Original Research Article

Higher Intrinsic Rate Constants of Alpha-amylase is Possible if Intrinsic Forward Rate Constant is ≠ Diffusion limited Rate of Encounter.

ABSTRACT

Background: Previous research has shown that ~~the intrinsic rate constants,~~ the intrinsic reverse and forward rate constants are higher **[larger?]** than the effective or apparent rate constants **[for what?]**. It is speculated that such intrinsic rate constants could be ~~higher~~ **[I do not understand what you are trying to say here. Do you mean inproved?]** if more appropriate mathematical equations were employed for their computation.

Methods: Theoretical, experimental (Bernfeld method), and computational methods.

Objectives: 1) To rederive the equations for calculating the intrinsic rate constants for forward (k_1) and reverse (k_2) reactions, 2) calculate the intrinsic rate constants, and 3) demonstrate that the probability (1/g) (or $\rho_{eq}(r)$) that an enzyme is at a distance from the substrate is ~~a~~ variable ~~constant~~. **[It can't be a constant if it is variable. Do you want to say "depends on the concentration" here?]**

Results and discussion:

The equations for the determination of intrinsic reverse **[Do you mean reversible? The next sentence implies you do]** first order and second order rate constants were re-derived. Unlike previous ~~research~~ findings, the intrinsic reverse first order rate (k_b) [**You used k2 previously. Are they the same?]** and forward second order rate (k_1) constants were ~~higher~~ **larger** than their apparent counterparts, but they were, however, very similar in magnitude. The intrinsic rate constants were much ~~higher~~ larger than previously reported values when enzyme (E) total concentration $[E_T]$ was « than substrate's total concentration $[S_T]$. The k_1 and apparent forward second order rate (k_f) values where $[E_T]$ is » $[S_T]$ were > where $[E_T]$ is < $[S_T]$. Therefore, the magnitude of the second order rate constant is a function of $[E_T]$. The values of k_1 and k_2 where $[E_T]$ « $[S_T]$ and *vice-versa* were respectively, ≈ 7.41 exp. (+6) L/mol. min and ≈ 81.34 exp. (+4) /min, and ≈ 15.76 exp. (+6) L/mol. min and ≈ 58.08 **exp ?** (+4) /min. The probability (1/*g*) (or $\rho_{eq}(r)$)) that an enzyme is at a distance away from the substrate with the possibility of mutual atraction has been found to **not** be ~~a variable~~ constant.

Conclusion: If the intrinsic forward rate constant (k_1) is ≠ diffusion limited rate (k_D) of encounter, the k_1 and k_2 values could be > values where $k_1 = k_D$. The probability (1/g) (or $\rho_{eq}(r)$) that an enzyme is at a distance from the substrate with the possibility of mutual attraction has been discovered to be a variable constant dependent on the concentration of the reaction mixture components and the enzyme's affinity for the substrate, and vice versa. ~~**Feature**~~ **Future** research may attempt to derive an equation for the determination of an intrinsic catalytic rate constant for the formation of a product. **[I would recommend using words in place of the symbols < or > here.]**

Keywords: *Aspergillus oryzea* alpha-amylase, apparent rate constants, higher intrinsic rate constants, intermolecular electrostatic potential energy, the probability of intermolecular distance.

1. INTRODUCTION

Both the intrinsic rate constants and the effective rate constants ***[for what]*** *are valuable to both chemical and process engineers. Those experts may reengineer devices that increase apparent or effective rates to attain values closer to intrinsic rates* ~~*so as*~~ *to achieve higher production at a lower cost. The issue of intrinsic rate constants has been of interest* ***for*** *many years* ~~*ago*~~ ~~*[2, 3] and recently*~~ *[1 - 4*~~*,*~~ *5].* **[I would suggest moving this and starting with it as shown to establish why it is important to establish these constants before going into detail about how to find them.]** Previous research has shown that the intrinsic ~~rate constants, the intrinsic~~ reverse and forward rate constants are ~~higher~~ **larger** than the effective or apparent rate constants [**for what? Also add a sentence or two to explain the different types of rate constants for this application. The following explanations would be easier**

to follw if the background material were presented here.] [1]. It is speculated in this research that ~~higher~~ **larger** intrinsic rate constants of alpha-amylase are possible **[produced?]** if the intrinsic forward rate constant, k_1, is ~~$\neq$~~ **not equal to the [it may be better to use words here and introduce the equations in parentheses for later reference]** diffusion limited rate of encounter, k_D. **[$k_1 \neq k_D$]** Such intrinsic rate constants could be ~~higher~~ **larger** than values reported ~~(~~using ~~the notion that~~ $k_1 = k_D$) in earlier research [1]. The concern expressed in the literature is that the undefined term g cannot be a consistent constant because it is concentration dependent [3]. This view is an offshoot of the view by the author [3] that K_C, the equilibrium constant only for concentrations, is, in fact, **[is only a true constant at 0 concentration since the activity coefficient ?]** not a true constant at all since U(r) **[it would also be useful to define this]** will always be concentration dependent. This view seemed to be confirmed by previous results [1], but only on the basis of the application of the diffusion coefficient, which decreases with increasing concentration of the substrate or product and, consequently, the ES. **[More explanation would help here. I assume this is related to a standard model for enzyme kinetics, but it is not totally clear which one. Terms like ES and g would make more sense to readers not expert in this problem if the model were summarized as suggested above]** In other words, with approach to terminal velocity (this differs with different concentrations of the substrate), whereupon an effective encounter is to be formed, and ultimately, ES formation, the values of g may differ. But there is a point in a given time and space for a given substrate concentration range with a specific concentration of the enzyme, where rectilinear motion of the bullet molecule **[I am not familiar with this term. Can you explain what determined the bullet molecule?]**, in this case, the much smaller enzyme, moves towards the much larger substrate molecule. Such a point is the specific distance between the enzyme, the bullet, and the target, the substrate, ~~for a given substrate concentration range~~.

The magnitude of g may differ from one concentration range to another, but not on the basis of individual concentration. The implication is that the author's [2, 3] undefined g (which appears to be the probability that a particle could be at a distance r from a target if re-expressed in the form of $1/g$ because, in the form, $g = \exp(U(\mathrm{r})/k_B T)$, g must always be greater than one, which is unlikely). Whichever is the case, g (or its reciprocal) can be taken to be a variable ~~constant~~, and it can be shown to be so. A simple analogy is the physical constant of acceleration due to gravity, which is not the same everywhere above the Earth's surface. Thus, the objectives of this study are as follows: 1) rederive the equations for calculating the intrinsic rate constants for forward (k_1) and reverse (k_2) reactions **[of….]** ; 2) calculate the intrinsic rate constants **[for….]**; and 3) show that the probability (1/g) (or $\rho_{eq}(r)$) that an enzyme is at a distance from the substrate is a variable constant.

2.0 Theory

2.1 Review of theory

In this review, two approaches, Shurr's [3] and Vijaykumar *et al*.'s [5], for the determination of intrinsic rate constants are under consideration. First, is a modified [1] Vijaykumar *et al.'s* approach [5], given as follows:

$$k_{\mathrm{d}}(\sigma) = \frac{4\,\pi\,(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})\,N_{\mathrm{A}}\,k_{\mathrm{off}}}{4\,\pi\,(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}} - k_{\mathrm{on}}} \tag{1}$$

Where, $k_d(\sigma)$, ~~R_E, R_S, D_E, and D_S are the 1^{st} order intrinsic rate constant for the dissociation of *ES*, Where $k_d(\sigma)$, R_E, R_S, D_E, and D_S are~~ **is** the first order intrinsic rate constants for ES dissociation, **R_E is the** hydrodynamic radius of enzyme, **[etc]** hydrodynamic radius of the substrate, translational diffusion coefficient of the enzyme, and translational diffusion coefficient of the substrate respectively; k_{off}, k_{on}, and N_A are the effective first order rate constants for ES dissociation; and Avogadro's number, respectively.

$$k_{\mathrm{a}}(\sigma) = \frac{4\,\pi\,(R_{\mathrm{E}} + R_{\mathrm{S}})\,(D_{\mathrm{E}} + D_{\mathrm{S}})\,N_{\mathrm{A}}k_{\mathrm{on}}}{4\,\pi\,(R_{\mathrm{E}} + R_{\mathrm{S}})\,(D_{\mathrm{E}} + D_{\mathrm{S}})\,N_{\mathrm{A}} - k_{\mathrm{on}}} \tag{2}$$

Where, $k_a(\sigma)$ and k_{on} denote the 2nd order intrinsic and effective 2nd order association rate constants, respectively. There is no problem with Equations (1) and (2). **[should I worry that (1) and (2) are the same?]** The modified Shurr's approach [3] given as:

$$k_2 = k_b\,(2 - R\,/R_0)\ (\text{Thus } k_2 \neq \boldsymbol{f}\,(g)) \tag{3}$$

Where, k_2, k_b, R, and R_0 are the 1^{st} order intrinsic rate constant for the dissociation of ES*, the* 1^{st} order effective rate constant for the dissociation of *ES*, the sum of R_E and R_S, and the intermolecular distance where electrostatic attraction begins. **[You could make it easier on your readers by defining terms as indicated above. Why is $k_2 \neq \boldsymbol{f}\,(g)$)?]**

$$k_1 = k_f\,g\,(2 - R\,/R_0)\ (\text{Thus } k_1 = \boldsymbol{f}\,(g)) \tag{4}$$

Where, k_1, k_f, and g are the 2nd order intrinsic rate constants for the formation of ES, the 2nd order effective rate constant for the formation of *ES,* and a dimensionless constant defined in two ways, viz: In line with Shurr's[3] definition, it is

$$g = \exp. (U(r)/k_B T) \tag{5}$$

The intermolecular potential energy, Boltzmann constant and thermodynamic temperature are denoted by U(r), k_B, and T, respectively.**[Can U(R) be negative as it is defined here? More explanation would help here]** According to the Vijaykumar *et al*. [5] method, $1/g$ (or $\rho_{eq}(r)$) is given as:

$$1/g \text{ (or } \rho_{eq}(r)) = \exp. (- U(r) /k_B T) \tag{6}$$

Where, $\rho_{eq}(r)$ is defined as the equilibrium probability that two particles are separated by a distance, r. Equation (6) is to address the fact that the maximum value of $\rho_{eq}(r)$ is one (1), whereupon $1/ \rho_{eq}(r)$ (or g) should always be ≥ 1 as expected if the equation, $g = \exp. (U(r) /k_B T)$, is upheld. This implies that g is no longer taken to be the same as $\rho_{eq}(r)$ as in the literature [1]. The concern about Eqs (5) and (6) is that there may be confusion if one realises that the maximum intermolecular potential energy is zero in line with the conservative field force principle [1]. In such a situation, nonequilibrium binding interactions cannot occur.

In previous research, it was assumed that k_D (k_D is the diffusion-limited rate constant, which determines the rate at which the two particles diffuse towards each other [5] may be = k_1 (or at least, $k_1 \simeq k_D$); this may be considered not out of place considering the assumption that enzymatic reaction may be diffusion dependent if $k_f \simeq k_D$ [3]. According to Shurr [3], diffusion dependence exists if the following conditions are met: a) $[S_T] \lesssim K_M$ and $k_3 \gg k_b$, and $k_3/k_D \simeq K_M$, b) $[S_T] \ll K_M$. Diffusion independence is the case if the following conditions hold: a) $[S_T] \gg K_M$, b) $k_3 \ll k_b$, c) $k_f \ll k_D$, and $k_3/k_D \ll K_M$. However, experimental determination of both parameters is needed in order to reach a well-informed decision on whether they are diffusion dependent or not. At the moment, it is not too clear whether or not the dependence on the other relations, such as: $k_3 \gg k_b$ (or $k_3 /k_D \simeq K_M$), $[S_T] \ll K_M$, *etc* [3], is on the basis of the all-or-none principle. Nonetheless, the assumption that g ($1/g$ as preferred in this research) is a variable constant can be shown as follows.

Recall the following equations (in a modified form) given by Shurr [3].

$$k_{\mathrm{b}} = \frac{k_{\mathrm{D}} k_2 \ \exp\left(-(U(r))/k_{\mathrm{B}}\right)}{f k_1 + k_{\mathrm{D}}/g} \tag{7}$$

$$k_{\mathrm{f}} = \frac{k_{\mathrm{D}} k_1}{f k_1 + k_{\mathrm{D}}/g} \tag{8}$$

In this research, k_1 and k_D are no longer seen to be equal in order to address the dimensional issue as in previous research [1] in which it was observed that $k_D = 4\pi(R_E + R_S)(D_E + D_S)$ is in any unit of volume per unit time rather than any unit of volume per mol. per unit time. There, R_E and R_S are the hydrodynamic radius of the enzyme and substrate, respectively, and D_E and D_S are the corresponding diffusion coefficients. It has, however, been shown that g is a variable constant in the literature [1], but on the assumption that k_1 and k_D are equal, which also presupposes that they must possess the same valid unit, where k_D is now given as $4\pi N_A(R_E + R_S)(D_E + D_S)$, where N_A is the Avogadro's number (see Levine [6]). Since Eqs (7) and (8) possess the same denominator, the following relations are possible.

$$\frac{k_{\mathrm{D}} k_1}{k_{\mathrm{f}}} = \frac{k_{\mathrm{D}} k_2 \ \exp\left(-(U(r))/k_{\mathrm{B}}\right)}{k_{\mathrm{b}}} \tag{9}$$

With the simple algebraic equation, $1/g$ (or exp (– (U(r)) /k_BT)) is given as:

$$1/g = \frac{k_{\mathrm{b}}\, k_1}{k_2\, k_{\mathrm{f}}} \tag{10}$$

As long as k_b, k_f, and, by extension, k_1 and k_2 are influenced by conditions such as temperature, pH, ionic strength, and the nature of the polymer, as in the case of starch from various sources, g cannot remain a consistent constant quantity. The rate constants may even vary given different concentration ([S_T]) ranges of the substrate for the same enzyme concentration, every other condition being constant. However, it is necessary to indicate the assumption by which the assay is undertaken given that where [S_T] « K_M (Michaelis-Menten constant), one may be operating on the basis of a reverse quasi-steady-state assumption (or approximation), if in particular, the concentration of the enzyme, [E_T] » [S_T]. It is also possible that [S_T] » [E_T], peculiar to the standard quasi-steady-state (or reactant stationary [7]) assumption, even though K_M may be **<** [S_T] with higher [E_T]. All scenarios are verifiable upon appropriate experimentation so that one can establish either diffusion dependence or independence. In this research, consideration is given to the displacement of the enzyme from what may be referred to as infinity to a point closer to the substrate where there may be mutual electrostatic perturbation. That point is at a distance from the substrate. Their mutual attraction begins with a reduction in (or even an outright absence of) random motion.

A dimensionless factor, *f* which was not given any name in the literature [2, 3], is re-stated here in modified form, first as:

$$f = -R(1/g)\int_{r<\infty}^{R}\frac{dr}{r^2} \tag{11}$$

Where, $R = R_E + R_S$, the reaction radius (regarded, for simplicity sack, as the sum of the hydrodynamic radii of the enzyme and substrate), and $R_{‹\infty}$ is the equivalent of R_0, intended to imply that it is **‹ the** concentration dependent maximum intermolecular distance, which R_∞ stands for. Therefore, Eq. (10) is restated as:

$$f = \left(1 - \frac{R}{R_0}\right)/g \tag{12}$$

2.2 Derivation of equations for intrinsic rate constants

Since the parameters of the rate constants are linked to translational diffusion and cognate diffusion coefficients, it is imperative to make it clear that if reactants remain in their fixed location, there cannot be encounter-complex formation, let alone any form of (bio/physico) chemical reaction. Because of mechanistic issues that are frequently applicable to organic reactions with or without biological catalysts, encounter-complex formation does not always translate to an immediate reaction. This is regardless of whether or not the ultimate variables and kinetic parameters are diffusion dependent. But when encountering complex formations, it must be diffusion dependent.

Substitution of Eq. (12) into Eq. (7) with the elimination of common factors gives, after rearrangement, the following.

$$k_2 = \frac{k_\mathrm{b}}{k_\mathrm{D}}\left(\left(\frac{R_0 - R}{R_0}\right)k_1 + k_\mathrm{b}\right) \tag{13}$$

Meanwhile, Eq. (10) can be rearranged to give an equation for k_1 and k_2 as follows:

$$k_2 = \frac{k_\mathrm{b}\,k_1}{k_\mathrm{f}/\mathrm{g}} \tag{14a}$$

$$k_1 = \frac{k_2\,k_\mathrm{f}}{g\,k_b} \tag{14b}$$

Substitution of Eq. (14a) into Eq. (13) gives:**[I think you mean 14 b here]**

$$k_2 = \frac{k_\mathrm{b}}{k_\mathrm{D}}\left(\left(\frac{R_0 - R}{R_0}\right)\frac{k_2\, k_\mathrm{f}}{g\, k_b} + k_\mathrm{b}\right) \tag{15}$$

Pulling like terms together gives another equation for k_2.

$$k_2 = \frac{k_\mathrm{D}}{\frac{k_\mathrm{D}}{k_\mathrm{b}} - \left(\frac{R_0 - R}{R_0}\right)\frac{k_\mathrm{f}}{g\, k_b}} \tag{16a}$$

Equation (16a) is simplified to:

$$k_2 = \frac{k_\mathrm{D}\, k_\mathrm{b}}{k_\mathrm{D} - \left(\frac{R_0 - R}{R_0}\right)\frac{k_\mathrm{f}}{g}} \tag{16b}$$

Unlike in Eq. (14a), Eq. (16b) contains both theoretically determinable parameters (with respect to k_D and *R,* for instance) and experimentally determinable parameters.

Rearrangement of Eq. (8) gives:

$$k_1 = \frac{k_\mathrm{f}\,(f k_1 + k_\mathrm{D}/g)}{k_\mathrm{D}} \tag{17}$$

$$k_1 = \frac{k_\mathrm{f}\, k_\mathrm{D}/g}{\left(k_\mathrm{D} - k_\mathrm{f}\left(1 - \frac{R}{R_0}\right)/g\right)} \tag{18a}$$

$$k_1 = \frac{k_\mathrm{f}\, k_\mathrm{D}}{\left(k_\mathrm{D}\, g - k_\mathrm{f}\left(\frac{R_0 - R}{R_0}\right)\right)} \tag{18b}$$

In the same vein, Eq. (18b) has both theoretically and experimentally determinable parameters, unlike Eq. (14b). The procedure for the determination of R_0 can be found in the literature [8] as applied elsewhere [1]; similarly, the determination of $U(R)$ can be found in the same literature [8]. It needs to be stated that the approach in this research is different since the Einstein-Stokes equation is applicable. All relevant equations are stated in the method section.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

As in the literature [1], *Aspergillus oryzea* alpha-amylase (EC 3.2.1.1) and soluble 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 in Mumbai, India. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd., Poole, England. Distilled water was purchased from a local market. The molar mass of the enzyme is = 52 kDa [9].

3.1.2 Equipment

An electronic weighing machine was purchased from Wenser Weighing Scale Limited, and a 721/722 visible spectrophotometer was purchased from Spectrum Instruments, China; a pH meter was purchased from Hanna Instruments, Italy.

3.2 Methods

3.2.1 Preparation of solution of reactants for the assay.

The enzyme was assayed according to the Bernfeld method [10] using gelatinised potato starch whose concentration range is 5–10 g/L; the weight average molecular weight of the insoluble potato starch is 6.454 exp. (+7) g/mol. [11]. The reducing sugar produced upon hydrolysis of the substrate using maltose as a standard was determined at 540 nm with an extinction coefficient equal to 181 L/mol.cm. A concentration equal to 1 g/100 mL of potato starch was gelatinised at 100 ^{o}C for 3 min and subjected to serial dilution after making up for the loss of moisture due to evaporation to give concentrations ranging between 5 and 10 g/L for assays in which $[S_T]$ » $[E_T]$, and between 0.3 and 3 g/L for assays in which $[E_T]$ » $[S_T]$. Aspergillus oryzea alpha-amylase was concentrated to 0.01 g/100 mL by dissolving 0.01 g of the enzyme (as the stock) in 100 mL of Tris HCl buffer at pH = 7. The assay of the enzyme, where $[S_T]$ » $[E_T]$ and $[E_T]$ » $[S_T]$, was carried out with an enzyme concentration » 1.667 and 20.0 mg/L, respectively. The duration of the assay was 3 minutes at 20 ^{o}C.

3.2.2 Determination of intermolecular distances for electrostatic attraction.

$$R_0 = R/\left(1 - S_{\mathrm{lope}-1}/S^2_{\mathrm{lope}-2}\right) \qquad (19)$$

$S_{\mathrm{lope-1}}$ is calculated by plotting the square of frequency, ζ^2 versus $1/R(R_\infty - R)$, whereas $S_{\mathrm{lope-2}}$ is calculated by plotting ζ versus $1/R_\infty$; $R_\infty = \sqrt[3]{(\mathrm{Vol.}_{(\mathrm{rxn})}/(n_S + n_E)\,N_A)}$, where n_S, n_E and $\mathrm{Vol.}_{(\mathrm{rxn})}$ are the number of substrate and enzyme molecules, respectively, and the reaction mixture volume which is = 2 mL. **[I don't think frequency was defined earlier. I should be explained]** The equation for the calculation of the frequency of effective collision is given as: $\zeta = 2\,\pi\,R\,(D_E + D_S)\,[N_{ES}]$ [12], where $[N_{ES}]$ is the number density of the enzyme substrate complex.

3.2.3 Determination of intermolecular potential energy, *U*(R).

This is as described in the literature [8], given here as:

$$U(\mathrm{R}) = -\frac{\phi\, e^2}{4\, \varepsilon_\mathrm{r}\, \varepsilon_0 R_0} \tag{20}$$

As explained in the literature [8], ϕ may be > 1 if there are strong long-range attractive forces in addition to short-range attractive forces. Besides, it is assumed that the enzyme begins with a translational velocity where strong electrostatic attraction begins, but not without the effect of solvent resistance and co-substrate crowding effect. The translational velocity u is determined in two ways [8] and shown for convenience as follows:

$$u = 2\, S_{\mathrm{lope}-1} \text{ɤ}_2 \sqrt[2]{\left(S_{\mathrm{lope}-1}\, (R_0 - R)/R_0\right)} \tag{21}$$

$$u = 2S_{\mathrm{lope}-1} \text{ɤ}_2 / S_{\mathrm{lope}-2} \tag{22}$$

The fraction (ɤ_2) of the total distance covered by an approaching smaller molecule compared to the larger molecule is:

$$\text{ɤ}_2 = \frac{\sqrt{M_\mathrm{S}/M_\mathrm{E}}}{\sqrt{(M_\mathrm{S}/M_\mathrm{E}) + 1}} \tag{23}$$

3.2.4 The probability that a molecule of an enzyme is at a distance, R_0, from the substrate

$$1/g \text{ (or } \rho_\mathrm{eq}(r)) = \exp(-\ 6\, \pi\, \eta\, R_\mathrm{E}\, u\, R_0/k_\mathrm{B}T) \tag{24}$$

Where, η and u denote the viscosity coefficient of the solvent and either Eq. (21) (or Eq. (22)) respectively. Based on conserved field force, the maximum potential energy is zero.

3.2.5 Determination of apparent rate constants.

The calculated outcomes of In ($[E_\mathrm{T}]/[E_\mathrm{F}]$), where $[E_\mathrm{F}]$ is the concentration of free enzyme, can be plotted versus $[S_\mathrm{T}]$ (1 – exp. (– $k\ t_\mathrm{ES}$)/k to yield a slope defined as: $(k_\mathrm{f} + k_3)/K_\mathrm{M}$, as described in the literature [13]. The latter is multiplied by the molar mass of maltose for reasons explained elsewhere [13]. In the literature, $k\ t_\mathrm{ES}$ is defined as follows [13]:

$$k\ t_{ES} = \mathrm{In} \frac{1}{1 - \frac{[E_\mathrm{T}] M_\mathrm{alt}}{[S_\mathrm{T}]} \mathrm{In} \frac{[\mathrm{E_T}]}{[\mathrm{E_F}]}} \tag{25}$$

Where, M_alt, k, and t_ES are the molar mass of malt, the pseudo-first order rate constant for substrate utilisation, and the duration of ES formation in this study. The right hand side of Eq. (24) can be plotted versus values of k calculated by a method described elsewhere [1, 13, 14] to give a slope, being the specific value of t_ES.

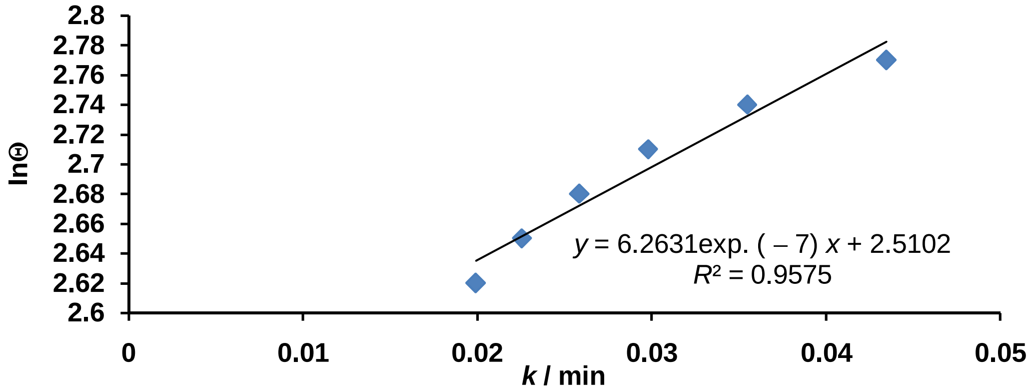
Statistical Analysis

The mean values of the two determinations were taken.

4. RESULTS AND DISCUSSION.

For reasons mentioned earlier in the text, previous research assumed that k_D could be equal to k_1. It is framed against the presence or absence of a causative factor(s).Those factors may originate from comparative relationships between independent variables and kinetic variables that this research generated in order to infer the ultimate diffusion dependence or independence. To generate data for needed calculations as evidence-based research (or, better yet, to avoid mystifying the younger ones, undergraduates, and interested individuals outside the field but related fields), a step-by-step approach with graphics (Figs 1, 2a, 2b, 3, and 4) was used. The graphs for the determination of v_{max} and slope needed for the calculation of the pseudo-first order rate constant for utilisation of substrate, k, are omitted.

In the determination of the duration t_{ES} of ES formation by the graphical method, there is a need to apply intuition in that a plot of Q versus k (Fig. (2a)) could be nonlinear if the substrate concentration range includes $[S_T]$ **<** 1 g/L, in particular, though not limit. **[what does this mean? Explain]** As a result, not less than six different concentrations of S must be used for the assay so that, from the plot (Figs (1) and (2b), the points (this cannot be 3 if six different $[S_T]$ were used and 5 if eight different $[S_T]$ were used) with the highest coefficient of determination, R^2 ($\gtrsim$0.9), can be used to determine t_{ES} as a slope. A time course experiment may be adopted for the determination of k for different concentrations with the same concentration of E instead of calculation in feature **[?]** research. Speculatively, this approach, may achieve linearisation of the plot.



$$\Theta \equiv ln\frac{1}{1-\frac{[E_T]M_{alt}}{[S_T]}ln\frac{[E_T]}{[E_F]}}$$

Fig. 1: Determination of the duration, t_{ES}, of ES formation for [S_T] » [E_T]. *M_{alt}, [E_T] and [S_T], and [E_F] are molar mass of maltose, total concentrations of enzyme and substrate, and concentration of free enzyme respectively.*

[this plot is not linear. How do you get E_F?]

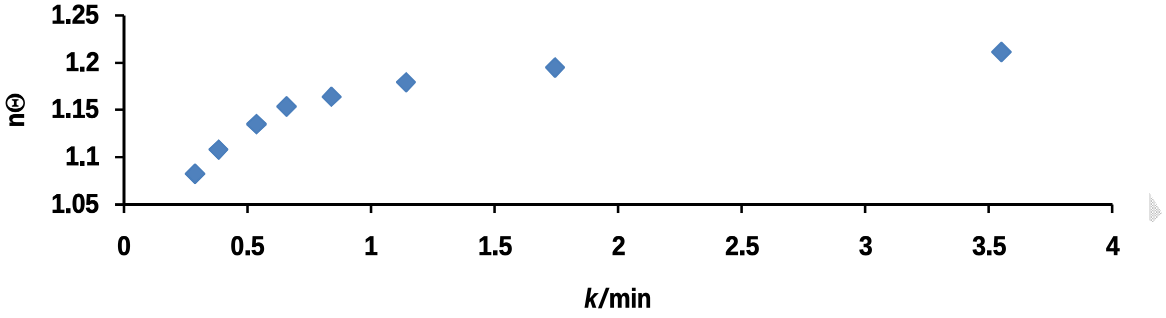


Fig. 2a. Plot showing nonlinear curve for the complete substrate concentration range for the determination of t_{ES}. *A better coefficient of determination is applicable in the range 1.2 → 3 g/L. Θ is defined in Fig 1.*

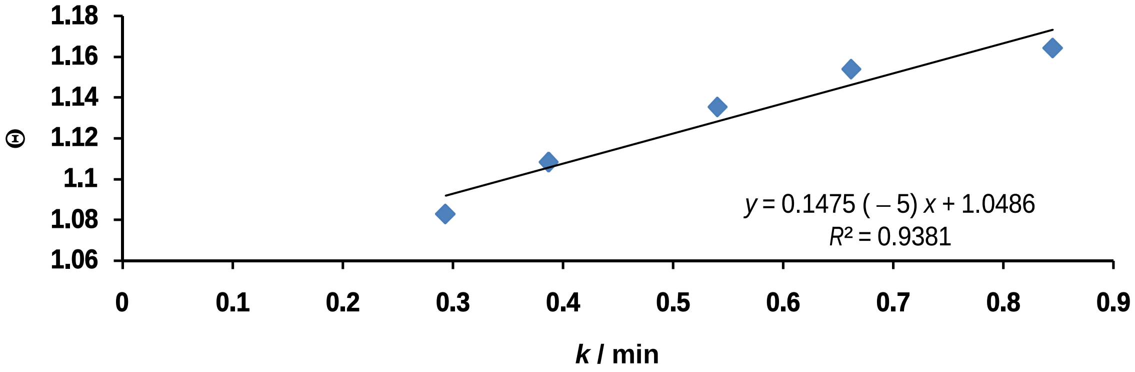


Fig.2b: Determination of the duration, t_{ES}, of ES formation, for [S_T] « [E_T]. *Definition of symbols, are as in Fig.1. The range of [S_T] used for the plot is 1.2→ 3 g/L. The full range, 0.3 → 3 g/L showed poor correlation = 0.8116 (R^2 = 0.6587) for what was a hyperbolic curve Fig. is not shown.*

Having determined the t_{ES} values for low and high [E_T], the determination of k_1 comes next. This is done as described in the method subsection. The plots (Fig. 3 and Fig. 4) show that the slope for low [E_T] is > the slope for high [E_T]. This means that the expected values of k_1 are different, being higher for low [E_T] than for high [E_T]. The reason is that with high [E_T], there is a greater trend toward a single turnover catalytic event [15] than for low [E_T]. With excess E, there is always free E in the bulk for the formation of ES.

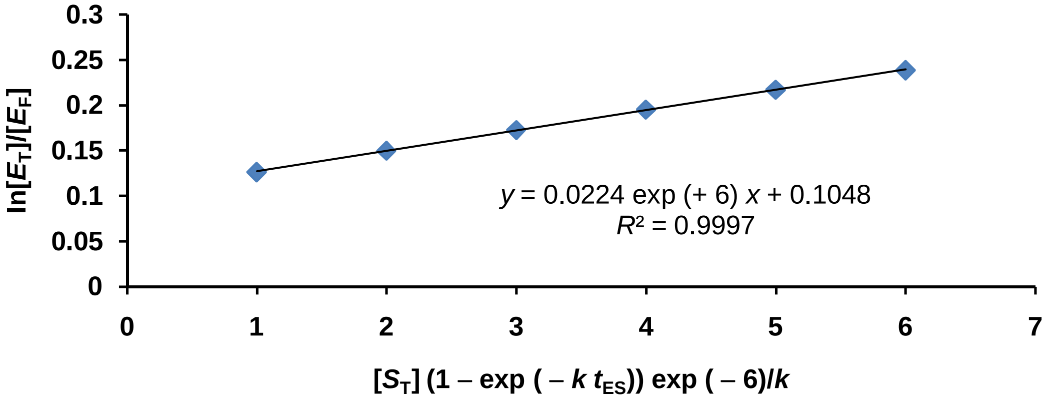


Fig.3: Determination of the 2nd order rate constant for [S_T] » [E_T]. [S_T], [E_T], [E_F], t_{ES}, *and* k *are the total concentrations of the substrate and enzyme respectively, concentration of free enzyme, duration of ES formation, and pseudo-first order rate constant for the utilisation of the substrate.*

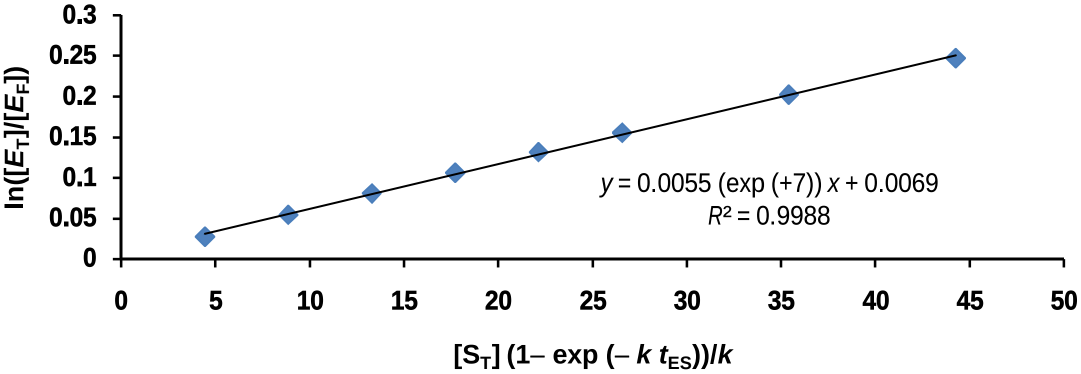


Fig. 4: Determination of the 2nd order apparent (or effective) rate constant where [S_T] « [E_T]. *Definitions of symbols are as in Fig. 2.*

As shown in Table 1, the kinetic parameters generated from the assay of two different concentrations of the enzyme are different as expected; this should be the case because one meets the condition that satisfies standard quasi-steady-state assumption (sQSSA) [16] or the recently acclaimed reactant stationary assumption (RSA) [7] which is regarded as one that does not require [S_T] to be » [E_T] to satisfy the condition for the validity of Michaelian kinetics and its cognate kinetic parameters on the basis of the steady-state assumption. The other scenario, however, met the condition that satisfies the criterion for the validity of reverse quasi-steady-state assumption (rQSSA) on the basis of [S_T] being « [E_T]. The values of k_2, g, K_M, k_3, and k_b where [S_T] > [E_T] were > the values where [S_T] « [E_T]. The value of k_1 where [S_T] > [E_T] is, however, < the value where [S_T] « [E_T]. Where [S_T] > [E_T] and [S_T] « [E_T], the values of k_f were > k_1; the difference was, respectively, ~ – 3.45 % and ~ – 17.555 % of k_1. In the same vein, following the same order, the value of k_2 > k_b; the differences for where [S_T] > [E_T] and [S_T] « [E_T] are respectively ~ 0.044 % and ~ 0.004 % of k_2. Unlike the report in the literature [1], the value of the apparent association rate constant, a 2nd order rate constant for the formation of ES, is > its intrinsic counterpart, k_1. This may be as a result of not equating k_D with k_1 in this research.

Table 1. Apparent and intrinsic rate constants and other physico-chemical parameters

Parameters	**For [S_T] » [E_T]**	**For [S_T] « [E_T]**
R_0/ exp. (– 8) m	9.4024	7.9868
$S_{\text{lope-1}}$/ exp. (– 9) m^2/s^2	1.5928	1000
$S_{\text{lope-2}}$/exp. (– 4) m/s	1.0032	105
u /exp. (– 5) m/s	3.0878	18.5219
U(R)/ exp. (– 22) J	– 1.3934	– 7.0996
1/g (or $\rho_{eq}(r)$)	0.9663	0.8376
Factor, f/exp.(–3)	163.7804	7.5973
K_M(or K_{eq}) / g/L	37.2390	10.7085
v_{max} / exp. (– 3) M/mL/min	0.6757	3.168
K_3 / exp. (+ 4) /min	2.1081	0.824
k_b / exp. (+ 4) /min	81.3073	58.0726
k_f / exp. (+ 4) L/g/min	2.2400	5.5000
k_f / exp. (+ 6) L/mol./min	7.6608	18.8100
k_D / exp. (+6) L/mol./s	52.2220	52.2220
k_1 / exp. (+ 6) L/mol./min	7.4054	15.7560
k_2 / exp. (+ 4) /min	81.3434	58.0753
K_3/k_D / L/g	434.2239	169.7265
(k_2 – k_b)/ min	361.3388 (0.0444 % of k_2)	27 (0.0046 % of k_2)
(k_1 – k_f) /exp. (+ 5) L/mol./min	–2.5540 (– 3.4488 % of k_1)	– 27.6590 (– 17.5546% of k_1)

The rate constants, k_f, k_b, k_3, k_2, and k_1 are the apparent 2nd order rate constant for enzyme-substrate formation, apparent first order rate constant, for the dissociation of ES into E and S, apparent first order rate constant (catalytic rate) for the formation of product, first order intrinsic rate constant for the dissociation of ES to E and S, and the second order intrinsic rate constant for the formation of ES respectively. Total enzyme concentration, [E_T], for [S_T] « [E_T] is » 3.8462 exp. (- 7) mol. /L and » 3.2051 exp. (- 8) mol./L for [S_T] » [E_T]. Arithmetic means of raw data from two *determinations were taken. $[S_T]$ for $[S_T] \ll [E_T]$ ranges between 0.3 → 3 g/L and, 5 → 10 g/L for $[S_T] \gg [E_T]$. t_{ES} = 6.2631 exp. (-7) min for $[S_T] \gg [E_T]$ and 1.4750 exp. (- 6) min for $[S_T] \ll [E_T]$. Data are approximations to 4 decimal places; absolute (raw) data were used for calculations.*

Unlike previous research [1], this study investigated two different concentrations of the same enzyme, *Aspergillus oryzea* alpha-amylase, to determine the effect of a much higher concentration of enzyme, which is in line with the usual reverse quasi-steady-assumption (rQSSA) requirement or where a single-turnover catalytic cycle is of interest. Thus, on the issue of criteria for either diffusion dependence or independence, the results for $[E_T] < [S_T]$ and for $[S_T] > [E_T]$ show that the rate of reaction for both was diffusion dependent since $[S_T] < K_M$ (Table 1); hence, as Table 2 shows, diffusion independence is not applicable because $[S_T]$ is neither $\gg K_M$ nor $\geq K_M$. In both scenarios, there was a case of diffusion independence in a situation in which $k_3 \ll k_b$, and consequently, diffusion dependence is not applicable because, $k_3 \ngtrsim k_b$. None of the scenarios gave values of k_f which are either $> k_D$ or $\simeq k_D$**,** leading to both rates being taken to be diffusion independent; this is also applicable to the ratio, k_3/k_D, which being $< K_M$ (Table 1) shows that the rates for the scenarios are again diffusion independent; the condition or criterion that k_3/k_D be $\simeq k_D$ is indeed not satisfied, hence diffusion dependence is not applicable in all scenarios (Table 2) An earlier study [1] discovered that the same enzyme had diffusion-controlled kinetic parameters because $k_3 > k_b$ and $K_M > [S_T]$. This is unlike in this research, in which the same enzyme's kinetic parameters can be ascribed to both diffusion independence because $k_3 < k_b$ and diffusion dependence because K_M is $> [S_T]$. This may be as a result of the different conditions of assay. **[Why?]**

Table 2. Classification of enzymatic action on the basis of diffusion dependence (Diff-Dep.) and independence (Diff-In-dep.), based on Shurr's [3] criteria where $[S_T] \gg [E_T]$ and $[S_T] \ll [E_T]$.

Criteria for Diff-In-dep.	**Results**	**Diff-dep.**	**Results**
$[S_T] \gg K_M$	NA	$[S_T] \lesssim K_M$	NA
$k_3 \ll k_b$	A	$k_3 \gg k_b$	NA
$K_f \ll k_D$	A	$k_3/k_D \simeq K_M$	NA
$k_3/k_D \ll K_M$	A	$[S_T] \ll K_M$	A
-	**-**	$K_f \simeq k_D$	NA

The alphabetic symbols, NA and A, means 'not applicable' and 'applicable' respectively.

One of the greatest challenges in any investigation for the determination of the intrinsic rate constant is the determination of the minimum interparticle distance (R_0) for the commencement of mutual electrostatic interaction between the substrate and the enzyme. This has been solved as recorded in the literature [8] and applied in recent research [1]. The 2nd challenge is the determination of total interaction energy that has also been solved [8, 17]. As Table 1 shows, the R_0 value for $[S_T] > [E_T]$ is > the value for $[S_T] \ll [E_T]$. This constitutes the basis for describing $\boldsymbol{g}$ and its reciprocal variant as a variable constant, just like the acceleration due to gravity, which is not constant in every location above the Earth's surface. The effect of different locations above the Earth's surface is analogous to the effect of different concentration ranges and regimens of the substrate and enzyme. This means that different g and, consequently, different U (R_0) will always exist. Thus, g (or $1/\boldsymbol{g}$) remains a variable constant (not a pseudo-constant), given Eq. (10).

There is no doubt that rate constants, including intrinsic rate constants, are important in research, industry, biological processes etc. So far, fundamental issues that concern the generated kinetic constants, some of which are variable constants, **[functions of ??? would be appropriate here]** have been discussed and analysed. Most of them are regular features in kinetic studies, in steady-state and pre-steady-state scenarios [15, 18-20]. However, the intrinsic rate constants seem to be a recent development, with much less attention given to amylolytic enzymes. Some research activities end up as purely theoretical papers [2, 3]. This research has given quantitative effect to theoretical input on the problem of intrinsic rate constants. There is a need, however, to add that work is going on in the area of the kinetics of cellulose hydrolysis by cellulases where, in one instance, the apparent processivity was observed to be typically smaller than the intrinsic processivity defined on the basis of a theoretical model using apparent rate constants [21]; this is quite similar to the procedure in this research. In order to understand the role of the catalytic site, the catalytic domain has been modeled as a one-dimensional stochastic "walker" that may only step in the forward direction as governed by an intrinsic rate constant, [22]. The latter is given as [22]:

$$k_{\mathrm{C}}^{(0)} = \nu e^{-\Delta U(g)} \qquad (26)$$

The interest in Eq. (26) cited in the literature is in its attribution to the intrinsic rate constant, the object of this research. There is a need, however, to state that thermal energy ought to be part of the exponent such that the equation takes the form (for probable feature application):

$$k_{\mathrm{C}}^{(0)} = \nu\, e^{-\Delta U(g)/k_{\mathrm{B}}T} \qquad (27)$$

Where, ν is some transition frequency and ΔU (g) is the activation energy in units of $k_{\mathrm{B}}T$ (k_{B} and T are the Boltzmann constant and thermodynamic temperature, respectively) of the intrinsic potential U (x) at position g. The implication is that there must be a way of defining quantitatively the intrinsic rate constant that should be known as the intrinsic catalytic rate constant for the formation of product considering the Arrhenius equation. Of course, this research investigated only the intrinsic reverse and forward rate constants. That constitutes the limit of the scope of this research.

Researchers in recent times are now applying a yet to be significantly understood fractal theory to the determination of total activity coefficient and what the authors [23] refer to as an intrinsic constant as applicable to the hydrolysis of recalcitrant cellulose. How the two parameters were determined with the results is not so clear considering the two different definitions of a constant, K, given as: 1) the initial rate coefficient at t = 0 and 2) the inherent specific activity on the insoluble substrate. The first definition is connected to the equation [23] given as:

$$k = K\, t^{-\mathrm{h}} \qquad (28)$$

Where, k, t, and h are the specific activity of the enzyme, time, and fractal factor.

The second equation [23] is given as:

$$P_{\mathrm{tot}} = K\, E_{\mathrm{p}}\, t^{(1-\mathrm{h})} \qquad (29)$$

Where, P_{tot} and E_{p} are the total generated product and the productively bound enzyme, respectively. It is hoped that researchers in this and similar fields show interest and come up with better explanations of how the intrinsic rate constant, in particular, can be determined. Indeed, gelatinised insoluble starch is far more soluble than raw starch in relative terms, just as the latter is far more soluble than cellulose. The question that cannot be answered now is whether or not fractal theory and its methods can be applied to the kinetics of gelatinised insoluble starch or raw starch, considering the observation that cellulose digestion shows the same time dependence of the specific activity coefficient as described for fractal systems. Interacting with other scholarly works is important, hence these comments, analysis, and proposition or view, and thus it is instructive to write that there is another view about the intrinsic nature or property of enzymes. "The measured kinetics of an enzyme-catalysed reaction in free solution, where the enzyme is solubilised, is generally, termed intrinsic" [24]. This may imply that the apparent rate constants are also intrinsic in contrast to what is referred to as observed kinetics and cognate rate constants or coefficients, as may be applicable.

In the light of emerging interest in intrinsic rate constants in recent times, the position taken in this research is that the kinetics of enzyme catalysed reactions in which the enzyme and substrate are in solution (the homogeneous case) and the enzyme and insoluble substrate are in the reaction mixture (the heterogeneous case) are different from the kinetics of either immobilised enzyme given free substrate, be it either soluble or insoluble or immobilised substrate. Otherwise, the kinetics and cognate rate constants for the immobilised reactants, either the enzyme or substrate, are apparent or observed but may be quantitatively and perhaps qualitatively different from those of the free enzyme or substrate.

While research has shown that the intrinsic Michaelis constant of the immobilised enzyme is very close to that of the enzyme in solution, such a constant for the immobilised enzyme tending towards the value for the enzyme in solution when activity is zero [25] is incomprehensibly unusual going by the definition of Michaelis constant. To be specific, the Michaelis constant is the substrate concentration at half the maximum velocity of catalysis or catalytic activity. In the light of this research, a reference to intrinsic Michaelis constant implies that there are intrinsic reverse, forward, and catalytic rate constant given the steady equation of Michaelis constant (K_M) given as: $K_M = (k_b + k_{cat})/k_1$. However, intrinsic rate constants are typically presented in the theory of diffusion-influenced processes as purely abstract, implicitly known variables [5]. This seems to suggest that they are not directly determined as in this research, in which k_b and k_1 were calculated by fitting relevant equations to experimental or apparent rate constants. Nonetheless the issue of diffusion [3, 5, 26-28] and rates in general but with occasional emphasis on intrinsic rate constants has been investigated for various reasons, which include modeling of cellular sensing [29], characterisation of enzymes of clinical importance [4], *etc*. Recently, it has been believed that explicit knowledge of these intrinsic rates is necessary in order to simulate complex biological processes [5]. All of these go to show that the importance of intrinsic rate constants cannot be overemphasised and that there should be a way for the determination of intrinsic catalytic rate constant (k_{cat}).

5. CONCLUSION

The equations for the determination of intrinsic reverse first order and second order rate constants were re-derived. Unlike previous research findings, the intrinsic reverse first order rate (k_b) and forward second order rate (k_1) constants were higher than their apparent counterparts, but they were, however, very similar in magnitude. The intrinsic rate constants were much higher than previously reported values when enzyme's (E) total concentration [E_T] was « than substrate's total concentration [S_T]. The k_1 and apparent forward second order rate (k_f) values where [E_T] is » [S_T] is > where [E_T] is < [S_T]. Therefore, the magnitude of the second order rate constant is a function of [E_T]. The values of k_1 and k_2 where [E_T] « [S_T] and *vice-versa* were respectively, ≈ 7.41 exp. (+6) L/mol. min and ≈ 81.34 exp. (+4) /min, and ≈ 15.76 exp. (+6) L/mol. min and ≈ 58.08 (+4) /min. The probability ($1/g$) (or $r_{eq}(r)$) that an enzyme is at a distance away from the substrate with the possibility of mutual attraction has been found to be a variable constant contingent upon the concentration of the components of the reaction mixture and the affinity of the enzyme for the substrate and *vice-versa*. Feature research may attempt to derive an equation for the determination of an intrinsic catalytic rate constant for the formation of a product.

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