Journal of Scientific Research & Reports
10(3): 1-10, 2016; Article no.JSRR.24321
ISSN: 2320-0227

SCIENCEDOMAIN *international*
www.sciencedomain.org

Derivation of Kinetic Parameter Dependent Model for the Quantification of the Concentration and Molar Mass of an Enzyme in Aqueous Solution: A Case Study on *Aspergillus oryzea* α-amylase

Ikechukwu Iloh Udema[1*]

[1]*Owa Alizomor Mixed Sec. Sch. Owa Alizomor, Ika North East/Ude International Concepts Ltd. (862217), B. B. Agbor, Delta State, Nigeria.*

Author's contribution

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

Article Information

DOI: 10.9734/JSRR/2016/24321
Editor(s):
(1) Surapong Pinitglang, Department of Food Science and Technology, School of Science and Technology, University of the Thai Chamber of Commerce, Thailand.
Reviewers:
(1) Eliton da Silva Vasconcelos, Federal University of Sao Carlos, Brazil.
(2) S. Thenmozhi, Periyar University, India.
Complete Peer review History: http://sciencedomain.org/review-history/13403

Original Research Article

Received 14th January 2016
Accepted 6th February 2016
Published 24th February 2016

ABSTRACT

Aim: The objective of the research was to verify a simple mathematical model for the determination of mass concentration and the molar mass of an enzyme in any solution using standard solution of *Aspergillus oryzea* alpha amylase and the action of *A. oryzea* alpha amylase on heat treated soluble potato starch.
Study Design: Experimental.
Place and Duration of Study: Chemistry & Biochemistry Department, Research Division of Ude International Concepts limited (RC: 862217). The research lasted for about 4 months between May and Sep, 2015, on a non – continuous basis.
Methodology: Bernfeld method of enzyme assay was used to generate data on catalytic activity of *A. oryzea* alpha amylase. One stock solution of the enzyme was prepared by dissolving 0.01 g of the enzyme in 100 ml of tris – HCl buffer, at *p*H 5 and temperature 20℃ and at pH 6.9 and temperature 37℃. In each case the stock solutions were di luted to the desired concentrations.
Results: The calculated mass concentration (Emp[E]) and the calculated molar mass (EmpM_{PROT})

**Corresponding author: E-mail: ikechukwu_udema99@yahoo.com, udemaikechukwu75@yahoo.com;*

of the enzyme were 107.99±7.32 mg/l and 54.58±4.87 kDa respectively at *p*H, 6.9 and temperature, 37℃ (all mean values were from 12 cal culations); The Emp[E] and EmpM_{PROT} values were 101.47±8.06 mg/l and 51.68±5.98 kDa respectively, at *p*H 5 and temperature 20℃ (all mean values were from 11 calculations).

Conclusion: The model can be used to determine the mass concentration of the enzyme in its crude extract and as a corollary, its molar mass can also be determined given accurate concentration and the strict adoption of the protocol described.

Keywords: Aspergillus oryzea alpha amylase; model formulation; maximum velocity; mass concentration calculation; molar mass calculation.

ABBREVIATIONS

Prot, protein; Prep, prepared; Emp, empirical; SD, standard deviation; QSSA, quasi steady state approximation; SQSSA, standard quasi steady state approximation

1. INTRODUCTION

Those who identifies with the truth, agree with the truth, and do not assume perfection are sometimes greater than the one that speaks the truth otherwise Mendel law of inheritance and other laws generated in similar circumstance may not have seen the light of the day; besides anyone who had never made mistake may after all not have discovered anything (A. Einstein).

Enzyme preparation leading to its purest state may be useful where the determination of primary structures and higher structures are of interest apart from other areas where the highest degree of purity is demanded. Industrialists have one thing in common and that is production at minimum cost so as to make reasonable profit. Therefore, where use of enzyme is applicable, its use in its purest state for large scale production of any commodity of interest may be at a prohibitive cost. Thus research in the past attempted to create a method for the quantification of enzyme concentration in its solution. Such singular objective is by no means an easy task, the belief in the simplicity of the process notwithstanding.

The principle of radial diffusion in substrate containing agar gel has been applied for the quantification of several enzymes [1,2]. Instead of agar as supporting medium Kohl and Johnson [3] used slide for the assay of staphylocoagulase. These do unlike Michaelis and Menten, who according to Johnson and Goody [4], had no way of knowing the enzyme concentration in their experiments; so all references were to relative amounts of enzyme added to the reaction mixtures.

Therefore, this research focuses on one objective, the serialized verification of a mathematical model for the determination of mass concentration and the molar mass of an enzyme in any solution using standard solution of *Aspergillus oryzea* alpha amylase and the action of *A. oryzea* alpha amylase on heat treated soluble potato starch. The reproduction of the mass concentration of the enzyme whose mass concentrations are known implies that the mass concentration of unknown concentration of the same enzyme (as in crude extract for instance) may be estimated with high degree of accuracy using the model. Use of known concentration of *Aspergillus oryzea* alpha amylase to verify or validate the model is therefore, relevant. While the kinetic parameter is vital to the verification of model, the scope of the investigation is strictly limited to the reproducibility of the mass concentration and molar mass of the enzyme.

1.1 Theoretical Background

Retrospective view of issues regarding models for the determination of kinetic parameters for enzyme catalyzed reaction has become very imperative in the light of mild criticism of Michaelis–Menten model and its transformations both of which are key models for the estimation of the concentration of enzyme in standard and non–standard solutions. Models for the quantification (and perhaps interpretation or analysis) of biochemical reactions and its outcome was formulated by earlier investigators some of which are, Michaelis and Menten [5] and Briggs and Haldane [6] who improved the work of earlier workers. Indeed, the work of Briggs and Haldane [6] contributed

to the formulation of Michaelis – Menten equation.

The model formulation takes into account a reaction in which a substrate S binds an enzyme to reversibly yield a complex called enzyme – substrate complex ES (or C for short) which, according to Hinch and Schnell [7] was first proposed in purely kinetics contest by Brown [8]. The complex can break down irreversibly to product (P) and free enzyme (E) which is free to undertake another catalytic cycle. The whole process is schematized as follows.

$$E + S \rightleftharpoons C \rightarrow E + P \quad (1)$$

In Eq. (1), the rate of formation of C is given as:

$$d[C]/dt = k\,[E]\,[S] \quad (2)$$

where k is the second order rate constant for the formation of C, t is the duration of assay, and [E] is the concentration of the free enzyme, while [S] is the concentration of the free substrate. The velocity (v_{-1}) of dissociation of C to E and S is given as:

$$v_{-1} = k_{-1}\,[C] \quad (3)$$

where k_{-1} is the first order rate constant for the dissociation of C to free E and S. The velocity (v) of product formation is given as:

$$v = k_2\,[C] \quad (4)$$

where k_2 is the rate constant for the formation of product.

In deriving what has been called Michaelis – Menten equation, it is assumed that [C] is approximately constant, often a brief transient phase. This is described as Briggs and Haldane approximation or standard quasi – steady state approximation [6] which suggests that the time scale at which the substrate is being consumed (or product is being formed), the concentration of enzyme-substrate complex is essentially not changing or $d[C]/dt \approx 0$ [9 – 11]. Besides, 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) [12]. It is believed however, that such condition is too strong or stringent and the classical QSSA is in fact valid providing that:

$$[E_T]/([S_T] + K_m) \ll 1 \quad (5)$$

This claim is however, in line with the procedure to be adopted for the determination of the concentration of enzyme in any solution.

The solution, be it standard solution used for verification/validation of model or non – standard solution of the same enzyme, the solution must be subjected to serial dilution such that the molar concentration of the enzyme should be lower than the molar concentration of the substrate if its molar mass is known. However, most soluble starch (though the so – called starch in this research was a mere suspension before gelatinization or heat treatment) may be modified starches with smaller molar mass than the parent native starches.

In the past, during the era of Michaelis – Menten, the authors laid the foundation upon which further research and improvement were carried out leading to the formulation of what became Michaelis – Menten equation. Nevertheless they showed that the velocity of enzyme catalyzed reaction was proportional to the relative amount of the enzyme [4]. While the usual practice is to plot v versus [S], the new model entails a plot of v versus reciprocal of dilution factors (d_f) in which the slope is equal to the velocity of hydrolysis of starch (or activity) at the highest concentration of the enzyme. This is akin to the determination of maximum velocity of catalysis without direct measurement, following assay of the enzyme. Thus when different velocities of enzymatic hydrolysis of gelatinized soluble potato starch is plotted against the reciprocal of different but corresponding dilution factors ($d_f > 1$), a straight line graph is created which shows that there is a linear relationship between the velocities and the reciprocal of d_f. For the avoidance of doubt, d_f is the number of times the final total volume of any solution is larger than unit volume of the stock being diluted. The slopes of such graphs can be used to determine the maximum velocity (V_{max}) and Michaelis – Menten constant (K_m) for the enzyme at its highest concentration by plotting the reciprocal of the slope versus reciprocal of the concentration of the substrate.

Thus, the slope is in line with Eq. (6) below.

$$v = k_r\,[E]/d_f \quad (6)$$

where k_r is a rate constant less than k_2. So, $k_r\,[E]$ is the slope, ***S*.**

For a given mass concentration of the enzyme and substrate, there should be an expression relating the total mass concentration ($[E]_{TMC}$) of the enzyme and the substrate concentration given as:

$$[E]_{TMC} = \zeta[S] \quad (7)$$

where ζ is a dimensionless proportionality constant. Equation (7) suggests that for any given concentration of the enzyme there should be a minimum starting – concentration of the substrate. The mass concentration of the enzyme can be converted to molar concentration. Thus,

$$[E]_T = \zeta[S]/M_{PROT} \quad (8)$$

where, as usual, $[E]_T$ and M_{PROT} are the molar concentration and molar mass of the enzyme respectively. Meanwhile, another Michaelis – Menten based equation for $[E]_T$ is:

$$[E]_T = ([S] + K_m)[ES]/[S] \quad (9)$$

But, [ES] is equal to v/k_2 such that,

$$[E]_T = ([S] + K_m)v/[S]k_2 \quad (10)$$

Equations (8) and (10) are similar and so,

$$\zeta[S]/M_{PROT} = ([S] + K_m)v/[S]k_2 \quad (11)$$

Rearrangement of Eq(11) gives:

$$v = \zeta k_2[S]^2 /M_{PROT}([S] + K_m) \quad (12)$$

By plotting v against β values that is $[S^2]/([S] + K_m)$, a new slope ($\boldsymbol{S_2}$) is given as:

$$\boldsymbol{S_2} = \zeta k_2/M_{PROT} \quad (13a)$$

$$\boldsymbol{S_2} = k_{2x}/M_{PROT} \quad (13b)$$

where k_{2x} is equal to ζk_2 and from Eq. (13b),

$$k_{2x} = \boldsymbol{S_2}M_{PROT} \quad (14)$$

Meanwhile, for the purpose of identification or operation, k_{2x} (Eq. 14) is defined as mass – mass rate constant. However, the operational use of k_{2x} is far more important than its, though convincing, but wordy definition. This is intended to preclude chanced based evolutionary outcome of the use of the model as opposed to well intended and thought out procedure that may yield fairly consistent and reproducible result. However, it should be emphasized that kinetic parameters such as v, K_m, V_{max} *etc* for the same concentrations of enzyme at the same *p*H, may differ due to differences in rate of heating and duration of heat treatment of starch. Nonetheless, the model may be used to determine every concentration used with reasonable degree of accuracy regardless of those differences in kinetic parameters that may be observed when the assay is repeated using the same set of different concentrations of the enzyme and substrate or another starch from different source.

Meanwhile, it is usual to define total molar concentration of the enzyme, $[E]_T$ as:

$$[E]_T = V_{max}/k_2 \quad (15)$$

Multiplication of both sides of Eq (15) by M_{PROT} gives mass concentration of the enzyme. However, the value of $[E]_{TMC}$ can also be defined in terms of mass concentration of the product of hydrolysis. This is where k_{2x} or Eq (14) becomes relevant as follows:

$$[E]_{TMC} = v_x M_{ALT}/k_{2x} \quad (16)$$

where v_x represents any velocity of hydrolysis and M_{ALT} is the molar mass of the product, maltose. In other words v_x could be V_{max} or less. If it is less than V_{max}, the equation becomes:

$$[E]_{TMC} = (K_m + [S])v_x M_{ALT}/\boldsymbol{S_2}M_{PROT}[S] \quad (17)$$

Equations (16) and (17) are not unreasonable because a careful look at Eqs (7) and (8) will unfold the fact that $[E]_{TMC}/[S]$ or $M_{PROT}[E]_T/[S]$ gives a dimensionless parameter, ζ in which $[E]_{TMC}$ and [S] are entirely different quantities. Also, $\{[E]_{TMC}/M_{ALT}\ t\ v_x\}$ yields dimensionless parameter, $1/tk_{2x}$, where t is a clear – cut duration of assay. Once again mass concentration of the product is totally different from mass concentration of the enzyme. Equation (17) can be transformed to give Eq (18) using Eq (14) in place of k_{2x} for the calculation of molar mass of the enzyme

$$M_{PROT} = (K_m + [S])v_x M_{ALT}/\boldsymbol{S_2}[S][E]_{TMC} \quad (18)$$

2. MATERIALS AND METHODS

2.1 Chemicals

Aspergillus oryzea alpha amylase (EC 3.2.1.1), soluble potato starch, and Bovine serum albumin (BSA) were purchased from Sigma – Aldrich,

USA. Hydrochloric acid, sodium hydroxide, sodium chloride, and copper (II) sulphate pentahydrate 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, while potassium iodide was purchased from Merck Germany. Distilled water was purchased from local market.

2.2 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.

2.3 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 – 6 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%. Different concentrations of the substrate used in all assays were prepared by adding different volumes of distilled water, 6 ml, 5 ml, 4 ml, 3 ml, 2 ml, and 0.0 ml to 4 ml, 5 ml, 6 ml, 7 ml, 8 ml, and 10 ml of heat treated starch respectively. The *p*H of tris – HCl buffer changes with temperature and this may affect the solubility of the substrate. This informed the need to use a neutral solvent to prepare different solutions of the gelatinized substrate.

One stock solution of the enzyme was prepared by dissolving 0.01 g of the enzyme in 100 ml of tris – HCl buffer, whose *p*H is 5. Different concentrations of the enzyme were prepared by serial dilution of its stock solution by 50-, 40-, 20, 10-, and 5-fold dilution of the stock solution of the enzyme. These correspond to mass concentration equal to 0.002 g/litre, 0.0025 g/litre, 0.005 g/litre, 0.01 g/litre, and 0.02 g/litre respectively. Another set of different enzyme concentrations were prepared by 80-, 40-, 20-, and 10-fold dilution of the stock enzyme solution prepared by dissolving 0.01 g of it in 100 ml tris – HCl buffer whose *p*H is 6.9 at 37℃. Assay of the enzyme was carried out according to Bernfeld method [13]. Hydrolytic activity of the enzyme was terminated at three minutes by adding 1 ml of 3, 5 – dinitrosalicylic acid solution to 2 ml reaction mixture composed of 1 ml substrate and 1 ml enzyme. Spectrophotometric readings were taken at 540nm and molar absorption coefficient 181.1 litre·mol^{-1}·cm^{-1} based on usual Beer – Lambert equation $A_{540nm} = \varepsilon C l$ where for the purpose of emphasis and clarity, ε, C, and l are molar absorption coefficient, molar concentration of product, and path length respectively while A_{540nm} is the absorbance. Kinetic parameters such as Michaelis – Menten constant (K_m) and maximum velocity (V_{max}) of hydrolysis were determined according to Lineweaver – Burk method [14] Equation (16) or (17) which requires the data generated from the use of Michaelis – Menten and Lineweaver – Burk methods is used to reproduce the mass concentrations of the enzyme. The choice of molar units for velocity of hydrolysis is due to the need for direct conversion to mass concentration units because 0.01 g of the enzyme was weighed and dissolved in 100 ml buffer. The molar mass of *Aspergillus oryzea* alpha amylase used according to Eq. (17) for conversion to mass concentration of the enzyme is 52.10 kDa [15]. Biuret method as described by the manufacturer of the reagent was used to verify the consistency or accuracy of the Wensar weighing Scale Ltd, Chennai.

2.4 Statistical Analysis

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

3. RESULTS AND DISCUSSION

The results are presented in molar and gram scale units because of the convenience implicit in mole concept and mass concentration units, which directly reflect the ultimate objective of the research. There is also need to minimize the inconvenience inherent in converting from international units to either molar or mass scale units. The starting point was to ensure that measurement taken was accurate. Thus result from Biuret assay of 0.01 g% of the enzyme indicated 0.111±0.0157 g/litre at a specific absorption coefficient, 0.045±0.0014 litre·g^{-1}·cm^{-1} when mean blank reading equal to 0.1145±0.00071 was used to correct absorbance at a wave length, 540nm. Using 0.115 blank reading for correction, the result is 0.1±0.02 g/litre.

The V_{max} values of the enzyme obtained from the use of measured v values in line with Lineweaver – Burk method [14] and from the

conversion of ***S*** values to corresponding V_{max} (i.e. ***S*** (K_m + [S])/[S]) and the corresponding concentrations of the enzyme at *p*H 5 and room temperature (20℃) are shown in Tables 1 and 2. The plots according to Lineweaver – Burk produced generally high r^2 values as shown in Table 1, but for the value where the concentration of stock enzyme solution is subjected to 20 – fold dilution. This was a reflection of the imperfection of the assay that is not necessarily unavoidable.

As shown in Table 1, there was increasing velocity of hydrolysis of the substrate with increasing concentration of the enzyme or decreasing dilution factor d_f. The ***S*** values at *p*H 5 and room temperature (20℃) also showed increasing trend with increasing concentration of the substrate (Table 2). The high coefficient of determination r^2 is a clear confirmation of the high correlation between the variables, *v* versus $1/d_f$ values (1/50, 1/40, 1/20, 1/10, and 1/5) and 1/*v* versus 1/[S]. Also the high values of correlation coefficient *r* point to the fact that the data are highly related (Tables 1 and 2). The mass concentrations of prepared solution of the enzyme (solutions prepared by serial dilution of stock and the stock solution) and the corresponding mass concentrations calculated based on the model using each of the different values of V_{max} at *p*H 5 and 20℃ are similar (Table 2).

The results of assay carried out at 37℃ and *p*H 6.9 in order to ascertain the reproducibility of the expected outcome of the use of the model, are indicated in Tables 3 and 4. As in Table 1 the increasing values of V_{max} correspond with increasing concentration of E. In other words, V_{max} is directly proportional to the concentration of the enzyme as expected. Also, there was increasing trend in ***S*** values with increasing concentration of S. If compared with values in Tables 1 and 2, one can observe that, r^2 (or *r*) values in Tables 3 and 4 containing other results of assay at 37℃ and *p*H, 6.9 are higher than values in Table 1. What is obvious is that there may have been improvement in the pipetting of aliquots of the solution of the enzyme and substrate coupled with more stable thermal environment provided by automated water bath fixed at 37℃. The corresponding mass concentrations, calculated, based on the model, were very similar to the mass concentrations of the enzyme prepared by dissolving 0.01 g in 100 mL of buffer as stock and by serial dilution of the stock.

In the plots, to determine ***S*** there was a clear – cut linear relationship, positive correlation, between *v* values and $1/d_f$ values (1/80, 1/40, 1/20, 1/10) with very high coefficient of determination. This is as should be expected given improved efficiency in pipetting of aliquots of reactants and the fact that rate of hydrolysis of substrate is proportional to the concentration of the enzyme at the initial stage of reaction (in which $K_m \neq (k_{-1} + k_2)/k_1$) and in particular, at low concentration of the enzyme.

Table 1. The maximum velocity of hydrolysis of different concentrations of the *A. oryzea* alpha amylase at *p*H 5 and room temperature (20℃), and the correspond ing empirical mass concentration calculated according to the model

Prep [E] (mg/l)	V_{max} (mU/l)	r^2	*r*	Emp[E] (mg/l)	EmpM_{PROT} (kDa)
2.0	363.91±2.62	0.994	0.997	2.10±0.02	52.04±0.40
2.5	424.14±70.75	0.948	0.974	2.45±0.41	48.52±8.09
5.0	714.55±1.80	0.878	0.937	4.12±0.01	40.87±0.10
10.0	1567.20±443.9	0.950	0.975	9.04±2.56	44.82±12.70
20.0	3025.50±38.18	0.994	0.997	17.5±0.22	43.26±0.55
100.0	19288.65±3755.37	0.984	0.992	111.3±21.67	55.16±10.74

*[E], [S], and V_{max} are mass concentration of the enzyme, mass concentration of the substrate, and maximum velocity of hydrolysis of the substrate. Prep and Emp represent prepared concentration of enzyme (weighing and dissolution in buffer) and empirically determined i.e. simple calculations based on model respectively. The value of $[E_o]$(stock) is 100 mg/litre. Each solution of the enzyme except stock solution was assayed at each concentration of substrate. Michaelis – Menten constant K_M ((22.978±8.314) g/litre) used is obtained by extrapolation from the plot of 1/**S** versus 1/[S]. Slope (**S**) from the plot, v versus $1/d_f$, is the velocity of hydrolysis at the highest concentration of the enzyme; but it is < its V_{max}. d_f is also the number of times the final volume of the diluted stock solution of the enzyme is larger than unit volume of the stock. **S** is plotted against β to give another slope S_2. The value of S_2 is (1189±92) U/g; the value of k_{2x} is M_{PROT}.1189 where M_{PROT} is the molar mass of the enzyme. 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)*

Table 2. The highest velocity (slope) of hydrolysis of different concentrations of substrate at *p*H 5 and room temperature (20℃) and the correspondi ng empirical mass concentration calculated according to the model

1st Slope(*S*) (mU/ml)	r^2	*r*	Emp [E] (mg/l)	[S] (g/l)	β (g/l)	EmpM_{PROT} (kDa)
2913.5±399.5	0.981	0.990	108±14.8	4	0.5931	56.20±7.71
3402.0±267.3	0.980	0.9899	104±8	5	0.8936	54.43±4.28
4296.0±407.3	0.992	0.996	114±11	6	1.242	59.34±5.63
4565.0±264.5	0.994	0.997	107±6	7	1.6345	55.91±3.24
4901.0±351.4	0.979	0.989	103±8	8	2.066	54.27±3.89
5922.0±606.7	0.993	0.996	107±11	9	3.0323	55.85±5.72

All parameters are as defined in Table one. Using calculated mass concentrations of the enzyme, based on the model, the mean calculated molar mass of the enzyme is presented as mean±SD; $\Sigma EmpM_{PROT}/n \pm SD$ = 51.68±5.98 kDa; $\Sigma d_f Emp[E]/n \pm SD$= ~ 101.47± 8.06 mg/l where n = 12; n is the sum of the number of calculations in Tables 1 and 2.

Table 3. The maximum velocity of hydrolysis of different concentrations of *A. oryzea* alpha amylase at *p*H 6.9 and 37℃ and the corresponding empirical mass concentration calculated according to the model

Prep [E] (mg/l)	V_{max} (mU/ml)	r^2	*r*	Emp[E] (mg/l)	EmpM_{PROT} (kDa)
1.25	327.44±0.08	0.969	0.984	1.24±0.03	46.64±0.12
2.50	658.32±4.95	0.948	0.974	2.45±0.02	49.90±0.04
5.00	1287.09±35.95	0.970	0.985	4.86±0.14	48.78±1.36
10.0	2813.99±138.69	0.982	0.991	10.62±0.52	53.32±2.63
100.0	30227.196±2989.537	0.940	0.9695	113.5±1	57.28±5.66

All parameters are as defined in Table

Table 4. The highest velocity (slope) of hydrolysis of different concentrations of substrate at *p*H 6.9 and 37℃ and the corresponding empirical mass concentration calculated according to the model

1st Slope(*S*) (mU/ml)	r^2	*r*	Emp[E] (mg/l)	[s] (g/l)	β	EmpM_{PROT} (kDa)
4071±168	0.999	0.9995	108±4	4	0.569	54.90±2.27
5631±4.24	0.997	0.9985	124±0	5	0.859	62.88±0.05
6187±42	0.999	0.9995	117±1	6	1.196	59.53±0.40
6207±35	0.999	0.9995	104±1	7	1.576	52.87±0.30
7512±7	0.999	0.9995	114±0	8	1.994	57.62±0.05
8673.5±195.9	0.999	0.9995	112±3	9	2.923	56.65±1.28

*Michaelis – Menten constant (K_M) (24.467±5.097g/l) is obtained by extrapolation from the plot of 1/**S** versus 1/[S]. Assay was at pH 6.9 and 37℃. The value of S_2 is 1794.5±30.41 U/g; the value of k_{2x} is M_{PROT}×1794.5. The experimental procedure is as summarized under Table 1. Using calculated mass concentrations of the enzyme, based on the model, the mean calculated molar mass of the enzyme is presented as mean±SD; $\Sigma EmpM_{PROT}/n \pm SD$ = 54.58±4.87 kDa; $\Sigma d_f Emp[E]/n \pm SD$ = ~ 107.99± 7.32 mg/l where n = 11; n is the sum of the number of calculations in Tables 3 and 4*

The maximum velocities were obtained by a method [14] stated earlier. The plots produced generally high r^2 values as shown in Table 3. The values of V_{max} obtained at 37℃ and *p*H, 6.9 from the plots of 1/***S*** *versus* 1/[S] was much higher than other values for lower concentration of the enzyme. This, justifies Eq. (6) in which k_r.[E] is defined as the highest velocity of hydrolysis at highest concentration of the enzyme at a given concentration of S.

The second slope S_2 at specified conditions of assay, *p*H 5 and 20℃, and *p*H 6.9 and 37℃ was determined for each set of conditions by plotting ***S*** against β (*i.e.* $[S]^2/(K_m + [S])$). The plots exhibited moderately high coefficient of

determinations, as shown in Fig. 1. Where the conditions are *p*H 6.9 and 37℃, r^2 is 0.926 and it is 0.956 when the conditions are *p*H 5 and 20℃ (the average room temperature within the period of assay).

As posited elsewhere by Marini [16] and from experience the main source of error is pipetting. This implies that with the use of highly advanced and automated pipette, and measuring cylinder, including thermostatically controlled hot plates or automated heater, it is possible to achieve very accurate velocities of hydrolysis of the substrate. This could ultimately influence the accuracy of the overall result. Thus the problem is not with the equation or model. Moreover, the values of mass concentrations obtained by calculation and serial dilution of stock solution are very similar. It is important to observe that there has been interest in 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 [17]. This is apart from the principle of radial diffusion in substrate containing agar gel [1,2] or slides [3]. The fact is that, while all enzymes are proteins, all proteins are not enzymes. Electrophoretic, chromatographic, and any other known methods when combined serve to produce highly purified enzyme but the quantity produced is far less than original quantity in the sample. Assay of different sources of the same enzyme may serve to show the relative amounts of the enzyme when comparison is made but it does not indicate the exact amount of the enzyme in any sample. Therefore, enzymes can be quantified using the new model so long as the substrate is known. Besides, the challenge arising from retardation of migration of solution component in agar medium is entirely not applicable to the current model which has additional advantage of being less time consuming compared to claims in literature as it affects other methods.

A departure from the usual practice of plotting *v* against [S] to the practice of plotting *v* against $1/d_f$, which reflects an indirect way of plotting against concentrations of highly diluted enzyme, exhibits high degree of linearity without which no further linearity may be expected in other plots. Therefore, theoretical fact such as standard quasi – steady state approximation [6] seems to be justified because it proposes that [E] must be much less than [S] or as expressed in Eq (5). What the situation might be if [E] is greater than [S] is not certain but such should require a different theoretical concept. At the moment, known methods for direct calculation of k_2 without molar concentration of the enzyme appears not to be largely available except the model according to Uludag – Demirer et al. [18] which relates what the authors called turnover number in the equation $(1/[P]).dP/dt = -k_3 + k_3([S]_0 - [S])/[P]$, to velocity of amylolysis and other extensive quantities, where [P], k_3, [S], $[S]_0$ and *t,* are concentrations of product, turnover number, free substrate, total

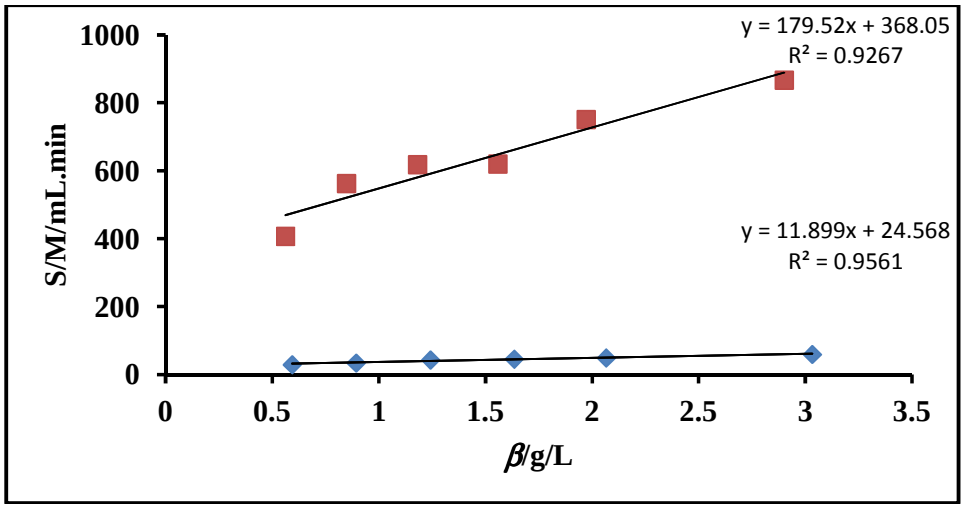

Fig. 1. The determination of the second slope (S_2) – "substrate based specific activity"

*Plot of slope (**S**) against β (described as fractional mass concentration of substrate because it is expressed as $[S]^2/(K_m + [S])$). **S_2** is used to calculate mass – mass rate constant as shown under Tables 1 and 4 or Eq (14).*

(■) and (◆) refer to conditions such as pH 6.9 and 37℃ and p H 5 and 20℃ (average room temperature) respectively

substrate, and duration of assay respectively. The parameter k_3 is intended to be the same as k_2 (or k_{cat}). However, unlike current model in this paper, it is not certain whether the parameter $k_3=V_{max}/[E]_T$. Most recently, research report shows that smartphone – based potentiometric biosensor has been applied in the quantitative analysis of human salivary alpha amylase in real human sample within 5 min [19]. Be it as it may, none of these methods can be used to determine both mass concentration of the enzyme and molar mass as the case may be.

Current model emanating from this research is multifunctional. The model may provide opportunity for the accurate estimation of the molar mass or molar concentration of the enzyme for any solution including crude extract of an enzyme of interest. To apply recent approaches, such as electrophoretic, chromatographic, and centrifugation techniques, as in literature [20–22], the sample must be in the highest state of purity unlike the case in this current research finding. Early pioneering work in the field of centrifugation for the determination of molecular weight of proteins is creditable to [23], an assertion that can best be described as a "gospel". This could be of great interest in industrial applications, such as fuel, textile, detergent, starch, food, and paper industries [24] which do not involve life object where invasive application at consumer level, may be the case. It may be used therefore, to compare different molar concentrations of the enzyme from different sources. Thus, when relevant parameters are substituted into Eq (18), the results, after calculation are 51.68±5.98 kDa as shown under Table 2 and 54.58±4.87 kDa as shown under Table 4. Manufactures presents different values of molar mass for the same enzyme (*A. oryzea* alpha amylase) as exemplified in the following values, 50.71 kDa as observed recently and 52.10 kDa as observed by other authors [17]. But all molar mass values are from Sigma Aldrich, USA. The calculated molar mass values (Tables 2 and 4) are similar to manufacture's molar mass (52.1 kDa and 50.71 k) and to ≈ 50 kDa for *A. oryzea* MIBA316 [20], and slightly lower than 57kDa for *A.oryzea* PP [25].

4. CONCLUSION

Substitution of the relevant data obtained for different parameters into the model equation consistently yielded, after calculation, results that were very similar to concentrations of prepared solutions of the enzyme. This consistency confirms the model which may therefore, be used, as described, for the accurate quantification of the concentration of any enzyme in crude extracts whose substrate is known. Important corollaries are: (1) The model may be used to verify the accuracy of results of assay and (2) Given accurate estimate of kinetic parameters generated as described the molar mass of the enzyme may be calculated. Whatever is the case, continuous re – verification is called for using other single substrate enzymes. A concentration of 0.2 g/litre as stock could be a starting point for the repeat of experiment.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Tauschel HD, Rudolph C, A new sensitive radial diffusion method for micro determination of alpha amylase. Anal. Biochem. 1982;120(2):262–266.
   DOI: 10.1016/0003 2697(82)90346-3
2. Farias DF, Carvalho AFU, Oliveira CC, Sousa NM, Rocha – Bezerrra LCB, Ferreira PMP, Lima GPG, Hissa DC. Alternative method for quantification of alfa-amylase activity. Braz. J. Biol. 2010;70(2):405–407.
   DOI: 10.1590/S1519-69842010005000008
3. Kohl JD, Johnson MG. Quantitative, radial diffusion slide assay for staphylocoagulase. Appl. Environ. Microbiol. 1980;39(2):339– 341.
   DOI: 10.1111/j.1745-4565.1984.tb00484.x
4. Johnson KA, Goody RS. Johnson KA, Goody RS. The original Michaelis constant: Translation of the 1913 Michaelis – Menten paper. Biochemistry. 2011;50(29):8264–8268.
   DOI: 10.1021/bi201284u
5. Michaelis L, Menten ML. Kinetic der invertinwirkung. Biochem. Z. 1913;49:333–369.
6. Briggs GE, Haldane JBS. A note on the kinetics of enzyme action. Biochem. J. 1925;19:338–339.
   DOI: 10.1042/bj0190338
7. Hinch R, Schnell SJ. Mechanism equivalence in enzyme-substrate

reactions: Distributed differential delay in enzyme kinetics. Math. Chem. 2004;35(3): 253–264.
DOI:10.1023/B:JOMC.0000033258.42803.6

8. Brown AJ, Enzyme action. J. Chem. Soc. Trans. 1902;81:373–388.
DOI: 10.1039/CT9028100373
9. Segel LA. On the validity of the steady state assumption of enzyme kinetics. Bull. Math. Biol. 1988;50:579–593.
DOI: 10.1007/BF02460092
10. Segel LA, Slemrod M. The quasi-steady state assumption: A case study in perturbation. SIAM Rev. 1989;31:446–477.
DOI: 10.1137/1031091
11. Agarwal A, Adams R, Castellani GC, Shouval HZ. On the precision of quasi steady state assumptions in stochastic dynamics J. Chem. Phys. 2012;137:1–11.
DOI: 10.1063/1.4731754
12. Schnell S, Maini PK. Enzyme kinetics far from the standard quasi-steady-state and equilibrium approximations. Math. Comput. Model. 2002;35:137–144.
DOI: 10.1016/S0895 – 7177(0100156 - x)
13. Bernfeld P. Amylases, alpha and beta. Methods Enzymol. 1955;1:149–152.
DOI: 10.12691/jaem-2-3-2
14. Lineweaver H, Burk D. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 1934;56(3):658–666.
DOI: 10.1021/ja01318a036
15. Sugahara M, Takehira M, Yutani K, Effect of heavy atoms on the thermal stability of alpha amylase from *Aspergillus oryzea*. Plos One. 2013;8(2):1–7.
DOI: 10.1371/journal.pone.0057432
16. Marini I. Discovering an accessible enzyme: Salivary α - amylase. Biochem. Mol. Biol. Edu. 2005;33(2):112–116.
DOI: 10.1002/bmb.2005.494033022439
17. 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.
DOI: 10.1016/j.vaccine.2012.03.035
18. 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.
DOI: 10.1590/S1984 82502009000400005
19. Zhang L, Yang W, Yang Y, Liu H, Gu Z, Smartphone-based point-of-care testing of salivary α-amylase for personal psychological measurement. Analyst. 2015;140(21):7399–7406.
DOI: 10.1039/c5an01664a
20. Kariya M, Shigemi Y, Yano M, Konno H, Takii YJ. Purification and properties of alpha-amylase from *Aspergillus oryzae* MIBA316 J. Biol. Macromol. 2003;2:57–60.
21. Kamran A, Bibi Z, Kamal M, Purification and molecular weight estimation of protease from thermophilic *Bacillus* species. Pak. J. Biochem. Mol. Biol. 2014;47(3-4):155–157.
22. Tabassum R, Khaliq S, Rajoka MI, Agblevor F, Solid state fermentation of a raw starch digesting alkaline alpha-amylase from *Bacillus licheniformis* rt7pe1 and its characteristics. Biotechnol. Res. Int; 2014.
DOI: 10.1155/2014/495384
23. Svedberg T, Fahraeus RJ. A new method for the determination of the molecular weight of proteins. Am. Chem. Soc. 1926;48(2):430–438.
24. Souza PM, Magalhães PO, Application of microbial alpha amylase in industry – A review Braz J. Microbiol. 2010;41:850–861.
DOI: 10.1590/S1517-83822010000400004
25. Evstatieva Y, Nikolova D, Ilieva S, Getov L, Savov V, Identification and characterization of α-amylase and endoxylanase, produced by *Aspergillus* mutant strains. Biotechnol. & Biotechnol. 2010;24:613–617.
DOI: 10.1080/13102818.2010.10817908

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/13403