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The Experimentally Determined Velocity of Catalysis could be Higher in the Absence of Sequestration

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Authors' contributions

This work was carried out in collaboration between both authors. Author IIU conceptualized and derived all equations, analysed and discussed the result while author AOO supervised the thesis from where the original concept was obtained. Both authors read and approved the final manuscript.

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ABSTRACT

Background: It is not unusual to observe calculated "total" free enzyme ([E]) in enzyme catalysed reaction, but this should include total enzyme-substrate complex ([ES_T]) which accounts for sequestration.
Objectives: 1) To show indirectly that the velocities of catalytic action can be higher than experimentally observed velocities without sequestration and 2) redefine the relationship between velocity of hydrolysis with Michaelian enzyme and [E], where concentration of substrate, [S_T] < Michaelis-Menten constant, K_M.
Methods: A theoretical research and experimentation using Bernfeld method to determine velocities of amylolysis with which to mathematically calculate [ES_T] and the enzyme-substrate complex ([ES]) prepared for product, P, formation.
Results: The [ES_T] is < [E]; [ES_T] and pseudo-first order constant, k decreased with increasing [S_T] and increased with increasing concentration of enzyme [E_T] while velocity amylolysis, v and maximum velocity of amylolysis, v_{max} expectedly increased with increasing [E_T] and [S_T].

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Conclusion: The fact is that the [ES_T] is lower than what is usually referred to as free enzyme ([E_T] – [ES]). Therefore, if the additional part of [ES_T] dissociated into product within the duration of assay, the velocity of amylolysis could be higher. The most important outcome and corollary when [K_M] > [S_T] is that $v \alpha 1/[E]$, $v \alpha [E][S_T]$ and a quadratic relationship exists between pseudo-first order rate constant and maximum velocity of amylolysis; separately, v is not α [E] and if $v \alpha [S_T]$ (if $\partial v/\partial[S_T]$ is constant with coefficient of determination = 1), then K_M is not applicable.

Keywords: Total enzyme-substrate complex; enzyme-substrate complex prepared for product formation; direct proportionality; rate constants; free enzyme; Aspergillus oryzea alpha-amylase.

1. INTRODUCTION

Most research activities on the catalytic activities of enzyme for whatever reason [1-5] are either at substrate concentration lower than or higher than the Michaelis-Menten constant (K_M) for a given concentration of the enzyme. Most often than not researchers or critics [6,7] of Michaelis-Menten kinetics ignore the fact that the concentration range used covers substrate concentration much lower than K_M and concentration much higher than K_M. Hardly in literature one finds scholars who employ linear transformation of Michaelis-Menten equation for the determination of kinetic parameters, K_M and maximum velocity of catalytic action (v_{max}) state the condition(s) for the generation of such kinetic constants which may satisfy either the standard quasi-steady-state approximation (sQSSA) or the reverse QSSA (rQSAA) that has become a regular issue in literature [8-10]. Another suggestion is that, if the enzyme E, converts substrate to product P, then the rate of change of the concentration of the product d [P]/dt, generally depends on the concentration of substrate [S], in a nonlinear pattern [11]. This suggestion is relevant when [S] approaches and greatly exceeds the K_M ([S] » K_m); but if [S] « K_m, linearity should be the case [12,13], and this is one of the bases for the formulation of the model intended in this research. The model is also based on the equation, $K_s = k_{-1}/k_1$, where k_{-1}, k_1, and K_s are the rate constant for the dissociation of the enzyme – substrate complex (ES) to E and S, 2nd order rate constant for the formation of ES, and equilibrium constant for the dissociation of ES respectively. This is not to imply that the model may not be possibly formulated if [S] »K_m, but in such a case, a different mathematical formalism may be needed.

The concern in this research is that in a homogeneous reaction mixture, the substrate is either smaller in molecular size than the enzyme (sucrose and invertase-EC. 3.2.1.26) or *vice versa* (a polysaccharide which may be soluble or partially insoluble in gelatinised form as in this research and alpha-amylase-EC. 3.2.1.1). If the substrate is smaller in size than the enzyme and binds to site other than the active site, the ES cannot undergo transformation into product; the enzyme may also be smaller than the substrate and binds the substrate with site other than the active site which cannot proceed to product formation. Besides, the introduction of the enzyme into a solution of the substrate (or rather partially soluble substrate best described as a colloid) does not lead to total involvement of the enzyme molecules in complex formation the use of magnetic stirrer notwithstanding *vis-à-vis* a situation in which the condition such as $[E_T]/(K_M+[S_T]) > 1$ [7] exists. It is therefore, proposed that the velocity of catalytic action may be higher than the experimentally observed velocities of the mobilised enzyme. This is against the backdrop of the concern that many metabolic enzymes are attached to particulate structures, which might affect individual rate constants and over-all rate due to such changes in geometry [14]. According to Shurr [14], over-all rate cannot be increased and will in general decrease by adsorbing the enzyme to large particulate structures. This is in addition to the effect of the viscosity of the medium. The objectives of this research are: 1) to show indirectly that the velocities of catalytic action can be higher than experimentally observed velocities without sequestration and 2) redefine the relationship between velocity of hydrolysis with Michaelian enzyme and [E] where [S] < K_M.

2. THEORY

Before the formulation of equations there is a need to examine the view about what may constitute valid kinetic constants. This is with the aim of taking appropriate stance regarding

appropriate kinetic constant when in particular $[S] < K_M$.

2.1 Issues Arising from Enzyme Kinetics at Substrate Concentration Less than K_M

In this section well known equations in literature are analysed before the derivation of equations that culminate in the equation for the determination of a probable velocity of catalytic action of the enzyme free from hindrance arising from wrong binding interactions which may generally be referred to as sequestration in particular. The 1913 Michaelis-Menten paper [15] (translated by Roger S. Goody and Kenneth A. Johnson [16]) contains the equation often given as

$$v = \frac{v_{\max}[S_T]}{K_M + [S_T]} \tag{1a}$$

Equation (1a) results from the assumption/condition that $[S_T] \rightarrow \infty$; but substrate concentration range is employed in every assay where kinetic parameters are to be determined. This implies that the lower part of the range consists of substrate concentration less than K_M. Perhaps this informed the alternative equation by Cornish-Bowden [17] which is given as

$$v = \frac{v_{\max}([S_T] - [P])}{K_M + [S_T] - [P]} \tag{1b}$$

Where, $[P]$ is the mass concentration of the product. Equation (1b) is very much applicable to the duration of assay much greater than millisecond time scale. Although literature contains views regarding the misuse of Michaelis-Menten formalism (sQSSA), what seems to be of less concern is the case where $[S_T]$ is « K_M. With reference to information in some standard text books, Bersani and Acqua [18] refute the claim that the "substantial" equilibrium in Michaelis-Menten equation of reaction is a real equilibrium. According to Bersani and Acqua [18] one of the main problems of the mathematical treatment of the sQSSA is the misinterpretation of the hypothesis that the complex time concentration has zero derivative. Perhaps this may mean that $\partial[C]/\partial t = 0$. The authors seem to be right; but if the species, *ES* formation and disintegration into either product or free substrate and free enzyme does not stop the formation of *ES* elsewhere in the reaction mixture as long as the concentration of the substrate, even at the lower end of the substrate concentration range employed for the assay is sufficiently higher than the concentration of the enzyme, $\partial[C]/\partial t = 0$ may hold but not ad infinitum regardless of the duration of assay. But this is on the condition that the time regime used is very short (« 1 s.; ms time scale or less is better.).

The concern that ought to be expressed by investigators is the transformation of Eq. (1a) into the form:

$$v = v_{\max}[S]/K_M \tag{2a}$$

$$v = k_2\,[E]\,[S]/K_M \tag{2b}$$

In Eq. (2b) $[E]$ is the concentration of free enzyme and k_2 is the rate constant for product formation. This equation can be found in standard text [19]. It is very clear that $k_2\,[E] \neq v_{\max}$ because $[E]$ (*i.e.* $([E_T] - [ES])) < [E_T]$. Worthy of note is the fact that $[S]$ is the concentration of free substrate. This makes Eq. 1b seemly relevant otherwise what was introduced into the reaction mixture is the $[S_T]$ at $t = 0$. Another concern that needs to be expressed is that if the original Michaelis-Menten equation should be applied only on the condition that $[S_T]$ » $[E_T]$ or in a much better way one invokes the highly frequent inequality in literature [20,21] $[E_T] / (K_M + [S_T])$ « 1 only within a very short duration of assay, then if the substrate concentration regime « K_M then, the occurrence of K_M in Eq. (2a) and Eq. (2b) is questionable. This is appropriate stance considering support in literature to the effect that "from a biophysical point of view, it seems reasonable to state that the enzyme–substrate complex *C* (*i.e. ES*) is in a QSS ($\partial[C]/\partial t = 0$) when the concentration of the substrate *S* is high enough, because the free enzyme *E* will immediately combine with another molecule of *S*" [21]. With reference to literature [8,9] Schnell and Maini [7] note that when there is an excess of enzyme *E*, this condition does not hold, where in particular, the duration of assay is longer than millisecond time scale. This is equivalent to a situation where $[S_T]$ « K_M; if so a different equation given in the work of Schnell and Maini [21] may apply. The equation is

$$v = \frac{v_{\max}[S_T]}{K_S + [S_T]} \tag{3}$$

The concern in this research remains Eq. (2a) and Eq. (2b) in particular where K_S should take the place of K_M. Equation (2b) can be restated as:

$$v_{exp} = k_2\,[E]\,[S_T]/K_S \quad (4)$$

Where, v_{exp} is a replacement for v and it is taken as the experimentally observed velocity of catalytic action. Mathematically, Eq. (4) is a joint variation of v_{exp} with respect to $[E]$ and $[S_T]$; the proposition in this research is that enzyme molecules may not be free for other reason other than the formation of *ES*.

Meanwhile Eq. (1a) can be traced back to the equation given as $[ES] = [E][S_T]/K_M$ where the concentration of the free enzyme $[E]$ is given as $[E] = [E_T] - [ES]$ where $[ES]$ is the concentration of the enzyme involved in enzyme-substrate complex formation. Substitution of the latter into $[ES] = [E][S_T]/K_M$ and rearranging the resulting equation gives Eq. (1a). It is not unusual to find in literature the assumption that $[S_T]$ could be very small as to be « K_M. If *ab initio* $[S_T] \ll K_M$ for a given enzyme concentration, then Eq. (1a) needs to be replaced by Eq. (3). However, the main issue is the fact that $[S_T]$ may be too low such that Eq. (3) can then be simplified to

$$[ES] = [S]\,[E_T]/K_S \text{ (or } v = k_2\,[E_T][S_T]/K_S) \quad (5)$$

In literature [19] can found "if $[E] \cong [E_T]$"; however, in this research Eq. (4) and Eq. (5) are seen as two distinct equations. One can make a mathematical statement that the velocity of enzymatic action is directly proportional to the total enzyme concentration. Equations (4) and (5) need to be re-written as follows:

$$v_{exp} = k_2\,[E]\,[S^*]/K_S \quad (6)$$

$$v_0 = k_2\,[E_T]\,[S^*]/K_S \quad (7)$$

The asterisk is a reminder that substrate concentration may be less than the total initial concentration ($[S_T]$) due to the phenomenon of sequestration [18] that is to be given detailed attention subsequently. Besides it is obvious that if k_2 is held constant in Eq. (6) and Eq. (7), the values of K_S should be different for the same concentration of substrate but different concentration of enzyme.

In a standard text book [19, 22] and journal [21] are respectively the equations: $v = k_2[E]\,[S]/K_M$ where $[E]$ and $[S]$ are the free enzyme and free substrate; $d[S_T]/dt = -k_1[E_T]\,[S_T] = -k_2[E_T]\,[S_T]\,/\,K_M$ which can be restated as $-d[S_T]/dt = v = k_2[E_T][S_T]/K_M$. What is new about these equations may be a correct question? But are the equations in line with the condition for the validity of any of QSSAs? The position of Stryer [19] is that under physiological condition $[S] \ll K_M$; then if this is the case rQSSA should be applicable and K_M should, therefore, not be the case. The equations were a result of the transformation of Michaelis-Menten equation in which $K_M + [S_T] \approx K_M$ because $[S_T] \ll K_M$. But this has mathematical implication, proportionality issue to be specific. Additional issues regarding the equations are reserved for results and discussion section.

2.2 On the Issue of Sequestration

In the light of sequestration there is a need to consider total enzyme-substrate complex ($[ES_T]$) formation which includes wrong complex formation and catalytically bound formation. As stated in manuscript under independent preparation, in time t_1 before the end of the chosen duration of assay, t_∞ ($0 < t_1 < t_\infty$) the total complex is taken as $[ES_T]$; therefore the free enzyme is $[E_T] - [ES_T] = [E_F]_{(1)}$. When a part of the total enzyme-substrate complex concentration is equal to $[ES]$ breaks into free enzyme, product, and substrate, the complex concentration left is $[ES_T] - [ES]$; in time t_∞ the free enzyme concentration is therefore, given as $[E_F]_{(2)} = [E_T] - ([ES_T] - [ES])$. It is obvious that $[E_F]_{(2)} > [E_F]_{(1)}$. Based on the preceding analysis one can state the equation of total pre-catalytic action molar concentration of *ES* (manuscript in preparation) as follows:

$$[ES_T] = [E_T] - \frac{[ES]}{\left(\frac{[E_T]}{[E_T]-K_S[ES]/[S_T]}\right)^{[S_T]/K_S} - 1} \quad (8)$$

3. MATERIALS AND METHODS

3.1 Chemicals

Aspergillus oryzea alpha amylase (EC 3.2.1.1) and potato starch were purchased from Sigma – Aldrich, USA. Tris, 3, 5 – dinitrosalicylic acid, maltose, and sodium potassium tartrate tetrahydrate were purchased from Kem light laboratories Mumbai, India. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd, Poole England. Distilled water was purchased from local market. The molar mass of the enzyme is ~ 52 k Da [23, 24].

3.2 Equipment

Electronic weighing machine was purchased from Wenser Weighing Scale Limited and

721/722 visible spectrophotometer was purchased from Spectrum Instruments, China; *p*H meter was purchased from Hanna Instruments, Italy.

3.3 Methods

The enzyme was assayed according to Bernfeld method [25] using gelatinized potato starch whose concentration ranges from 5-10 g/L. Reducing sugar produced upon hydrolysis of the substrate using maltose as standard was determined at 540 nm with an extinction coefficient equal to ~181 L/mol.cm. Concentration equal to 1 g/100 mL of potato starch was gelatinized at 100ºC for 3 min and subjected to serial dilution after making up for the loss of moisture due to evaporation. Concentration equal to 0.01 g/100 mL of *Aspergillus oryzea* alpha amylase was prepared by dissolving 0.01 g of the enzyme in 100 mL of Tris HCl buffer at *p*H = 6. Concentrations equal to between 20-and 60- fold dilution of the stock were assayed. The rest was stored in a freezer.

The kinetic parameters and subsequently rate constant for product formation and release in particular, were first determined according to Lineweaver-Burk method [26]. The total *ES* molar concentrations were calculated with Eq. (8). The rate constant, k_2 and the *ES* destined for product formation were calculated with $v_{max}/[E_T]$ and v/k_2 (where v is the velocity of amylolysis) respectively.

3.4 Statistical Analysis

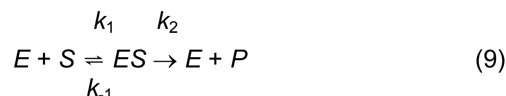
All values of velocities of amylolysis obtained are expressed as mean ± SD. Each parameter is an average value from four determinations. The SD values were calculated according to the method by Hozo [27]. The mean values were used to determine other parameters.

4. RESULTS AND DISSCUSION

The claim that the derivative of C (or *ES*) is equal to zero [18] when the time is equal to a certain time of defined magnitude seem to ignore the fact that different time regimes give different velocities for the same concentration of enzyme and substrate [28]. The reason is quite basic and cannot be ignored; given two durations of assay, t_1 and t_2 (where $t_1 < t_2$) for the same concentration of substrate (this concentration may be $< K_M$), the concentration of the parent substrate, if in particular a polysaccharide is the case but not limited to that, available to the enzyme in time t_1 may be disproportionately > than what may be available to the same enzyme concentration in time, t_2. This presupposes substrate depletion such that not just product per unit time but substrate availability per unit time should also be considered. The conclusion reached elsewhere [28] is that there will always be a different kinetic constant for different duration of assay. Regardless of the time regime, the maximum velocity of a given enzyme concentration cannot be attained if the substrate concentration regime is well-below the K_M which, is simply the usual substrate concentration within substrate concentration regime employed for the assay of a given solution of an enzyme, be it a standard solution or nonstandard solution, for the attainment of half maximum velocity of a catalytic action.

The maximum velocity occurs at a substrate concentration $> K_M$ but it is $\neq 2\ K_M$. Until hyperbolic curve relating velocity to substrate concentration, without disproportionate substrate depletion within the chosen duration of assay, is attained, no claim to Michaelian kinetics or sQSSA can be valid. Thus Michaelis-Menten equation is valid if the K_M lies between the $[S_T] < K_M$ and $\gg K_M$. The equation does not directly link velocities and cognate substrate concentration regime with the actual maximum velocity which is usually an extrapolated value by direct linear plot, conventional Lineweaver – Burk plot, and nonlinear plot. In other words the equation is not relevant when the derivative of C is = zero (or when $C = [E_T]$). It is relevant when C is increasing with increasing $[S_T]$ within a duration of assay that must not lead to substantial substrate depletion; thus an adoption of time regimes which tend to ∞ is a clear diversion from the true meaning of Michaelian kinetic equation; a higher concentration of an enzyme will take shorter duration than lower concentration of the same enzyme for the transformation of the same concentration of the substrate. A maximum velocity is strictly a function of substrate concentration available and cannot be ascertained if there is substrate depletion due to longer duration of assay and indeed the velocity should decrease as t (duration of assay) $\rightarrow \infty$.

Proceeding further requires consideration for the equations referred to in literature [18].



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \quad (9)$$

$$K_M = \frac{[E][S]}{[ES]} = \frac{[E_T] - [ES]][S]}{[ES]} \quad (10)$$

A correct claim, in line with Michaelian principle is that both S and [ES] in Eq. (9) above are different from zero, and that when $t \to \infty$, [S] and [ES] $\to$ 0. However, until a hyperbolic curve relating v to [S] is the case, regardless of the value of v_{max}, the value of the K_M cannot be regarded as the Michaelis – Menten constant. If not, enzyme-substrate complex dissociation constant should be the case in line with rQSSA. Anything on the contrary should represent a misuse of Michaelis-Menten equation. Besides, the claim as observed in literature [18] is that Eq. (10) is valid when [ES] reaches its maximum value. As long as it means the concentration of ES when the maximum velocity is attained, then, the maximum value of [ES] is = [E_T]; this means that K_M may be = 0 in Eq. (10). Another view is that in the deterministic rQSSA, it is assumed that the enzyme is in high concentration [29]. In this approximation, two time scales are considered. Starting with an initial condition ([S], [E], [C], [P]) = ([S_0], [E_0], 0, 0) the enzyme concentration is [E] $\approx$ [E_0] during the initial transient phase [29]. Since there is almost no complex during this time, an approximate model such as d[S]/dt = $-$ k_1 [E_T][S] which can be restated as [22] $-$ d[S]/dt = k_2[E_T][S]/K_M is the case. It appears therefore, that the phenomenon of sequestration may run into conflict with the demand of rQSSA. However, for the purpose of this research, the occurrence of free enzyme, E and free substrate, S, ([E_T] –[ES]) and ([S_T] – 342 [P]), respectively implies that the correct mathematical expression should be $v \propto [S]/[E]$ for a given concentration of the enzyme and different concentrations of the substrate. The equation $v = k_2[E][S]/K_x$ where K_x is intended to be the enzyme-substrate complex dissociation constant (K_s) on the ground that [S_T] < K_M (and this is supposed to be in line with the demand of rQSSA) is a mathematical statement showing that v is directly proportional to the product of [E] and [S]; separately, however, it is clearly not correct to state that v is directly proportional to E with increasing concentration of substrate. The velocity of hydrolysis could increase with increasing [E_T] given a known concentration of the substrate and with increasing concentration of the latter, given a known concentration of the enzyme. The data generated from such an assay (Table 1) is explored for the investigation of all issues including the important issue of sequestration and validity of kinetic parameters.

Fig. 1 confirms that v is directly proportional to [E][S] or (v_{max} – v)[S] but the K_s (or K_M) values (Table 2) calculated from the slopes is not equal to the value from the double reciprocal plots. The challenge of the lack of software for nonlinear regression notwithstanding, it was possible to show that with higher dilution factor the slope, $\partial v/\partial$[S] (Table 2) was higher than for lower dilution factor; this implies that with much lower concentration of the enzyme, the condition for sQSSA was satisfied to a greater extent with dilution factor (df) equal to 1/60 than 1/40; much lower dilution of the enzyme, yielded a higher slope which suggests a non-saturating substrate concentration in line with conditions which is defined by rQSSA. The plot of v versus [S](v_{max} – v) gave values of $1/(\partial v/\partial([S](v_{max} - v)) = K_s$ (or K_M) which are different from values from double reciprocal plot. But it needs to be stated that such a plot is only suitable where K_M > [S].

Figure 2 confirms that v is separately proportional to [S] and inversely proportional to [E] or (v_{max} – v). Thus a plot of v versus [S]/[E] gave a linear relationship with positive correlation (Fig. 2). It is in line with decreasing trend of [E] (Table 2) with increasing concentration of the substrate.

Table 1. The velocities of amylolysis of starch with different concentration of *Aspergillus oryzea* alpha amylase

[S]/g/L	Dilution factor 1/60	Dilution factor 1/40	Dilution factor 1/30	Dilution factor 1/20
	v/μM/min/mL	v/μM/min/mL	v/μM/min/mL	v/μM/min/mL
5	75.46 ± 0.75	158 ± 0.69	274.25 ± 0.73	638.65 ± 0.55
6	90.15 ± 0.76	185.10 ± 0.37	320.20 ± 0.73	693.50 ± 1.43
7	104.50 ± 0.90	217.10 ± 0.37	336.80 ± 0.88	762.00 ± 1.22
8	123.35 ± 20	247.35 ± 0.96	393.85 ± 0.88	828.28 ± 0.29
9	125.00 ± 1.96	246.66 ± 0.57	425.15 ± 0.76	830.05 ± 0.39
10	127.01 ± 1.22	253.99 ± 0.34	431.20 ± 0.33	930.00 ± 1.22

The results are approximations to two decimal places

Table 2. Calculated concentration of free enzymes, slopes (∂ *v*/∂ [S]), and calculated K_s (or K_M)

Dilution factor 1/60	Dilution factor 1/40	Dilution factor 1/30	Dilution factor 1/20
[*E*]/exp(– 8) mol/L	**[*E*]/ exp(– 8) mol/L**	**[*E*]/ exp(– 8) mol/L**	**[*E*]/ exp(– 8) mol/L**
2.81	3.82	4.85	5.79
2.73	3.66	4.58	5.46
2.66	3.46	4.49	5.05
2.56	3.34	4.17	4.65
2.55	3.27	3.99	4.64
2.54	3.23	3.95	4.05
∂ *v*/∂ [S]/mol/g.min	∂ *v*/∂ [S]/mol/g.min	∂ *v*/∂ [S]/mol/g.min	∂ *v*/∂ [S]/mol/g.min
10.9	19.81	33.4	55.21
$1/(\partial v/\partial([S](v_{max}-v))/g/L$	$1/(\partial v/\partial([S](v_{max}-v))/g/L$	$1/(\partial v/\partial([S](v_{max}-v))/g/L$	$1/(\partial v/\partial([S](v_{max}-v))/g/L$
40.00	28.57	16.13	8.4
K_s (or K_M)/g/L	K_s (or K_M)/g/L	K_s (or K_M)/g/L	K_s (or K_M)/g/L
34.72	~ 23.24	~ 15.44	~ 7.69
v_{max} / μM/min/mL	v_{max} / μM/min/mL	v_{max} / μM/min/mL	v_{max} / μM/min/mL
610.87	903.34	~ 1124.48	1605.39

$1/(\partial v/\partial([S](v_{max}-v))$ = K_s (or K_M) from the plot of v versus $[S](v_{max}-v)$ and ∂ v/∂ [S] is the slope from the plot of v versus [S]

There may be a corollary arising from inverse relationship between *v* and [*E*]. In this regard, a better equation of proportionality is given and numbered as follows:

$$v = \phi\, k_2 [S_T] / (v_{max} - v) \quad (11)$$

Where, ϕ is a proportionality constant and the product of $\phi\, k_2$ is the slope ($S_{lope\ -1}$). An expansion of Eq. (11) gives the following:

$$v^2 - v_{max}v + \phi\, k_2 [S_T] = 0 \quad (12)$$

The parameter *v* from Eq. (12), can be stated as

$$v = \frac{v_{max} \pm \sqrt[2]{v_{max}^2 - 4\,\phi\, k_2[S_T]}}{2} \quad (13)$$

In Eq. (13) $\phi\, k_2$ can be replaced by the slope from the plot of *v* versus $[S_T]/(v_{max} - v)$. Given that $v = -\,d[S]/dt = k\,[S_T]$, then substitution into Eq. (13) and rearrangement gives

$$k = \frac{v_{max} \pm \sqrt[2]{v_{max}^2 - 4\, S_{lope-1}[S_T]}}{2[S_T]} \quad (14)$$

Where, *k* is the pseudo-first order rate constant for the transformation of the substrate. To be dimensionally valid the unit of maximum velocity should be in g/L.min ($342.v_{max}$). Equation (14) is similar to equation in literature [30].

Based on Eq. (14), which is another method for the calculation of pseudo-first order rate constant for the hydrolysis of substrate, the rate constants as function of different [S_T] were calculated for different concentration of the enzyme. The calculation showed a strange and fascinating result in that while the magnitude of the lower root (approximated values) showed increasing trend with increasing concentration of the enzyme, the values (Table 3) were approximately constant (though there was increasing trend, but the differences which were not indicated due to approximation, were mainly by 3-4 decimal places) with increasing concentration of the substrate for each concentration of the enzyme. There is no intuitive explanation for now. However, the positive roots (high and low) showed increasing trend with increasing concentration of the enzyme and a regular decreasing trend in higher root values with increasing concentration of the substrate for each concentration of the enzyme (Table 3).

Even if magnetic stirrer is employed in the assay of an enzyme, the introduction of an aliquot of an enzyme solution into the aqueous substrate solution/suspension (if insoluble) does not lead to total participation of the enzyme in the amylolytic action. Most often than not [*E*] is regarded as the free enzyme; this suggests that some of the enzyme molecules are free from active site-substrate complex. This is the case considering that [*E*] = [E_T] – [*ES*], but the fact is that just before terminating the assay at the end of a chosen duration of assay does not stop the

formation of other complexes which may not have dissociated into product and substrate. As Table 3 shows the values of the total enzyme-substrate complex, $[ES_T]$ is < $[E_T - [ES]$ in Table 2. The difference is therefore, the concentration of free enzyme without any complex formation before product release. Generally there was a decreasing trend in the values of $[ES_T]$ with increasing concentration of the substrate contrary to expectation unlike the trend with increasing concentration of the enzyme (Table 3). As stated elsewhere (submitted manuscript) increasing $[ES]$ implies that less amount of free enzyme form complex at sites other than active site and associated carbohydrate binding modules (CBMs), initially known as cellulose binding domains (CBDs) [31-33]; "CBMs have been considered as contiguous amino acid sequences with discrete folds within the modular structures of carbohydrate active enzymes and cellulosomal scaffoldins (proteins that mediate the assembly of multiprotein cellulase–hemi cellulase complexes)" [34].

As per the effect of increasing concentration of the substrate, it seems, perhaps, that an approach to saturation (higher $[S_T]$) reduces a tendency to sequestration due to higher rate of turn-over. There are the possibilities that the enzymes bind the substrate at sites other than active site via strong hydrogen bonding in particular, some enzyme molecule make effective catalytic contact with the substrate before other molecules, and there are also the possibility of enzyme self-solvation, G_{22} the degree of dilution notwithstanding. The occurrences of non-catalytic oriented ES seem to be in line with the current phenomenon of enzyme sequestration [35-38] and what have been referred to as "sequestration

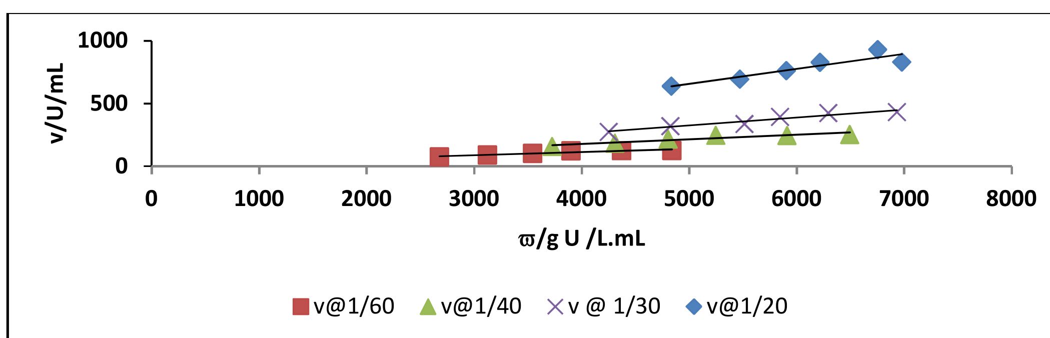


Fig. 1. Plots of experimental mean velocities (v_{exp}) versus ϖ ($[S_T].(v_{max} - v)$) for illustrating linear trend

The symbols (■), (▲), (×), and (◆) denote assays with different concentrations of the enzyme in terms of the dilution factors equal to 1/60, 1/40, 1/30, and 1/20 respectively. The corresponding slopes ($1/k_x$) are 0.025 L/g, ~ 0.035 L/g, 0.062 L/g, and 0.119 L/g

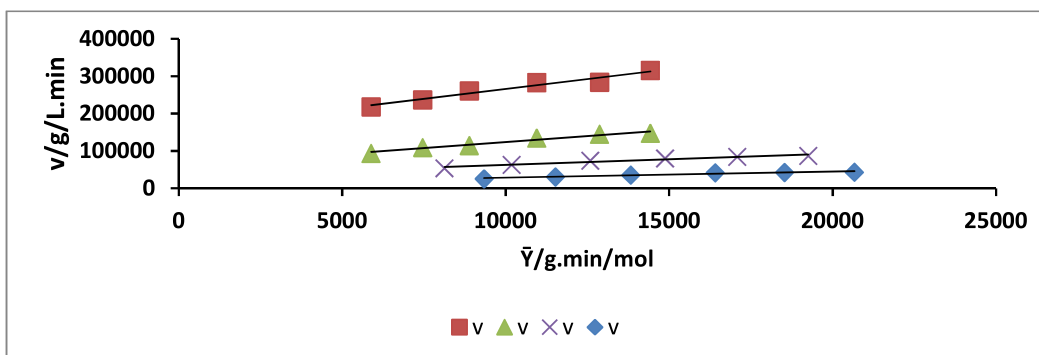


Fig. 2. Plots of experimental mean velocities (v_{exp}) versus Ȳ (*i.e.* $[S_T]/(v_{max} - v)$) for illustrating linear trend

The symbols (■), (▲), (×), and (◆) denote assays with different concentrations of the enzyme in terms of the dilution factors equal to 1/20, 1/30, 1/40, and 1/60 respectively. The corresponding slopes (ϕ s) are: 10.54 exp (– 6), ~ 6.50 exp (– 6), 2.98 (exp – 6), and 1.64 (exp – 6) respectively

Table 3. Rate constants and total enzyme-substrate complex

Dilution factor	Dilution factor	Dilution factor	Dilution factor
1/60	**1/40**	**1/30**	**1/20**
***k*/min**	***k*/min**	***k*/min**	***k*/min**
~ 41.78 exp (– 3)	61.78 exp (– 3)	~ 76.90 exp (– 3)	~ 109.79 exp (– 3)
7.85 exp (– 6)	9.65 exp (– 6)	16.91 exp (– 6)	19.20 exp (– 6)
34.81 exp (– 3)	51.48 exp (– 3)	~ 64.08 exp (– 3)	91.49 exp (– 3)
7.85 exp (– 6)	9.65 exp (– 6)	16.91 exp (– 6)	19.20 exp (– 6)
~ 30.70 exp (– 3)	44.12 exp (– 3)	~ 54.96 exp (– 3)	~ 78.42 exp (– 3)
7.85 exp (– 6)	9.65 exp (– 6)	16.91 exp (– 6)	19.20 exp (– 6)
~ 26.11 exp (– 3)	38.61 exp (– 3)	~ 48.06 exp (– 3)	68.61 exp (– 3)
7.85 exp (– 6)	9.65 exp (– 6)	16.91 exp (– 6)	19.20 exp (– 6)
23.21 exp (– 3)	34.32 exp (– 3)	~ 42.72 exp (– 3)	~ 60.99 exp (– 3)
7.85 exp (– 6)	9.65 exp (– 6)	16.91 exp (– 6)	19.20 exp (– 6)
20.88 exp (– 3)	30.88 exp (– 3)	~ 38.45 exp (– 3)	~ 54.89 exp (– 3)
7.85 exp (– 6)	9.65 exp (– 6)	16.91 exp (– 6)	19.20 exp (– 6)
k_2/min	**k_2/min**	**k_2/min**	**k_2/min**
~ 19.059 exp (+ 3)	~18.790 exp (+ 3)	~ 17.540 exp (+ 3)	~ 16.700 exp (+ 3)
[ES_T]/exp (– 8) mol/L	**[ES_T]/exp (– 8) mol/L**	**[ES_T]/exp (– 8) mol/L**	**[ES_T]/exp (– 8) mol/L**
~ 1.98	~ 2.88	4.31	5.11
~ 2.01	~ 2.85	~ 3.69	4.87
2.02	~ 2.95	3.37	~4.84
~ 2.16	~ 3.00	~ 3.58	4.84
1.89	~ 2.64	~ 3.53	4.53
~ 1.71	~ 2.46	~3.29	4.78

The parameters, k, k_2, and [ES_T] are the rate constants for the hydrolysis of the starch and the formation of the product, and total enzyme-substrate complex respectively

hypothesis"[18]. However, sequestration for the purpose of this research is not about saturating the active site, but about binding to sites other than active site and to what has been generally referred to as carbohydrate binding modules. Carbohydrate-binding modules, CBMs, and starch binding domain, SBD promote the association of the enzyme with the substrate [34, 39,40]. There is also the occurrence of surface binding site, SBS which is an allosteric regulator of the enzyme activity via binding of oligosaccharide products to the SBS [41].

From the foregoing, it is clear that CBMs and SBDs enhance the catalytic action of the hydrolases by aiding the concentration of the substrate around the enzyme molecules' catalytic domain such that there cannot be any question of substrate unbinding as a promoter of enzyme function as claimed elsewhere [42]. Therefore, the reasonable deduction is that sequestration occasioned by CBMs, SBDs, SBSs and active sites are essentially different from other sites that neither enhance nor participate in the transformation of the substrate. To be substantially bound to the active site and sites other than the active sites, implies that a large fraction of the enzyme as shown in Table 3 may not be available for immediate action/function; this is what Blüthgen, et al. [38] refers to as the sequestration of the enzyme which is concomitant with the sequestration of the substrate that becomes less available for the enzyme. However, extended duration of assay enables complexes destined for product release to complete the process before the termination of the assay with suitable reagent. Sequestration seems to suggest that the substrate concentration is < K_M. This is not likely to be the case due to what Blüthgen, et al. [38] referred to as Goldbeter and Koshland [43] zero-order ultrasensitivity to emphasize that the enzymes need to be operating in their zeroth order *i.e*., substrate-saturated regime expected if standard quasi-steady-state assumption (sQSSA) condition is to be satisfied as may be applicable to the phosphorylation of isocitrate dehydrogenase [36] and muscle glycolysis [44]. Upon termination of the assay at the expiry of the duration of assay, there are also other complexes that were yet to be transformed into product and free enzyme. Thus this could be

another source of unaccounted sequestration that reduces the velocity or amount of product formed per unit time. Nonetheless, this is regardless of whatever QSSAs that is applicable. The fact is that the enzyme can sequester a significant amount of substrate by binding to it, making this sequestered fraction of the substrate no longer accessible to other enzyme molecules [18.]. Most importantly, is the choice of the concept of total substrate concentration, $\hat{S}$ given as $S + C$, which takes into account sequestrated and free substrate [18]; this is similar to issues raised elsewhere [45]. This is very much applicable to total free enzyme which should also include sequestrated enzymes unable and yet to transform substrate to product.

The message that needs to be taken into account is that attainment of full saturation of the enzyme does not mean that all the enzyme molecules are involved in active site-substrate complex formation; there may be complexes formed with the substrate with sites other than the CBMs, SBDs, SBSs and active sites. Perhaps, the immobilisation of the enzyme in a way that can expose mainly the CBMs, SBDs, SBSs and active sites can increase the experimental values of the velocity of amylolysis. Additional approach may be to design the reactor in a way that extracts the product as soon as they are formed such that in line with Le Chatelier's principle, the concentration of the *ES* can always be sustained given that the effect of molecular crowding can also be minimised.

There is also a likely biological or therapeutic application of sequestration; concern for balanced diet must include the realisation that what may be a balanced diet for a diabetic or any other health challenge may not be the same for any person without health challenge. In this regard, diet formulation for a diabetic for instance, may include harmless substances with strong affinity for the enzyme that has a very strong potential to sequestrate the enzyme (alpha-amylase in the intestine for instance); in this way the concentration of the reducing sugar, glucose in particular, may be drastically reduced to a level tolerable by the glucose homeostatic mechanism. This should also call for an appropriate QSSA model given that the argument has always been that the concentration of enzyme *in vivo* is much higher than what in practice by choice is the case *in vitro*. "Intracellular concentrations of enzyme are usually higher or at least of the same magnitude as their substrates and, consequently, a significant fraction of *S* can be bound as *C* complexes" [7]. If the enzyme is considerably sequestrated by non-dietary substances leading to $[E_T]$ being « $[S_T]$, then rQSSA should be applicable.

5. CONCLUSION

Verifiable equations, the main equations and equation arising as a corollary were given. The fact is that the concentration of total enzyme-substrate complex ($[ES_T]$) is lower than what is usually referred to as free enzyme; the latter is composed of both undissociated enzyme-substrate complex and free enzyme. Therefore, if in addition, extra part of $[ES_T]$ dissociated into product within the duration of assay, the velocity of amylolysis could be higher. The most important outcome and corollary is that $v \propto 1/[E]$, $v \propto [E][S_T]$ with increasing concentration of $[S_T]$ and a quadratic relationship exists between pseudo-first order rate constant and maximum velocity of amylolysis; separately, v is not $\propto [E]$ and if $v \propto [S_T]$ (and if $\partial v/\partial[S_T]$ is always constant with coefficient of determination = 1), then K_M is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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