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Substrate conversion and product inhibition of mannuronate lyase from *Haliotis* *

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ABSTRACT

Purified preparations of alginate lyase from *Haliotis tuberculata* were investigated by recording the progress of product formation when acting on well-characterized alginates and alginate fragments. An apparent endpoint level of maximal conversion was reached within hours. This level not only reflected the initial substrate concentration, but was also dependent on the enzyme dose. Addition of more enzyme or substrate during incubation revealed that the apparent inhibition was not of an irreversible nature. However, the degree of conversion was clearly affected by product inhibition. Testing of substrates enriched in polymannuronic (MM) or heteropolymeric (MG) blocks showed that the product inhibition observed could be fully ascribed to the latter type, resulting in a delayed and reduced conversion. A similar inhibition was observed by adding only oligomer fractions obtained by separation of enzymatic breakdown products from a MM substrate. A numerical model based on integrated Michaelis–Menten equations will be reported separately.

INTRODUCTION

Alginates are salts of alginic acid, a linear copolymer of (1 → 4)-linked β-D-mannuronic acid (M) and (1 → 4)-linked α-L-guluronic acid (G). The uronic acid residues are organized in blocks of polymannuronic acid (MM), polyguluronic acid (GG), and heteropolymeric sequences containing both (MG)[1].

Alginate lyases are enzymes that catalyze a β-elimination reaction, cleaving the alginate chain and creating an unsaturated uronic acid at the new non-reducing end[2]. Since this unit will absorb strongly in the UV region at 230–240 nm, alginate lyase activity may easily be detected as an increased absorbance at these wavelengths[3]. Alginate lyases have been found in a variety of organisms, including marine molluscs, echinoderms, bacteria, and fungi[4,5]. Different lyases may be expected to be specific for different types of alginate blocks or uronic acids. However, as pointed out earlier[2], the term specificity must be used with caution for enzymes acting on copolymers with non-repeating structures. Roughly, alginate

* Kinetics and Specificity of Alginate Lyases, Part II. For Part I, see ref. 2.

lyases may be classified as mannuronate (M-) lyases, defined by an incapability to cleave polyguluronic blocks, or guluronate (G-) lyases when polymannuronic blocks are left intact. In this sense, lyases from marine molluscs appear generally to be endo-enzymes of the mannuronate type[4]. This has been documented for the alginate lyase of abalone (*Haliotis* sp.) by Nakada and Sweeny[3], Boyen et al.[4], Haugen et al.[2], and others.

Attempts have been made to study the action of alginate lyases by isolation and characterization of the oligomeric end-products[6,7]. As an alternative, enzyme kinetics may be studied by a classic Michaelis–Menten approach[2]. Apparently, Michaelis–Menten kinetics were obeyed and kinetic parameters could be identified also for the *Haliotis* mannuronate lyase acting on substrates enriched in homopolymeric blocks[2]. However, proper definitions of substrate and product concentrations in the Michaelis–Menten sense remains as a fundamental problem for polymeric substrates of irregular structure such as alginates. Moreover, the two approaches presented above cannot be connected if the kinetic analysis is restricted to the Michaelis–Menten initial phase situation of excessive substrate levels and no product formation. As pointed out by Haugen et al.[2], specificity defined by kinetic parameters may not coincide with that defined on the basis of extensive degradation. The correlation between initial enzymatic specificity and the nature of the apparent end-products can only be revealed by analysing the complete process of substrate conversion. Particularly, analysis of progress curves may be applied to identify and characterize enzyme inhibition mechanisms[8].

Such a type of study is also necessary to predict reaction rates and product yields obtainable in vitro. It should be noted that alginate lyases have a variety of practical applications: as a tool in carbohydrate chemistry to quantify and characterize alginates in solution[9], in seaweed biochemistry and technology to produce protoplasts[5] for cell and tissue-culture studies, and to simplify separation of DNA and RNA.

The purpose of this investigation was to analyse progress curves of an alginate lyase acting on well-characterized substrates in order to identify the enzymatic specificity and inhibition mechanisms affecting reaction rates, degree of conversion, and product composition.

EXPERIMENTAL

Enzymes.—Alginate M-lyase was prepared from *Haliotis tuberculata*, freshly collected at Roscoff, Brittany. Preparative purification was performed by ammonium sulphate precipitation and ion-exchange chromatography as described by Boyen et al.[4]. The purified enzyme was found to be stable when stored as frozen stock solutions, diluted to 1 UA/mL unless otherwise stated.

Substrates.—The different alginate substrates applied in the following studies are summarized in Table I. Alginates from *Laminaria digitata* were supplied by Protan A/S. Alginate fragments of extreme block composition were prepared as

TABLE I

Composition [a] of alginates and alginate block fragments applied in tests

Sample/source	F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}
L. digitata	0.59	0.41	0.43	0.16	0.16	0.25
A. nodosum FMI	0.88	0.12	0.86	0.02	0.02	0.10
"MG" type	0.63	0.37	0.34	0.29	0.29	0.08
"MM" type	0.92	0.08	0.88	0.04	0.04	0.04
"GG" type	0.05	0.95	0.0	0.05	0.05	0.90

[a] Data obtained by NMR spectroscopy[11].

described by Haug et al.[1,10]. The one with a high content of guluronic acid (GG) was prepared from *Stilophora rhizoides*, that with a high content of mannuronic acid (MM) was obtained from *Sargassum* sp., while fragments with high transition (MG) came from *Ascophyllum nodosum*. All fragments had an average $dp_n > 20$. The uronic block composition was estimated by nearest-neighbour frequencies determined by NMR spectroscopy[11], see Table I.

Assays.—Alginate lyase *activities* were determined at 20°C and 50 mM Tris–HCl buffered to pH 7.5, as described earlier[2]. Volumes of buffer (2 mL), substrate solution (0.5 mL), and enzyme (0.5 mL) were mixed directly in a 10-mm quartz cuvette, to give concentrations of 1% NaCl and 0.1% (w/v) alginate substrate. Unless otherwise stated, the standard reference substrate was alginate from *L. digitata*, containing at least 25% of both MM, MG, and GG blocks (Table I). The initial increase in absorbance at 230 nm was recorded continuously with a Shimadzu UV-260 Spectrophotometer in order to verify linearity; the enzyme was diluted and the recordings were repeated if necessary. Enzyme activity is reported in units (UA) defined as the increase in absorbance units per min.

Enzyme kinetics and progress curves.—Progress curves were recorded in the same experimental system. Volumes of buffer (4 mL) and alginate test solution (2 mL) were mixed, and the background absorbance was recorded. All concentrations given refer to those of the test solution unless otherwise stated. A small volume (100 μL unless otherwise stated) of a stock solution (1 UA/mL) of purified *Haliotis* alginate M-lyase was added to all samples, and the enzyme background recorded separately in a blank sample without alginate. Absorbance recordings were repeated until a stable level was reached[9].

Separation and testing of products.—Crude "product" solutions were produced by increasing the enzyme and substrate concentrations to 0.33 UA/mL and 3.3 mg/mL of alginate respectively. After incubation overnight at room temperature to obtain maximal conversion, the reaction was stopped and the enzyme inactivated by rapid boiling (5 min) and cooling. Stock solutions thus obtained are denoted product (P) solutions.

The P solution obtained from a high MM substrate from *A. nodosum* FMI (Table I) was treated according to Haugen et al.[2], separating the oligosaccharide

products according to size on BioGel P4 (2 serial columns, each at 26 × 850 mm) eluted with 0.05 M Na_2SO_4. Collected fractions were pooled, dialyzed, freeze-dried, and weighed, and the actual sugar content was determined by the phenol–sulphuric acid method of Dubois et al.[12]. Based on these data, stock solutions of each fraction pool were made at a concentration of 3.3 mg/mL of sugar, that is at the same level as the unfractionated P solutions.

The potential inhibitions of crude and fractionated P solutions were tested by adding fixed amounts of the corresponding stock solutions to a standard substrate concentration of 0.05%. 0.1 UA of enzyme was then added and progress curves were recorded as described above.

RESULTS AND DISCUSSION

Progress curves.—Fig. 1 shows the characteristic kinetics observed when 0.01–0.2 UA of M-lyase was added to test samples of 0.1% *L. digitata* alginate. The differences in enzymatic activities were directly reflected in the differences of the initial increase of the curves. In all cases, a stable level of enhanced absorbance was reached within 2 h. This equilibrium level was strongly dependent on the amount of enzyme used. Such an effect might be expected to disappear at higher enzyme-to-substrate ratios. However, a reduction in substrate concentration by a factor of 5 gave curves of similar shape (results not included). Even at this low concentration (0.02% of alginate), it was not possible to establish an enzyme level where the substrate could be considered as completely degraded. It should be noted that the enzymatic activity was stable for weeks when incubated under similar conditions in the absence of substrate (results not included).

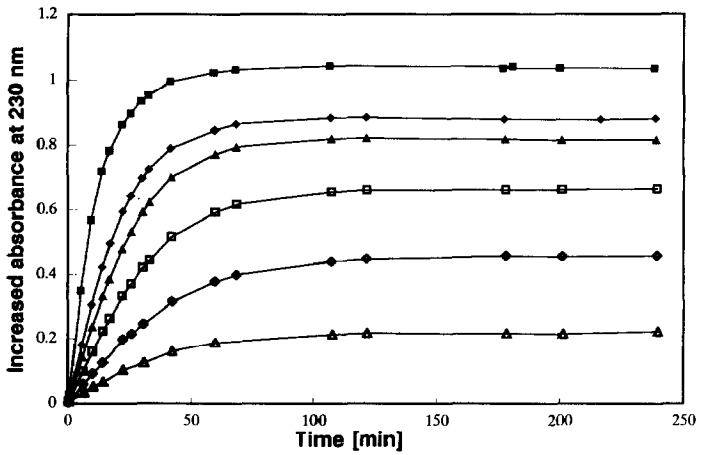

Fig. 1. Progress curves for *Haliotis* lyase acting on 0.1% alginate from *L. digitata*, with enzyme doses corresponding to (top to bottom) 0.2, 0.1, 0.08, 0.06, 0.04, and 0.02 UA, respectively.

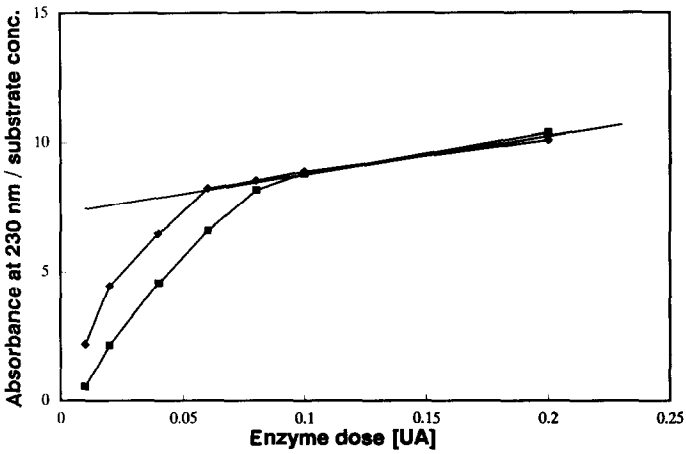

Fig. 2. Maximal substrate conversion obtained after 3 h for *Haliotis* lyase acting on 0.02% (♦) and 0.1% (■) alginate from *L. digitata*, shown as a function of enzyme dose.

Degree of conversion.—The apparently stable endpoint levels of maximal product formation obtained above may be plotted as a function of enzyme concentration. In order to compare the two experiments, data have been re-scaled as degree of conversion (absorbance per initial substate concentration) in Fig. 2. Both curves approach the same linear relationship for higher concentrations of enzyme. At lower concentrations of substrate, this linear range was extended towards lower concentrations of enzyme. For alginate concentrations up to 0.1%, 0.08 UA of M-lyase was sufficient to reach the linear range (Fig. 2). Within the linear range, the degree of conversion appeared to be constant for a given enzyme concentration. In other words, the apparent endpoint levels of product formation were directly proportional to the initial concentration of substrate. This has been routinely verified by observing linear calibration curves when product levels recorded at 3 h are plotted as a function of concentration for a given alginate at constant enzyme dose[9]. A change in enzyme concentration would alter the factor of proportionality.

Fig. 2 shows that a complete conversion of substrate could not be achieved. As illustrated in Fig. 1, the enzymatic reaction was limited by an enzyme-dependent factor. The reversibility of this inhibition or inactivation may be revealed by adding enzyme or substrate in several steps.

Additional enzyme / additional substrate.—Fig. 3A illustrates the effect of adding more enzyme after 2 h of incubation[13]. Product formation responded rapidly. Most important, a doubling of the enzyme concentration after 2 h gave curves approaching those of the same total enzyme dose given initially. Thus, the apparent endpoint levels were unaffected by a multistep addition of enzyme as long as the final total dose was constant.

Fig. 3B shows the response when the substrate concentration was doubled after 2 h (cf. Larsen et al.[13]). Despite the fact that the reaction had ceased at a level far

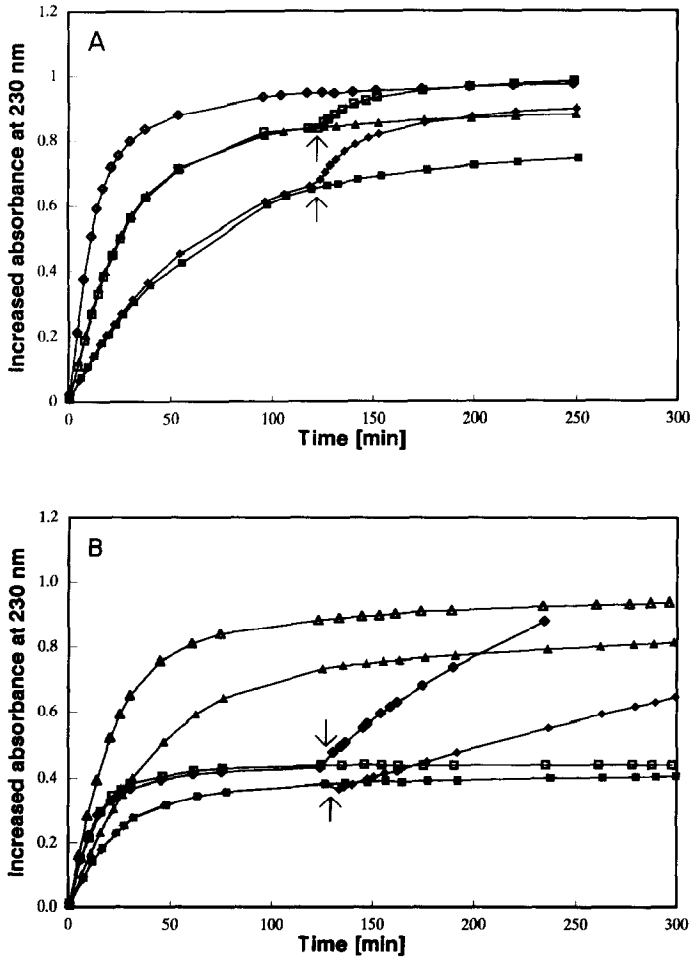

Fig. 3. Progress curves for *Haliotis* lyase acting on alginate from *L. digitata*, including data published by Larsen et al.[13]: A, Arrows indicate a doubling of enzyme doses after 2 h of incubation, with initial enzyme doses corresponding to (top to bottom) 0.2, 0.1, and 0.05 UA, respectively; substrate concentration was 0.1%. B, Arrows indicate a doubling of substrate concentration after 2 h of incubation, from 0.05 to 0.1%; enzyme concentrations were 0.1 (open symbols) and 0.05 UA (closed symbols).

from maximal conversion, addition of fresh substrate led immediately to an increased formation of product. The enzyme present can therefore not be considered as inactivated or irreversibly inhibited. It should also be noted that the reaction induced by substrate addition at 2 h progressed at a much lower rate than the initial one (Fig. 3B). The reaction was in fact too slow to reach a stable endpoint within the recorded period. This effect can only be explained by a reduced amount of enzyme accessible to the new substrate.

Product inhibition.—The results of Fig. 3 may possibly be explained by a reversible product inhibition. Different amounts of P solution were added at the

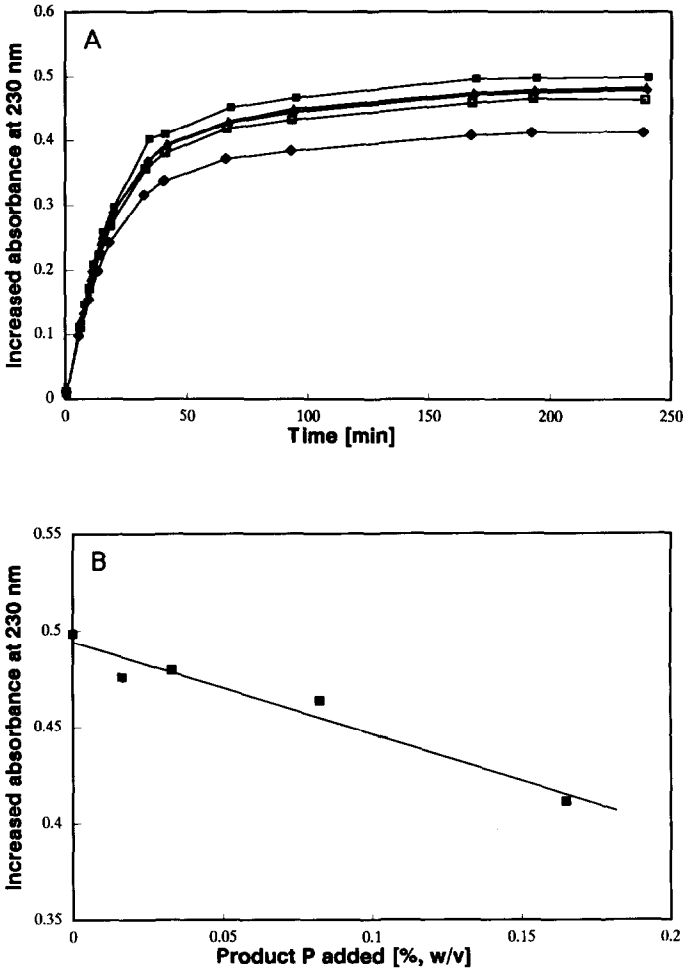

Fig. 4. Inhibition of *Haliotis* lyase (0.1 UA) acting on alginate from *L. digitata* by initial addition of product solution P: A, Progress curves obtained with 0.05% substrate and 0.1 UA of enzyme, with P solution corresponding to (top to bottom) 0, 0.1, 0.2, 0.5, and 1.0 mL, respectively, included in the standard 6-mL test volume. B, Maximal substrate conversion shown as a function of P concentration in the test solution.

start of incubation of 0.1 UA of *Haliotis* M-lyase acting on the standard *digitata* substrate (0.05%). The recorded progress curves obtained after correction of the initial blank values are shown in Fig. 4A. A gradually reduced formation of product was observed at increasing amounts of P. Fig. 4B shows the effect on the final endpoint levels, illustrating a product inhibition that may be roughly described as a linear function of the product P added. The highest dose of P corresponds to 3.3 times the initial substrate concentration on a weight/weight basis.

Mannuronate lyases such as the *Haliotis* lyase leave the polyguluronic blocks of the alginate unattacked[2,9], and such blocks will therefore tend to dominate the

smaller molecules accumulated as the alginate is broken down. However, addition of amounts of GG-blocks (Table I) in excess gave no significant inhibition in experiments similar to that of Fig. 4 (results not included).

Specificity.—The effects reported above cannot be explained without taking the alginate block structure and the corresponding enzyme specificity into consideration. This block specificity may be revealed by testing the fragments given in Table I as substrates. We have already demonstrated that the relative conversion recorded after 3 h under standardized conditions could be ascribed to a 70% conversion of MG-blocks and 0% conversion of GG-blocks, relative to that of MM[9,14].

However, the possible influence of block length on the degree of conversion must also be taken into consideration[9]. This will not affect the comparison of different enzyme doses for a given type of fragment, but rather its ideality as a homogeneous model substrate. The "MM" block type was therefore replaced with the *A. nodosum* FMI alginate to obtain much longer substrate molecules at the same MM content. Table I also indicates that the guluronate contamination was more dominated by the inactive GG form in the latter case. Fig. 5A illustrates the progress curves observed for different enzyme doses acting on a solution of the MM-rich FMI alginate. The differences in initial slopes correspond well with the differences in enzymatic dose. Most important, all progress curves seem to approach the same limit at increased periods of incubation. Our "MM" block substrate (Table I) showed a qualitatively similar pattern (results not included). This is as expected for an enzyme reaction obeying simple Michaelis–Menten kinetics.

The "MG" block fragments gave completely different results when tested as a 0.1% substrate, see Fig. 5B. Although the rate of product formation decreased gradually, no final endpoint level was observed within 6 h. The progress curves of different enzyme doses did not at all approach a similar degree of conversion. It appears that the effects on the degree of conversion obtained in the progress curves of the *L. digitata* alginate (Fig. 1) are as expected for a mixture of MM and MG blocks (Fig. 5).

Product inhibition at the block structure level.—Product inhibition may be revealed by adding increasing amounts of product to fresh "MM" and "MG" fragment substrates, as shown for intact alginate in Fig. 4A. In this case, the same fragments were used to produce two types of product P solution, correspondingly denoted P_{MM} and P_{MG}. Thus, four different combinations were studied.

The effects observed on the reaction rates of the "MM" substrate (0.05%) and 0.1 UA of enzyme were small and hardly of significance even for P_{MM} and P_{MG} doses up to 0.0825% (progress curves not included). Also, see below.

The results obtained for the "MG" substrate under similar conditions are shown in Fig. 6. P_{MM} gave the most significant inhibition even at the early stages of product formation (Fig. 6A). Qualitatively, this is as expected for a competitive product inhibition. Compared to this, the effects of P_{MG} were much smaller (Fig. 6B). A quantitative treatment will be given elsewhere[15].

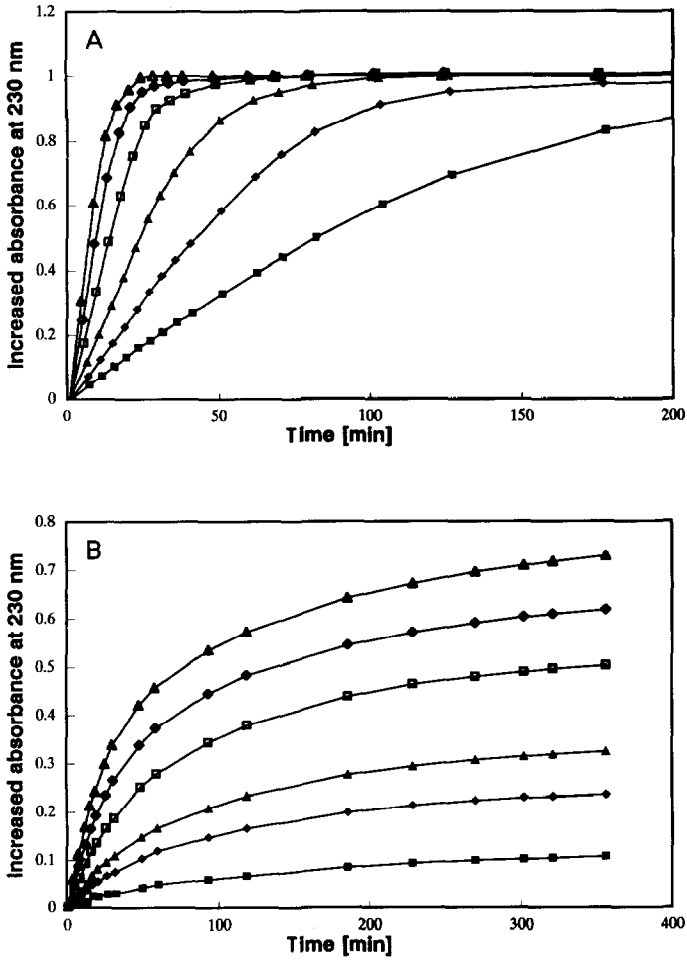

Fig. 5. Progress curves for *Haliotis* lyase acting on alginate substrates, with enzyme doses corresponding to (top to bottom) 0.2, 0.15, 0.1, 0.06, 0.04, and 0.02 UA, respectively; note different time scales: A, With MM-rich substrate from *A. nodosum* FMI, 0.05%. B, With MG-rich substrate ("MG"; Table I), 0.1%.

Fig. 7 gives a summary of the effects on the apparent endpoint levels for both block fragment substrates and P solutions. It is concluded that only the breakdown of polymannuronic blocks will give products that may seriously inhibit the action of the *Haliotis* mannuronate lyase on heteropolymeric MG blocks.

Inhibition by product fractions.—The exact nature of the product responsible for this inhibition is not known. Obviously, it is not the product as defined by the number of cuts in the polymeric chain, but rather some molecular fraction formed by the action of the enzyme. The strongest inhibitor solution obtained after maximal enzymatic breakdown, type P_{MM}, was therefore fractionated according to

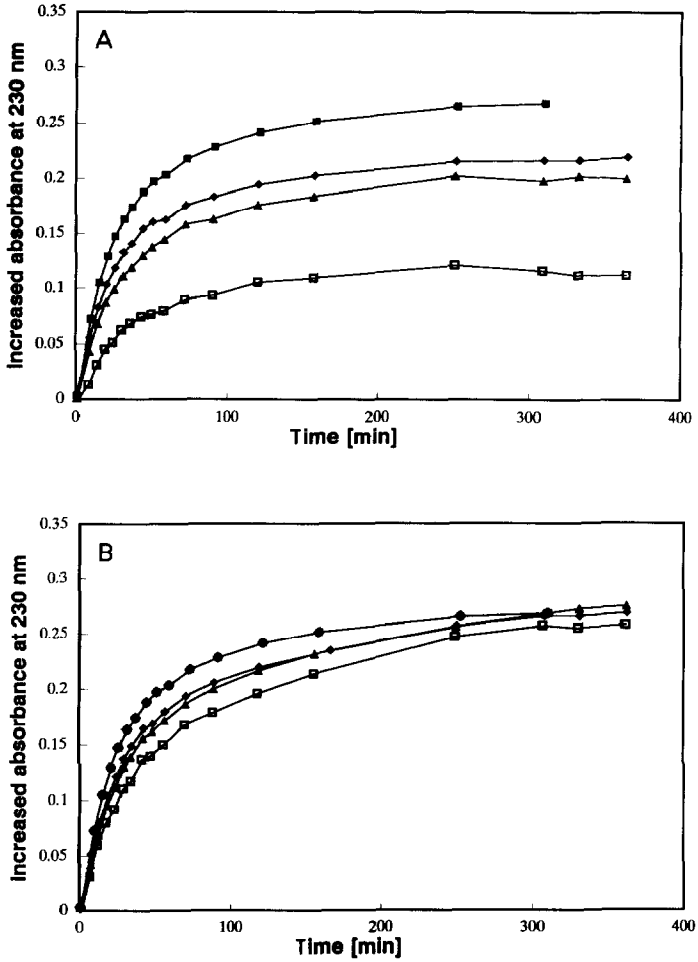

Fig. 6. Inhibition of *Haliotis* lyase (0.1 UA) acting on "MG" substrate (Table I; 0.05%) by initial addition of product solution P_x. Progress curves were recorded with P_x solution corresponding to (top to bottom) 0, 0.1, 0.2, and 0.5 mL, respectively, included in the standard 6-mL test volume: A, $P_x = P_{MM}$ produced by breakdown of "MM" type (Table I). B, $P_x = P_{MG}$ produced by breakdown of "MG" type (Table I).

size by gel filtration, giving the chromatogram shown in Fig. 8A. Fractions were analysed by the phenol–sulphuric acid method. A significant amount of polymeric material was eluted directly in the void volume (Fig. 8A). Clearly, the end-products must be considered as a mixture of lower oligomers[6,7]. The dominant peak was found in the range of fractions 92–103, corresponding to a chain length of 4 units, followed by fractions 106–116 identified as trisaccharides[16].

The separated fractions were pooled as illustrated in the diagram and tested at fixed concentrations corresponding to 0.033 and 0.0825% in the test solution

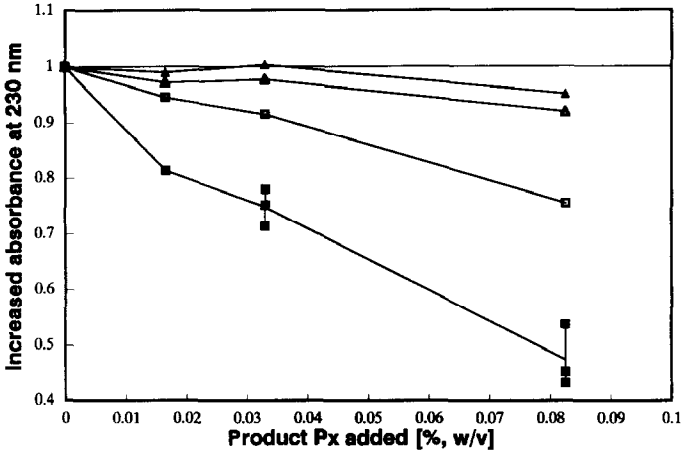

Fig. 7. Inhibition of *Haliotis* lyase (0.1 UA) acting on "MM" (open symbols) and "MG" (closed symbols) substrate (Table I; 0.05%) by initial addition of product solution $P_x = P_{MM}$ (■, □) or $P_x = P_{MG}$ (▲, △). Maximal substrate conversion relative to absence of inhibitor is shown as a function of P_x concentration in the test solution.

containing fresh "MG" block substrate (0.05%). The recorded inhibition of the corresponding unfractionated material has been included in Fig. 7. Fig. 8B illustrates the inhibition effects observed after fractionation, relative to that of the unfractionated P_{MM} added to the column. The polymeric material of fraction P1 had no effect, and was most probably dominated by the guluronate contamination initially present in our FMI alginate (Table I). All oligomeric fractions P2–P5 reduced enzyme conversion significantly, with the highest specific inhibition obtained for the larger molecules eluted in P2 and P3. The integrated results of Figs. 8A and 8B do not fully explain the quantitative inhibitory effect of the unfractionated material P_{MM}. Some losses of material during handling, separation, and dialysis cannot be excluded. Still, the data of Fig. 8B support the view that oligomers formed by cleavage of MM-blocks will act as strong competitive inhibitors for the enzymatic action on heteropolymeric MG sequences, and that is the major reason for the enzyme-dependent maximal conversion of natural intact alginates illustrated in Fig. 1.

Conclusions.—Qualitatively, we have shown that the action of the *Haliotis* mannuronate lyase may be explained at the block structure level of alginates. The enzyme remained unaffected by polyguluronate blocks. The breakdown of polymannuronate blocks creates products that may competitively inhibit the conversion of heteropolymeric (MG) blocks, to an extent where the apparent endpoint of product formation is seriously affected. This is typical of inhibitors of high affinity approaching the extreme "irreversible" situation[8]. These observations may not

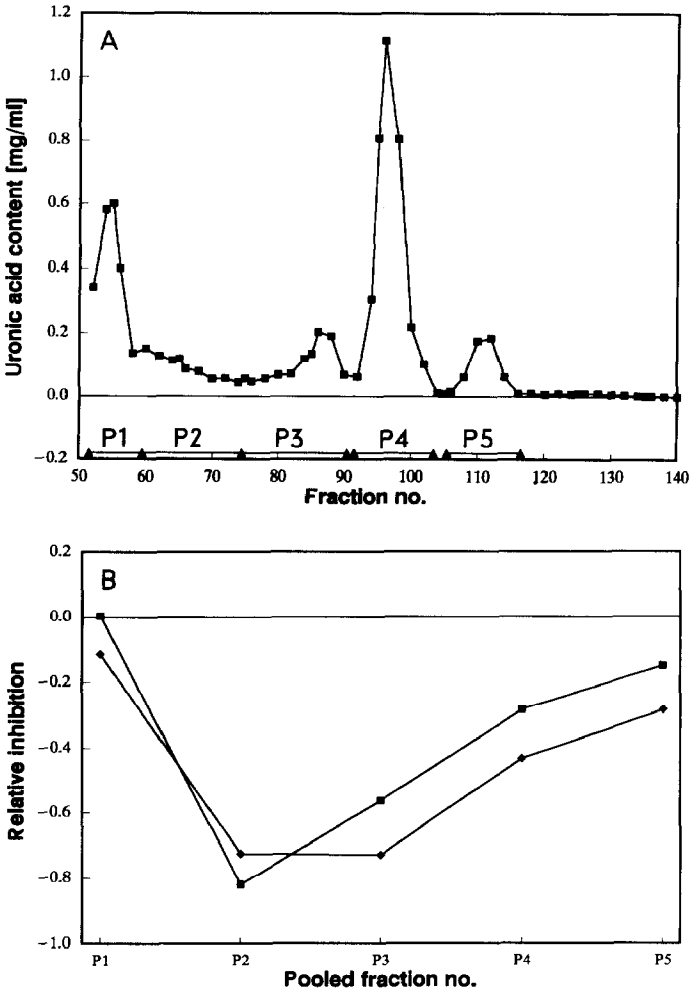

Fig. 8. Product solution P_{MM} separated by gel filtration: A, Chromatogram, showing uronic acid content of collected fractions estimated by the phenol–sulfuric acid method[12]; fractions were pooled as P1–P5, as indicated. B, Inhibition of *Haliotis* lyase (0.1 UA), acting on "MG" substrate (Table I; 0.05%), by adding pooled fractions of Fig. 9A at concentrations corresponding to 0.033% (■) and 0.0825% (◆) in the test solution. Reduction of maximal conversion is given relative to that of unfractionated P_{MM} at the same concentration (−1).

only be of interest to carbohydrate and enzyme chemists; they are also of importance for the practical or commercial application of such alginate lyases[9,14].

A quantitative analysis will be given separately[15].

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