

Analysis of Kinetic Data of Pectinases with Substrate Inhibition

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Abstract Enzyme kinetics data play a vital role in the design of reactors and control of processes. In the present study, kinetic studies on pectinases were carried out. Partially purified polymethylgalacturonase (PMG) and polygalacturonase (PG) were the two pectinases studied. The plot of initial rate vs. initial substrate concentration did not follow the conventional Michaelis-Menten kinetics, but substrate inhibition was observed. For PMG, maximum rate was attained at an initial pectin concentration of 3 g/l, whereas maximum rate was attained when the initial substrate concentration of 2.5 g/l of polygalacturonic acid for PG I and PG II. The kinetic data were fitted to five different kinetic models to explain the substrate inhibition effect. Among the five models tested, the combined mechanism of protective diffusion limitation of both high and inhibitory substrate concentrations (semi-empirical model) explained the inhibition data with 96–99% confidence interval.

Key words: Kinetics, substrate inhibition, pectinases, *Aspergillus niger*

Pectin and other polysaccharides contribute to the firmness and structure of plant cells. Pectolytic enzymes degrade pectic substances, which are multiple forms due to the complex nature of their substrates. *Aspergillus niger* is used for the industrial production of pectolytic enzymes. This fungus synthesizes polymethylgalacturonase (EC 3.2.1.41), pectinlyase (EC 4.2.2.10), polygalacturonase (EC 3.2.1.15), and pectinesterase (EC 3.1.1.15) [1–3]. Pectolytic enzymes have a wide range of applications in food and biotechnological industries [4–8]. Several researchers have performed kinetic studies on substrate and product inhibition [9–13]. Extensive work has also been done on kinetics in batch and continuous reactors in various systems [14, 15], yet very little literature is available on pectinases. Pectinases possess a wide range of applications, including commercialization

of the process. In order to develop the process, kinetic data are necessary to design a suitable reactor for the degradation of pectic substances.

Polymethylgalacturonase (PMG) and polygalacturonase (PG) were purified to an appreciable amount. Pectinlyase (PL) could not be purified, therefore, kinetic studies were carried out with partially purified PMG and PG. The plot of initial rate vs. initial substrate concentration did not follow the conventional Michaelis-Menten kinetics, however, substrate inhibition was observed. Hence, an attempt was made to fit the experimental data to different existing kinetic models (both derived and semi-empirical) and to explain the substrate inhibition phenomenon.

THEORY AND MODELS USED FOR SUBSTRATE INHIBITION

Any one of the following reasons can reduce the rate of enzymatic action:

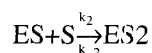
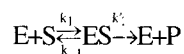
- a structural analog of the substrate binds to the active site of the enzyme either reversibly or irreversibly.
- an inhibitor may bind to a portion of an enzyme and modify the three-dimensional structural conformation of the enzyme which, in turn, reduces its catalytic ability.

Enzyme activity can be inhibited in the presence of several inhibitors like chloroform, benzenoids, organophosphates, sulfonyl fluorides, metal chelators, or peptide chloromethyl ketones [16]. The other kind of inhibition mechanism is substrate and product inhibition where the concentrations of the substrate or product are increased to levels at which enzymatic action is retarded. In this case, the substrate or product may bind to an alternate site and the resulting enzyme substrate or enzyme product complex is inactive. In another case, the enzyme has more than one active catalytic site, and the presence or absence of the substrate at that site might alter the catalytic activity [17]. This kind of inhibitory behaviour plays a significant role in chemotherapy. Various aspects of inhibition have been discussed in many reviews [17–21].

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Multiple Substrate-Enzyme Complex

Haldane [22] proposed the following mechanism:

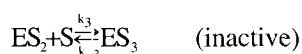
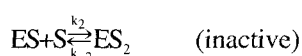
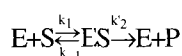


By this mechanism, the enzyme forms an inactive enzyme-substrate complex involving two molecules of substrate per enzyme molecule. The rate expression is given as:

$$V=V_m/[1+K_1/S+S/K_2] \quad (1)$$

where $K_1=k_{-1}/k_1$ and $K_2=k_{-2}/k_2$

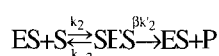
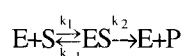
Yano *et al.* [23] have generalized the above case by assuming the formation of multiple inactive enzyme-substrate complexes. The mechanism is as follows:



The rate expression for the above model is given by:

$$V=V_m/[1+K_1/S+\Sigma(S/K_j)] \quad (\text{Model 3}) \quad (2)$$

Webb [24] has assumed the identical active sites can be represented by the following reactions:



The rate expression is given by:

$$V=V_m S (1+\beta S/K'_m)/[S+K_m+S^2/K'_m] \quad (\text{Model 2 for } \beta=1)(3)$$

where $K_m=(k_{-1}+k_2)/k_1$ and $K'_m=(k_3+\beta k_2)/k_3$

If $\beta > 1$, this implies that product formation rate increases. When $0 < \beta < 1$, the rate of formation of the SES complex will be slower than the ES complex. A special case is considered by Haldane [22] when $\beta=0$. Under these conditions, Equation (3) is modified to:

$$V=V_m S/[K_s+S] [1+S/K_1] \quad (\text{Model 1}) \quad (4)$$

Empirical Models

This is a purely empirical model. The inhibition effect caused by the substrate can be explained by incorporating the term $\exp(-S/K_s)$ into Michaelis-Menten kinetics [25]. The rate expression is (Model 4) given by:

$$V=[V_m S/K_s+S] \exp(-S/K_s) \quad (5)$$

Edwards [26] proposed this model by combining the mechanism of diffusion-controlled substrate supply with inhibitory substrate concentration. The rate expression (Model 5) is given as:

$$V=V_m [\exp(-S/K_s) - \exp(-S/K_s)] \quad (6)$$

MATERIALS AND METHODS

Materials

Galacturonic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as the standard substrate for calculating the activities of PMG and PG. Polygalacturonic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as the substrate for PG. The other chemicals used were of analytical grade and procured in India.

Organism

Aspergillus niger NCIM 548 was obtained from the National Chemical Laboratory, Pune, India. The organism was maintained on potato-dextrose-agar slants containing (g/l): potato, 200; dextrose, 25; agar, 20.

Production of Pectolytic Enzymes

The microbiological parameters were optimized, viz., slant age, inoculum age, and amount of inoculum for the enhanced production of pectolytic enzymes [27]. The spores from 81.4 h (optimized condition) slant were dispersed into 10 ml of sterile water. One ml of this suspension, containing approximately 10^5 – 10^7 spores, was used as an inoculum in a 500-ml Erlenmeyer flask containing 100 ml of seed culture medium containing (g/l): dextrose, 34.6; ammonium sulfate, 8.42, KH_2PO_4 , 2.0. The medium was autoclaved at 121°C for 20 min and inoculated after cooling to 30°C. The initial pH of the medium was maintained at 4.5. The culture was incubated at 30°C for 66.7 h (optimized condition) on a rotatory shaker maintained at 160 rpm. A seed of 15.78% (v/v, optimized value which is equivalent to 3.58 kg dry weight of cell per litre of fermentation broth) was transferred aseptically to the production medium that contained (g/l): dextrose, 34.6; ammonium sulfate, 8.42; KH_2PO_4 , 2.0; corn, 20.97 [28]. The initial pH of the medium was maintained at 4.5. The culture was incubated at 30°C for 5 days on a rotatory shaker maintained at 160 rpm. The culture was harvested on the fifth day, centrifuged, and the supernatant was stored in a sterile container at 4°C until further use.

Assays

PMG was assayed by conducting an enzymatic reaction with pectin as a substrate. This reaction was conducted by adding 0.074 ml of culture filtrate containing enzyme to

0.2 ml of the substrate solution (2.4 g/l in a sodium acetate buffer at pH 5.3). The reaction was stopped by adding 0.5 ml of copper reagent. The reducing groups formed were estimated by the Nelson-Somogyi method [29, 30]. The enzyme activity was expressed in terms of a unit (U). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μ mol of product per unit volume of enzyme solution per unit time at assay conditions. PG was assayed by the same procedure as described for PMG, but polygalacturonic acid was used as the substrate (2.4 g/l in the sodium acetate buffer at pH 6.6).

Protein content in the enzyme sample was estimated by the method of Lowry *et al.* [31].

Purification of Pectinases

At the end of the growth phase, the fermentation broth was centrifuged at 5,000 rpm for 20 min. The supernatant was precipitated by acetone (1:1 volume ratio). After storage for 2 h, the precipitate was centrifuged and dissolved in 0.005 M Na_2HPO_4 (pH 7.0) and repeatedly chromatographed on a DEAE-cellulose column (3.5 \times 24 cm) at a flow rate of 0.5 ml per minute. Elution of the pectolytic enzymes was carried out effectively by using the linear gradient elution technique with a buffer increasing in ionic strength from 0.005 M to 0.2 M [32].

Kinetic Studies

The initial concentration of pectin and polygalacturonic acid varied between 0.5 g/l and 4.5 g/l during kinetic studies of PMG and PG. The total amount of enzyme added to the reaction mixture was maintained constant. Kinetic experiments were performed by varying initial substrate concentration, and initial rates (enzyme activity) were measured. Five models were arbitrarily selected for comparison from the models discussed in the previous section. The functions of the different models are summarized in Table 1. The substrate inhibition data were applied to these five functions and compared with each other. The kinetic parameters were estimated by nonlinear multiple regression analysis, using the commercial package MATLAB. The initial rates were simulated by using the estimated kinetic parameters, and compared with the experimental values. The fitness of the model was checked by conducting a statistical Fischer's test.

Table 1. Different kinetic models used to fit the substrate inhibition data.

S. No	Rate equation	Reference
Model 1	$V = V_m S / [K_s + S] [1 + S/K_i]$	24
Model 2	$V = V_m S (1 + S/K_i) / [S + K_s + S^2/K_i]$	24
Model 3	$V = V_m S / [K_s + S + (S^2/K_i) (1 + S/K_i)]$	23
Model 4	$V = [V_m S / (K_s + S)] \exp(-S/K_i)$	25
Model 5	$V = V_m [\exp(-S/K_i) - \exp(-S/K_s)]$	26

Statistical Analysis

The F-statistic value is calculated as [33]:

$$F = (\text{mean square regression}) / (\text{mean square error})$$

$$= \frac{SSR / (p - 1)}{SSE / (N - p)} \quad (8)$$

where:

SSR is sum of squares due to regression

SSE is sum of squares due to error

(p - 1) is the degrees of freedom associated with SSR

(N - p) is the degrees of freedom associated with SSE

SSR and SSE are calculated as follows:

$$SSR = \sum_{i=1}^N (Y(x_i) - \mu)^2 \quad (9)$$

$$SSE = \sum_{i=1}^N (Y_i - Y(x_i))^2 \quad (10)$$

RESULTS AND DISCUSSION

As a part of the process of development, the generation of kinetic data becomes very necessary. Inhibition kinetics plays a vital role in theoretical and applied biology, and it is very useful to have a clear knowledge about the interaction of the enzyme and the substrate molecules. Since it was not possible to purify the pectinlyase, the kinetic studies were carried out with partially purified PMG and PG. The rate profile showed a substrate inhibition pattern and different kinetic models were

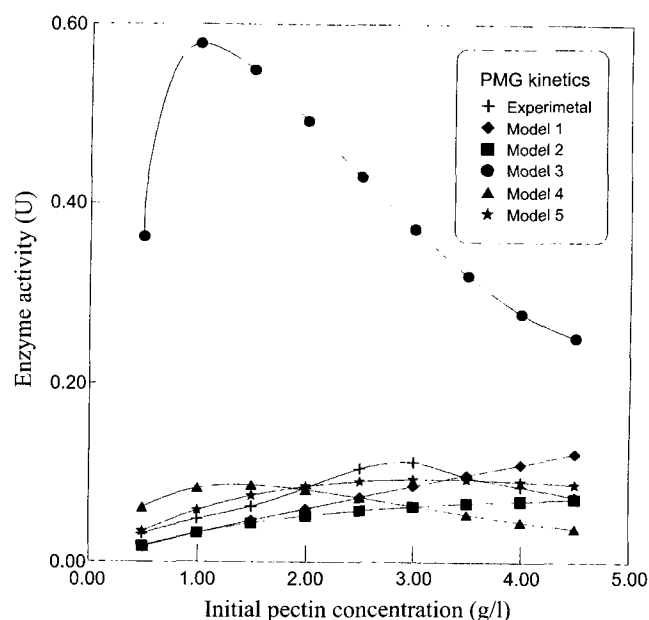


Fig. 1. Plot of simulated model activities and experimental activities vs. initial pectin concentration (PMG).

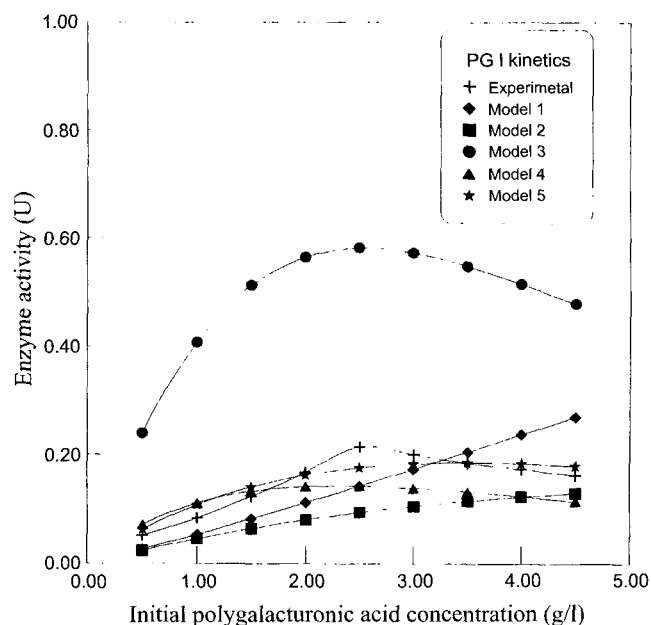


Fig. 2. Plot of simulated model activities and experimental activities vs. initial pectin concentration (PG I).

applied to the experimental data to explain the inhibition effect.

Purification of Pectinases

The active fractions eluted from a DEAE-cellulose column were separated into three fractions using the gradient elution technique. PG was separated into two fractions; namely, PG I and PG II in fractions of 5 and 8, respectively. PMG

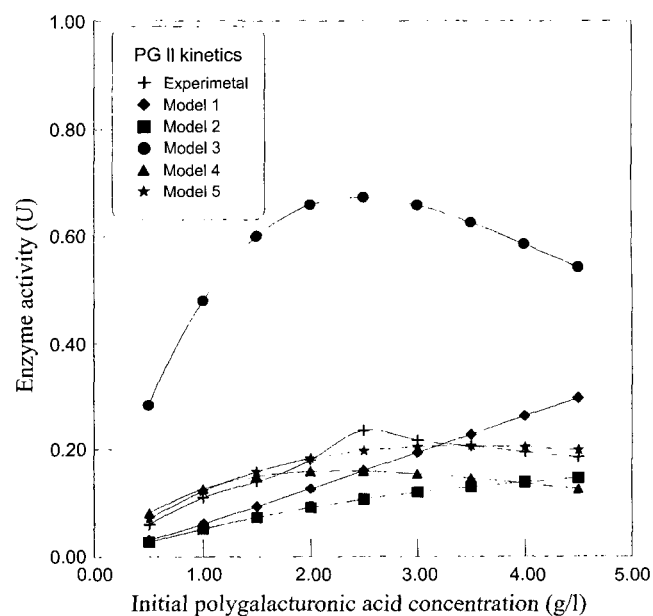


Fig. 3. Plot of simulated model activities and experimental activities vs. initial pectin concentration (PG II).

Table 2. Kinetic parameters estimated for PMG.

S. No	V_m ($\mu\text{M}/\text{min}$)	K_s (g/l)	K_i (g/l)	K (g/l)
Model 1	0.0833	2.2348	3.8087	-
Model 2	0.0829	2.2305	2.2225	2.238
Model 3	2.0779	2.2296	2.261	2.246
Model 4	0.4173	2.2249	2.2452	-
Model 5	2.241	2.9947	3.3521	-

was purified 14-fold, while PG I and PG II were purified 10- and 10.7-fold, respectively. Although many experiments were carried out, it was not possible to purify pectinlyase. Hence, further experiments were carried out only for PMG, PG I, and PG II.

Kinetic Studies

The enzyme kinetics was analyzed and an enzyme activity profile of the initial substrate concentration was produced. This is shown in Figs. 1 to 3 where the data correspond to PMG, PG I, and PG II, respectively. The plot clearly indicated that kinetic studies on pectinase did not follow conventional Michaelis-Menten kinetics. An inhibition effect was observed for PMG, PG I, and PG II. The kinetic data were applied to five different models (Table 1) to explain the inhibition phenomena. Models 1 to 3 were based upon various assumptions in enzyme-substrate complex formation, while models 4 and 5 were semi-empirical models used to investigate the exponential dependency of the substrate on the enzyme kinetics.

Parameter Estimation and Simulation

The parameters in the model equations were estimated by nonlinear multiple regression analysis using a commercial software package MATLAB. The kinetic parameters were estimated for all the five models, which are shown in Tables 2-4 for PMG, PG I, and PG II, respectively. The enzyme activities were simulated using the estimated kinetic parameters. Both the experimental and model predicted activities are shown in Figs. 1 to 3 for PMG, PG I, and PG II, respectively. It was observed that model 3 was not a good fit compared to other models, implying that an assumption of multiple enzyme-substrate complex formation in a pectinase system is unwarranted.

The simulated values for models I and II (Figs. 1-3) did not show decrease in activity at high substrate concentration,

Table 3. Kinetic parameters estimated for PG I.

S. No	V_m ($\mu\text{M}/\text{min}$)	K_s (g/l)	K_i (g/l)	K (g/l)
Model 1	0.1879	3.7799	2.742	-
Model 2	0.1809	3.7659	3.7303	3.7949
Model 3	2.0608	3.7363	3.9517	3.8351
Model 4	0.6915	3.7458	3.7774	-
Model 5	3.2482	3.241	3.7865	-

Table 4. Kinetic parameters estimated for PG II.

S. No	V_m ($\mu\text{M}/\text{min}$)	K_s (g/l)	K_i (g/l)	K (g/l)
Model 1	0.217	3.6968	3.0266	-
Model 2	0.2021	3.6778	3.6289	3.718
Model 3	2.389	3.655	3.8144	3.7266
Model 4	0.7697	3.6524	3.6923	-
Model 5	3.1882	3.1481	3.7533	-

because of inhibition. If an enzyme can have more than one active catalytic site, the presence or absence of substrate molecules at one site will affect the activity of other sites. This phenomenon is called allosteric inhibition. Model 2 was developed for such systems by assuming two identical active sites with the intermediate SES as inactive (i.e., the value of $\beta=1$). These assumptions could not explain the kinetic data for the pectinase system. The only difference between Model 2 and model 1 was the value of β ($\beta=0$ in the case of model 1). This suggested that the SES complex formed a product at a lower rate than the ES complex, but this assumption could not explain the enzymatic reaction. Models 1 and 2 predicted almost similar activities at a low substrate concentration, but varied much at high substrate concentrations. Even though models 1 and 2 did not fit well with experimental data, they were a better fit than model 3. This was observed for all the three enzyme components (Figs. 1–3). Hence, allosteric inhibition and Haldane's assumption do not hold well for pectinases.

The semi-empirical models 4 and 5 were tested to explain the inhibition effect. Model 4 was a semi-empirical model, which was obtained by incorporating the exponential inhibition dependence of the substrate into a Michaelis-Menten model. The simulated activity profile was similar to the experimental one, but model 4 predicted the inhibition effect at a low substrate concentration, which was not true in reality (Figs. 1–3). It was observed that model 5 held up well, compared to other models. It should be noted that model 5 was a semi-empirical model. The simulated activity value by model 5 was found to be close to the experimental values, when compared to other models (Figs. 1–3). Based on the simulated results from model 5, it can be predicted that the combined mechanism of protective diffusion limitation of high and

Table 5. Comparison of lack of fitness of models for PMG.

S. No	MSR	MSE	F	P>F	Confidence level (%)
Model 1	0.005	0.00099	5.036	0.062	93.8
Model 2	0.000867	0.0015	0.5781	0.092	90.8
Model 3	0.0314	0.2509	0.125	0.095	90.5
Model 4	0.0012	0.0018	0.6692	0.095	90.5
Model 5	0.0016	0.000175	9.1031	0.032	96.8

Table 6. Comparison of lack of fitness of models for PG I.

S. No	MSR	MSE	F	P>F	Confidence level (%)
Model 1	0.0281	0.0047	5.9774	0.055	94.5
Model 2	0.0036	0.0091	0.3904	0.093	90.7
Model 3	0.0318	0.2140	0.1487	0.095	90.5
Model 4	0.002	0.0031	0.6524	0.095	90.5
Model 5	0.0073	0.000528	13.8333	0.0002	99.98

inhibitory substrate concentration explains the substrate inhibition observed in this study. The disadvantages of these models are that one cannot predict the maximum substrate concentration at which the enzyme exhibits no activity.

The fitness of the model was also tested by conducting the statistical Fischer's test. The statistical analyses of the five models are given in Tables 5–7 for PMG, PG I, and PG II, respectively. The calculated F values for model 5 corresponding to PMG, PG I, and PG II are 9.1031, 13.8333, and 21.3896, respectively. The primary criterion is that the calculated 'F' value should be several times greater than the tabulated 'F' value in order to create a good model [33]. If the F-value is greater than tabulated F, the null hypothesis is rejected at the α level of significance, and infers that the variation accounted for by the model is significantly greater than the unexplained variation. For model 5, the null hypothesis was rejected at 0.032, 0.0002, and 0.0001 levels of significance for PMG, PG I, and PG II, respectively. This shows that the regression level (model 5) was significant at the levels of 96.8%, 99.98%, and 99.99% for PMG, PG I, and PG II, respectively.

CONCLUSIONS

The enzymes PMG, PG I, and PG II exhibited inhibition kinetics. The kinetic data were fitted to five different general substrate inhibition models to explain the data. It was observed that model 5 fitted well with the experimental data. The statistical analysis also suggested that model 5 explains the substrate inhibition data well, when compared to other models considered in this study.

Table 7. Comparison of lack of fitness of models for PG II.

S. No	MSR	MSE	F	P>F	Confidence level (%)
Model 1	0.0334	0.0053	6.2751	0.053	94.7
Model 2	0.0045	0.0106	0.4215	0.093	90.7
Model 3	0.0404	0.2926	0.1382	0.095	90.5
Model 4	0.0024	0.0036	0.6773	0.095	90.5
Model 5	0.0088	0.00041	21.3896	0.0001	99.99

NOMENCLATURE

E	: enzyme (g/l)
ES ₁ , ES ₂ ...ES _n	: enzyme-substrate complex (g/l) ²
K _s , K _m , K' _m , K _i , K	: kinetic constants (g/l)
k	: rate constants (g/l-min or min ⁻¹)
N	: number of experiments
P	: number of parameters
S	: initial substrate concentration (g/l)
V	: initial rate (mM/min)
V _m	: maximum rate (mM/min)
Y(x _i)	: predicted response (mM/min)
Y _i	: experimental response (mM/min)

Greek Symbols

β	: constant
μ	: mean of predicted values

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