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Determination of Molar Mass and Its Relationship with Free Energy of Activation: A Case Study on Human Salivary Alpha Amylase

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Author's contribution

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

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ABSTRACT

Aims: The objectives of the research were (i) to show that the mass concentration, molar mass of one-active site enzyme, and consequently, the type of human salivary alpha amylase (HSαA), can be determined using kinetic parameter dependent model, (ii) to show that the free energy of activation for hydrolysis of substrate is usually related to molar mass.
Study Design: Experimental.
Place and Duration of Study: Department of Biochemistry, Ambrose Alli University and Research Division of Ude International Concepts Limited (RC 862217) B. B. Agbor Delta, Nigeria. The research spanned between June, 2008 and August, 2016.
Methodology: Bernfeld method of enzyme assay was used. Assays were carried out on diluted commercial human salivary alpha amylase (HSαA) and crude extract of HSαA.
Results: Mean values of calculated mass concentration and molar mass of the enzyme were 171.09±0.64 mg/l and 61.92±0.64 kDa respectively (from 20 calculations). The concentration of crude extract of HSαA was 94.5±0.4 mg/l. With the old model the free energy of activation (ΔG_a) value is 51.19±0.12 kJ/mol (n = 20). Using directly the new model the value is 51.21±0.08 kJ/mol.

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Conclusion: It is concluded that the model can be used to accurately determine the concentration of the enzyme and its ΔG_a using either standard solution or crude extract. Therefore, as a corollary, the molar mass of such enzyme can be determined given well defined concentration of the enzyme. The enzyme purchased from Sigma – Aldrich, USA, may be the glycosylated HSαA.

Keywords: Human salivary alpha amylase; mass concentration of enzyme; molar mass; Gibbs free energy of activation.

1. INTRODUCTION

> *"Imperfection keeps man busy in search of better alternatives: Anyone who has never made a mistake may not have discovered anything – Albert Einstein. Consequently, if assay to determine molar mass of HSαA using model applied to other homologue is not a new research, this paper may be turned down-My experience."*

The need to know the quantity of enzyme in crude extract has become very important in the light of the use of enzyme in most industrial applications such as food, fermentation, textile, paper, detergent, and pharmaceutical industries and with advancement in biotechnology, application has assumed clinical, medicinal, and analytical chemistry relevance [1,2]. The most widely used industrial enzyme is alpha amylase (E.C. 3.2.1.1). Bacterial sources of the enzyme are under intense research for reasons which include stability/structure for function [3,4]. This should not be surprising because these sources of the enzyme provide after extraction, the enzymes whose use is not exclusive of the environment that may inhibit its function which is of major interest to the users in an industrial setting. It has been observed that some manufacturers do not indicate the molar mass of bio-molecular polymers such as enzymes and starch in particular sold to customers.

Meanwhile, mastication and physical characteristics of food influence the secretion rate of saliva and its amylase activity; the total protein content of saliva has been reported to range from 0.1 to 1.0 mg/mL [5] and therefore, the report of concentrations of up to 7 mg/mL and by implication a range of 0 to 7.5 mg/ml must be in error [6]. This is a problem that needs resolving. However, modern methods of purification, electrophoresis, chromatography, centrifugation *etc* can not only purify the enzyme but its molar mass can also be determined [7,8]. One of the earliest methods is Svedberg method [9]. Recently also, smart phone technology has been applied to the detection of alpha amylase in saliva within 5 minutes [10]. This method cannot however, determine the molar mass of the enzyme. Several authorities including manufactures have reported different molar masses of human salivary alpha amylase. Some manufacturers are not specific as to the class of salivary alpha amylase prepared and sold to consumers or researchers. There are non-glycosylated and glycosylated human salivary alpha amylases whose molar masses were reported as 56 kDa and 59 kDa respectively [11]. Ramasubbu et al. [12] observed that HSαA is composed of 496 amino acids with a molecular weight ~ 56000. Review report by Takeuchi [13] shows relative molecular masses of 56000 and 62000 for the two forms of the enzyme. The work of Takeuchi [13] shows that the relative molar masses of type A and type B of the enzyme are 61000 and 64000 respectively but acknowledged that "the relative molecular masses of glycoprotein with high carbohydrate contents are overestimated by SDS-polyacrylamide gel electrophoresis and so, the relative molecular mass of salivary amylase B with about 9% carbohydrate may be overestimated". Also, Bank et al. [14] reported 62 kDa for glycosylated and 56 kDa for non-glycosylated enzymes. Human salivary Amylase of 56 kDa (family B) or 62 kDa (family A), the difference in molecular weight due to the presence or absence of a carbohydrate moiety, were indicated by two manufacturers, OriGene EU, Germany and OriGene Technologies Inc, USA. The sample commercial human salivary alpha amylase used in this investigation was purchased from Sigma -Aldrich, USA about two years ago. It is not certain whether, the glycosylated enzyme which may be more stable is sold by the company! Since saliva is from human sources it is not unlikely that there may be minor differences in the size of the carbohydrate moiety linked to the enzyme's primary structure apart from instrumental differences used by investigators.

There has been effort in the past to relate molar mass of an enzyme to free energy of activation which was analyzed by plotting mean group values of free energy of activation versus the mean group values of molar mass. The result clearly showed an asymptotic decrease of the activation energy with the increase in the molecular mass of the enzyme [15]. However, this does not imply that a relationship has never existed between free energy of activation and molar mass as may be shown under theoretical background. A different approach to be applied herein in the case of HSαA has been applied using *Aspergillus oryzea* alpha amylase as a case study [16]. The importance and advantage of the model lie in the fact that it is multifunctional such that various parameters, mass concentration, molar mass and free energy of activation of any solution of an enzyme, be it standard or non-standard solution as applicable to a crude extract of one active site-enzyme with known substrate, can be determined. The objectives of the research were: (i) to show that the mass concentration, molar mass of one-active site enzyme, and consequently, the type of HSαA, can be determined using kinetic parameter dependent model, (ii) to show that the free energy of activation for hydrolysis of substrate is always related to molar mass of the enzyme.

1.1 Theoretical Background

1.1.1 Review of earlier contributions

The review of research in the past deserves the mention of the paper entitled "Die kinetic der Invertinwirkung" by Michaelis and Menten [17] published in Biochemische Zeitschrift. The mathematical formulation in its earlier form according to Michaelis and Menten and translated into its modern form without losing the original form by Johnson and Goody [18] is:

$$V = C\Phi [S] / ([S] + K) \quad (1)$$

where $C.\Phi$ is equal to V_{max} (the maximum velocity of enzymatic hydrolysis), Φ is the total enzyme concentration, and K represents K_S just as C stands for modern rate constant, k_{cat}, and K_S may stand for enzyme – substrate (ES) complex dissociation constant otherwise called Michaelis – Menten constant (K_m). However, not just Johnson and Goody [18], but another worker [19] reports that other workers have revisited the work of Michaelis – Menten model using 1H NMR spectroscopy by which their classic work on invertase reaction dynamics was studied.

According to Johnson and Goody [18], Michaelis and Menten did not realize that double reciprocal plot is a means to obtaining a linear extrapolation to infinite substrate concentration. It was Lineweaver and Burk [20] that realized the value of the double reciprocal plot. However, there is emerging interest in the linearization of Michaelis-Menten like equation, that is, what has been described as type 3 equation characterized by the nature of a hyperbola can easily be turned into a linear form [21]. This reference is important because the path of linearity must be followed in the application of the model. However, when an enzyme E converts substrate S to product P, the rate of change of concentration of the product, d[P]/dt, generally depends on the saturating concentration ([S]) of substrate, through the now familiar Michaelis-Menten hyperbolic curve characteristics of a nonlinear response. This however, appears to challenge the need for linear rate of transformation. Incidentally there has been harsh criticism of linear transformation of Lineweaver-Burk equation as pointed out elsewhere [22]. There is yet another criticism to the effect that in computer age the linear transformations are not proper way for analyzing kinetic data in spite of their visual appeal [23]. "The common problem with these transformations is the fact that transformed data usually do not satisfy the assumptions of linear regression, namely that the scatter of points around the straight line follows a Gaussian distribution, and that the standard deviation is equal at every value of the independent variable" [23]. However, Butterworth et al. [24] opined that reliable estimates of the kinetic parameters can be achieved if rate measurements are performed within the early stages of the reaction before the emergence of complications emanating from a combination of product inhibition and substrate exhaustion. The important issue is linearity. Therefore, the decision to use highly diluted solution of the enzyme should not be unreasonable. Thus the equation in the work of Gunawardena [25] herein referred to as Eq (2) below need not be in contention.

$$d[P]/dt = \{V_{f,max}([S]/K_{fM}) - (V_{r,max}[P]/K_{rM})\}/\{1 + ([S]/K_{fM}) + ([P]/K_{rM})\} \quad (2)$$

Although the variables were not defined by the author but it can be understood as an equation of rate of product formation (d[P]/dt) with elements of dissociation constant for forward (K_{fM}) and reverse (K_{rM}) reactions when product inhibition

sets in. According to Gunawardena [25] Eq. (2) turns to Eq. (3) below when [P] is zero. But d[P]/dt is an expression of apparent rate or velocity of hydrolysis and so, it is not certain what the origin of [P] should be. Could it be the initial concentration which may be infinitesimal in quantity?

Modern form of Michaelis-Menten equation which is widely cited by several authors including Gunawardena [25] and Reuveni et al. [26] is also,

$$v = V_{max}\,[S]/(K_m + [S]) \quad (3)$$

where v is the velocity of enzyme catalyzed reaction when the active sites are not fully occupied by the substrate molecules, [S] is the molar concentration of the substrate, and V_{max} is the maximum velocity of the enzyme catalyzed reaction when the all the active sites are occupied by the substrate molecules. It is on the basis of Eq (3) that Lineweaver and Burk [20] derived the famous Lineweaver-Burk equation. Michaelis-Menten model and its transformation are vital for the derivation of models for the estimation of the concentration of enzyme in standard and non-standard solutions. 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 [27] was first proposed in purely kinetics context. 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.

$$E + S \rightleftharpoons C \rightarrow E + P \quad (4)$$

The parameter [C] is assumed to be approximately constant, often a brief transient phase. This is described as Briggs and Haldane [28] approximation or standard quasi-steady state approximation (sQSSA) which suggests that enzyme-substrate complex is nearly unchanged or $d[C]/dt \approx 0$ [29-31]. The classical sQSSA is in fact valid providing that:

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

Equation (5) is a useful guide because, assay at very low concentration of the enzyme yields better result. This has been corroborated by Butterworth et al. [24] who posited that "initial rate measurements are better performed (in order to maintain linear product release with time) using reaction mixtures containing enzyme concentrations that approximate those found *In vivo*" in which the concentration of the enzyme is much lower than that used *in vitro* by most investigators. Meanwhile, Eq. (6) [16] below has been found to be useful for the determination of the mass concentration of an enzyme.

$$[E]_{TMC} = (K_m + [S])\,S_{L\text{-}1}\,M_{ALT}/S_{\mathbf{L\text{-}2}}\,M_{PROT}\,[S] \quad (6)$$

where $S_{L\text{-}1}$ and $S_{L\text{-}2}$ are the first and second slopes respectively; the values of $S_{L\text{-}1}$ are obtainable when velocity of hydrolysis (activity, v) is plotted versus reciprocal of dilution factors while the second slope ($S_{L\text{-}2}$) is obtainable when $S_{L\text{-}1}$ is plotted versus β (*i.e.* $[S^2]/([S]+K_m)$); M_{PROT}, K_m, [S], M_{ALT}, and $[E]_{TMC}$ are the molar mass of the enzyme, Michaelis-Menten constant, concentration of the substrate, molar mass of the product, maltose, and total mass concentration of the enzyme.

Equation (6) represents a modified/reversed form of earlier equation [32] found to be deficient.

$$"M_{PROT} = (K_m + [S])v_x M_{ALT}/S_{L\text{-}2}[S][E]_{TMC}" \quad (7)$$

where v_x may be activity of the diluted solution of the enzyme or the highest activity (the first slope, $S_{L\text{-}1}$) at the highest concentration of the enzyme.

$$\Delta G_a = RT\,\ln\,(k_B M_{ALT} T/h S_{L\text{-}2}\,M_{PROT}{}^2) \quad (8)$$

where ΔG_a, R, k_B, T, and h are free energy of activation, gas constant, Boltzmann constant, thermodynamic temperature, and Planck's constant respectively. However, a well known equation is $\Delta G_a = RT\ln(k_B T/hk_2)$, where k_2 is the rate constant. But $k_2 = M_{PROT}V_{max}\,/[E]_{TMC.}$ Substitution of this into the former equation gives:

$$\Delta G_a = RT\,\ln([E]_{TMC} k_B T/h M_{PROT} V_{max}) \quad (9)$$

The difference between Eq. (8) and Eq. (9) is that while Eq. (8) can be used to determine ΔG_a without initial information about the mass concentration of the enzyme, it is not so using Eq. (9). Therefore, regardless of different approach of Pawlowski PH and Zielenkiewicz [15], there has always been a relationship as shown in Eq. (9). With different values of M_{PROT} for different homologues of the same enzyme of the same mass concentration, ΔG_a should be directly proportional to natural log of molar mass. With Eq. (8), it should be directly proportional to natural logarithm of the molar mass [22].

2. MATERIALS AND METHODS

2.1 Chemicals

Alpha amylase (8.5 mg solid – A103 – 1KU) from human saliva was purchased from Sigma – Aldrich, USA. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd, Poole England, and potato starch was purchased from Sigma, USA. Tris was purchased from Kiran Light Laboratories, USA, 3, 5-dinitrosalicylic acid and maltose were purchased from Kem light laboratories, India. Sodium potassium tartrate tetrahydrate was purchased from Kermel, China, while calcium chloride was purchased from Lab Tech Chemicals, India. 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. *PH* meter was purchased from Hanna Instruments, Italy.

2.3 Methods

Stock solution of soluble potato starch was prepared by mixing 3 g in 50 ml of tris - HCl buffer at *p*H 7.4 subjected to heat treatment at 100^{o}C for three minutes, cooled to room temperature, and final volume was made by topping the volume with buffer to 100 ml to give 30g%. A solution of the enzyme was prepared by dissolving 8.5 mg of the enzyme in 50 ml buffer containing first 5 mM calcium chloride and then 0.1% sodium chloride to give 0.17 g/l solution. Four different concentrations were prepared by carrying out dilutions by 100-, 50-, 25-, and 12.5-fold to give 0.0017 g/l, 0.0034 g/l, 0.0068 g/l, and 0.0136 g/l respectively. Assay of the enzyme was carried out according to Bernfeld method [33] and kinetic parameters such as Michaelis-Menten constant and maximum velocity of hydrolysis were determined according to Lineweaver - Burk method [20]. Crude extract from the researcher was obtained by spitting into a 50 ml beaker. Saliva sample was collected about few hours after meal; 20ml of saliva mixed with calcium salt, buffer (TrisHCl, *p*H = 6.9), sodium chloride, and distilled water to give 1:5 diluted saliva. This was finally diluted to give 1:20 diluted saliva as working stock. The working stock was separately subjected to 1:20, 1:10, 1:5, and 1:2.5 dilutions to give four different unknown concentrations. The different solutions of unknown concentrations were immediately assayed one after the other, at room temperature. Spectrophotometer readings for the determination of amount of maltose yielded were taken at 540nm and the extinction coefficient was 181.1/M.cm. Rate constant (sometimes called turnover number) for the release of product is calculated by usual method, $V_{max}/[E_0]$. Calculation of mass concentration and molar mass for testing the model are according to Eq (6) and Eq (7) respectively.

2.4 Statistical Analysis

All values are expressed as mean±SD. Microsoft Excel was used to calculate SD. Internet based Simple Interactive Statistical Analysis was used to carry out *t*-test for significant difference between results obtained from old model and new model.

3. RESULTS

This presentation is clearly focused such that much attention cannot be given to data on kinetic issues albeit they are indispensible to the determination of mass concentration, molar mass, and other physico-chemical parameters. In order to determine what has been termed “mass-mass rate constant” k_{2x}, $S_{L\text{-}1}$ values were plotted versus “fractional mass concentration” of the substrate $[S]^2/(Km + [S])$ to give the second slope. The product of second slope, $S_{L\text{-}2}$ and the molar mass (the choice of which is difficult because it is not certain if the manufacturer sold either non glycosylated or glycosylated enzyme) of the enzyme gives k_{2x}. Nonetheless, with known mass concentration the model is used to determine the molar mass of the enzyme. The graph is presented as Fig. 1 (with moderately high coefficient of determination, r^2 = ~ 0.91) and value of $S_{L\text{-}2}$ is shown under Fig. 1.

As presented in Table 1, and as expected, there were increasing trend in the rate of hydrolysis of starch (activity in mM/ml.min) with increasing concentration of the substrate and enzyme. Just as the K_m can be obtained from the combination of the slope and the intercept of the double reciprocal plot of activity versus [S], the slope $S_{L\text{-}1}$ is also obtained by extrapolation and represents the highest activity *v* at the highest concentration of the enzyme. The reproduction of stock mass concentrations according to Eq. (6), were made possible by multiplying the values

obtained by the corresponding dilution factor. Mention must also be made of the observation that the reproduced concentrations of the enzyme were similar to the diluted concentrations of the enzyme. The results including average from all determinations under Table 1 were very similar to the stock concentration equal to 170 mg/l. In the same vein, average molar mass from all determinations (20 in all) shown under Table 1 and other molar masses according to Eq. (7) were very similar to the literature value of 62 kDa [14]. The ΔG_a values were determined using the recent and old models. The results were very similar. Indeed with respect to molar mass and ΔG_a values obtained using old and recent model there were no significant difference ($P > 0.05$) between the values obtained from the old and recent model.

4. DISCUSSION

As stated earlier in this paper, the main goal is highly restricted to the determination of mass concentration and molar mass in a manner dependent on kinetic parameters. Thus attention may not be paid to elaborate comparison of current velocities (v) of hydrolysis of heat treated starch, Michaelis – Menten constant (K_m) and rate constant for the formation and realize of product (k_2). Nevertheless the k_2 value, 10500/min (175/s) reported for human salivary alpha amylase in literature [34] at 25^{o}C and pH 6.9 is ~ 7/10th of the value reported in this research at 37^{o}C and pH 7.4 as indicated under Table 1. It is abundantly clear that the reason for the difference in the values of k_2 is as a result of different pH and temperatures in particular under which the measurement were made, given as it were, that as in this experiment gelatinized potato starch was used as substrate. However, it should be made known that what is called gelatinized potato starch in aqueous solvent does not yield homogenous mixture as in this experiment but tends towards partial homogeneity upon heat treatment. It is not also clear if Ramasubbu et al. [34] used heat-treated starch as substrate. Dutta et al. [35] reported K_m values of different polysaccharides namely

Table 1. Model based calculated mass concentration, molar mass of human salivary alpha amylase and Gibbs free energy of activation

[S] (mg/l)	6	7	8	9	[E] (Measured) (mg/l)
$[E]_{cal}$ (mg/l)	1.72±0.12	1.69±0.00	1.74±0.00	1.74±0.05	1.7 ≡ 0.17/100
v (mM/ml.min)	0.29±0.03	0.30±0.00	0.32±0.00	0.329±0.01	
M_{PROT} (kDa)	62.61±6.48	61.17±0.00	63.52±5.95	63.29±1.00	
$[E]_{cal}$ (mg/l)	3.43±0.12	3.39±0.06	3.32±0.00	3.28±0.00	3.4 ≡ 0.17/50
v (mM/ml.min)	0.58±0.02	0.60±0.01	0.61±0.00	0.62±0.00	
M_{PROT} (kDa)	62.61±2.16	61.97±1.10	60.54±0.00	59.73±0.00	
$[E]_{cal}$ (mg/l)	6.81±0.20	6.89±0.11	6.75±0.05	6.87±0.58	6.8 ≡ 0.17/25
v (mM/ml.min)	1.15±0.05	1.22±0.02	1.24±0.01	1.3±0.11	
M_{PROT} (kDa)	62.07±2.70	63.00±1.03	61.54±0.50	62.2±2.65	
$[E]_{cal}$ (mg/l)	13.90±0.18	13.44±0.67	13.53±0.00	14.04±0.00	13.6 ≡ 0.17/12.5
v (mM/ml.min)	2.35±0.03	2.426±0.030	2.487±0.00	2.65±0.00	
M_{PROT} (kDa)	63.42±0.81	62.46±0.77	61.71±0.00	63.63±0.00	
$[E]_{cal}$ (mg/l)	174.82±1.72	171.67±6.38	169.0±1.90	177.03±0.00	170* (stock)
S_{L-1} (mM/ml.min)	29.53±0.29	30.40±1.13	31.06±0.35	33.51±0.04	
M_{PROT} (kDa)	63.76±0.63	61.80±2.30	60.85±0.69	63.73±0.076	

The velocity (v) at each mass concentration of substrate was plotted against $1/d_f$ such as 1/100, 1/50, 1/25, and 1/12.5 to give slope (S_{L-1}) presented as Mean ± SD. Assays were in duplicate. The values of $1/S_{L-1}$ were plotted against 1/[S] to give V_{max} = 43.18±0.03 mM/ml.min (≡ 15727.68±10.93/min) and K_m = 2.86±0.04 g/l. Also S_{L-1} values were plotted against β which is equal to $[S]^2/(K_m + [S])$ as shown in Fig.1. As usual K_m and V_{max} are Michaelis-Menten constant and maximum velocity. Using V_{max} values obtained from two Lineweaver-Burk plots, the calculated concentration ($[E]_{Cal}$) of the enzyme is 0.175±0.006 g/l. Mean of $[E]_{TMC}$ and M_{PROT} from 20 determinations are 171.09±2.06 mg/l and 61.92±0.64 kDa respectively. The measured mass concentration of the enzyme is 0.17 g/l. The concentration of alpha amylase in human saliva crude extract is 94.5±0.4 mg/l. Values are approximations to the nearest decimal figure. With the old model the ΔG_a value is 51.19±0.12 kJ/mol (n = 20). Using directly the new model (Udema 2016b), the value is 51.21±0.08 kJ/mol. With respect to molar mass and ΔG_a, there was no significant difference ($P > 0.05$) between values obtained from new model and those from old methods

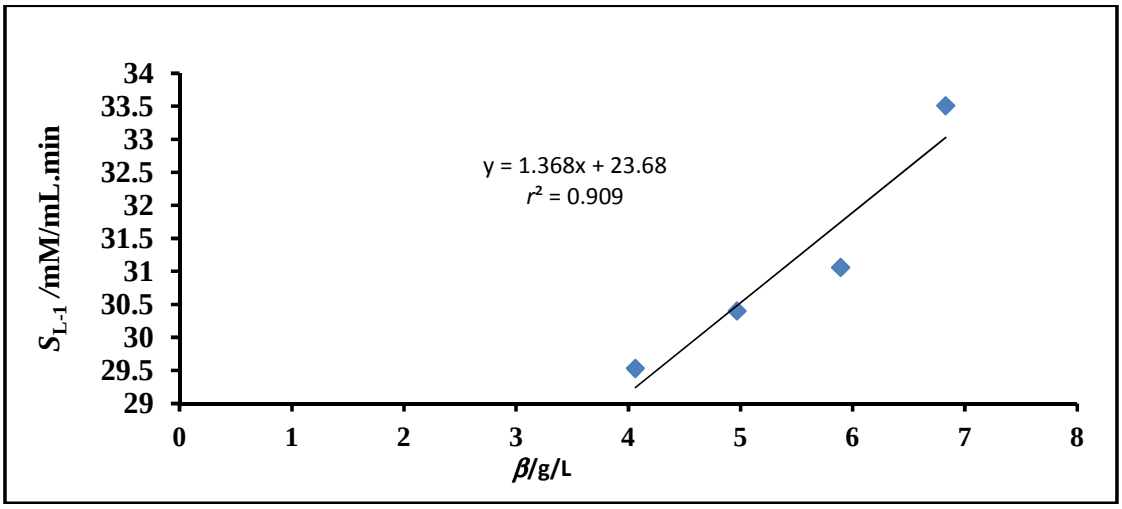

Fig. 1. S_{L-1} versus β

S_{L-1} values as in Table 1, is the slope (gradient) obtained from the plot of velocity of hydrolysis of starch versus reciprocal of dilution factors such as 1/100, 1/50, 1/25, and 1/12.5 and $\beta = [S]^2/(K_m+[S])$. The meaning of β is explained in the text. The equation of straight line (inset) shows the gradient (the second slope, $S_{L-2} = 1.368\times10^{-3} \pm 0.04$ mol/mL.min.g)

amylose, soluble starch, amylopectin, and glycogen. But for the purpose of this investigation, K_m values such as 1.82 g/l, 1.96 g/l, 2.86 g/l for amylose, soluble starch, and amylopectin [35] respectively are of interest because they are generally comparable to report in this investigation. Of particular note is almost total similarity between K_m value for amylopectin and heated potato starch used in this investigation. Unfortunately, no mention was made about the nature (native or heat treated) of the substrates by the researchers.

As Table 1 shows, the calculated mass concentrations of the enzyme were very similar to the measured concentration as stock or diluted stock. The factors that made it possible in the light of the model are intrinsic to the enzymes. The intrinsic factors are kinetic in nature and therefore, they are characteristics of the enzyme. Hence they are largely biochemical in nature unlike pure physical factors such as relative solubility, mobility, density, rate of diffusion, mass or gravitational properties *etc* that characterize, as the case may be, other known methods such as Svedberg centrifugation technique [9,36-37], electrophoresis, chromatography, etc [38,39]. Nonetheless, there is recent opinion that new techniques such as electrospray/ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) [9,39] which are claimed to have revolutionized biological mass spectrometry (MS), have diminished the relevance, though indispensible chromatographic techniques and other traditional methods [40]. The success of these hi-tech instrumentations though expensive and intimidating, requires the highest possible state of purity that should necessitate the use of purification technique otherwise the success of the method can be compromised by dirty and contaminated samples [40]. The current kinetic parameter dependent model does not require such degree of purity though manufacturer-purified commercial enzyme was used to verify the model.

The calculated molar mass (~61.64 kDa) based on the model are similar to the molar masses reported for glycosylated enzyme 62000 [13-14,41]. The manufacturers, OriGene EU Germany and OriGene Technologies, USA, reported value equal to 62 kDa and 57 kDa for glycosylated and nonglycosylated enzyme. Ramasubbu et al. [12] report, being 56 kDa (for the nonglycosylated enzyme), is about 4 kDa less than current result which is however, less than overestimated value of 64000 (~ 63.63 kDa) reported by Takeuchi [13]. The value of the molar mass, 61.92±0.64 kDa obtained in this research is very close to values such as 59 kDa [11,42]. A supportive evidence to the probable molar mass equal to 62 g/mol is the report, using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for purification, that "human salivary alpha-amylase family A (HSA-A) was converted to family B (HSA-B) in human

saliva. This conversion did not occur in the supernatant of saliva which had been centrifuged at 105,000 x g for 60 min. An enzyme which catalyzed the conversion existed in the insoluble fraction of human saliva [41]. The enzyme reduces the molecular weight of HSA-A (62,000) to the same molecular weight (58,000) as that of HSA-B, without forming any intermediate [41]. There is also an observation that "SDS-PAGE revealed that the purified amylase comprised two isoamylases with estimated molecular weights of 55 kDa and 59 kDa" [42]. Another study has also shown that "the predominant form of α-amylase purified from saliva of various races and genders is nonglycosylated with the same molecular weight of 55,881.2, which is 1885.8 lower than the calculated value based on the DNA-predicted sequence" [43]. It is probable that some researchers may not have taken into cognizance of the occurrence of an enzyme which converts glycosylated enzyme to the lower molar mass nonglycosylated enzyme. The duration of purification process, the type of instrumentation and possible contamination with the sediment after centrifugation may lead to partial digestion of the glycosylated enzyme. According to Itol et al. [41], no degradation of the glycosylated form occurs in the supernatant. Therefore, it is likely that if the crude extract before centrifugation is kept briefly before refrigeration, the degrading enzyme, which is said to be stable below 40^oC, may convert some of the glycosylated enzyme to nonglycosylated enzyme. This leaves one to conclude not preemptively, that the molar concentration of human salivary alpha amylase prepared and marketed by Sigma – Aldrich, USA may be about 62 kDa.

The second aspect of the investigation was the successful attempt to reproduce the mass concentration of the enzyme, both as the stock concentration and diluted solution concentrations. The calculated mass concentrations were similar to the measured values of the purchased enzyme. The calculated mass concentration of amylase from researcher's saliva according to the model using 57 kg/mol and 62 kg/mol of the enzyme, HSαA is ~ 0.103 g/l and ~0.095 g/l which is approximately within the protein concentration ranging from 0.1 to 1mg/ml as formally cited by Butterworth et al. [24]. The result using researcher's saliva is < the range reported by Perry et al. [6] which has been seen as an overestimation [24]. The values are also within the concentration range, 0.042-0.244 g/l reported by Jacobsen and Hensten-Pettersen [44]. Reported concentrations of HSαA for 10 individuals showed variation [44].

The main limitation or constraint is the emotional stability of the researcher whose performance can improve with the use of highly sterilized and automated pipette, water bath, hot plates *etc*. One of the sources of error has been identified by Marini [45]. Pipetting error is an example [45]. To avoid product inhibition which might be another source of error, [S] should be moderately high compared to [E] which must be sufficiently dilute as advised elsewhere [24] and in line with the opinion 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. It is observed however, that the slope in Fig. 1 is not the same with almost every three points in the curve. This can be accounted for and solution suggested subsequently.

The observed deviation of the highest point in Fig. 1 may be ascribed to purely instrumental error arising from pipetting. Secondly the gelatinized potato starch is not a true solution and it is probable that the gelatinized starch was not uniformly distributed in its "false-solution" after cooling to room temperature before incubation in water bath set at 37^oC. With 3 points where [S] = 6 g/l, 7g/l, and 8 g/l, r^2 is 0.992 ($S_{L\text{-}2}$ = 0.835 exp (-3) mol/mL.min.g); where [S] = 6 g/l, 8g/l, and 9 g/l, r^2 is 0.986 ($S_{L\text{-}2}$ = 1.473 exp (-3)) mol/mL.min.g); where [S] = 6 g/l, 7 g/l, and 9 g/l, r^2 is 0.902 ($S_{L\text{-}2}$ = 1.355 exp (-3) mol/mL.min.g); where [S] = 7 g/l, 8 g/l, and 9 g/l, r^2 is 0.903 ($S_{L\text{-}2}$ = 1.674 exp (-3) mol/mL.min.g). It is obvious that the slope is not the same with every set of 3 [S]. The reason apart from issue of error of instrumental origin [45] is that enzyme catalyzed reaction is far more mechanistic or systematic than elementary reaction outcome such as effervescence which occurs at rates dependent strictly on the effect of concentration of dilute acid on say powdery calcium trioxocarbonate (IV) salt. Such saturation phenomenon may not be totally absent even at the most linear part of the plot of v versus [S] let alone at points closer to and above the K_m. Thus both linear and non linear approaches in the determinations of kinetic data are not free from saturation effect. In the first place, it should be recalled that for the model to be workable, $[E_T]$ /($[S_T] + K_m$) should be « 1 as indicated in Eq. (5). This ensures that substrate exhaustion is

avoidable while at the same time, product inhibition should be very low. It is also important to point out the fact that very high coefficient of determination r^2 (or correlation coefficient, r) does not justify presumed accuracy of result from any double reciprocal plot because, there may be negative intercept (or very low intercept) that result from disproportionate concentration of substrate at a lower part of a given concentration range. This was not the case in this investigation. However, the use of few data points seemed to be a set back and there was no attempt to use the same enzyme for the repeat of experiment. This however, informed the use of much larger number of different concentrations (6 different concentrations) of the substrate in subsequent experiments with another homologue [16,22]. It must be pointed out however, that the higher the concentration of the substrate at very low concentration of the enzyme, there could be trend toward saturation resulting to slight loss of very vital linearity. Linearity is a *sine qua non* for the effective and accurate determination of parameter of interest. Perhaps, it is appropriate to determine kinetic parameter, K_m in particular, using a number (say 7-9) of different [S]. Subsequently, very low different concentrations of the enzyme may then be assayed using different [S] whose values should include K_m as one of the concentrations or highest concentration, if it is high.

It must be stated however, that smartphone-based potentiometric biosensor has been applied in the quantitative analysis of human salivary alpha amylase in real human sample within 5 minutes [10]. Therefore, the advantage of this method lies in its speed unlike any other method including the current kinetic parameter dependent model which has additional value of being used to determine the molar mass of the enzyme. Thus if the concentration of an enzyme can be reproduced by the new model, the concentration of a crude extract of the same kind cannot be an exception.

5. CONCLUSION

The kinetic parameter dependent model reproduced, upon application, the mass concentration of stock and diluted solution of the stock. The model was also used successfully to determine the molar mass of the enzyme. The old and new or recent model can be used to determine the Gibbs free energy of activation for the hydrolysis of gelatinized starch with no significant difference ($P > 0.05$). Since the calculated molar mass based on the model is similar to that frequently reported for glycosylated human salivary alpha amylase, it leaves one without fear of contradiction that the enzyme purchased from Sigma-Aldrich, USA, may be the glycosylated human salivary alpha amylase. The model is therefore, multi – functional. Further research to re-verify the applicability of the model to other hydrolases including disaccharidase (maltase/sucrase) and any other microbial or mammalian amylase may be worthwhile.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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