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Numerical model for alginate block specificity of mannuronate lyase from *Haliotis* †

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Abstract

Purified preparations of alginate (mannuronate) lyase from *Haliotis tuberculata* have been investigated by recording the progress of product formation when acting on well-characterised alginates and alginate fragments. We have earlier shown that maximal conversion is affected by enzyme dose as well as by initial substrate concentration. This is related to a strong, reversible product inhibition, dominant in the enzymatic breakdown of the heteropolymeric (MG) fraction of the alginate compared to that of polymannuronic (MM) blocks. Polyguluronic (GG) structures were found to be inactive both as substrate and as inhibitor. A numerical two-substrate model is now presented in an attempt to analyse data by simply assigning different kinetic constants to each block fraction of the accessible substrate. Including reversible product inhibition, the general model is expressed by a set of six coupled nonlinear differential equations. If the steady-state assumption of the Michaelis–Menten initial rate analysis is accepted, these equations may be integrated directly. This approach could only adequately describe data for poly-M substrates showing no significant product inhibition. The steady-state assumption could not be maintained for results obtained with substrates rich in heteropolymeric MG blocks. Instead, the kinetic parameters were estimated by a nonlinear minimalisation of the sum squares of residuals. In this form, the model could fully describe our experimental observations.

† Kinetics and Specificity of Alginate Lyases, Part III. For Part II, see ref 9.

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1. Introduction

Polysaccharides as substrates for polymer-modifying or polymer-cleaving enzymes constitute a highly complex field. This is closely related to the irregular structure of such molecules. First of all, wide and variable molecular weight distributions and the possibility of simultaneous or multiple enzyme attacks, including endo- or exo-enzymes, make such basic concepts as a molar substrate concentration most difficult to apply. Secondly, it is evident that some specificity must be present, in the sense that some restricted part of the polymer will represent a binding site with affinity for the enzyme molecule. However, even this concept may be difficult to apply to polymers having irregular or nonrepeating structures [1].

This is valid also for the apparently simple case of linear copolymers. One example is chitosan, where the acetylation pattern gives a *random* molecular structure that has been characterised by NMR methods and which can only be described by statistical methods [2,3]. Consequently, enzymatic breakdown must also be described by similar methods, and the term specificity defined by the statistical description of sequences corresponding to the number of monomers necessary to form a binding site, apparently a hexasaccharide (Stokke, unpublished results).

Alginates represent an entirely different molecular structure, with a *block*wise organization of the D-mannuronic (M) and L-guluronic (G) residues in homopolymeric MM and GG as well as heteropolymeric MG sequences [4]. The polymer chain will be cleaved by alginate lyases [5]. Such enzymes are now experimentally available from several sources, at high purity and with clear differences in specificity [6–8]. It is evident that alginate and alginate lyases constitute a useful and illustrative model system in understanding the role of polysaccharide molecular structure both in biological processes and industrial applications.

The basic question is, of course, whether an enzyme specificity in this case may simply be assigned to each of the block structures of alginate. Such a three-substrate model is principally a simplification, neglecting both the variability in, and limitations of, block lengths. Also, alginates contain four different glycosidic linkages, since an M–G bond is essentially different from that of G–M [1]. Still, the simplest possible block substrate model should be tested as an approach to the analysis of experimental data and the evaluation of the applicability and limitations of the classical theory of enzyme kinetics [1].

We have earlier presented characteristic experimental data for the alginate lyase of abalone (*Haliotis* sp.) [9]. Briefly, analysis of progress curves revealed that the apparent endpoint of maximal conversion not only reflected initial substrate concentration, but also depended on enzyme dose. Addition of more enzyme or product during incubation showed that the apparent inhibition was reversible. Conversion was clearly affected by product inhibition. Testing of substrates enriched in MM or MG blocks showed that this inhibition could be ascribed to the latter heteropolymeric type [9]. The purpose of the work presented here was to compare these data with the simplest possible mathematical model, assigning

Table 1
Composition [a] of alginates and alginate fragments applied in tests

Code, sample/source	F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}
mm, *A. nodosum* FMI alginate	0.88	0.12	0.86	0.02	0.02	0.10
mg, "MG"-enriched alginate from *A. nodosum*	0.63	0.37	0.34	0.29	0.29	0.08
pyr, "MM"-enriched alginate from *M. pyrifera*	0.80	0.20	0.65	0.15	0.15	0.05
dig, *L. digitata* alginate	0.59	0.41	0.43	0.16	0.16	0.25

[a] Data were obtained by NMR spectroscopy[10]. Also see ref 9.

different kinetic constants to the two types of substrate accessible to this enzyme, the MM and MG blocks.

2. Experimental

See ref 9. The estimated sequence compositions [10] of the different alginate substrates are summarised in Table 1. All enzyme studies were performed at room temperature in 50 mM Tris–HCl, buffered to pH 7.5, and 1% (w/w) NaCl. Briefly, product formation was measured as the increase in absorbance at 230 nm, continually recorded in a Shimadzu UV-260 spectrophotometer until a stable level was reached. Substrate and enzyme background absorbance were determined separately and subtracted. This excess absorbance at 230 nm is proportional to the fraction of unsaturated uronic acids formed at new nonreducing ends [1]. Other details are given elsewhere [9]. Under these conditions, maximal breakdown of a 0.1% solution of alginate to oligomers dominated by tetrasaccharides gave an increased absorbance of ca. one unit [9]. Experimental data obtained at constant substrate level and different enzyme concentrations are labelled MM-e, MG-e, etc., whereas constant enzyme, different substrate levels are labelled MM-s, MG-s, and likewise for the other alginates included (Table 1).

3. Theory

General assumptions.—The finding that tetrasaccharides of alginate fragments are an accumulating product during lyase cleavage of alginates [1,9,11,12] suggests that there is a minimum degree of polymerisation of the substrate required for the action of the enzyme. There are 2^4 different sequences within a tetrasaccharide unit of a binary statistical copolymer. In principle, all those 16 different units should be represented by their respective kinetic constants in a detailed description of the enzymatic depolymerisation of alginate. This will, however, lead to a model with such a flexibility that any attempt to fit its large number of parameters to available experimental data would both be impractical and useless. Instead, we

have found it essential to reduce the degrees of freedom by minimising the number of substrate types to a level determined by the available data.

This empirical approach also implies that the block structure of alginate is emphasised to an extent where all minor sequences are considered negligible, corresponding to "infinite" block lengths. Information about the chemical structure of alginate suggests that the four different glycosidic linkages are grouped in three different block types [4]. Since the GG-blocks proved to be unaffected by *Haliotis* M-lyase [9], the rough approximation of two types of substrate (MM and MG) within an alginate chain will be adopted in this paper. It is assumed that the *a* and *b* types of substrate mutually compete for the active site of the enzyme and that reversible product inhibition [9] may occur:

$$e + a \underset{k_{-1}}{\overset{k_{+1}}{\longleftrightarrow}} \underset{y_1}{ea} \xrightarrow{k_{+2}} \underset{y_2}{eP_a} \underset{k_{-3}}{\overset{k_{+3}}{\longleftrightarrow}} e + P_a \tag{1a}$$

$$e + b \underset{k_{-4}}{\overset{k_{+4}}{\longleftrightarrow}} \underset{y_3}{eb} \xrightarrow{k_{+5}} \underset{y_4}{eP_b} \underset{k_{-6}}{\overset{k_{+6}}{\longleftrightarrow}} e + P_b \tag{1b}$$

In Eq. *1*, e is the enzyme concentration, P_a and P_b are the respective products formed by conversion of the two substrates a and b, k_{+m} and k_{-m} ($m = 1$–6) are the rate constants, and y_n ($n = 1$–4) are shorthand notations for the intermediates. Following standard procedures in analysing enzyme kinetics, the kinetic equations for the intermediates are:

$$\begin{aligned}
\frac{\partial y_1}{\partial t} &= k_{+1}ea - k_{-1}y_1 - k_{+2}y_1 \\
\frac{\partial y_2}{\partial t} &= k_{+2}y_1 + k_{-3}eP_a - k_{+3}y_2 \\
\frac{\partial y_3}{\partial t} &= k_{+4}eb - k_{-4}y_3 - k_{+5}y_3 \\
\frac{\partial y_4}{\partial t} &= k_{+5}y_3 + k_{-6}eP_b - k_{+6}y_4
\end{aligned} \tag{2}$$

Conservation of total enzyme yields:

$$e_0 = e + y_1 + y_2 + y_3 + y_4 \tag{3}$$

and the conservation of the two types of substrates as well as introducing $y_5 = P_a$ and $y_6 = P_b$ yields:

$$\begin{aligned}
a_0 &= a + l(y_1 + y_2) + y_5 \\
b_0 &= b + l(y_3 + y_4) + y_6
\end{aligned} \tag{4}$$

In Eq. *4*, it is assumed that the substrate binding to the active site effectively inhibits l neighbouring glycosidic linkages for attack by other enzymes. Although there are experimental data [9] suggesting that $l = 4$, this parameter is formally

kept adjustable. Eqs. 2–4 yield the following set of coupled nonlinear differential equations:

$$\begin{aligned}
\frac{\partial y_1}{\partial t} &= k_{+1}(e_0 - y_1 - y_2 - y_3 - y_4)[a_0 - l(y_1 + y_2) - y_5] - k_{-1}y_1 - k_{+2}y_1 \\
\frac{\partial y_2}{\partial t} &= k_{+2}y_1 + k_{-3}(e_0 - y_1 - y_2 - y_3 - y_4)y_5 - k_{+3}y_2 \\
\frac{\partial y_3}{\partial t} &= k_{+4}(e_0 - y_1 - y_2 - y_3 - y_4)[b_0 - l(y_3 + y_4) - y_6] - k_{-4}y_3 - k_{+5}y_3 \\
\frac{\partial y_4}{\partial t} &= k_{+5}y_3 + k_{-6}(e_0 - y_1 - y_2 - y_3 - y_4)y_6 - k_{+6}y_4 \\
\frac{\partial y_5}{\partial t} &= -k_{-3}(e_0 - y_1 - y_2 - y_3 - y_4)y_5 + k_{+3}y_2 \\
\frac{\partial y_6}{\partial t} &= -k_{-6}(e_0 - y_1 - y_2 - y_3 - y_4)y_6 + k_{+6}y_4
\end{aligned} \tag{5}$$

Steady state.—Eqs. 5 may be solved directly by assuming steady state for all intermediates, that is $\partial y_n/\partial t = 0$ $(n = 1\text{–}4)$. This is equivalent to the approach generally applied in the Michaelis–Menten analysis of initial reaction rates. The fraction of substrate bound in the intermediate stages are then constant, yielding expressions for product formation rates

$$\begin{aligned}
v_a &= \frac{(V_f^a/K_{ma})a}{1 + a/K_{ma} + P_a/K_{mPa} + b/K_{mb} + P_b/K_{mPb}} \\
v_b &= \frac{(V_f^b/K_{mb})b}{1 + a/K_{ma} + P_a/K_{mPa} + b/K_{mb} + P_b/K_{mPb}}
\end{aligned} \tag{6}$$

where the conventional Michaelis–Menten parameters, V_f^a, K_{ma}, V_f^b, K_{mb}, K_{mPa}, and K_{mPb} have been introduced. The relation to the respective kinetic constants is identical to that of the single substrate model as presented by Cornish-Bowden [13]:

$$\begin{aligned}
V_f^a &= \frac{k_{+2}k_{+3}e_0}{k_{+2} + k_{+3}} \\
K_{ma} &= \frac{(k_{-1} + k_{+2})k_{+3}}{(k_{+2} + k_{+3})k_{+1}} \\
K_{mPa} &= \frac{k_{+3}}{k_{-3}}
\end{aligned} \tag{7}$$

and analogously for V_f^b, K_{mb}, and K_{mPa}. In our case, the total product formation rate is simply $v = v_a + v_b$. It should be noted that, at single substrate steady-state

conditions, the Michaelis–Menten equation (equivalent to Eq. *6*) may be directly integrated to give the time t as an analytical function of the product (or substrate) concentration, and that this expression may be linearised in various ways to simplify graphic analysis of experimental data [13].

Nonsteady state.—The following strategy for obtaining the parameters in Eq. *5* was adopted. The measured product was postulated to be $P = P_a + P_b = y_5 + y_6$. All parameter estimations were carried out by minimising the sum squares of residuals, SSR, between the observed and the calculated total product, using the simplex algorithm [14]. The length of the polymer in the active site l is set to 4 in the calculations reported here. Initial calculations carried out either for constant substrate and varying enzyme concentrations, or constant enzyme and varying substrate concentrations, with all the kinetic constants freely adjustable, showed, however, a not too good consistency between different substrates. The following constraints were therefore introduced. Estimates of the rate constants were obtained for the MM-type substrate by minimising SSR for the data obtained on the poly-M substrate in the MM-e and MM-s experiments simultanously. The amount of substrate, the ratio between the two types of substrate, enzyme concentrations in the MM-e, and substrate concentrations in the MM-s set of experiments were additional adjustable parameters. Correlations with the nominal concentrations are evaluated below. The rate constants obtained for the MM-substrate, k_{+m}, k_{-m} ($m = 1$–3) were kept as constraints in minimalisations based on the MG-e, MG-s, and whole-alginate time-progress curves. Attempts to fit all rate constants to the experimental data obtained for the different substrates *simultaneously* were also carried out.

When product was added to the mixture before addition of enzyme, the initial product was treated as an adjustable parameter, and the experimentally observed time-progress curves were equated with the excess sum of products formed.

4. Results

Polymannuronate MM blocks.—The *Ascophyllum nodosum* FMI alginate preparation has been applied as a model substrate for poly-M blocks, containing 86% MM and 4% heteropolymeric MG according to NMR analysis [9]. Since the GG fraction was inactive both as a potential substrate and as an inhibitor [9], the MM content may be considered as 95–96% of the accessible substrate.

Fig. 1 shows the experimental data for the MM-e (A) [9] and MM-s (B) experiments. The progress curves obtained in the latter case all showed the same initial slope, representing initial substrate saturation. The continuous lines of Fig. 1 depict the least-squares fit of our general two-substrate model to the MM-e and MM-s experiments simultanously. The broken lines depict the fit of the MM-e and MM-s experiments based on the global fit of the two-substrate model (see below). The corresponding estimates for the kinetic constants are listed in Table 2. Despite the applied two-substrate model, the numerical estimates of the two substrate concentrations (Table 3) show that there is only a small fraction of the

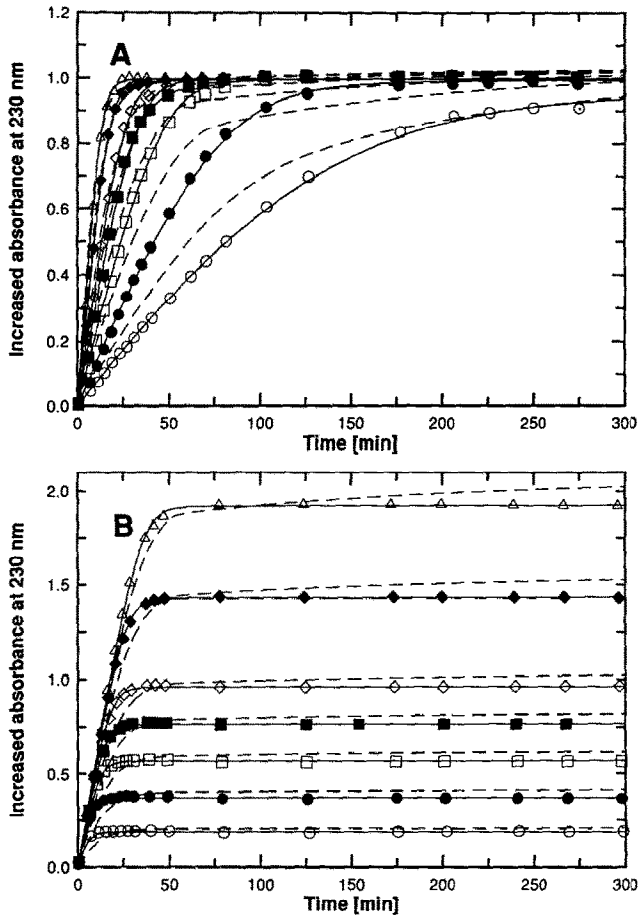

Fig. 1. A: Exptl code MM-e. Effect of different initial doses (e_0) of *Haliotis* M-lyase acting on 0.05% polymannuronate (MM) substrate. Symbols indicate experimental data obtained [9] with $e_0 = 0.02$ (○), 0.04 (●), 0.06 (□), 0.08 (■), 0.1 (◇), 0.15 (◆), and 0.02 UA (△). B: Exptl code MM-s. Effect of different initial concentrations (s_0) of MM substrate exposed to *Haliotis* M-lyase (0.1 UA). Symbols indicate experimental data obtained with $s_0 = 0.01$ (○), 0.02 (●), 0.03 (□), 0.04 (■), 0.05 (◇), 0.075 (◆), and 0.10% (△). Continuous curves indicate the theoretical estimates based on MM-e and MM-s experiments simultanously. Broken lines depicts global fit (see text).

b-type substrate. This implies that the inaccuracy of the numerical estimates of the constants k_{+m} and k_{-m} ($m = 4$–6) in Table 2 is larger than for the *a*-type substrate, simply because of the lack of sensitivity to these values. Correlations between nominal and fitted estimates of enzyme levels and substrate concentrations are presented below together with the corresponding data for the other alginate substrates.

The simple single-substrate integrated Michaelis–Menten model, assuming steady-state conditions, was also adapted to the same data sets according to the linearised graphic procedure of Cornish-Bowden [13]. The calculated curves thus

Table 2
Kinetic parameters estimated by fitting the mathematical two-substrate model (Eq. 5) to independent data sets identified by the experimental code given. The corresponding theoretical progress curves are shown in Figs. 1–4. Global values given at the bottom were obtained by simultaneous least-squares fit to all data sets.

Exptl code	k_{+1}	k_{-1}	k_{+2}	k_{+3}	k_{-3}	k_{+4}	k_{-4}	k_{+5}	k_{+6}	k_{-6}
MM-e/MM-s	23	0.34	2.7	18	0.058	1.0	1.0	0.09	9.4E-4	3.4
MG-e						3.8	1.2	0.16	3.2E-3	0.72
MG-s						4.2	19	1.0	1.2	2.7E+2
MG-i						3.3	1.7	1.8	0.34	3.0E+1
Pyr-e						6.9	18	0.44	9.7E-3	3.4E+2
Dig-e						6.9	12	0.50	5.2E-2	6.2E+4
Global	6.2	0.50	4.2	51	7.5E-3	6.0	8.3	0.10	2.8E-2	5.2E+4

obtained closely resembled those of our general model shown in Fig. 1 (results not included). No significant product inhibition was revealed, in accordance with the very large estimates for k_{+3}/k_{-3} (Table 2).

Initial addition of excessive levels of product inhibitor q_0 had no clear effect on the curved parts of the progress curves for the polymannuronate substrate (results not included). The final maximal conversion was, however, somewhat suppressed when tested on short MM blocks containing 8% of heteropolymeric MG [9]. This effect cannot be directly explained within the limits of the homogeneous single-substrate model.

Heteropolymeric MG-enriched alginate. — Unfortunately, a completely heteropolymeric MG substrate is not experimentally available. Our enriched alginate sample contains 58% MG and 34% MM according to NMR data [9], i.e., a relative MM contamination of 38% of the accessible substrate. This contamination is most serious, since it is evident that MM blocks will be broken more rapidly than MG [9], and thus tend to dominate the first stages of the reaction.

Fig. 2 shows data recorded for the MG-enriched substrate, the MG-e series (A) [9] and the MG-s series (B). The continuous lines depict the least-squares fit of the

Table 3
Initial substrate concentrations a_0 and b_0 estimated by fitting the mathematical two-substrate model (Eq. 5) to independent data sets identified by the experimental code given. The corresponding theoretical progress curves are shown in Figs. 1–4

Exptl code	Independent fit			Global fit
	a_0	b_0	b_0/a_0	b_0/a_0
MM-e/MM-s	1.00	0.29	0.29	0.04
MG-e	0.99	0.54	0.55	
MG-s	0.42	1.31	3.10	1.89
MG-i	0.20	0.18	0.93	
Pyr-e	0.85	0.51	0.60	1.36
Dig-e	1.12	0.88	0.79	1.18

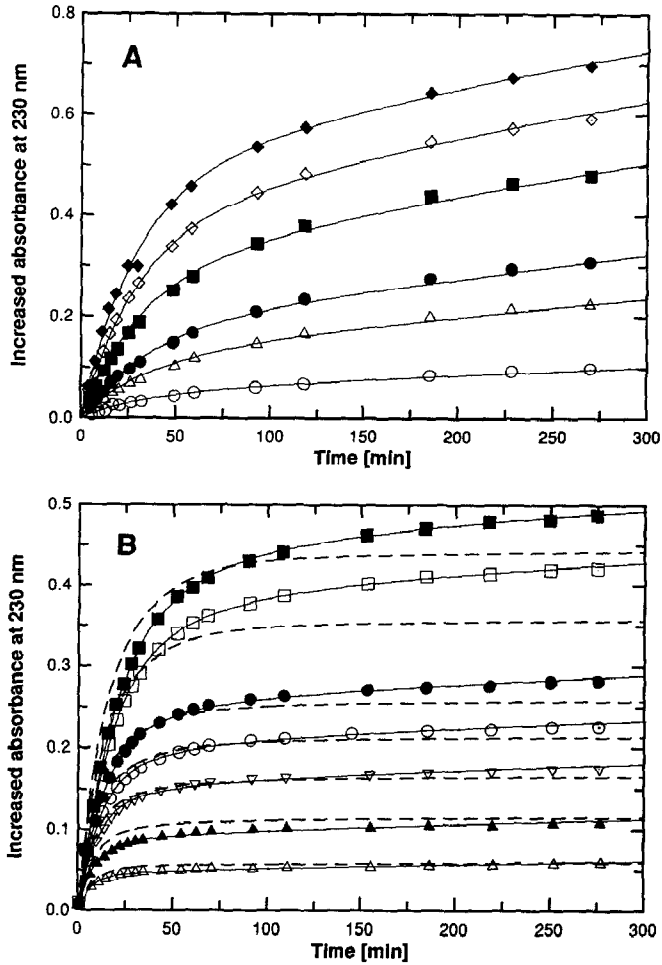

Fig. 2. Exptl code MG-e. Effect of different initial doses (e_0) of *Haliotis* M-lyase acting on 0.05% heteropolymeric MG-enriched alginate. Symbols indicate experimental data obtained [9] with $e_0 = 0.02$ (○), 0.04 (△), 0.06 (●), 0.1 (■), 0.15 (◇), and 0.2 UA (◆). B: Exptl code MG-s. Effect of different initial concentrations (s_0) of heteropolymeric MG-enriched substrate exposed to *Haliotis* M-lyase (0.1 UA). Symbols indicate experimental data obtained with $s_0 = 0.01$ (△), 0.02 (▲), 0.03 (▽), 0.04 (○), 0.05 (●), 0.075 (□), and 0.10% (■). Continuous curves indicate the theoretical estimates within the MG-e and MG-s experiments. Broken lines indicate global fit (see text).

two-substrate model where the rate constants for the MM-part of the MG substrate are constrained to the values obtained for the MM-e and MM-s fit. The actual fit shows that both cases could be adequately described by this model. The numerical estimates for the kinetic model parameters are given in Table 2, and those for the amounts of substrate in Table 3. The dotted lines depicting the global fit to the MG-s experiments show poorer agreement with the data than the constraint fit based on the MG-s experiments.

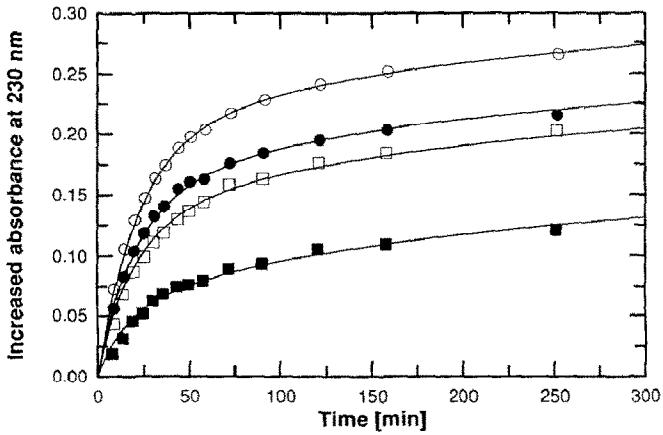

Fig. 3. Exptl code MG-i. Effect of different initial inhibitor concentrations (q_0) added to 0.1 UA of *Haliotis* M-lyase acting on 0.05% heteropolymeric MG-enriched substrate. Symbols indicate experimental data obtained with $q_0 = P_{MM}$ solutions corresponding to 0 (○), 0.1 (●), 0.2 (□), and 0.5 mL (■), respectively, included in a standard 6-mL test volume [9]. Continuous curves indicate the respective theoretical estimates.

Fig. 3 illustrates progress curves obtained for the same enzyme dose and 0.05% of the MG-enriched substrate at increasing initial amounts of product inhibitor q_0, produced as described by Østgaard and Larsen [9]. The nominal inhibitor concentrations consisted of the series 0, 0.1, 0.2, and 0.5 mL of a pre-made stock solution, while the corresponding parameter estimates of the model were 0, 0.047, 0.069, and 0.144 expressed in units of absorbance. The correlation is evident. The other parameter estimates describing the fitted lines of Fig. 3 are listed in Tables 2 and 3.

Alginates.—Algal alginates generally contain significant amounts of both MM and MG blocks. A substrate representing an intermediate balance of MM and MG blocks compared to those above was prepared from alginate of *Macrocystis pyrifera* (Table 1). Fig. 4A illustrates the two-substrate model fitted to the Pyr-e data. It is possible that the restricted length of such block-substrate preparations may, to some extent, reduce the enzymatic degree of conversion [9,15]. Fig. 4B shows the results obtained for different concentrations of enzyme acting on an unhydrolysed alginate obtained from *Laminaria digitata* at a polymer concentration of 0.1% [9]. There was good agreement between the fitted curves and the data for both alginates when substrate-dependent rate constants were allowed (Fig. 4). The global fit shows poorer agreement with the experimental data, in particular for Pyr-e experiments. The corresponding parameter estimates are given in Tables 2 and 3. Nominal and estimated enzyme concentrations are compared below.

Comparative evaluation.—Qualitatively, the set of estimated model parameters in Table 2 clearly shows that all results for substrates containing significant amounts of MG blocks (substrate b_0) are associated with a strong product inhibition (small k_{+6}/k_{-6}). This is the major factor in the enzyme-dependent

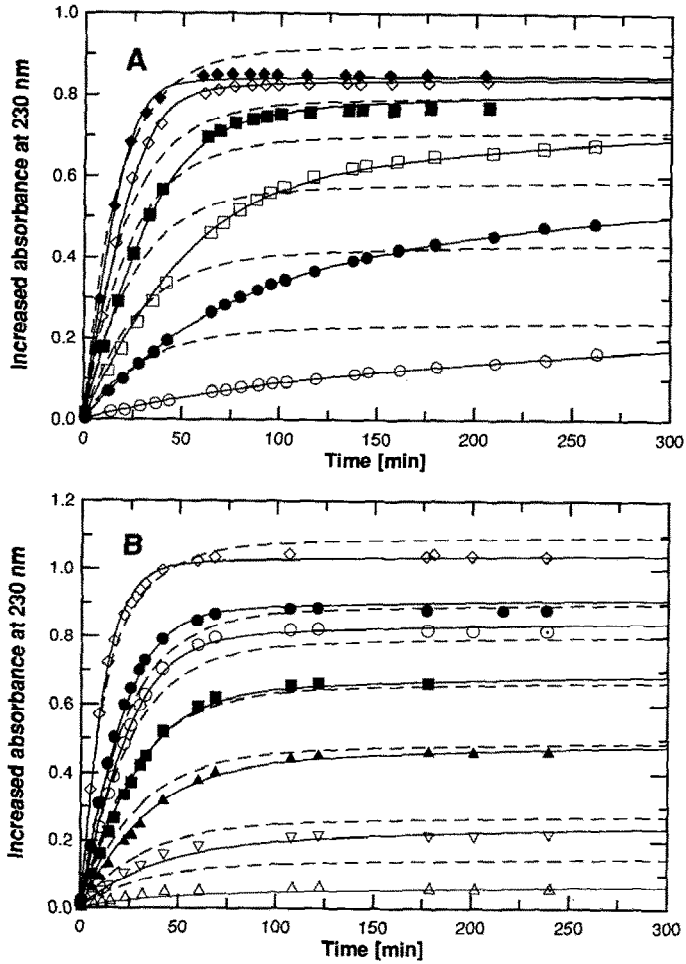

Fig. 4. A: Exptl code Pyr-e. Effect of different initial doses (e_0) of *Haliotis* M-lyase acting on 0.05% alginate fragment isolated from *Macrocystis pyrifera*. Symbols indicate experimental data obtained with $e_0 = 0.02$ (○), 0.04 (●), 0.06 (□), 0.1 (■), 0.15 (◇), and 0.2 UA (◆). B: Exptl code Dig-e. Effect of different initial doses (e_0) acting on 0.1% alginate from *Laminaria digitata*. Symbols indicate experimental data obtained with $e_0 = 0.02$ (△), 0.04 (▽), 0.06 (▲), 0.08 (■), 0.1 (○), 0.15 (●), and 0.2 UA (◇). Continuous curves indicate the theoretical estimates based on the Pyr-e and Dig-e experiments. Broken lines depict the global fit to the Pyr-e and Dig-e experiments.

degree of conversion obtained in Figs. 2A, 3A, 4A, and 4B. The consequences may be clarified as in Fig. 5, where the global parameters estimated from all the data have been applied to calculate the concentrations of intermediate enzyme-complexes, y_n ($n = 1$–4), during incubation as well as the two products for one set of MG-s data (Fig. 2B). The results are presented on a logarithmic scale to clarify changes in all factors in the same diagram. Clearly, the steady-state assumption of intermediates cannot be applied in such a case, not even for the low degrees of conversion during the initial stages (Fig. 5).

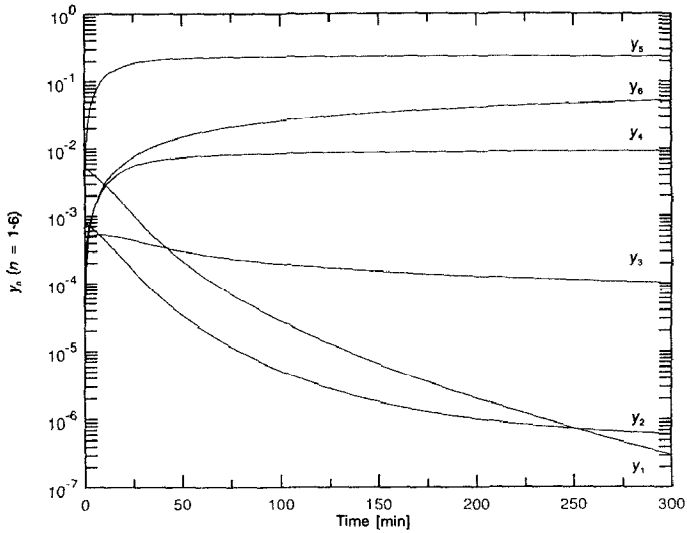

Fig. 5. Progress curves obtained by the two-substrate model for global fit rate constants and parameters values corresponding to expt MG-e for $e_0 = 0.1$ UA. Different lines indicate intermediates and final products, y_n ($n = 1$–6) as indicated. Concentration units are in excess absorbance at 230 nm. Please note logarithmic scale.

A quantitative comparison of data sets from different experiments must be done with some caution. Data have been accumulated over a long period of time. Some changes in substrates (shelf life, water content), in enzyme (losses during freezing, storage, thawing), or in external factors (room temperature, quartz cuvette, spec-

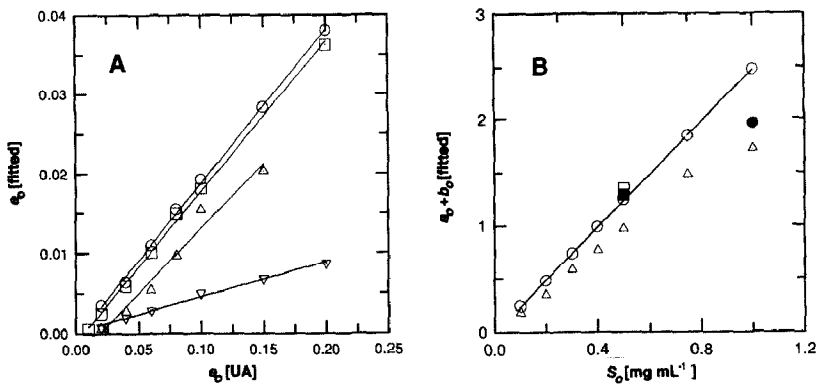

Fig. 6. A: Estimated enzyme doses versus nominal enzyme doses obtained from the two-substrate model for the enzyme from *Haliotis* acting on alginate substrates of the MM-type (○), MG-type (▽), alginate from *M. pyrifera* (△), and alginate from *L. digitata* (□). Each depicted line is the least-squares fit of a linear correlation for the four different substrates. B: Estimated substrate levels versus nominal substrate levels obtained from the two-substrate model for the enzyme from *Haliotis* acting on alginate substrates in the MM-s (○), MM-e (■), MG-s (△), Pyr-e (□), and Dig-e (●) experiments, respectively. The line depicts the linear least-squares fit within the MM-s experiment.

trophotometer) cannot be excluded. Fig. 6A shows the correlation between nominal enzyme concentrations and the numerical estimates for the least-squares fits for the substrates of the MM-type (Fig. 1A), MG-type (Fig. 2A), *M. pyrifera* (Fig. 4A), and *L. digitata* alginate (Fig. 4B). Fig. 6A indicates a linear correlation between the nominal and estimated values. Furthermore, this correlation is nearly identical, within the experimental uncertainty, for three of these substrates, whereas the MG-e experiment departed from the three others. Fig. 6B shows the correlation between the nominal substrate concentrations and the estimated sum of $a_0 + b_0$ for the substrates of MM and MG type. The correlation between fitted and nominal values is clearly linear. The minor differences in slopes of the two fitted lines may reflect differences in the amount of convertible sequences within the different substrates. This is in good agreement with earlier observations, generally showing a 70% maximal conversion of MG-blocks compared to that of MM-blocks when 13 widely different alginates were tested [16].

The large number of free parameters estimated for each independent experiment cannot be considered as independent. The significance of the differences in the same kinetic constants estimated from different data sets (Table 2) is therefore difficult to evaluate, particularly when considering the potential experimental errors mentioned above. As a comparison, a simultaneous adaptation of all data sets was attempted, assuming identical kinetic parameters in all experiments and accepting nominal enzyme concentrations as constant for all experiments and nominal substrate concentrations constant within one type of substrate. In the latter case, a proportionality scaling factor was allowed as a parameter to obtain a_0 and b_0 directly from the nominal mass concentration of substrate. The ratios between the two substrates, a_0/b_0, were kept constant for each type of substrate, but allowed to adopt different values for the various substrates. Data from the MG-e experiment were not included as the basis for this global fit because of lack of conformity to the other observations (Fig. 6A). The global fit thus obtained (dotted lines in Figs. 1, 2, and 4) reveals generally poorer agreement with the observations than fits based on only one set of data, in particular for the Pyr-e experiment. Although there is a correlation between the fitted a_0/b_0 ratio and the ratio of the experimentally determined diads, $F_{MM}/(F_{MG} + F_{GM})$, the quality of this global fit appears to be affected by limiting experimental factors. We would, however, like to stress that the overall ability of the global fit to reproduce the general trends as observed is preserved, particularly the enzyme-dependent final substrate conversion which cannot be explained in terms of the single-substrate Michaelis–Menten description. The final kinetic constants thus obtained have been included at the bottom of Table 2 and compositional data are listed in Table 3.

5. Discussion

This paper is based on published (Figs. 1A, 2A, 3, and 4B) [9] as well as new data (Figs. 1B, 2B, and 4A). A major point has been to concentrate on the mathematical treatment and modelling of these observations. Consequently, since

product formation is measured in units of absorbance, substrate concentration is simply treated as a purely theoretical term expressed in the same units to fulfill conservation of substrate plus product in a mass balance. Although studies of end-products [9] may be used to calibrate the absorbance recordings according to the number of new ends formed, a direct chemical interpretation in molar values is a nontrivial problem in our case. First, we have a polymeric substrate subject to multiple attacks. Secondly, although the physical end-products may be oligomers, the product recorded corresponds to the number of new non-reducing ends [9]. The initial substrate concentration $s_0 = a_0 + b_0$ should only be considered as a parameter representing the theoretically maximal conversion, so that $P \rightarrow s_0$ as $s \rightarrow 0$, without any physical interpretation. Obviously, s_0 will be proportional to the initial alginate concentration for a specific alginate substrate.

The product measured simply represents the number of cuts of the molecular chain. It is very difficult to accept that new molecular ends may act directly as an enzymatic inhibitor. Most probably, the inhibition is due to some oligomer product fraction [9]. The formation of such a fraction will depend on the chain length, polydispersity, and block length distribution of the substrate during incubation, as well as the inter- and intra-molecular substrate competition for binding to available enzyme molecules. It cannot be expected to be directly proportional to the measured product values. This assumption therefore probably represents the most drastic simplification included in our model. It is striking that the model is still able to explain the major features of our observations (Figs. 1–4).

As stated in the Introduction, we wished to compare our observations with the simplest possible, acceptable mathematical model. Since the amount of data available is limited, it is necessary to restrict the degrees of freedom of the model to a level where it may be truly tested or even rejected. Clearly, the major advantage of this model is analytical, by illustrating to what extent the action of an alginate lyase may be explained by simply defining the alginate as a sum of three different potential substrates, corresponding to its total block composition, and assigning kinetic constants to each block type. The true molecular block distribution within and between molecules is thus neglected. For alginates containing shorter block lengths, transition zones become increasingly important, and the model correspondingly less valid. So far, it does not seem possible to obtain experimental data good enough to quantify such a discrepancy.

Alternatively, the opposite approach is still possible, by starting with the monomers at the molecular level. Such a model may include all possible monomer sequences. Based on data and assumptions on block length distributions and size of enzyme site, progress curves may be generated by Monte-Carlo simulation. The number of unknown and adjustable parameters will necessarily be very large, and the model will therefore easily adapt to all data. Nevertheless, this type of approach is definitely useful to obtain an understanding of the enzymatic mode of action and kinetics at the molecular level. It has been applied successfully in studies of chitosan (Stokke, unpublished results), where more experimental data on the relative rate of hydrolysis by lysozyme, or enzyme site specificity, are available [17].

A two-substrate system may in many cases be quite simple to analyse, since both reactions may be considered to be independent. The presence of a strong product inhibition, as in our case, introduces a strong coupling factor, since the product of one reaction will inhibit the other [9]. The situation is complicated by the fact that we are unable to distinguish between the products of the two reactions; only their sum is recorded. Moreover, both substrates will be present in the same molecular chain. It is likely that this situation may be quite common for heteropolysaccharide substrates, where structural irregularities are likely to interfere with enzyme specificity.

The most important aspect of our study is that a strong product inhibition in a two-substrate system of this kind will severely restrict the applicability of the classic Michaelis–Menten type of steady-state assumption (see Fig. 5). At weak inhibition, this assumption may still be valid for a high degree of substrate conversion, and applied to solve the differential equations of the model by direct integration [13]. As illustrated by our general model, solutions may be found even for the nonsteady state situation employing numerical methods.

Finally, we want to stress that our findings should not only be of academic interest. The factors restricting alginate lyase conversion have practical consequences for preparative applications of this enzyme in seaweed biotechnology [18,19], in routine testing of alginates and alginate structure [15,16], as well as in the future production of purified alginate block structures.

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