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Lineweaver Burk Plot, Rate Constant, and Mathematical Relationship between Molar Mass and Free Energy of Activation: "To be or not to be"

Ikechukwu Iloh Udema[1,2,3*]

[1]Research Division of Ude Concept International Limited (RC 862217), B. B. Agbor, Delta State, Nigeria.
[2]Owa Alizomor Mixed Sec. Sch., Owa Alizomor, Ika North East, Delta State, Nigeria.
[3]Idumukpo Qtr, Onicha Uku, Ubulu-Uku, Aniocha South, Delta State, Nigeria.

Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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Short Research Article

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ABSTRACT

Aims: Verify a derived kinetic parameter dependent model for the determination of Gibbs free energy of activation and consequently rate constant and to show that despite criticism against the process, Lineweaver Burk plot and the rate constant, both process and term remain indispensible for the determination of Gibbs free energy of activation for a nonstandard solution of an enzyme in particular.
Study Design: Experimental investigation involving *in vitro* assay of *Aspergillus orzyzea* alpha amylase using gelatinized soluble potato starch for test backed with control test without substrate as the blank.
Place and Duration of Study: Research Division of Ude Concept International Limited (RC: 862217), B. B. Agbor, Delta State, Nigeria. The investigation lasted for one year as part of a series of research between 2013 and 2016.
Methodology: Bernfeld method of enzyme assay was used. Controls were free from substrate.
Results: The result of investigation showed that Gibbs free energy (ΔG_a) values were 59.20±0.11

**Corresponding author: E-mail: udemaikechukwu75@yahoo.com, udema_ikechukwu99@yahoo.com;*

kJ/mol (from old model; n = 6) and 59.43±0.19 kJ/mol (from new model; n = 2) while rate constant (k_2) was 10385.08±55.13/min (n = 2) at pH, 5 and temperature, 20℃; The ΔG_a values, 57.50±0.29 kJ/mol (from old model; n = 6), 57.82±0.04 kJ/mol (from new model; n = 5), and 57.58±0.16 (from old model; n = 5) and k_2 values, 23536.77±3045.794/min(n = 6) and 20210.95±1344.04/min (n = 5) were reported at pH 6.9 and 21℃.

Conclusion: The Gibbs free energy (ΔG_a) and rate constant (k_2) values calculated using the old and new models were similar. The derived model which suggested that $\Delta G_a \propto \ln (1/M_{PROT})^2$ at a given temperature stands verified. Thus the continuous use of Lineweaver Burk plot and the kinetic parameter, k_2 as defined remains very necessary due to vital and useful results obtainable when they are used.

Keywords: Aspergillus oryzea alpha amylase; rate constant; Gibbs free energy of activation; model; molar mass.

1. INTRODUCTION

Efforts have been directed to the determination of concentration of enzyme in aqueous solution of enzymes [1–5; Udema 2016, manuscript under consideration]. Each of the method cited in literature has its merit or advantage. According to Kohl and Johnson [1] the quantitative radial diffusion slide assay for staphylocoagulase does not require purification of the enzyme but only small quantities, 7µL, are needed for each test. Furthermore, the method is said to be suitable for an objective comparison of the relative amounts of coagulase produced by different cultures of *Staphylococcus aureus* [1]. Despite the purported use of a mixture of different protease inhibitors to prevent false negative and false positive reactions in the same clotting assay, an assay based on a direct and specific measurement of staphylothrombin activity based on the use of chromogenic substrate, is regarded as a better option considering the fact that short assay time is involved and there is a possibility of screening large number of strains or staphylocoagulase samples in a quantitative way [2]. Tauschel et al. [3] method involves the use of "Reactone Red" 2 B – amylopectin as the substrate; the authors [3] reported that the logarithm of enzyme activity ranging from 1 mU/ml to 1100 U/ml is linearly correlated with the diameter of diffusion zone on agarose gel medium and no dilution of the enzyme is needed unlike method described by Udema in manuscript awaiting approval.

Another approach which also entails the creation of radial diffusion zone, requires the use of *Aspergillus oryzea* alpha amylase (EC 3.2.1.1) for the construction of standard curve to show the relationship between enzymatic concentration and the area of clear radial diffusion zones and another enzyme barley alpha amylase is used to validate the model; this is reported as an improvement on Tauschel et al. [3] method such that in the modified approach, a clear radial diffusion zone can be observed after 4 hours of incubation at 20℃ [4]. Also, the quantification of proteins through another method such as Ni^{2+} chelation technique which is seen to be generally applicable to all proteins as to eliminate the need for different assays for different proteins had been carried out by Jalen et al. [5]. The most recent hi-tech driven approach is the use of smartphone technology for the quantification of human salivary alpha amylase within 5 minutes Zhang et al. [6].

All the aforementioned methods have single function, the determination of the quantity of enzyme without precluding the measurement of activity. Beyond this, there is no evidence to proof that the methods can be used to determine rate constant, maximum velocity of amylolysis and Michaelis – Menten constant (K_m) let alone the molar mass of the enzyme. It should be made known however that various linear transformation of Michaelis – Menten equation and consequently the extrapolated parameters, K_m and V_{max} are severely criticized as being in accurate [7,8]. Recently, attempt had been made to relate molar mass of enzymes to Gibbs free energy of activation. Recent methods, electrospray/ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) has been described as techniques which have revolutionized biological mass spectrometry (MS) by which the molar mass of macromolecules are determined [9]. Once again this method cannot be used to estimate kinetic parameters.

To answer the question whether the Gibbs energy (ΔG_a) of activation significantly depends

on the molecular mass (M_{mE}) of the enzyme a single-factor analysis of variance was performed. To analyze the relationship between ΔG_a and M_{mE}, the mean group values ΔG_{ai} were plotted versus the mean group values M_{mEi}. The result clearly showed an asymptotic decrease of the activation energy with the increase in the molecular mass of the enzyme. A quantitative explanation of the phenomenon was proposed based on a developed theoretical model describing the interactions of enzyme and product which culminated in part to the equation: $\Delta G_a = \Delta U_{loc} + \phi / M_{ME}^{5/3}$ where U_{loc} is one aspect of energy of the interaction field (U_{Field}) composed of global interactions (glo) and local interactions (loc); ϕ is a coefficient [10].

The two objectives of this research were to verify a derived kinetic parameter dependent model that has a multifunctional attribute of being applicable to the determination of Gibbs free energy of activation and consequently rate constant *etc* and to show that despite "two hot elephants' criticism" against the process, Lineweaver Burk plot and the rate constant, both process and term remain indispensible for the determination of Gibbs free energy of activation for a nonstandard solution of an enzyme in particular. It is understood however, that it is impossible for now to determine ΔG_a, measured in J/mol, without knowledge of the molar mass of the enzyme. But through the model it is possible to determine ΔG_a and consequently, the rate constant (k_2) given that the molar mass of the enzyme is known. From the value of $\Delta G_a = \mathrm{In} k_B T/hk_2$ where k_B, T, and h are Boltzmann constant, absolute temperature, and Planck's constant respectively, the k_2 value and consequently the mass concentration of the enzyme can be calculated. However, there is a model according to Uludag – Demirer et al. [11] which relates what the authors called turnover number in the equation (1/[P]). $\mathrm{d}P/\mathrm{d}t = -k_3 + k_3([S]_0 - [S])/[P]$, to velocity of amylolysis and other extensive quantities, where [P], k_3, [S], $[S]_0$ and t, are concentrations of product, turnover, free substrate, total substrate, and duration of assay respectively. It is not certain whether the parameter $k_3 = V_{max}/[E]_T$.

2. BRIEF REVIEW OF EARLIER MODEL

As in the submitted manuscript the mass concentration of the enzyme ($[E]_{TMC}$) is:

$$[E]_{TMC} = V_{max} M_{ALT} / \boldsymbol{S_2}\, M_{PROT} \quad (1)$$

where V_{max} represents maximum velocity of hydrolysis and M_{ALT}, and M_{PROT} are the molar mass of the product, maltose, and protein or the enzyme. The second slope ($\boldsymbol{S_2}$) is obtained by plotting $[S^2]/([S]+K_m)$ which is represented by β versus the first slope ($\boldsymbol{S}$) which is the first slope obtainable when velocity of hydrolysis (v) values are plotted versus the reciprocal of the number of times (otherwise called dilution factor (d_f)) the stock solution of the enzyme is diluted. If v is less than V_{max} then $V_{max} = v\,(K_m + [S])/[S]$. The highest velocity $\boldsymbol{S}$ could be in place of v.

$$[E]_{TMC} = (K_m + [S]) v M_{ALT} / \boldsymbol{S_2}\, M_{PROT}\, [S] \quad (2)$$

It may also be the stock mass concentration if Eq. (3) below is used.

$$[E]_{TMC} = (K_m + [S])\, \boldsymbol{S}\, M_{ALT} / \boldsymbol{S_2}\, M_{PROT}\, [S] \quad (3)$$

It should made clear that $\boldsymbol{S}$ as defined is not equal to $V_{max,}$, rather it is the highest velocity obtainable if the stock solution of the enzyme is assayed.

3. DERIVATION OF THE MODEL LINKING MOLAR MASS TO FREE ENERGY OF ACTIVATION FOR PRODUCT RELEASE

Since Eqs (1), (2) and (3) define total mass concentration of the enzyme it can be converted to number of moles if divided by M_{PROT}. Hence,

$$[E]_{TMC} / M_{PROT} = [E]_T = V_{max}\, M_{ALT} / \boldsymbol{S_2}\, M_{PROT}^2 \quad (4)$$

where $[E]_T$ is the molar concentration of the enzyme. From Eq (4),

$$M_{PROT}^2 = V_{max}\, M_{ALT} / \boldsymbol{S_2}\, [E]_T = k_2 M_{MALT} / \boldsymbol{S_2} \quad (5)$$

Meanwhile,

$$k_2 = k_B T \exp(-\Delta G_a / RT) / h \quad (6)$$

Substituting Eq(6) into Eq(5) gives after rearrangement

$$M_{PROT}^2 = k_B T \exp(-\Delta G_a / RT)\, M_{ALT} / h\, \boldsymbol{S_2} \quad (7)$$

Further rearrangement of Eq (7) gives

$$\exp(-\Delta G_a / RT) = M_{PROT}^2\, \boldsymbol{S_2}\, h / M_{ALT} k_B T \quad (8)$$

Natural logarithm of Eq (8) gives

$$-\Delta G_a / RT = \mathrm{In}\,(M_{PROT}^2 \boldsymbol{S_2} h / M_{ALT} k_B T) \quad (9)$$

Further rearrangement gives

$$\Delta G_a = RT\ln (k_B M_{ALT} T/h \boldsymbol{S_2} M_{PROT}^2) \quad (10)$$

4. MATERIALS AND METHODS

4.1 Materials

Chemicals: *Aspergillus oryzea* alpha amylase (EC 3.2.1.1) and soluble potato starch were purchased from Sigma – Aldrich, USA. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd, Poole England. Tris 3, 5 – dinitrosalicylic acid, maltose, and sodium potassium tartrate tetrahydrate were purchased from Kem light laboratories Mumbia, India. Distilled water was purchased from local market.

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.

4.2 Methods

Stock solution of soluble potato starch was prepared by mixing 1 g in 100 ml of distilled water and subjected to heat treatment at 100℃ for 3 minutes, cooled to room temperature, and decrease in volume due to evaporation was corrected by topping the volume with distilled water to 100 ml to give 1.0 g%. Assay of the enzyme was carried out according to Bernfeld method [12] and kinetic parameters such as Michaelis – Menten constant and maximum velocity of hydrolysis were determined according to Lineweaver – Burk method [13]. Spectrophotometer readings for the determination of amount of maltose yielded were taken at 540 nm and the extinction coefficient was 181.1/M.cm.

4.3 Statistical Analysis

All values are expressed as mean ± SD. Microsoft Excel was used to calculate SD.

5. RESULTS AND DISCUSSION

As Table 1 shows, the k_2, values obtained from different means were very similar. Such mean values involve the use of V_{max} obtained from $\boldsymbol{S}([S]\pm K_m)/[S]$ and directly from the plot of $1/v$ and $1/\boldsymbol{S}$ versus $1/[S]$ as described under Table 1. Also under Table 1 are similar mean values of ΔG_a obtained from two approaches. The report in Table 2 showed similar values for k_2 and for ΔG_a. However, the mean k_2 values 23536.77±3045.794/min and 20210.95±1344.04/min are shown under Table 1 at pH 6.9 and 20℃ and 10385.08 ±455.13/min at pH 5 and 20℃ under Table 2. The vital factor, the second slope ($\boldsymbol{S_2}$) was determined by plotting β *i.e.* $[S]^2/([S]+K_m)$ versus the first slope ($\boldsymbol{S}$) obtained by plotting v versus reciprocal of dilution factor.

But these values are much lower than the jumbo value (10^5/min) reported for amylase by Butterworth et al. [14]. However, the values obtained from this research fall within the range 10 – 1000/s (600 – 60000/min) cited by Gao et al. [15]. In order to achieve the objectives of this investigation data were generated according to well known methods. Incidentally the data must be substituted into Lineweaver Burk equation so that a double reciprocal plot can generate relevant kinetic parameter needed for the verification of a model which relates Gibbs free energy of activation to molar mass of an enzyme. Unfortunately Lineweaver plot had been battered with a barrage of criticism. For instance, the Lineweaver-Burk transformation had been reported to give a deceptively "good" fit, even with unreliable points [7]. "The marked inferiority of the Lineweaver-Burk plot strongly suggests that it should be abandoned as a method for estimating K_m and V_{max} from unweighted points, whether the points are fitted by eye or by the method of least squares. The undeserved popularity of the Lineweaver-Burk method may well be based upon just this ability to provide what seems to be a good fit even when the experimental data are poor" [7]. The good fit resulting from the plot of $1/v$ versus $1/[S]$ [7] always implies that there is high positive correlation coefficient between the two variables even though a "scattergram" resulting from irregular trend in the increasing values of v or its trend towards nonlinearity is the case. What might account for poor experimental data should be of major concern. Since v values are plotted versus [S], the smaller concentration within the concentration range of the substrate used may give rise to error if within the duration of assay such concentration is insufficient for the same concentration of enzyme assayed with much higher [S]. Such scenario could result to $1/v$ being disproportionately higher than $1/v$ for other higher [S]. This could result in much smaller intercept or negative intercept, $1/V_{max}$.

Table 1. Determination of free energy of activation of *A. Oryzea* alpha amylase at pH 6.9 and room temperature (21℃), on the basis of the new an d old model

[S] (g/l)	1ST SLOPE(*S*) (mU/ml)	k_2	*R*	ΔG_a (kJ/mol)
5	7990.2±537.8	22667.12	0.999	57.57
6	9145.0±485.0	29654.01	0.9899	56.91
7	9968.0±301.9	21689.69	0.996	57.68
8	11040.0±92.0	21742.07	0.997	57.69
9	12360.0±671.8	22356.11	1	57.60
10	13755.0±132.2	23111.60	0.9995	57.52
PREP [E] (mg/l)	1.67	2.5	5.00	10.00

*Prep represents prepared concentration of enzyme (weighing and dissolution in buffer). The value of $[E_o]$ (stock) is 100mg/l. Each solution of the enzyme except stock solution was assayed at each concentration of substrate. Michaelis – Menten constant K_m (22.09±9.31g/l) (n = 2) used is obtained by extrapolation from the plot of 1/**S** versus 1/[S]. **S** is plotted against β to give another slope S_2. The value of S_2 is (2474.5±33.9) U/g; Empirical values are presented as mean ± S.D. **N.B.:** Units/ml = micromoles maltose yielded/1ml enzyme × 3min. The original unit of v, V_{max}, and **S** is mmol/ml/min (or M/ml/min). k_2 = Mean ±S.D. = 23536.77±3045.794/min (n = 6); Calculated ΔG_a value according to old model i.e. Eyring – Polanyi model, $\Delta G_a = RTIn(k_BT/hk_2)$ = 57.50±0.29kJ/mol(obtained using k_2 from V_{max} obtained from **S**([S]+K_m)/[S]; n = 6); ΔG_a value according to New Model-Eq.(10) = 57.82±0.04 kJ/mol; n =2. ΔG_a obtained using all k_2 values from all direct V_{max} values obtained from the plots of 1/v and 1/**S** versus 1/[S] = 57.58±0.16kJ/mol. The average of such k_2 values was 20210.95±1344.04/min; n = 5(number of different concentrations of enzyme)*

Table 2. Determination of free energy of activation of *A. oryzea* alpha amylase at pH 5 and room temperature (20℃), on the basis of the new an d old model

[S] (g/l)	1ST SLOPE(*S*) (mU/ml)	k_2	*R*	ΔG_a (kJ/mol)	
4	2913.5±399.5	10288.79	0.990	59.22	
5	3402.0±267.3	9967.37	0.9899	59.30	
6	4296.0±407.3	10863.78	0.996	59.09	
7	4565.0±264.5	10236.35	0.997	59.23	
8	4901.0±351.4	9936.83	0.989	59.30	
9	5922.0±606.7	11017.34	0.996	59.05	
PREP [E] (mg/l)	2.0	2.5	5.0	10.0	20.0

*Abbreviations, parameters, preparation of solution of the enzyme, and the determination of kinetic parameters are as stated under Table 1 and previously defined in the text and methods subsection. The value of $[E_o]$ (stock) is 100mg/l. Michaelis – Menten constant K_M =22.978±8.314g/l. The value of S_2 is 1189±92 U/g; n = 2; Results are presented as described under Table 1. The data on **S** values (Udema, submitted manuscript) were presented in the submitted manuscript to Journal of scientific Research and Report. The data were used as supporting evidence to multiple applicability of the model. k_2 = 10385.08±455.13/min; n = 2; $\Delta G_a = RTIn(k_BT/hk_2)$ = 59.20±0.11kJ/mol (obtained using k_2 from V_{max} obtained from **S**([S]+K_m)/[S]; n=6). ΔG_a value according to New Model-Eq. (10) = 59.43±0.19kJ/mol; n=2*

For linearity, [E] should be sufficiently low [14] and such linearity is most probable at much earlier part of the reaction. Hence the duration of such assay must not be longer than 3 minutes so as avoid substrate exhaustion even at low [E] and probable premature product inhibition. In agreement with this position is the assertion that standard quasi steady state approximation (SQSSA) as a basis for the determination of kinetic parameters is only valid when the enzyme concentration is much lower than either the substrate concentration or Michaelis – Menten constant (K_m) [16]. It is believed however, that such condition is too strong or stringent and the SQSSA is in fact valid providing that:

$$[E_T]/([S_T] + K_m) \ll 1 \quad (11)$$

Support for this position includes the suggestion that enzyme preparations are diluted so that no more than 44% of the starch will be hydrolyzed in the allotted time [17].

For both parameters, K_m and V_{max}, when the error is small (SD = 0.05), all the methods are extensively accurate (i.e. close to the ''true'' value) and precise (i.e. small standard deviations, small variances) [8]. Therefore, only at linear portion of the curve could error be minimal or almost nonexistent. However, as the magnitude of the error increases, the limitations

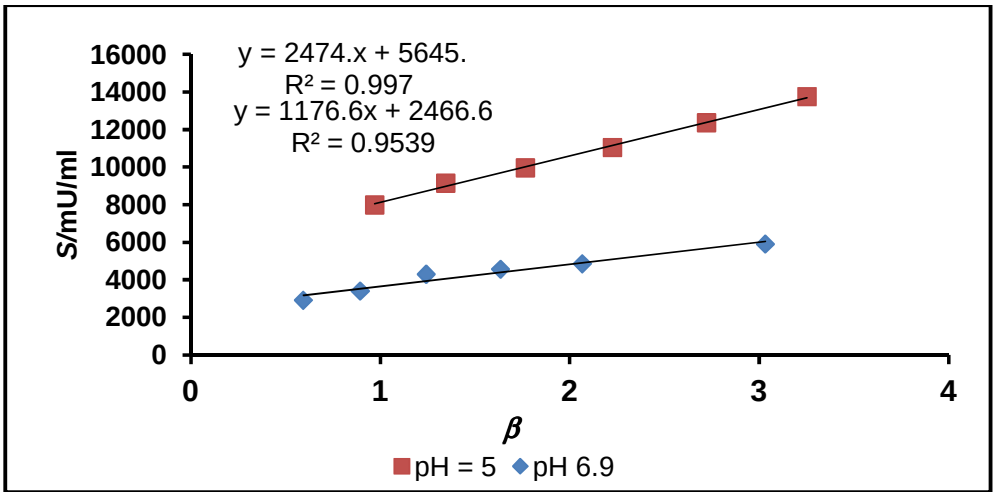

Fig. 1. Determination of second slope (S_2): a plot of first slope (S) versus $[S]^2/([S]+K_m)$

of the graphical methods are highlighted. One may need to add that sources of error are pipetting, measurement of volume *etc* [18]. Whilst nonlinear regression proves to be the most accurate and precise method of calculation, the double reciprocal plot is clearly the worst procedure both in terms of accuracy and precision [8]. The point of view is that unweighted nonlinear regression is the most reliable method for determining enzyme kinetic parameters. On the other hand, when used without a weighting factor, double reciprocal plot is the less satisfactory procedure [8]. The authors [8] seem to question how one would expect correct linearity in double reciprocal curve without error when the enzymes' active sites are totally occupied by the substrate, a situation that gives hyperbolic curve when a plot of v versus [S] is carried out.

The issue to ponder about is that no one is sure if double reciprocal plot at [S] < K_m can give upon extrapolation accurate V_{max} and K_m if compared with values obtained from nonlinear plots. Nonetheless, as the result from this research showed, there is very strong agreement between the values of ΔG_a obtained from the old and new model; the old being dependent on k_2 and the new being dependent on $\boldsymbol{S_2}$, put to question the barrage of criticism leveled against linear regression method for the determination of first V_{max} and then k_2. It is therefore, inconceivable to jettison the continuous use of linear regression in the light of current results in support, however, of the advice that students should continue the use of graphical methods in enzyme kinetics since like this result, they provide information of great value both to monitor the pattern of the experimental data during their measurement and, in particular, as a powerful diagnostic tool in inhibition studies [8].

This paper is in two parts, one of which is the determination of "rate constant" (also called turnover number by Butterworth *et al*. [14] which for the purpose of this investigation is the rate of formation of product and release of such product as detected by visible spectrophotometer at a wavelength of 540nm. Tables 1 and 2 show data for rate constant (k_2). This definition of k_2 notwithstanding, there has been recent criticism of the term rate constant as being inappropriate. For instance there is proposition against the use of the terms "turnover rate" and "catalytic constant" (k_{cat}, perhaps, k_2) in an interchangeable manner [19]. Besides "turnover frequency" (TOF) reportedly called turnover number, N, defined as molecules reacting per active site in unite time is different from N [19]. TOF is also implicated to have been wrongly used as a rate constant since the rate of reaction (r = TOF $\times$ [Cat] where definitely [Cat] is the concentration of the enzyme.) depends on the catalyst (enzyme) concentration [19]. But this is very similar to the usual relationship between V_{max} and the so called rate constant, k_2 (V_{max} = $k_2[E]_T$). Besides, TOF from strict terminological point of view is a frequency, with units of 1/time and could depend on concentration of reactants and products even at saturation, and in this sense it is closer to a rate than to a kinetic constant [19]. It seems the terms rate constant and kinetic constant are different despite the exemplification of kinetic parameters as K_m, V_{max}, k_2, and k_2/K_m.

Incidentally, another researcher has his/her definition of turnover rate (k_{turn}). The parameter k_{turn} is defined as the reciprocal of the mean turn over time $\langle T_{turn} \rangle$ - the average time it takes a single enzyme to produce a single molecule of product [20]. This definition is similar to the definition stated earlier because $1/k_2$ is at least dimensionally similar to $\langle T_{turn} \rangle$. The earlier definition is therefore "a working definition" for this investigation. Moreover, Reuveni et al. [20], defined a relationship between k_{turn} and k_2 as:

$$k_{turn} = k_{cat}\,[S]/([S] + K_m) \quad (12)$$

$$k_{cat}\ (\text{or } k_2) = ([S] + K_m)/[S]\,\langle T_{turn} \rangle \quad (13)$$

It is obvious that $k_{cat.} > k_{turn}$. A close look at Eq (13) reveals something similar to Michaelis – Menten formalism. If velocity v of hydrolysis of gelatinized starch as in this investigation is plotted directly versus concentrations of the enzyme $[E_T]([E_T]>[ES])$, so long as E is sufficiently dilute, a straight line graph should be expected; the slope (k_s) should be a constant of proportionality similar to k_{cat} (k_2) in terms of the mathematical relationship between v (and $\boldsymbol{S}$ in particular, for the purpose of this research in which $k_s = \boldsymbol{S}/[E]$) and [E]; but $k_s < k_{cat}\,(V_{max}/[E_T])$ because at each [E] or $1/d_f$, $v < V_{max}$ (just as maximum velocity obtainable from the plot of $1/\boldsymbol{S}$ versus 1/[S] is » $\boldsymbol{S}$). Thus the following could be a possibility in the light of Eq (13) according to Reuveni et al. [20]: $k_{cat} = ([S] + K_m)\,v/[S][E_T] = ([S] + K_m)k_s/[S]$. It seems therefore, that $k_s = 1/\langle T_{turn} \rangle$. From the foregoing, issues for and against k_2, a choice as to the continuous use of it should not be out of place for the simple reason that its dimension is of the nature of frequency and its application in this investigation gives the expected result "just as two wrongs cannot give correct output and two accurate formulation for the same parameter cannot give widely different solution".

Gibbs free energy of activation of hydrolysis of starch is very important because it enables one to predict the capacity and effectiveness of an enzyme to catalyze the hydrolysis of its substrate. However, both substrate and enzyme can affect the rate of hydrolysis of the substrate as it is known that different sources of starch present different rate of hydrolysis by the same enzyme [21]. Thus the resistance offered by the nature of the starch for instance to the hydrolytic action of the enzyme constitute an "energy barrier" to which according to Low et al. [22] an input of free energy of activation facilitates the reduction of the magnitude of the energy barrier. The values reported in this research for *Aspergillus oryzea* alpha amylase, at lower *p*H and temperature, are higher than values reported for the hydrolysis of native (52.2 kJ/mol), gelatinized (heat treatment at 100℃) (45.17 kJ/mol), native normal rice (46.62 kJ/mol), and gelatinized normal rice at 100℃ (46.67 kJ/mol), at higher *p*H and temperature with 0.81nM porcine alpha amylase [21]. Therefore, investigation into what the magnitude of free energy of activation should be for any enzyme cannot be a waste of time and resources. This research clearly verifies in a different way, the claim that the result of an investigation clearly showed an asymptotic decrease of the activation energy with the increase in the molecular mass of the enzyme [10]. This is very apparent from Eq. (10) which upon rearrangement gives $\Delta G_a = RT\ [\ln(k_B T M_{ALT}/\boldsymbol{S_2} h) - 2\ln M_{PROT}]$. However, looking at the latter equation, $-2\ \ln M_{PROT}$ is equivalent to $2\ \ln(1/M_{PROT})$. Therefore, with increasing molar mass of different homologues of the enzyme, there may be decreasing values of ΔG_a. Therefore, at a definite temperature, the free energy of activation varies linearly with the natural logarithm of the molar mass of the enzyme.

6. CONCLUSION

A new mathematical model was derived for the determination of Gibbs free energy of activation (ΔG_a) for the hydrolysis of heat treated soluble potato starch at 100°C. The calculated ΔG_a value of the enzyme based on the new model was very similar to value obtained according to the old model, Eyring – Polanyi model. The values of ΔG_a from new model and from the old were used to calculate the rate constant (k_2). The k_2 values obtained were not largely different. The implication is that an unknown concentration of an enzyme, as may be case for a crude extract or nonstandard solution of the enzyme, can then be determined, given the value of V_{max} (which does not require initial information about the mass or molar concentration of the enzyme) because $[E_T] = V_{max}/k_2$. Thus the model which suggests that $\Delta G_a \ \alpha\ 2\ln 1/M_{PROT}$ at a given temperature stands verified.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Kohl JD, Johnson RS, Quantitative radial diffusion slide assay for staphyloco-agulase. Appl Environ Microbiol. 1980;39(2):339 – 341.
2. Engels W, Kamps MA, van Boven CP, Rapid and direct Staphylocoagulase assay that uses a chromogenic substrate for identification of *Staphylococcus aureus.* J. Clin. Microbiol. 1981;14(5):496–500.
3. Tauschel HD, Rudolph C. A new sensitive radial diffusion method for micro-determination of alpha amylase. Anal. Biochem. 1982;120(2):262–266.
4. Farias DF, Carvalho AFU, Oliveira CC, Sousa NM, Rocha-Bezerrra LCB, Ferreira PMP, et al. Alternative method for quantification of alpha amylase activity. Braz. J. Biol. 2010;70(2):405–407.
5. Jalen Š, Smilović V, Fidler K, Podobnik B, Marušić M, Komel R, et al. New assay for quantification of PEGylated proteins during *in vitro* permeability studies. Acta. Chim. Slov. 2014;61:615–622.
6. Zhang L, Yang W, Yang Y, Liu H, Gu Z, Smartphone-based point-of-care testing of salivary α-amylase for personal psycho-logical measurement. Analyst. 2015; 140(21):7399–7406
7. Dowd JE, Riggs D. A comparison of estimates of Michaelis-Menten kinetic constants from various linear transfor-mations. J. Biol. Chem. 1965;240(2):863–869.
8. Ranaldi F, Vanni P, Giachett E. What students must know about the deter-mination of enzyme kinetic parameters. Biochemical Education. 1999;27:87–91.
9. Strupat K, Molecular weight determination of peptides and proteins by ESI and MALDI. Methods Enzymol. 2005;405:1–34.
10. Pawlowski PH, Zielenkiewicz P. Theoretical model explaining the relationship between the molecular mass and the activation energy of the enzyme revealed by a large-scale analysis of bioinformatics data. 2013;60(2):239–247.
11. Uludag – Demirer S, Duran J, Tanner RD, Estimating the turnover number in enzyme kinetic reactions using transient and stationary state data. Braz. J. Pharm. Sci. 2009;45(4):636–642.
12. Bernfeld P. Amylases, alpha and beta. Methods. Enzymol. 1955;1:149–152.
13. Lineweaver H, Burk D. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 1934;56(3):658–666.
14. Butterworth JP, Warren FW, Ellis PR. Human alpha amylase and starch digestion: An interesting marriage. Starch/Stärke. 2011;63:395–405.
15. Gao J, Ma S, Major DT, Nam K, Pu J, Truhlar DJ. Mechanisms and free energies of enzymatic reactions. Chem. Rev. 2006;106:3188-3209.
16. Schnell S, Maini PK. Enzyme kinetics far from the standard quasi-steady-state and equilibrium approximations. Math. Comput. Model. 2002;35:137–144.
17. Huggins H, Russell PS. Colourimetric determination of amylase. Ann. Surg. 1948;128(4):668–678.
18. Marini I. Discovering an accessible enzyme: Salivary α - amylase. Biochem. Mol. Biol. Edu. 2005;33(2):112–116.
19. Kozuch S, Martin JML. “Turning Over” definitions in catalytic cycles. ACS Catal. 2012;2:2787−2794.
20. Reuveni S, Urbakhc M, Klafterc J. Role of substrate unbinding in Michaelis–Menten enzymatic reactions. Proc. Natl. Acad. Sci. U.S.A. 2014;1–6.
21. Slaughter SL, Ellis PR, Butterworth PJ. An investigation of the action of porcine pancreatic alpha amylase on native and gelatinized starches. 2001;1525:29–36.
22. Low PS, Bada JL, Somero GN. Temperature adaptation of enzymes: Roles of the free energy, the enthalpy, and entropy of activation. Proc. Natl. Acad. Sci. U.S.A. 1973;70(2):430–432.

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