at purification were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the procedure of Laemmli and Favre (1973). Slab gels of 14% (w/v) acrylamide/0.7% (w/v) bis(acrylamide) pH 8.8 and of 3% (w/v) acrylamide/0.08% (w/v) bis(acrylamide) pH 6.8 were used for the running gels and stacking gels, respectively. Prior to electrophoresis protein samples were treated for 10 min at 90° C in denaturing sample buffer (Laemmli and Favre 1973). Proteins were stained with Coomassie brilliant blue R-250.

Non-denaturing polyacrylamide gel electrophoresis of enzymatically degraded alginate was performed in glass tubes as described by Bucke (1974). Polysaccharides were stained with toluidine blue-O (Dunne and Buckmire 1985).

Analytical methods

Protein was determined according to Read and Northcote (1981) using bovine serum albumin (fraction V, Sigma) as standard. Total carbohydrate was assayed by the phenol-sulphuric acid method (Dubois et al. 1956). ^{1}H-nmr spectroscopy of alginates was carried out on samples prepared as described by Grasdalen et al. (1979). 400-MHz ^{1}H-nmr spectra were recorded with a Bruker AM-400 spectrometer. Monomeric composition and diad frequencies of alginates were calculated from the spectra as described previously (Grasdalen et al. 1979).

Enzyme assays

Alginate lyase activity was routinely determined with sodium alginate (Manucol LH) as substrate under the following conditions, unless otherwise noted: 0.5 ml of substrate solution (6.6 mg alginate and 17.5 mg NaCl per ml 50 mM phosphate buffer pH 7.0) was mixed with 0.1 ml of enzyme-containing solution and incubated at 37° C for up to 20 min. The reaction was stopped by boiling the mixture for 2 min. The degradation products of alginate were determined with the thiobarbituric acid (TBA) method according to Weissbach and Hurwitz (1959). One unit of enzyme activity (U) corresponded to the release of 1 nmol β-formylpyruvate/min under the conditions described. 10 nmol β-formylpyruvate gave an A_{548} of 0.29 in the TBA assay (Preiss and Ashwell 1962). In some cases, enzymatic degradation of alginate was assayed by following the increase in absorbance at 235 nm and by measuring the decrease of the specific viscosity of the reaction mixtures in a Micro-KPG-Ubbelohde-viscosimeter (capillary diameter 0.32 mm) at 25° C. Protease activity was determined photometrically with azocasein as substrate (Prestidge et al. 1971) and on nutrient agar with 5% (w/v) skim milk or 0.15% (w/v) elastin as substrates, respectively. Mannuronan C-5 epimerase activity in mucoid strains of *Pseudomonas aeruginosa* was studied essentially as described by Piggott et al. (1981). In short, bacteria were grown in yeast extract peptone medium (Wicker-Böckelmann et al. 1987) at 37° C for 22 h, before they were collected by centrifugation (20,000 × *g*, 20 min), resuspended in half of the original volume of 50 mM phosphate buffer pH 7.0 and broken by sonication (5 × 30 s, 90 W). Cell debris was removed by centrifugation at 7,000 × *g* for 10 min. The clear cell extracts, cell-free culture supernatants and 80% ammonium sulfate precipitates of culture supernatants were used as a source for epimerase activity. The epimerase assay mixtures contained 4 mg of poly(ManA) dissolved in 1.8 ml of cell fraction and 0.2 ml 7.2 mM $CaCl_2$. After incubation of the mixtures at 30° C for 18 h, the reaction was stopped by heating at 100° C for 2 min. Subsequently, the presence of guluronate was determined after addition of 2 µg of purified alginate lyase protein from *K. aerogenes* in 1 ml 0.15 M

phosphate buffer pH 7.0 containing 0.9 M NaCl. At different time intervals for up to 30 min at 37° C samples were removed, boiled for 2 min and assayed for unsaturated uronic acid by the TBA method (Weissbach and Hurwitz 1959).

Polysaccharides

Commercial sodium alginate (Manucol LH) and propylene glycol alginates (Manucol Ester E/LH, 65% esterified, and Manucol Ester E/RE, 83% esterified) were obtained from Kelco-AIL-International Ltd., Hamburg, FRG. Homopolymeric block regions poly(ManA) and poly(GulA) were prepared from sodium alginate (Manucol LH) by partial acid hydrolysis (Haug et al. 1974). Bacterial alginate containing 7.7% (w/w) O-acetyl groups was purified from mucoid strain *P. aeruginosa* 7293 (Wicker-Böckelmann et al. 1987). Bacterial alginate was deacetylated by alkali according to Evans and Linker (1973). Hyaluronate (potassium salt), chondroitin sulfate (sodium salt), pectate (sodium salt), heparin (sodium salt), glycogen, dextran 60 and pullulan were purchased from Serva, Heidelberg, FRG. Mycolaminaran was purified from *Phytophthora cactorum* (Wang and Bartnicki-Garcia 1973). Manucol LH, chondroitin sulfate, pectate, heparin, and dextran 60 were further purified by centrifugation of 2% (w/v) aqeous solutions at 40,000 × *g* for 30 min, dialysis against deionized water and freeze-drying.

Results

Isolation and purification of an alginate lyase from Klebsiella aerogenes

The expression of alginate lyase activity of *K. aerogenes* grown in nutrient broth medium was dependent on the presence of alginate (Fig. 1). Enzyme activity appearing in the late-logarithmic growth phase was detected to be cell-bound and in cell-free culture fluids. Alginate lyase activities steadily increased during the stationary growth phase to maximum values followed by a drop in enzyme activity on prolonged incubation. Protease activity in cell-free culture fluids could not be detected employing agar plate assays with skim milk and elastin substrates or by using a photometric assay with azocasein as substrate. This indicated that the observed decrease of lyase activity was probably not due to proteolytic inactivation. During 30 h incubation in alginate-containing growth medium the pH value dropped from 7.4 to 5.8, whereas in alginate-free cultures no change of pH occurred.

Purification of alginate lyase was started from stationary cells of *K. aerogenes* by sonication of the bacteria which resulted in an approx. 50-fold increase in enzyme activities compared with unbroken cells. The data of subsequent purification steps are summarized in Table 1. Half of the amount of cellular protein was precipitated with 50% ammonium sulfate from cell extracts after sonic disruption, whereas alginate lyase remained soluble and was obtained by increasing the ammonium sulfate concentration to 90%. Subsequent anion-exchange chromatography at pH 8.5 separated alginate lyase activity from 95% of the other proteins that bound to the column, whereas the enzyme had no affinity to the ion-exchanger. The lyase-containing fractions were devoid of carbohydrate indicating the absence of an enzyme-carbohydrate association that has been reported for

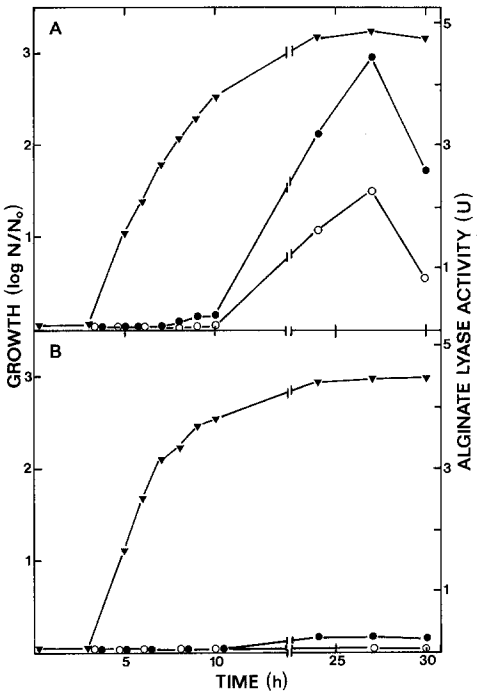

Fig. 1. Growth (▼) and alginate lyase activity in cell-free culture fluids (○) and in whole cells (●) of *Klebsiella aerogenes*. Bacteria were grown at 37° C in nutrient broth medium in the presence (**A**) and absence (**B**) of 1% (w/v) alginate. The initial cell density (N_0) was 1.8×10^6/ml. At different times 1.5 ml samples were withdrawn for microscopic determination of cell numbers (*N*) in a Neubauer counting chamber and for measurement of alginate lyase activity in culture supernatants as well as in whole cells recovered by centrifugation and resuspension in the original volume of 50 mM phosphate buffer pH 7.0

the extracellular alginate lyase (Boyd and Turvey 1977). Further purification of alginate lyase was obtained by cation-exchange chromatography at pH 7.0. The main protein fraction did not bind to the ion-exchanger, whereas alginate lyase eluted at approximately 0.15 M NaCl on application of a linear salt gradient. Purification of the enzyme was more effective when a stepwise salt gradient was employed. The final purification step was gel filtration on a column of Sephacryl S-200. Alginate lyase activity eluted as a single symmetrical peak. Comparison of the enzyme activity peak with standard proteins used for calibration of the column yielded a native molecular weight of 28,000 for alginate lyase. The purification of alginate lyase was checked by dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 2). The last purification step revealed a single protein band corresponding to an apparent molecular weight of 31,600.

General properties of alginate lyase

For the characterization of alginate lyase of *K. aerogenes* a commercial algal alginate (Manucol LH, approx. mean molecular weight 88,000) with a mannuronate/guluronate ratio of 1.4 was employed as substrate. Alginate lyase was found to require high salt concentrations for maximal enzyme activity. Highest enzyme activities were obtained at 0.1 – 0.3 M with monovalent cations (Na^+, K^+) and at 0.05 to 0.1 M with magnesium ions.

Table 1. Purification of intracellular alginate lyase from *Klebsiella aerogenes*

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Cell extract	116	12,992	327	39.7	100	1
50–90% ammonium sulfate fraction	18	9,668	129	74.9	75	1.9
DEAE-Sepharose Cl-6B	20	3,110	4.7	658.9	24	16.6
CM-Sepharose Cl-6B	5	627	0.3	2,239.3	4.8	56.5
Sephacryl S-200	10	363	NM[a]	–	2.8	–

[a] NM, not measurable

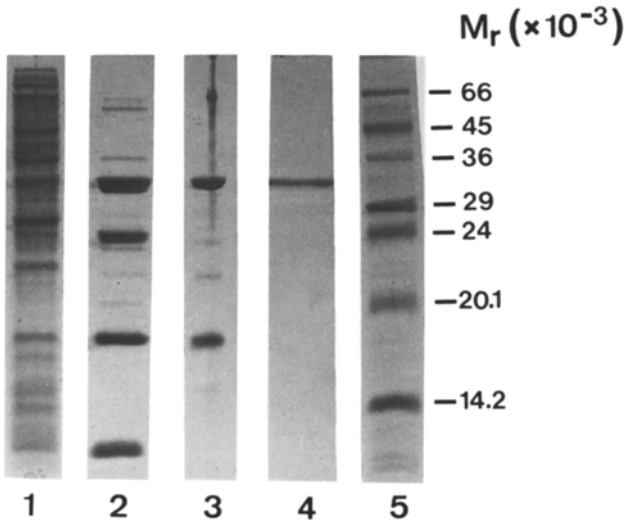

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of alginate lyase preparations at different purification steps. *Lane 1*: 50–90% ammonium sulfate fractionation; *lane 2*: anion-exchange chromatography; lane 3: cation-exchange chromatography; *lane 4*: gel filtration; *lane 5*: standard proteins (Sigma): bovine albumin, 66,000; ovalbumin, 45,000; glyceraldehyd-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,200

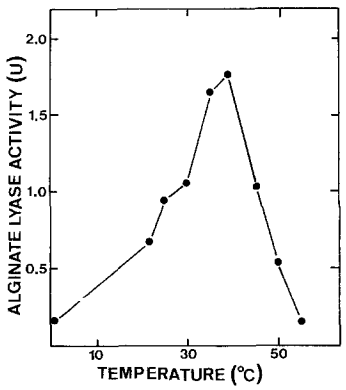

Fig. 3. Effect of temperature on alginate lyase activity

Alginate lyase activity revealed a broad pH optimum with a sharp drop of activity below pH 6.0 and above pH 7.5. Identical results were obtained whether phosphate, Tris or Hepes buffers were used.

Alginate lyase activity showed a sharp temperature optimum around 37°C (Fig. 3). At 0°C and 55°C a weak residual enzyme activity of approximately 9% was still measurable. The sulfhydryl reagents dithiothreitol and 2-mercaptoethanol, tested in final concentrations between 0.1 and 6.0 mM, had no influence on alginate lyase activity. This indicated that disulfide bonds within the enzyme protein were not essential for lyase activity.

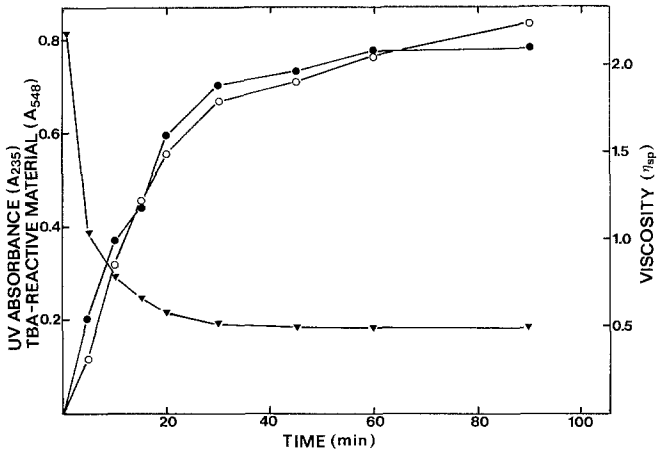

Fig. 4. Time course of depolymerization of alginate (Manucol LH) by purified alginate lyase as demonstrated by increase in TBA-reactive material (●) and in ultraviolet light absorbance (○) as well as decrease of specific viscosity (▼). The final concentration of alginate in the reaction mixture was 3 mg/ml

The time course of alginate depolymerization by alginate lyase could be followed by an increase in ultraviolet light absorption, formation of TBA-positive material and decrease in specific viscosity (Fig. 4). This suggested that the purified enzyme degraded alginate by a lyase rather than a hydrolase reaction. At a substrate concentration of 3 mg/ml the reaction had almost ceased after 30 min (Fig. 4). The rapid initial rate of alginate degradation was also visualized on non-denaturing polyacrylamide gels [6% (w/v) acrylamide/0.12% (w/v) bis(acrylamide) pH 8.3] by a change of polydisperse high-molecular-weight alginate with low electrophoretic mobility to low-molecular-weight products with increasing mobility depending on the incubation time with alginate lyase (not shown).

Substrate specificity of purified enzyme was tested on native and chemically modified alginates as well as on homopolymeric block regions of alginate. As can be seen in Table 2, the enzyme was most active on poly(GulA) compared with poly(ManA) indicating that the enzyme is a poly(GulA) lyase. Native algal alginate with a mannuronate/guluronate ratio of 1.4 proved to be a better substrate than a deacetylated bacterial alginate with a mannuronate/

Table 2. Depolymerization of different alginate preparations by alginate lyase from *Klebsiella aerogenes*. Substrates were oligomeric block regions [poly(ManA), poly(GulA)], algal alginate (Manucol LH), O-acetylated, and deacetylated bacterial alginate from mucoid strain *Pseudomonas aeruginosa* 7293 and propylene glycol alginate with 65% and 83% of carboxyl groups esterified. The final concentration of all substrates was 3 mg/ml in the reaction mixture

Substrate	F^a_M	F^a_G	F^a_{MM}	F^a_{GG}	Enzyme activity		
					U[b]	ΔA_{235}/30 min[c]	$\Delta\eta_{sp.}$/30 min[d]
Poly(ManA)	0.80	0.20	0.75	0.15	0.06	0.05	0
Poly(GulA)	0.08	0.92	0.01	0.85	1.98	2.70	0.01
Algal alginate	0.59	0.41	0.47	0.29	2.20	2.30	1.54
Bacterial alginate, acetylated	0.84	0.16	0.69	0.01	0.27	0.26	ND
Bacterial alginate, deacetylated	0.84	0.16	0.69	0.01	0.95	1.30	ND
Propylene glycol alginate							
65% esterified	ND[e]	ND	ND	ND	0.42	0.84	0.28
83% esterified	ND	ND	ND	ND	0.13	0.22	0.04

[a] Relative frequency of occurrence of mannuronate (F_M), guluronate (F_G), mannuronate diads (F_{MM}) and guluronate diads (F_{GG}) in the substrate molecules calculated from ^{1}H-n.m.r. spectra according to Grasdalen et al. (1979)
[b] Enzyme activity determined by the TBA method
[c] Enzyme activity determined by increase in ultraviolet light absorbance
[d] Enzyme activity determined by decrease of specific viscosity
[e] ND, not determined

guluronate ratio of 5.3, thus confirming the preference of the enzyme for guluronate-rich alginate. Native bacterial alginate with 7.7% (w/w) O-acetylation was only weakly degraded by alginate lyase, in contrast to the same alginate after removal of acetyl groups. Increasing degree of esterification of alginate with propylene glycol resulted in a weak depolymerization by alginate lyase.

Other polysaccharides such as hyaluronate, pectate, heparin, chondroitin sulfate, dextran, glycogen, pullulan, mannan, and mycolaminaran were not degraded by the purified alginate lyase.

Assay of mannuronan C-5 epimerase activity in mucoid Pseudomonas aeruginosa with purified alginate lyase

According to Piggott et al. (1981), mucoid *Pseudomonas aeruginosa* possesses an extracellular mannuronan C-5 epimerase involved in alginate biosynthesis whose activity could be measured by using a guluronic acid-specific lyase for the detection of the conversion of mannuronate to guluronate in poly(ManA) as epimerase substrate. Employing essentially the same reaction conditions of Piggott et al. (1981) three clinical mucoid, i.e., alginate-synthesizing strains of *P. aeruginosa* (strains 4335, 5386b and CF1/M1) were tested for the presence of epimerase activity. Cell-free culture fluids, ammonium sulfate concentrated supernatants and extracts from broken cells were incubated with poly(ManA) at 37° C for 18 h. After addition of poly(GulA) lyase from *K. aerogenes* no release of TBA-reactive material could be measured, i.e. no increase in guluronate content of poly(ManA) as potential epimerase substrate had taken place. In contrast to earlier results of Piggott et al. (1981), addition of $CaCl_2$ (final conc. 7.2 mM) to the epimerase assay mixtures did not result in the detection of enhanced epimerase activities.

Discussion

Within the family of *Enterobacteriaceae* only *Klebsiella* spp. revealed alginolytic activities (Davis and Ewing 1964). An extracellular alginate lyase with poly(GulA) specificity has been discovered in culture fluids of *K. aerogenes* (Boyd and Turvey 1977). Using the same strain and identical growth conditions as described by Boyd and Turvey (1977) it was demonstrated in the present study that most of the alginate lyase activity was located intracellularly (Fig. 1).

The present study focused on the purification and the characterization of the intracellular alginate lyase enzyme, which was recovered from sonicated cells of the stationary growth phase. The most effective purification steps were achieved by successive anion- and cation-exchange chromatography. At pH 8.5 alginate lyase did not bind to the positively charged DEAE groups of the ion-exchanger, indicating the absence of a net negative charge of the enzyme at this pH value. This is in accord with the observation that the isoelectric point of the major alginate lyase activity in crude bacterial extracts of *K. pneumoniae* was determined to be 8.9 (Caswell et al. 1986). The basic properties of alginate lyase led to an effective fractionation of enzyme binding at pH 7.0 to the negatively-charged CM groups of the cation-exchanger used for the subsequent purification step, whereas most of the other proteins had no affinity to the ion-exchanger. Electrophoretically homogeneous enzyme protein was obtained after the last purification step of gel filtration. The apparent molecular weight of 28,000 determined by gel filtration for the purified native enzyme and of 31,600 determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the denatured enzyme indicate that active alginate lyase probably consists of a single polypeptide chain. Other bacterial alginate lyases have been reported to have molecular weights of 50,000 (Davidson et al. 1976), 35,000 and 100,000 (Doubet and Quatrano 1984), 29,000 (Romeo and Preston 1986a), and 58,000 (Wicker-Böckelmann et al. 1987).

The dependence of optimum lyase activity on relatively high salt concentrations has also been determined for the extracellular alginate lyase of *K. aerogenes* (Boyd and Turvey 1977). The pH optimum around 7.0 and the temperature optimum around 37° C for alginate lyase of *K. aerogenes* did not deviate significantly from the values reported for

other bacterial alginate lyases (Preiss and Ashwell 1962, Davidson et al. 1976, Linker and Evans 1984). Enzymatic depolymerization of algal alginate by purified enzyme occurred by an eliminase rather than a hydrolase reaction as deduced from the usual criteria for alginate lyases of causing production of ultraviolet light-absorbing material and release of TBA-reactive products during the cleavage reaction of alginate (Preiss and Ashwell 1962). In general, end products of alginate lyases are a series of oligosaccharides containing an unsaturated uronic acid residue on the non-reducing end of the oligosaccharide chain (Preiss and Ashwell 1962). The initial rapid rate of alginate degradation by purified enzyme suggested that the alginate lyase revealed endolytic activity cleaving the glycosidic linkages in a random fashion as has also been demonstrated for the extracellular lyase of *K. aerogenes* (Boyd and Turvey 1977).

In tests for substrate specificity purified alginate lyase preferentially depolymerized poly(GulA) relative to poly(ManA) (Table 2), indicating that the purified enzyme revealed poly(GulA) lyase activity. Depolymerization of native algal alginate was comparable to that of poly(GulA), although algal alginate contained only 41% guluronate residues vs. 92% in poly(GulA) (Table 2). The action of extracellular alginate lyase of *K. aerogenes* on poly(GulA) has been reported to be inhibited by triguluronate, which was not degraded any further by the enzyme (Boyd and Turvey 1978). A similar effect may also occur with the intracellular lyase due to higher concentrations of inhibitory cleavage end-products from poly(GulA) degradation compared with that of algal alginate.

Chemical modifications of alginate such as bacterial alginate with partially O-acetylated mannuronate residues and propylene glycol-esterified alginate proved to be poor substrates for alginate lyase of *K. aerogenes*, suggesting a role for intact mannuronate and carboxyl moieties in substrate recognition and/or cleavage reaction. This inhibition on enzyme action by O-acetylation and carboxyl esterification of alginate substrate seems to be a common property of some bacterial alginate lyases (Linker and Evans 1984; Romeo and Preston 1986b; Wicker-Böckelmann et al. 1987). Several neutral and acidic polysaccharides other than alginates were not depolymerized by purified alginate lyase, demonstrating a high substrate specificity of the enzyme.

Poly(GulA) lyases have been used for assays of D-mannuronan C-5 epimerase as the enzyme involved in the final step of bacterial alginate biosynthesis. Crude preparations of poly(GulA) lyase from *K. aerogenes* and *Beneckea pelagia* have been employed for detecting extracellular calcium-dependent epimerase activities in *Azotobacter vinelandii* (Currie and Turvey 1982) and a mucoid strain of *Pseudomonas aeruginosa* (Piggott et al. 1981), respectively. In all studies either mannuronic acid-rich alginate or poly(ManA) served as epimerase substrate. The progress of epimerase reaction, i.e. the conversion of mannuronate to guluronate residues at the polymer level, was followed by an increase in susceptibility of the epimerase substrate to the action of a poly(GulA) lyase. In the present study purified poly(GulA) lyase from *K. aerogenes* was used to detect mannuronan C-5 epimerase activity in three different mucoid strains of *P. aeruginosa*. Under reaction conditions similar to those described by Piggott et al. (1981) epimerase activity could not be detected either intracellularly or extracellularly in all strains. Our results are in accord with those of Singh et al. (1987) who also reported that mucoid *P. aeruginosa* was devoid of mannuronan C-5 epimerase activity. They used a sensitive enzyme assay based on the release of ^3H from poly D-[5-^3H]-mannuronate during epimerase reaction, which could only be demonstrated in *A. vinelandii* and not in *P. aeruginosa*. It was concluded that the pathways of alginate biosynthesis in *A. vinelandii* and *P. aeruginosa* may be different.

After submitting the present paper, work was published on the molecular cloning and heterologous expression of an alginate lyase gene from *Klebsiella pneumoniae* PG1 (Caswell et al. 1989). The authors reported a molecular weight of 28,000 for the alginate lyase, whose major enzyme activity was found extracellularly in their strain.

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