Biochimica et Biophysica Acta, 358 (1974) 341–354

BBA 67277

ISOLATION AND CERTAIN PROPERTIES OF ALGINATE LYASE VI FROM THE MOLLUSK *LITTORINA* SP

LYUDMILA A. ELYAKOVA and VALERY V. FAVOROV

Pacific Institute of Bioorganic Chemistry, Far East Science Centre, Academy of Sciences of the USSR, Vladivostok-22 (U.S.S.R.)

(Received February 4th, 1974)

SUMMARY

One of the alginate lyases, designated as alginate lyase VI, isolated from the hepatopancreas of the mollusk *Littorina* sp., has been purified 360 times after fractionation with ammonium sulphate and chromatography on DEAE-Sephadex, SE-Sephadex and CM-cellulose. The enzyme proved to be homogeneous when subjected to disk electrophoresis in polyacrylamide gel. According to thin-layer gel filtration data, the enzyme molecular weight was 40 000; pH 5.6 was optimum for enzyme activity. The enzyme is stable within a pH range from 4 to 8, and is inactivated completely within 1 h at 50 °C. Alginate lyase VI splits alginic acid and its oligomers of various compositions at different rates and does not hydrolyze other polysaccharides. Alginate lyase VI is ostensibly an endotranseliminase, preferably splitting the glycoside bond between two mannuronide residues in the alginic acid.

INTRODUCTION

Alginic acid is a widely used polysaccharide consisting of D-mannuronic and L-guluronic acids. Despite large successes in establishing the structure of the said compound by chemical procedures [1, 2], close attention is being paid to alginate lyases, which in a definite way split the linear chain of this polymer, as instruments for structural studies. Alginate lyases (EC 4.2.2.3) have been known for a long time [3]. Thus, in the mollusk *Holiotus*, two alginate lyases with exo- and endo-type activities [4], have been detected, both possessing different specificity with respect to the composition of alginic acids.

For the structural studies of alginic acid, alginate lyases from the mollusk *Dollabella auricula* were used after 15-food purification by fractionating with salts and gel filtration [5]. It was established that the rate and degree of hydrolysis for alginates with different compositions varies considerably.

Also widely known are alginate lyases of bacterial origin. Thus, Williams et al. [6] attained a 10-fold enrichment of the enzyme from *Agarbacterium alginicum* by fractionation with different salts [6]. As a result of four-fold gel filtration of the enzyme preparation from *Pseudomonas* [7], one of the enzymes, hydrolyzing oligoguluronide only, was enriched 21-fold. In *Clostridium alginoliticum*, 2–3 alginate lyase fractions

were observed with the gel filtration of the summary preparation [8]. These fractions produced approximately the same set of oligomers.

Several zones possessing alginate lyase activity were detected with electrophoresis in starch gel of the enzyme preparation from the mollusk *Dollabella* after fractionation with ammonium sulphate [5].

Electrophoresis in polyacrylamide gel showed that the alginate lyases from *Littorina* sp. were heterogeneous [9]. It was demonstrated that the fractions possessed different specificities with respect to oligomers of different composition, and varied in the decrease in viscosity of the alginic acid solution. From these results, one may assume the existence of alginate lyases specific to uronide linkages with exo- and endo-type activities, the use of which may prove to be quite fruitful in structural investigations.

This work describes the isolation of homogeneous alginate lyase; certain alginate lyase properties have also been revealed.

MATERIALS AND METHODS

Substrates

Alginic acid was isolated from the brown seaweed *Laminaria cichorioides* by sodium carbonate extraction after removing pigments with methanol and neutral sugars with 0.5% hydrochloric acid. Alginic acid oligomers were isolated after partial acid hydrolysis with sulphuric acid in accordance with Haug et al. [2]. The alginic acid and oligomer compositions were determined by the standard procedure [10]. The alginic acid viscosity was 23.4 dl/g, and the relationship of mannuronic acid:guluronic acid (M/G) was 1.37. Oligomers enriched with guluronic acid (G-blocks) had an M/G ratio equal to 0.06. Oligomers with an alternating sequence of uronic acids (MG-blocks) had an M/G ratio equal to 1.27. Oligomers enriched with mannuronic acid (M-blocks) had an M/G equal to 15.

Galactan from larch and mannan from wheat were purchased from the Koch-Light Laboratories, England; α-methyl-D-mannoside from Reanal, Hungary; and CM-cellulose and gum arabic from Serva, West Germany. Glucuronomannan [11] and oligogalacturonide [12] were kindly made available by the laboratory of carbohydrate chemistry of our institute. Gulono-γ-lactone was obtained from Gee Lawson, England; glucuronic acid from Fluka, G.F.R.; Sephadex G-25, DEAE-Sephadex A-50 and SE-Sephadex C-50 from Pharmacia, Sweden; and CM-cellulose (CM-52) from Whatman, England. Mannuronic and guluronic acids were isolated in accordance with Haug and Larsen [10].

Enzyme

A lyophilized aqueous extract from the hepatopancreas of the mollusk *Littorina* sp. was centrifuged for 10 min at 15 000 rev./min and subsequently used as a crude enzyme preparation.

Enzyme activity

Alginate lyase activity was determined by the increase in absorbance in the incubation mixture at 235 nm [5]; by the increase of the reducing power; and by the decrease in solution viscosity. To determine the alginate lyase activity, a substrate

solution (0.2 ml, 2 mg/ml) and an 0.2 M acetate buffer solution (0.1 ml), pH 5.6, were added to the enzyme (0.1 ml). The mixture was incubated for 1 h at 37 °C with the subsequent addition of an 0.05 M sodium carbonate solution (1.6 ml) and photometering at 235 nm followed by the estimation of the increased reducing power in accordance with Nelson [13] in an aliquot mixture (1 ml). The results were corrected by substrate and enzyme blanks. Photometric measurements were carried out in a SF-4A spectrophotometer in quartz cuvettes (1 cm). The enzyme quantity that splits 1 μM of uronide bonds per min at 37 °C was taken as the unit enzyme activity. In calculating the activity by the increase in the reducing power (Nelson's procedure [13]), $\varepsilon_{750\ nm}$ was taken to equal $1.05 \cdot 10^4$ $M \cdot cm^{-1}$, estimated by the calibration curve plotted with glucuronic acid as the standard, and $\varepsilon_{235\ nm}$ was taken to equal $5.05 \cdot 10^3$ $M \cdot cm^{-1}$ [14].

Viscometric estimations were made in an Ostwald viscometer with a capillary of 0.73 mm at 27 °C; the alginic acid concentration was 1 mg/ml and its relative viscosity 3.5. Enzyme activity was determined graphically with the coordinates $1/\ln\eta_{rel} - t$ (s) [15].

Chromatography

Column chromatography was performed in accordance with the recommendations of the manufacturers.

Determination of protein

Protein concentration in fractions (column profile) was determined by absorbance at 280 nm. Quantitatively, protein concentrations were measured by the method of Lowry et al. [16], using bovine serum albumin as a standard. The protein in the alginase preparation eluted from a CM-cellulose column was determined after concentration by polyethylene glycol through a cellophane membrane.

Disc electrophoresis

Disc electrophoresis was run in a 7.5% polyacrylamide gel in Tris–glycine buffer solution, pH 8.3 [17]. The protein was stained with Coomassie blue in 12.5% trichloroacetic acid with subsequent washing in 6% acetic acid.

pH optimum

The dependence of enzymatic activity on pH was determined in an acetate–phosphate–borate (0.05 M each) buffer. Enzymatic activity was estimated according to standard procedure, photometering at 235 nm.

pH stability

An enzyme aliquot (0.2 ml) was incubated in the above buffer solution at a given pH for 3 h at 37 °C. Then a 1 M acetate buffer solution (0.4 ml), pH 5.6, and a substrate solution (0.4 ml, 2 mg/ml) were added to the enzyme solution with subsequent incubation of the mixture for 1 h at 37 °C, photometering at 235 nm.

Thermostability

The enzyme aliquot in a 0.05 M acetate buffer solution, pH 5.6, was placed in a closable capillary pipette and incubated at a given temperature for 1 h. Then the

mixture was maintained for 1 h at 5 °C, and the enzyme activity was determined in accordance with the standard procedure, photometering being carried out at 235 nm.

Molecular weight

Molecular weight was estimated by thin-layer gel filtration [18] on a G-75 Sephadex (superfine) in a 0.05 M acetate buffer solution, pH 5.6, with 0.2 M sodium chloride. The standards used were cytochcome *c*, myoglobin, chymotrypsinogen, hen egg white and bovine serum albumin, and aldolase from the MS II set ('Serva', G.F.R.).

Ion effect

The effect was determined at a 0.001 M ion concentration in the incubation mixture. The assay was run in a 0.05 M acetate buffer solution, pH 5.6, with M-blocks (1 mg/ml) as substrates, according to standard procedure, photometering at 235 nm.

Action of alginate lyase VI on substrates and related compounds

The action of alginate lyase VI (0.1 nl) on the substrate and related compounds (0.4 mg/ml) 0.2 ml, was determined in a 0.05 M acetate buffer solution, pH 5.6, with 0.2 M sodium chloride. The increased absorbance at 235 nm and the incubation mixture reducing power were determined in accordance with standard procedure.

Kinetics

Enzymatic reactions were carried out in cuvettes (1.00 cm), using a double-beam recording spectrophotometer (UV-VIS Specord, Karl Zeiss, G.D.R.) in a running thermostated block at 25 °C. The reaction was recorded at 235 nm. The initial rates were estimated graphically. The apparent Michaelis constants were determined by the method of Lineweaver and Burk. The effect of the substrate analogues and related compounds (0.2 mg/ml) on the initial rate was determined with M-blocks (0.2 mg/ml) as substrates.

RESULTS

Enzyme isolation

All operations were performed in a cold room. The precipitate, after $(NH_4)_2SO_4$ (50–100%) fractionation of the crude preparation (14 g), was dissolved in an 0.02 succinate buffer solution (100 ml), pH 5.6, and chromatographed on a Sephadex G-15 column (4 cm × 65 cm). The enzyme was layered onto a DEAE-Sephadex A-50 column (3.6 cm × 65 cm) and eluted with a linear gradient of a succinate buffer solution (0.02–0.15 M), pH 5.6, 1 l each (see Fig. 1). The fractions with highest activity were combined and transferred to an 0.02 M phosphate buffer, pH 7.0. Sodium azide (0.2 g/l) was used in this and all subsequent stages to provide protection from bacteria. The enzyme preparation thus obtained from 45 g of lyophilic powder (900 ml) was applied to a SE-Sephadex C-50 column (3 cm × 45 cm) and eluted with a NaCl linear gradient (0–0.11 M, 1 l) in an 0.02 M phosphate buffer solution, pH 7.0 (Fig. 2). Fractions (58–100, 545 ml) were desalted and applied to a CM-52 column (2.2 cm × 44 cm). The alginate lyases were eluted with NaCl (0–0.11 M, 1 l each) gradient in an 0.02 M phosphate buffer solution, pH 7.0 (Fig. 3). Alginate lyase VI Fractions (138–158 220 ml) was desalted and rechromatographed under the same conditions; fractions

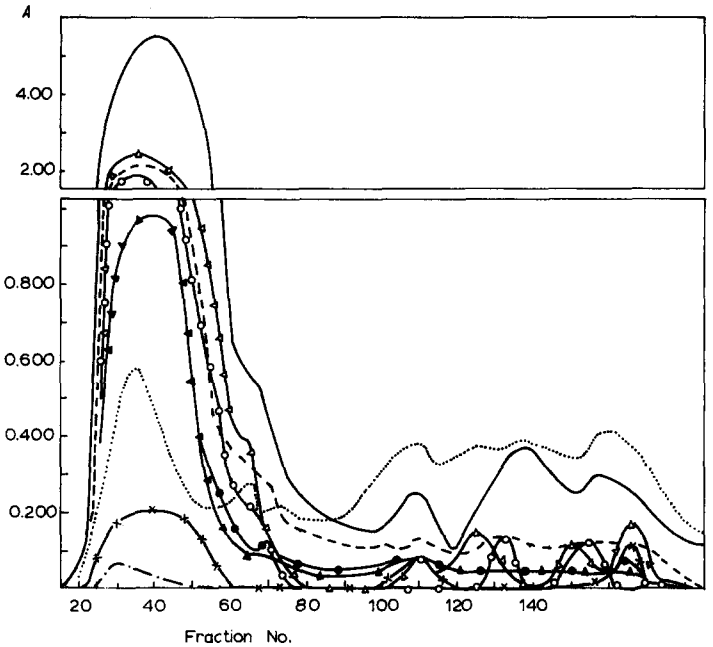


Fig. 1. Ion-exchange chromatography on DEAE-Sephadex A-50. The column (3.6 cm × 65 cm) was eluted with a linear gradient from 0.02 to 0.15 M, 1 l each, of a sodium succinate buffer solution. Fractions (10 ml) were collected for 15 min. ···, protein ($A_{280\ nm}$). Activity on: ———, M-blocks (Nelson); – – –, M-blocks ($A_{235\ nm}$); ○—○, Alginate (Nelson); ●—●, alginate ($A_{235\ nm}$); △—△, MG-blocks (Nelson); ▲—▲, MG-blocks ($A_{235\ nm}$); ×—×, G-blocks (Nelson); ·—·, G-blocks ($A_{235\ nm}$).

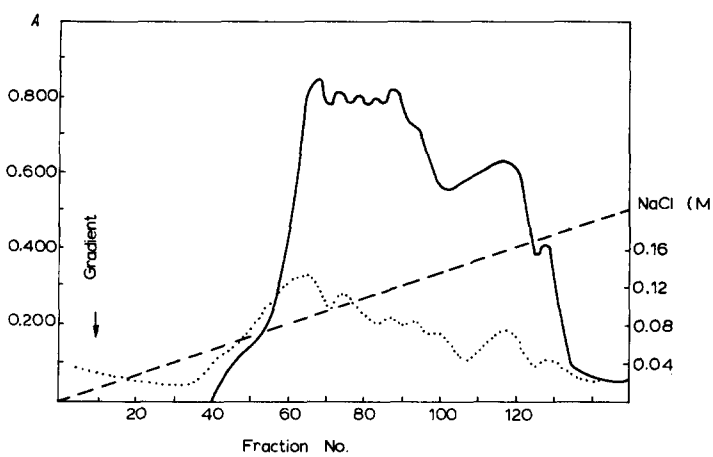


Fig. 2. Ion-exchange chromatography on SE-Sephadex C-50. The column (3 cm × 45 cm) was eluted with a linear gradient of sodium chloride (0–0.11 M) in a 0.02 M sodium phosphate buffer solution (pH 7.0), 1 l each. The preparation (900 ml) was applied in 0.02 M phosphate buffer solution. Fractions (13 ml) were collected for 20 min. ···, protein ($A_{280\ nm}$); ———, M-blocks ($A_{235\ nm}$); – – –, sodium chloride gradient.

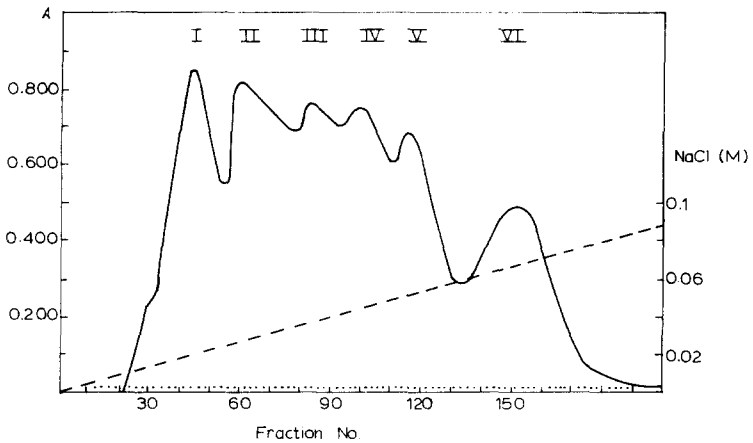


Fig. 3. Ion-exchange chromatography on CM-52 cellulose. The column (2.2 cm × 44 cm) was eluted with a linear gradient of sodium chloride (0–1.1 M) in a 0.02 M sodium phosphate buffer solution, 1 l each. Fractions (7 ml) were collected for 15 min. ···, protein ($A_{280\ nm}$); ———, activity (M-blocks, $A_{235\ nm}$); – – –, sodium chloride gradient.

with the highest activity were combined (73 ml). Table I shows the results of alginate lyase VI purification.

Homogeneity

The degree of purification was controlled by disc electrophoresis in 7.5 % poly-

TABLE I

ISOLATION OF ALGINATE LYASE VI FROM THE HEPATOPANCREAS OF THE MOLLUSK *LITTORINA* SP.

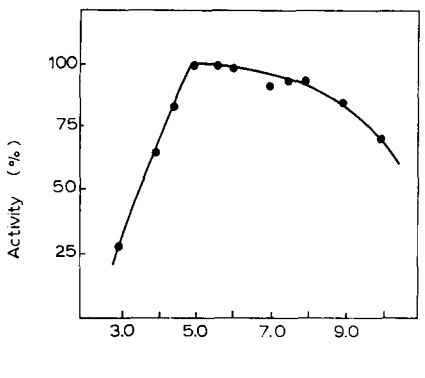
Isolation stage	Vol. (ml)	Protein		Activity*			Yield	Purification
		mg	mg/ml	units	units/ml	units/mg	(%)	(-fold)
1 Initial preparation	450	15 750	35	18 400	41	1.17	100	1
2 Precipitation with $(NH_4)_2SO_4$ gel filtration on Sephadex G-25	600	5 420	9	10 900	18.2	2.0	59	1.7
3 Chromatography on DEAE-Sephadex (Peak I)	900	390	0.43	9 050	10.0	232	49	20
4 Chromatography SE-Sephadex (Peak I)	545	74	0.135	5 800	10.6	78.5	31	67
5 Chromatography CM-cellulose (Peak VI)	214	0.54	0.0025	152	0.71	280	0.83	240
6 Rechromatography of alginate lyase VI on CM-cellulose	73	0.34	0.0046	141	1.92	415	0.77	360

* The activity was determined on M-blocks, photometering at 235 nm.

acrylamide gel at each purification stage. Following rechromatography on CM-cellulose, alginate lyase VI showed only one narrow band in the upper part of the gel.

Stability

Alginate lyase VI stored at −20 °C retains activity for at least three months. Approximately 10% of the activity is lost in ten days in the presence of sodium azide at +4 °C. When frozen, alginate lyase VI loses activity by 20–25%. Figs 4 and 5 show the dependence of alginate lyase VI stability on pH and temperature.



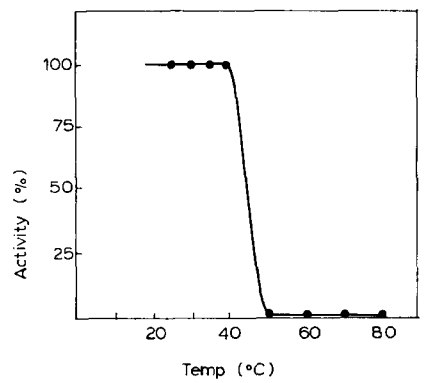


Fig. 4. pH stability of alginate lyase VI. The enzyme was incubated for 3 h at 37 °C in an acetate–phosphate–borate buffer at various pH values. The activity was determined with M-blocks at pH 5.6, photometering at 235 nm.

Fig. 5. Alginate lyase VI stability at different temperatures. The enzyme was incubated in an 0.05 M acetate buffer solution, pH 5.6, at various temperatures for 1 h. The activity was determined with M-blocks, photometering at 235 nm.

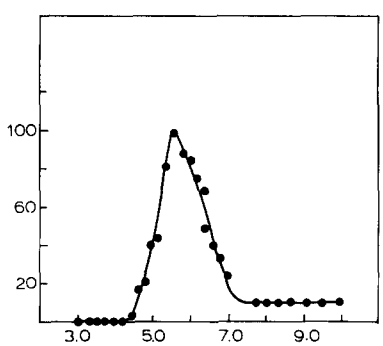


Fig. 6. pH-effect on the enzymatic activity of alginate lyase VI. The activity was determined in an acetate–phosphate–borate buffer solution with M-blocks, photometering at 235 nm.

pH optimum

Fig. 6 shows the interdependence of alginate lyase VI activity and pH.

Determination of molecular weight

The molecular weight of alginate lyase VI was determined by the results of

thin-layer gel filtration. The values of R_F were plotted in the graph against the logarithm of the molecular weights of proteins with known molecular weight [18]. The results are shown in Fig. 7.

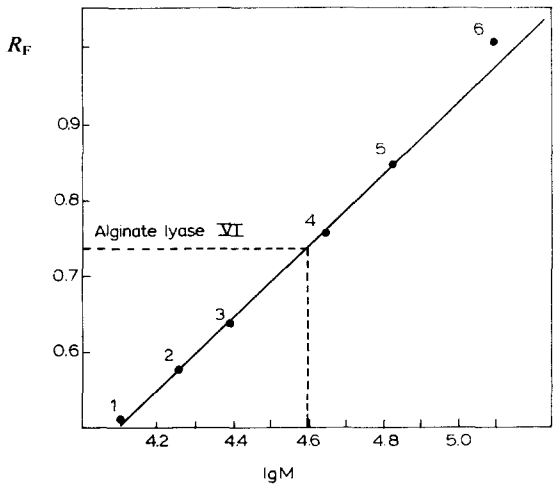


Fig. 7. Determination of the molecular weight of alginate lyase VI by thin-layer gel filtration on Sephadex G-75 (superfine). Markers used: 1, cytochrome *c*; 2, myoglobin; 3, chymotrypsinogen; 4, bovine serum albumin; 5, hen egg albumin; 6, aldolase.

TABLE II

EFFECT OF ALGINATE LYASE PREPARATIONS ON VARIOUS SUBSTRATES AT DIFFERENT PURIFICATION STAGES

Substrate	Activity (units)							
	G-blocks		MG-blocks		Alginate		M-blocks	
	235 nm	Nelson	235 nm	Nelson	235 nm	Nelson	235 nm	Nelson
Starting preparation	7450	8150	11 900	8150	13 800	11 800	18 400	23 300
$(NH_4)_2SO_4$–G-25	450	680	2 580	1900	5 850	4 650	10 900	14 700
DEAE-Sephadex	490	770	2 350	2100	3 460	2 910	9 050	7 950
SE-Sephadex (Peak I)	0	0	1 620	1390	2 200	1 520	5 800	4 500
SE-Sephadex (Peak II)	165	315	370	260	169	218	1 470	1 300
CM-cellulose Peak I	6	0	96	71	100	49 (57*)	148	131
II	6	0	152	117	149	81 (93*)	290	268
III	9	0	115	87	102	60 (167*)	267	228
IV	9	0	101	87	96	49 (157*)	180	171
V	9	0	122	92	125	55 (202*)	119	89
VI	9	0	155	101	132	89 (194*)	152	172

* Viscosity.

Substrate specificity

The results of the action of alginate lyase fractions, at various purification stages, upon oligomers with different compositions and on alginic acid are given in Table II.

TABLE III

EFFECT OF METAL IONS AND CERTAIN REAGENTS ON ALGINATE LYASE VI

Concentration: 10^{-3} M; M-blocks (1 mg/ml); 0.05 M acetate buffer solution (pH 5.6), 37 °C, 1 h; results are given as a percentage with respect to the control experiment.

Substance	Activity (%/control)	Substance	Activity (%/control)
Without admixtures	100	Li^+	112
Sulphate	102	NH_4^+	112
Nitrate	96	Na^+	100
BF_4^-	115	K^+	115
Borate	103	Cs^+	140
Benzoate	103	Ba^{2+}	155
Oxalate	105	Mg^{2+}	140
Galacturonate	103	Co^{2+}	180
Citrate	102	NaN_3	120
Dodecylsulphate	56		
Dithioglycolate	103		
p-Chloromercuribenzoate	88		
Cu^{2+}	0		
Hg^{2+}	0		
Zn^{2+}	16		
Cd^{2+}	72		

TABLE IV

CONCENTRATION DEPENDENT EFFECT OF DIFFERENT IONS ON ALGINATE LYASE VI

Ion concn (M)	Activity (%/control)								Sodium acetate pH 5.6
	Na^+	K^+	Co^{2+}	Ba^{2+}	Ca^{2+}	Zn^{2+}	Cu^{2+}	Hg^{2+}	
1	92	105							118
0.75	130	137							135
0.50	130	137							150
0.25	143	153							150
0.125	137	154							150
0.100	130	153							130
0.075	135	128							
0.050	130	124	15	63	52				100
0.025	100	104	34	67	70				
0.010	100	96	104	63	160				
0.0075			132	89	174				
0.0050			117	158	158	11	0		
0.0010			100	106	132	11	19	41	
$5 \cdot 10^{-4}$						54	48	35	
$1 \cdot 10^{-4}$						90	86	50	
$5 \cdot 10^{-5}$						81	90	60	

Effect of ions and other substances

The effect of certain ions and various substances on the enzyme reaction of alginate lyase VI was investigated. The results are shown in Table III. The concentration dependence of ion action on enzyme reaction is demonstrated in Table IV.

Effect of alginate lyase VI on substrates and some related compounds and its influence on the initial reaction rate

Table V shows the effect of alginate lyase VI on certain substrates and related

TABLE V

EFFECT OF ALGINATE LYASE VI ON DIFFERENT SUBSTRATES AND RELATED COMPOUNDS

Substrate (0.2 mg/ml); enzyme (0.1 ml); 0.005 M acetate buffer solution, pH 5.6, (0.4 ml); 1 h, 37 °C; analysis in accordance with standard procedure.

Substrate	Activity	
	$A_{235\ nm}$	$A_{750\ nm}$
M-blocks	0.670	1.280
Alginate	0.440	0.830
MG-blocks	0.210	0.300
G-blocks	0.010	0.030
CM-cellulose	0	0
Mannan	0	0
Galactan	0	0
α-Methyl-D-mannoside	0	0
Glucuronomannan [11]	0	0
Gum arabic	0	0
Galacturonide [12]	0	0

TABLE VI

EFFECT OF CERTAIN SUBSTANCES ON THE INITIAL RATE OF ALGINATE LYASE VI REACTION

M-blocks (0.2 mg/ml); admixtures (0.2 mg/ml); NaCl–0.2 M, 0.05 M acetate buffer solution, pH 5.6. Results are given as a percentage with respect to the control experiment.

Substance	Activity (%)
Without admixtures (control)	100
α-Methyl-D-mannoside	101
Gulono-γ-lactone	101
Galacturonic acid	100
Glucuronic acid	104
Mannuronic acid	91
Guluronic acid	91
Glucurone (lactone)	110
Mannan	93
Galacturonide	102
Reaction products*	89

* Exhaustive hydrolysis of the M-blocks was carried out; after having subjected the enzyme to heat denaturation, the reaction products were introduced into a newly prepared incubation mixture.

TABLE VII

K_m AND V VALUES FOR DIFFERENT SUBSTRATES

Substrate	K_m (M)	V ($mM \cdot min^{-1} \cdot mg^{-1}$)
M-blocks	$1.9 \cdot 10^{-4}$	34
Alginate	$1.9 \cdot 10^{-4}$	24
MG-blocks	$5.0 \cdot 10^{-4}$	14

compounds. The results obtained in determining the influence of related compound on the initial rate of the splitting of M-blocks are shown in Table VI.

K_m and V

The Michaelis–Menten kinetic parameters of the alginate lyase VI reaction with M-blocks, MG-blocks and alginic acid are given in Table VII.

Determination of the type of action of alginase VI

Periodically, the decrease in viscosity of the incubation mixture and the increase in reducing power were measured. The results are shown in Fig. 8.

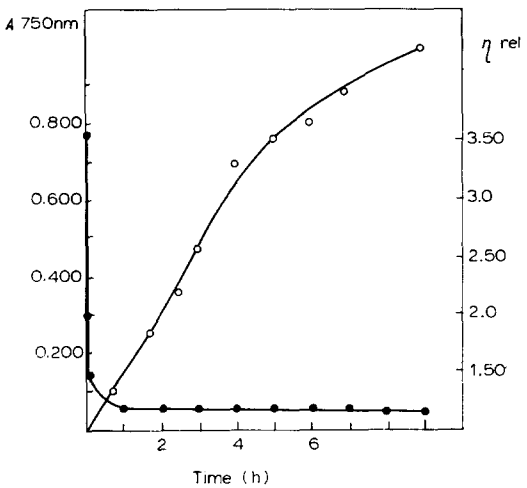


Fig. 8. Alginic acid hydrolysis by alginate lyase VI. Changes in the viscosity and reducing power of alginate solution caused by alginate lyase VI in 0.2 M sodium chloride solution in 0.05 M acetate buffer solution, pH 5.6. Alginate concentration: 1 mg/ml; relative viscosity, 3.5. Decrease in viscosity (●—●) was determined at 37 °C in an Ostwald viscometer. Increase in reducing power (○—○) was determined in accordance with Nelson.

DISCUSSION

From a preliminary comparative study, the gastropod mollusk *Littorina* sp. was chosen as the alginate lyase source [19]. The possibility of obtaining an enriched alginate lyase preparation by means of affinity chromatography on a biogel–alginate

column was shown. A large number of alginate lyase fractions was found using disc electrophoresis [9]. In order to isolate one of the alginate lyases in the homogeneous state, we thought it more expedient to use the methods of fractionation with ammonium sulphate and ion-exchange chromatography. It was established that with 50% saturated $(NH_4)_2SO_4$ a large protein portion precipitates to concentrate the alginate lyase activity in a 100% saturated precipitate. Having used a more gentle gradient than the one applied by Nakada and Sweeny [4] for *Holiotus* alginase, we obtained a more effective enrichment of the main alginate lyase peak on DEAE-Sephadex chromatography (see Fig. 1).

A considerable rise in alginate lyase specific activity and separation into two peaks was obtained with chromatography on SE-Sephadex (see Fig. 2). In the procedure, a large amount of inactive protein was eluted prior to gradient application; it is not shown in Fig. 2. After being transferred to the phosphate buffer solution (900 ml) without lyophilization, leading to a 25% loss in activity, a preparation of the main alginate lyase peak obtained with chromatography on DEAE-Sephadex was applied to the SE-Sephadex column.

In chromatographing the main peak, obtained on SE-Sephadex, on a microcrystalline cellulose CM-52 column, the alginate lyases were separated into several peaks, which we designated in the order of their exit at elution (see Fig. 3). The said fractions show rather perceptible differences when tested with the alginate, MG- and M-blocks as substrates (see Table II). Peak VI, after rechromatography on a CM-52 column, proved to be homogeneous according to disc electrophoresis data. After staining the protein with Coomassie blue, we discovered a single narrow band in the gel upper region; the activity was localized in the same range. Alginate lyase VI showed an optimum pH 5.6 at (see Fig. 6).

Alginate lyases from other sources have optimum pH values in the range 7–8 [20]. For the enzyme that splits alginate fractions enriched with glucuronic acid, the highest activity was observed at pH 4.0 [4]. At the same time, for polygalacturonide transeliminases, the usual pH optimum was in the 5–6 range [21, 22].

Alginate lyase VI is stable within a rather wide pH range from 4 to 8 (see Fig. 4); it was inactivated completely after 1 h at 50 °C (see Fig. 5). When kept for as long as 3 months in the solution at +4 °C, the enzyme loses its activity by 20%.

Apart from Zn^{2+}, Cu^{2+} and Hg^{2+}, which essentially inhibit alginate lyase VI, numerous ions activate the enzymatic reaction. Na^+ and K^+, similar to the acetate buffer solution, show an activating effect up to an 0.2 M concentration and with increased concentrations, their action becomes reversed. The bivalent cations Ba^{2+}, Ca^{2+} and Co^{2+} are activators at lower concentrations (see Table IV). Apparently, the ionic strength of the solution is more important for enzyme activity, since it affects substrate conformation to a considerable extent [23].

The anions studied, such as benzoate, oxalate, citrate, galacturonate, sulphate, phosphate, borate and others, have no marked effect on enzymatic activity. It should be noted that *p*-chloromercuribenzoate and dithioglycolic acid do not considerably decrease the enzymatic activity of alginate lyase VI (see Table III). From this, one may assume that sulphydryl groups are not essential for the enzymatic reaction.

The apparent kinetic parameters V and K_m and the inhibition constant were calculated (see Table VII). The K_m value is of the same power as the value obtained for polygalacturonide transeliminase [22]. The rate at which alginate lyase VI splits

oligomers enriched with mannuronic acid is the highest. The chain length has no substantial effect on the reaction rate. The somewhat higher reaction rate with the high molecular alginate compared to that with MG-blocks of approximately the same M/G ratio (see Table V) may be explained by the fact that alginate lyase VI preferably splits the linkage between two mannuronic acid residues. Alginic acid, having a block structure, is more likely to involve a larger number of such linkages than MG-blocks, wherein monomer alteration is supposedly regular.

Alginate lyase VI hydrolyzed none (see Table V) of the examined uronide-containing polysaccharides and compounds containing mannose when the reaction was tested in accordance with Nelson and measured photometrically at 235 nm; neither were the G-blocks split.

Mannuronic acid, guluronic acid, mannan, enzymatic reaction products and G-blocks proved to somewhat decrease the reaction rate (see Table VI). The effect of G-blocks was examined in greater detail to determine the effective inhibition constant K_p as equal to $2.85 \cdot 10^{-4}$ M. It is noteworthy that gulono-γ-lactone does not affect the reaction rate.

When alginate lyase VI acts on the alginate, the solution viscosity decreases rapidly. The increase in reducing power, by which one can judge the number of ruptures in the macromolecule, took place at a longer incubation (see Fig. 8).

The above-said allows us to assume that the alginate lyase isolated has endo-type activity and predominantly breaks the linkage between the two mannuronic acid residues.

A polymannuronide lyase preparation, similar in respect to enzymatic properties to alginate lyase VI, was obtained from the mollusk *Dollabella* [5]. The use of an homogeneous enzyme with well-determined specificity and type of action may be helpful in polysaccharide studies. Investigations are being continued to clarify the specificity and mechanism of action of alginate lyase VI.

ACKNOWLEDGEMENT

The translation of the paper from the Russian by Joseph C. Shapiro is hereby acknowledged.

REFERENCES

1 Reds, D. and Samuel, J. W. B. (1967) J. Chem. Soc. (C) 2295–2298
2 Haug, A., Larsen, B. and Smidsrod, O. (1967) Acta Chem. Scand. 21, 691–704
3 Oshima, K. (1931) J. Agric. Chem. Soc. Japan 7, 328–331
4 Nakada, H. and Sweeny, P. (1967) J. Biol. Chem. 242, 845–851
5 Nishizawa, K., Fujibayashi, S. and Kashiwabara, Y. (1968) J. Biochem. 64, 25–37
6 Williams, A. K. and Eagon, R. G. (1962) Can. J. Microbiol. 8, 649–654
7 Kashiwabara, Y., Suzuki, H. and Nishizawa, K. (1969) J. Biochem. 66, 503–512
8 Kaiser, P., Le Borgne, L., Hardisson, C., Pigman, G. and Vigal, G. (1968) C.R. Acad. Sci. Ser. D 267, 376–379
9 Favorov, V. V. (1973) Int. J. Biochem. 4, 107–110
10 Haug, A. and Larsen, B. (1962) Acta Chem. Scand. 16, 1908–1918
11 Khomenko, V. N., Pavlenko, A. F., Solov'eva, T. F. and Ovodov, Yu. S. (1971) Khim. Prirodn. Soedinen. 393–396
12 Solov'eva, T. F., Arsenyuk, L. V. and Ovodov, Yu. S. (1969) Carbohydr. Res. 10, 13–18
13 Nelson, N. (1944) J. Biol. Chem. 153, 375–381

14 Tsujino, J. (1963) Agric. Biol. Chem. 27, 236–242
15 Hultin, E. and Wanntorp, I. (1966) Acta Chem. Scand. 20, 2267–2277
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
17 Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
18 Andrews, P. (1965) Biochem. J. 96, 595–605
19 Favorov, V. V. and Vaskovsky, V. E. (1971) Comp. Biochem. Physiol. 38B, 689–696
20 Franssen, F. and Jenniaux, Ch. (1965) Cahiers Biol. Marine 6, 1–21
21 Ishii, S. and Yokotsuka, T. (1972) Agric. Biol. Chem. 36, 146–153
22 Hatanaka, C. and Ozawa, J. (1969) Ber. Ohara Inst. Land. Biol. XV, 15–23
23 Smidsrod, O. and Haug, A. (1968) Acta Chem. Scand. 22, 797–810