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KINETICS AND SPECIFICITY OF ALGINATE LYASES: PART I, A CASE STUDY

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ABSTRACT

Purified preparations of alginate lyase from *Klebsiella aerogenes* and *Haliotis* sp. were investigated for activity and degradation patterns with alginate and alginate fragments having different compositions and sequences. With fragments approaching homopolymers of guluronate and mannuronate, Michaelis–Menten kinetics were obeyed and kinetic parameters could be obtained. Degradation of alginates containing all four possible linkages in various proportions, followed by isolation of the fragments and identification of the end groups by n.m.r. spectroscopy, indicated that the enzyme preparations can attack more than one type of linkage. The results are discussed with reference to the concept of specificity for enzymes with copolymeric substrates having non-regular distributions of units.

INTRODUCTION

Alginate lyase activity has been detected in several microorganisms[1–4], marine molluscs[5–7], and marine brown algae[8]. These enzymes catalyze a β-elimination reaction that cleaves the chain (**1**) and creates an unsaturated uronic acid residue (**2**) at the new non-reducing end. The activity of the enzyme may be detected easily by any method (*e.g.*, viscosity) that measures reduction in molecular weight or increase in the number of reducing or non-reducing end groups. In spite of these possibilities, few kinetic data are available for these enzymes. Attempts to characterize the action of the enzymes have been concentrated mainly on the isolation and partial characterization of the oligomeric end-products[4,9]. The results clearly demonstrate that the end-product is a mixture of lower oligomers with various d.p.

Since alginate is a linear copolymer[10] of (1→4)-linked β-D-mannuronic acid (M) and (1→4)-linked α-L-guluronic acid (G), it is conceivable that the lyases may be specific for the type of uronic acid. The terms "mannuronate lyase" and "guluronate lyase" have been used to suggest that this concept applies, at least to some of the enzyme preparations[11,12]. The specificity of the enzyme is defined, not by iden-

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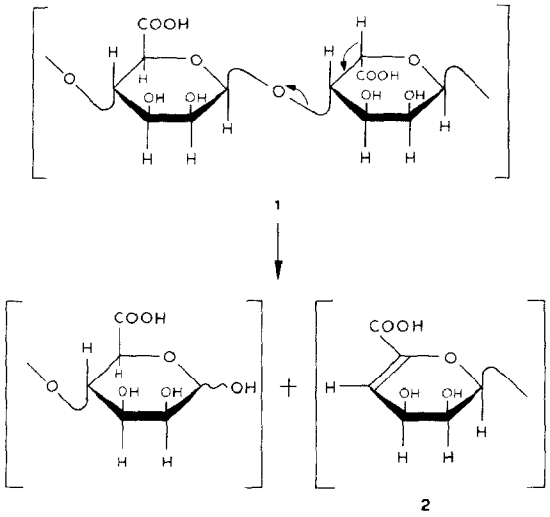

tification of the actual unit bound and/or attacked, but by its ability to degrade blocks of uronic acid that approach poly-M and poly-G. These terms indicate an analogy with glycosidases since they are based on the identity of the sugar and the configuration of the linkage. However, this represents only two of the four possible glycosidic linkages in an alginate (M–M, G–G, G–M, M–G). Thus, designation of the specificity will require knowledge of the identity of the two uronic acids associated with the linkage cleaved by the enzyme. Such data have been obtained during an investigation of the feasibility of using these enzymes for studying the distribution of the lengths of homopolymeric blocks in alginates.

We now report data obtained with the "specific" lyases prepared from *Klebsiella aerogenes* (G-ase) and *Haliotis* sp. (M-ase) as a basis for discussing the relevance of kinetic parameters and the concept of specificity for lyases acting on binary copolymers.

EXPERIMENTAL

Enzyme preparations. — *K. aerogenes* was grown and the extracellular enzyme was prepared essentially as described by Boyd and Turvey[11]. The final product was dialyzed against 10mM phosphate buffer (pH 7), freeze-dried, and stored at −20°.

Abalone hepatopancreas acetone powder (Sigma) was used to prepare the *Haliotis* enzyme. The powder (1 g) was leached with 25mM Tris buffer (pH 7, 20 mL) for 1 h at 4° and insoluble material was removed by centrifugation followed by filtration (Millipore GF/C). The solution was applied directly to a column (2.5 × 90 cm) of Sepharose CL-6B (Pharmacia) and eluted with 25mM Tris buffer (pH 7) containing 0.2M KCl at 4°. The elution was monitored by the absorbance at 280 nm. Active fractions were combined, dialyzed, and freeze-dried. The product was

purified as described for the *Klebsiella* preparation. The yield was 280 mg/g of acetone powder.

SDS gel electrophoresis of each enzyme preparation gave one main band with a mol. wt. of ~43 000. Each preparation contained small amounts of other proteins.

Substrates. — The preparation of alginate and alginate fragments has been described[13–15]. Alginates from *Laminaria digitata* were kindly provided by Protan A/S. All substrates were characterized by their content of uronic acids determined by the phenol–sulfuric acid method[16] and the nearest-neighbor frequencies by n.m.r. spectroscopy[17].

Kinetic experiments were carried out with alginate fragments of extreme composition, namely, UUS (high content of guluronic acid, prepared from *Stilophora rhizoides*), LLS (high content of mannuronic acid, same source as UUS), and L1.4 [fragment with high transition (MG, GM) frequency, prepared from *Ascophyllum nodosum*]. The composition and nearest-neighbor frequencies for these fragments are given in Table I.

The n.m.r. data indicated that the average d.p. was >20 for these fragments, so that $F_{GM} = F_{MG}$.

The composition and the nearest-neighbor frequencies of the alginates from *L. digitata* and the outer cortex of *L. hyperborea* are given in Table II.

Enzymic degradation and separation of fragments. — A solution of alginate (200 mg) in 30mM phosphate buffer (pH 7, 200 mL) containing 1% of NaCl was incubated for 48 h at 37° with the appropriate enzyme preparation (20 mg). No further increase in absorbance could be detected after this time. The solution was concentrated to 10 mL, made 0.1M with respect to Na_2SO_4, and filtered if necessary,

TABLE I

COMPOSITION OF ALGINATE FRAGMENTS

Fragment	F_M	F_G	F_{MM}	F_{MG}	F_{GG}
UUS	0.06	0.94	0.03	0.03	0.91
LLS	0.90	0.10	0.90	0	0.10
L1.4	0.62	0.38	0.33	0.29	0.09

TABLE II

COMPOSITION OF ALGINATE USED IN DEGRADATION EXPERIMENTS

Alga	F_M	F_G	F_{MM}	F_{MG}	F_{GG}
L. digitata	0.57	0.43	0.42	0.15	0.28
L. hyperborea	0.23	0.77	0.13	0.10	0.67
D. antarctica	0.66	0.34	0.45	0.21	0.13

and the products were eluted from a column (2.2 × 180 cm) of BioGel P4 (200–400 mesh) with 0.1M Na_2SO_4 at ~25 mL/h.

Enzyme kinetics. — Kinetic experiments were performed in 0.05M phosphate and 0.05M Tris-HCl, each containing 1% of NaCl. Buffer (2.0 mL) was mixed with substrate solution (0.5 mL) in a 10-mm quartz cuvette. Enzyme solution (0.5 mL) was added, and increase in absorbance at 230 nm was recorded each 0.2 min during 10–15 min, using a Shimadzu Spectrophotometer UV-260. The amount of enzyme used was 0.25 mg for UUS and LLS, and 1.0 mg for L1.4. Substrate concentrations were in the range 0.08–1.0 mg/mL, the lower limit being determined by the absence of an initial, linear part of the curve.

All substrate concentrations refer to the amount of uronic acid determined by the phenol–sulfuric acid method[16] and calculated as the sodium salt of the "anhydrouronic acid" monohydrate (mol. wt. 216). Enzymic activity is reported as absorbance units (AU) per min.

RESULTS AND DISCUSSION

The extracellular lyase from *Klebsiella aerogenes*has been classified as a guluronate lyase[9] based on its ability to degrade G-blocks, prepared from alginate, but not M-blocks. A G-block preparation from *Stilophora* was used to study the kinetics. The results obtained in the two buffer systems are given in Fig. 1 as Lineweaver–Burk plots. The lines were fitted to the experimental points using the least squares method. The values for K_m and V_{max}, calculated from the intercepts, were 0.11 mg/mL and 0.014 AU/min, respectively, for phosphate buffer, and 0.059 mg/mL and 0.019 AU/min, respectively, for Tris buffer. Thus, the enzyme is considerably more efficient in the Tris buffer, with a K_m of 2.7×10^{-4}M.

Fig. 2 shows the results obtained with the abalone poly-M lyase, using an M-block (LLS) as the substrate. The difference in efficiency in the two buffer systems is even more pronounced, with K_m and V_{max} values of 0.20 mg/mL and

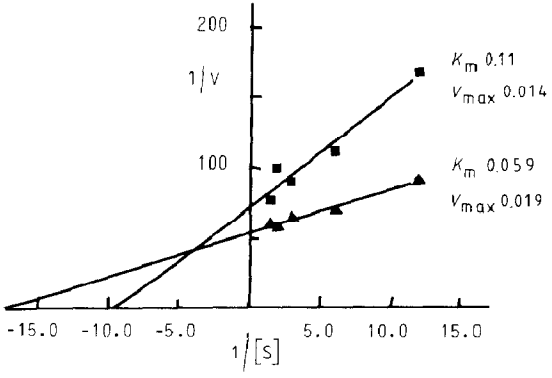

Fig. 1. Kinetics for the *Klebsiella* enzyme with poly-G as substrate in phosphate (■) and Tris (▲) buffers.

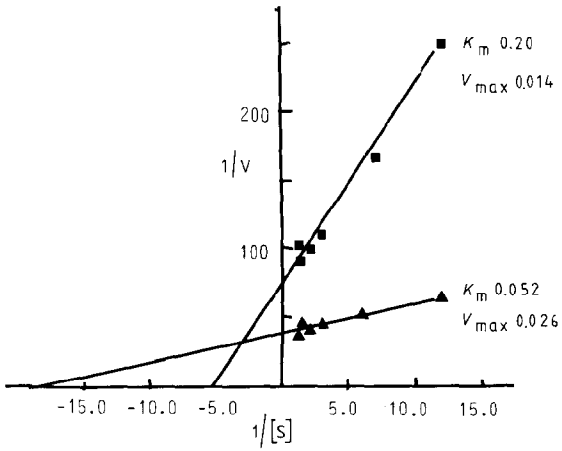

Fig. 2. Kinetics for the *Haliotis* enzyme with poly-M as substrate in phosphate (■) and Tris (▲) buffers.

0.014 AU/min, respectively, in phosphate buffer, and 0.052 mg/mL and 0.026 AU/min, respectively, in Tris buffer (K_m 2.4×10^{-4}M).

The above substrates contained mainly one type of linkage. In order to ascertain the requirement for maximal activity, several substrates with various compositions and sequences were used. The total amount of each polymer substrate was adjusted to give a final concentration of M and G units of 0.33 mg/mL.

The results shown in Table III clearly indicate that, for the *Klebsiella* enzyme, the initial rate observed depends only upon the concentration of G units in solution. For the *Haliotis* enzyme, the addition of poly-G does not influence the reaction. On the other hand, with substrates having different distributions of uronic acids, the initial rates observed were significantly lower. Thus, the *Klebsiella* enzyme may attack either one type of linkage or different types at different rates.

In order to distinguish between these possibilities, it is necessary to identify the uronic acid residues joined by the glycosidic linkage to be broken. This information may be obtained by n.m.r. analysis of the fragments produced by the enzymic attack. Substrates of different uronic acid composition were degraded by the

TABLE III

DEGRADATION OF VARIOUS SUBSTRATES WITH ENZYMES FROM *Klebsiella* AND *Haliotis* in Tris buffer[a]

Alginate	*Initial rate (AU/min)*	
	Klebsiella	*Haliotis*
UUS	0.141	
LLS		0.192
LLS/UUS 60:40	0.148	0.188
L. digitata	0.154	0.154
D. antarctica	0.148	0.145
L1.4	0.168	0.104

[a]Concentration of M and G units was 0.33 mg/mL.

TABLE IV

COMPOSITION OF THE REDUCING END-GROUPS RELEASED DURING LONG-TERM DEGRADATION

				Reducing end-groups[a]			
				Klebsiella		*Abalone*	
Alginate	F_{GG}	F_{MM}	F_{MG}	*G*	*M*	*G*	*M*
L. hyperborea	0.67	0.13	0.10	23.9	<0.1	3.7	11.4
L. digitata	0.28	0.42	0.15	21.7	0.3	5.3	13.0

[a]Percentage of the total uronic acid.

enzymes until no further increase in absorbance could be detected. The fragments were isolated by gel filtration and each was analyzed for uronic acid composition of the end groups by ^{1}H-n.m.r. spectroscopy. The fractions with d.p. 2–5 were probably monodisperse, whereas longer fragments were definitely polydisperse. A detailed report of these experiments will be published elsewhere, but some of the results are given in Table IV. The recovery of uronic acids was 90 ±2% in each experiment.

The fragments with d.p. <10 produced by the *Klebsiella* enzyme had a G unit at the reducing end and generally both G and M end-groups could be detected in the fragments produced by the *Haliotis* enzyme. Also, with the high-G substrate (*L. hyperborea* alginate), there appeared to be a preference for G end-groups down to about a tetramer. With the *L. digitata* alginate, which contains ~60% of M units, the end groups were mainly M units except in the dimer fraction.

The identity of the uronic acid residues converted into unsaturated, non-reducing end-groups (δ units) by the enzyme cannot be determined directly. However, an estimate of the relative proportions of M and G units converted can be obtained by using the n.m.r. data to calculate the decrease in the proportions of M and G units as a result of the enzyme action. The results in Table V show that the identity of the unit converted into an unsaturated uronic acid is strongly dependent on the sequence of the substrate. The *Klebsiella* enzyme attacks almost entirely G–G linkages in a high-G substrate, whereas the enzyme cleaves mainly G–M linkages in *L. digitata*.

When used with substrates of a composition close to a homopolymer of G or M units, the reactions catalyzed by the lyase enzymes from *Klebsiella* and *Haliotis* appear to obey Michaelis–Menten kinetics so that values for K_m and V_{max} may be obtained and used to characterize the enzyme preparations on a dry-weight basis.

The results of Figs. 1 and 2 emphasize the importance of the buffer system. Both enzyme preparations are considerably more effective in Tris than in phosphate buffer. According to the ratio of the selectivity coefficients (V_{max}/K_m) in Tris and phosphate buffer, the efficiency is higher by a factor of 2.5 for *Klebsiella* enzyme and 7.1 for the *Haliotis* enzyme.

The above conclusions rest on the assumption that the M units present in the poly-G preparation, and vice versa, do not contribute significantly to the rates observed. If the linkages to these units are attacked at the same or similar rates as those of the homopolymer parts, then the substrate concentration used for the calculations would be low by as much as 10%. Whereas it is clear that the ability of the *Klebsiella* enzyme to cleave M–M linkages is low, as is that of the *Haliotis* to cleave G–G linkages, no conclusions can be drawn for M–G and G–M linkages. If these linkages are attacked at rates that differ significantly from that for M–M or G–G, or not at all, the initial rates observed with substrates containing both types of units should vary with the sequence of the substrates. The results in Table III demonstrate that this applies to the *Haliotis* enzyme. The concentration of M units is the same in each experiment and the addition of a poly-G preparation to give an M/G ratio of 60:40 did not influence the rate of breakdown. This ratio was selected as an approximation to the composition of the other three substrates used. Consequently, the presence of G–G linkages will not interfere with the activity of the *Haliotis* enzyme. Although they are very similar in gross composition, the other three preparations in Table III are degraded at significantly different rates. It is concluded that the efficiency of the enzymic reaction depends on the recognition of more than one uronic acid residue in the chain. The rate of reaction appears to decrease with increasing transition frequency (F_{MG}), but this does not imply that M–G or G–M linkages are not attacked, but that their rates of cleavage must be significantly lower than that of the M–M.

This result has important consequences for the acquisition of kinetic data and for the nomenclature to be used with this type of substrate–enzyme interaction. Substrates with a mixed composition should not be used for kinetic studies unless it has been demonstrated that the rate of reaction is independent of sequence.

When, as with the *Haliotis* enzyme, the observed rate of reaction depends on the identity of more than one unit in the alginate chain, the specificity/selectivity cannot be based on the one-unit concept. It is conceivable that enzymes may exist that can break only one of the four possible linkages in the alginate molecule (M–M, M–G, G–M, and G–G). The specificity in this context should be based on a dimer unit. The observation made by Currie[4] of an "alternase" may be an example of such an enzyme being able to break only the M–G or the G–M linkage.

Under the same conditions (Table III), the initial rates observed for the various alginates were the same for the *Klebsiella* enzyme, suggesting that the enzyme either recognizes only a G unit or has a low selectivity for the neighboring unit.

N.m.r. analysis (Table IV) of the reducing end-groups accumulated during prolonged degradation of two different substrates by the *Klebsiella* enzyme shows that few M end-groups are found in the polymer fractions resistant to enzymic attack. In the remaining fractions, which account for most of the uronic acids, only G end-groups were detected. This result strongly suggests that only G–G and G–M linkages are cleaved by the enzyme.

The fragments produced by the *Haliotis* enzyme contain both M and G units at the reducing end (Table IV). With a high-M substrate (*L. digitata* alginate), M reducing end-groups preponderate. This result also indicates that the distribution depends on the composition and/or sequence of the alginate used as substrate.

Specificity defined by kinetic parameters may not coincide with that defined on the basis of extensive degradation. The kinetic approach provides information on the linkage(s) having the highest rate constant, whereas final end-group analysis will be independent of their rate constants. Thus, the kinetic data indicate that the *Klebsiella* enzyme will not attack the M–M linkage and, in addition, exclude the M–G linkage as a substrate. Based on the end-group analyses, no similar conclusion can be drawn for the *Haliotis* enzyme.

By using the n.m.r. data for each fraction and their yields, the total recovery of each of the uronic acids may be calculated and the proportions converted into unsaturated, non-reducing end-groups can be determined (Table V). The errors in these calculations may be large and the results should be considered as qualitative only. With a high-G substrate (*L. hyperborea* alginate), practically all of the non-reducing end-groups stem from G units, whereas the opposite is true for a high-M substrate. Thus, it is concluded that the *Klebsiella* enzyme will cleave both G–G and the G–M linkages. Furthermore, the results for different substrates (Table III) indicate that the difference in rate constants for the scission of these linkages is small.

For a high-M substrate (*L. digitata* alginate), the main conversion occurring with the *Haliotis* enzyme is of an M unit into an unsaturated moiety. Combining this with the results of the n.m.r. analysis demonstrates that the enzyme has a preference for the M–M linkage. The proportion of the M–M diad is high in this alginate (Table II). However, since there is a significant proportion of reducing G-units in each fraction, the enzyme must also be able to attack the G–M linkage. The results obtained with LLS and L1.4 (Table III) suggest that there must be a considerable difference in rate constants for scission of the M–M and G–M linkages.

The high-G alginate *(L. hyperborea)*, with its low content of M–M diads, may be an atypical substrate for the *Haliotis* enzyme. The preponderance of reducing M-units in the dimer and trimer fractions probably reflects an effective degradation of the few M-blocks present. G reducing-end-groups occur at a higher frequency in fractions of higher d.p., suggesting that the transition diads may serve as

TABLE V

ESTIMATED PERCENTAGE OF M AND G CONVERTED INTO UNSATURATED, NON-REDUCING END-GROUPS

Alginate	Klebsiella		Haliotis	
	M	*G*	*M*	*G*
L. hyperborea	5	95	45	55
L. digitata	85	15	100	0

substrates. The high incidence of G→δ conversions with this substrate (Table V) indicates that it is the M–G linkage which is attacked.

The results obtained for extended degradation with the *Haliotis* enzyme do not support the kinetic observation that the G–G linkages are not attacked. Assuming that the main band observed in electrophoresis is the only active lyase in the preparation, the results suggest that this enzyme is capable of breaking M–M, M–G, and G–M linkages, although possibly at considerably different rates. The possibility that this enzyme preparation is a mixture of two active enzymes should not be overlooked.

The results presented here demonstrate that the term specificity should be used with caution for enzymes that act on copolymers having non-repeating structures.

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