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Equation Validating Kinetic Parameters Must be Dimensionally Valid: General Equation of Rate Constant in Focus

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Author's contribution

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

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ABSTRACT

Objectives: The objectives of this research are 1) to examine the units of parameters in quasi-steady state approximation (QSSA) equations (inequalities) with a view to confirming or otherwise the dimensional consistency, 2) to slightly modify the general equation of rate constant or *ES* as molar concentration of enzyme involved in complex formation, 3) to justify or otherwise the shift from free substrate to total substrate intended to extend the validity of standard QSSA otherwise called total QSSA,
Study Design: Theoretical and Experimental.
Place and Duration of Study: Department of Chemistry and Biochemistry, Research Division of Ude International Concepts LTD (862217), B. B. Agbor, Delta State, Nigeria; Owa Alizomor Secondary School, Owa Alizomor, Ika North East, Delta State, Nigeria. The research, including the derivation of equations, lasted between 10th Jan 2018 and 3rd Mar 2018.
Methodology: Bernfeld method of enzyme assay was used. Assays were carried out on *Aspergillus oryzea* salivary alpha-amylase.
Results: The total mass concentration of substrate as against free substrate values were < total

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substrate concentration at the time, t = o; both rate constant and total substrate (concerning mass conservation) showed increasing trend with increasing concentration of substrate, S.
Conclusion: The number of moles of each product, cannot be > the number of moles of enzyme-substrate complex. The molar concentration of bound enzyme can be calculated (Eq. 16). Equation (34) divided by molar mass gives a similar result as Eq. (16). The general rate equation needs the molar mass as denominator to be dimensionally consistent (Eq. (36)). The shift from the free substrate to "total substrate" seems valid going by the similarity between [S_T] - M_P and Eq. (19), Eq. (23), and Eq. (32b). The kinetic constants seem to satisfy mainly the condition for validity of rQSSA.

Keywords: Aspergillus oryzea alpha-amylase; quasi-steady state approximation; total concentration; free substrate concentration; general rate equation; Michaelis menten substrate constant; enzyme-substrate dissociation constant.

1. INTRODUCTION

There have been efforts by contemporary researchers to improve on the contribution of previous investigators. For instance Tzafriri [1] noted that Borghans et al. [2] extended the validity of the standard quasi-steady-state approximation (sQSSA) by employing a change of variable from S to the total substrate concentration, $S + C = S_T - P$ in which respectively, are the free substrate, enzyme-substrate complex as in the original definition but to be redefined as may be shown shortly, total substrate in the system and product. But the question that needs to be answered is, what is the difference between total substrate implied in $\hat{S} = S + C = S_T - P$ and total substrate concentration of the system, S_T?

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

$$E_T = E + C \quad (2)$$

$$S_T = S + C + P \quad (3)$$

The issue as stated earlier and in a submitted manuscript is that C in Eq. (1) cannot stand for the mass concentration of the substrate and mass concentration of the enzyme forming the complex at the same time. In Eq. (2) it may serve as mass concentration of the enzyme forming a complex with the substrate because, in the equation, the substances are of the same chemical species, and expresses mass conservation law for the enzyme only. The unit may be g/L or mol/L. It seems most authors are only interested in all kinds of QSSA and ignored fundamental concept, the stoichiometry of equation of reaction established many years ago [3] and continued to be relevant and completely unavoidable. Recently, equation for substrate mass conservation was derived [4] and there is also, the concept of mass-energy balance [5]. Which ever be the case, Lavoisier principle which states that matter is neither created nor destroyed in the non-nuclear reaction must be upheld. Thus in Eq. (3), C cannot represent the combined mass of the substrate and enzyme which gave the complex otherwise the concentration of the total substrate should be higher by the concentration of the free enzyme which combined to form the complex.

If C in Eq. (2) is usually v/k_2 whose product with a molar mass of the enzyme is the mass concentration of the enzyme forming a complex with the substrate the same cannot be said of Eq. (3). However, by the stoichiometric ratio of 1:1 for the formation of the enzyme-substrate complex, as indicated elsewhere [4] and for the fact that the molar mass of the substrate and the enzyme may not be the same, C in Eq. (3) should separately represent the mass concentration of the substrate which formed a complex with separate mass of the enzyme. Therefore, Eq. (2) and (3) which are similar to submission elsewhere (submitted manuscript) are restated as:

$$E_{Tmc} = E_{mc} + C_{2mc} \quad (4)$$

$$S_{Tmc} = S_{mc} + C_{3mc} + P_{mc} \quad (5)$$

where E_{Tmc}, E_{mc}, and C_{2mc} respectively stand for mass concentrations of total enzyme, free enzyme and mass concentration of the enzyme involved in complex formation (in Eq. (4)) and S_{Tmc}, S_{mc} , C_{3mc}, and P_{mc} stand respectively for mass concentrations of total substrate at t = 0, free substrate, substrate involved in complex formation with the enzyme, and mass concentration of the product detected by the

spectrophotometer: But it must be made clear that if a polysaccharide is a substrate, there may be fragments such as large oligosaccharides [4] maltodextrins [6].

For the extension of the domain of validity of sQSSA, which called for the change from free substrate to total substrate, the total substrate, $\hat{S}_{mc} = S_{mc} + C_{mc} = S_{Tmc} - P_{mc}$. It seems $\hat{S}_{mc}$ is the sum of all untransformed, free substrate, substrate fragments, and substrate forming complex with the enzyme but yet to be transformed. Thus, based on mole concept, C in Eq. (2) and Eq. (3) may be the same because the stoichiometric ratio is 1:1 with further understanding that for every amylolytic action only one molecule of reducing sugar, maltose or glucose is released as the case may be by the kind of hydrolase. Therefore, there is always a fragment until the time, $t \rightarrow \infty$. Hence to ensure mass balance and dimensional consistency, the molar concentration of the spectrophotometrically determined product must be multiplied by its known molar mass to give $[P_{mc}]$ as in Eq. (5): If $[S_{Tmc}]$ is divided by its molar mass, then the right-hand side of Eq. (5) must be divided by the molar mass of the substrate. So much has been said about the condition for validity of various equation of validation of kinetic parameters such as equation for total QSSA (tQSSA) in which the alternative equation for the rate constant (to be shown later in the text) has been implicated [1]. But there is need to consider the dimensional accuracy of the equation in question to justify its use in the equation used to validate kinetic parameters at high enzyme concentration (*i.e.*, where $[E_T] \gg [S_T]$).

Therefore, the objectives of this research are 1) to examine the units of parameters in QSSA equations with a view to confirm or otherwise the dimensional consistency, 2) to slightly modify the general equation of rate constant or *ES* as molar concentration of enzyme involved in complex formation, 3) to justify or otherwise the shift from free substrate to total substrate intended to extend the validity of sQSSA otherwise called tQSSA.

1.1 Theoretical Background

It is imperative to always respect and recognize pioneering work in any theoretical consideration; hence this section begins with the scheme below advanced in its original form as observed elsewhere [7]. In a paper by Baici [7] the work of Briggs and Haldane [8] was indicated in its original form as follows:

$$\begin{array}{ccccccc} & & & & k_1 & k_3 & \\ \mathrm{A} & + & \mathrm{E} & \rightleftharpoons & \mathrm{AE} & \rightarrow B & + \mathrm{E} \\ (a-x) & & (\mathrm{e}-p) & & k_2 & p & x \end{array} \quad (6)$$

The symbols of the original paper are used here: a, the initial concentration of A; x, the concentration of product B at a time t; e, the total concentration of E; k_1, k_2 and k_3, rate constants (today's customary k_1, k_{-1} and k_2 respectively); and p, the concentration of the AE complex. Briggs and Haldane suggested that the rate of change of p, the concentration of the AE complex, is negligible compared with the rate of change of x and (a - x). This means that the number of moles of any product must be different from the number of moles of the enzyme-substrate complex from which it is formed. This may be possible with a disaccharide whose hydrolysis gives two molecules of reducing sugar. The parameter $a - x$ means that a part of the substrate equal to x formed a complex with the enzyme. For a one-active site enzyme, the quantity of the enzyme on mole basis that bind the substrate cannot be < or > x. The latter, $a - x$, may be valid if x (in mol/L) of the substrate yielded x mol/L of the product. This expression is applicable if one product is yielded. But if for instance, the substrate is a disaccharide, two moles of the product, reducing monosaccharide, for every mole of the substrate should be produced. Then for every x mol of product x/2 mol of the substrate is hydrolyzed. If a polymer is the substrate, *e.g.* starch molecule, several molecules or reducing sugar and fragments of polysaccharides, mixtures of different saccharides, i.e., maltodextrins [6] should be formed. Therefore, total masses of the fragments, oligosaccharides, short and long, and reducing sugars may be taken into account such that, $x \sum M$ where $\sum M$ is the sum of the molar masses of all fragments and detected product (reducing sugar) excluding the mass of the enzyme given as xM_2, should be total mass of all product stated earlier.

The fundamental issue is that the number of moles of the substrate that disappeared to bind with the enzyme at the active site cannot be larger than the number of moles of the enzyme that formed the enzyme-substrate complex. Therefore, one may wish to know if $x = p$ in Eq. (6); if in time, t ($1/k_2$), one molecule of reducing sugar is formed from one molecule of polysaccharide, then one molecule of the fragment (ϕ-2) - if ϕ is the degree of

polymerization - and maltose, as the reducing sugar, are also formed. When t » $1/k_2$ but « $t \rightarrow \infty$ and [*ES*] of enzyme formed a complex, the number of moles of the substrate which formed a complex with the enzyme cannot be > [*ES*]. It is only the substrate, polysaccharide in this research (but it could be a polypeptide) that is transformed into various products illustratively designated as P_1, P_2, $P_3 \ldots P_R$ where $P_1 \rightarrow P_3 \ldots$ are fragments from various polysaccharides and P_R is the reducing sugar; all yielded within a duration, *t.* Expectedly as stated earlier $[P_1] = [P_2] = [P_3] \ldots [P_R] = v\,t$ (which is $\equiv t\,k_2\,[ES]$). It should take some times per minute = k_2 otherwise called turnover number, for enzyme concentration = [*ES*] to yield $[P_R]$ in time *t* or a determined molar concentration per unit time, *v*. This analysis may justify the assertion that *p* should not be different from *x*. This theoretical background will aid the derivation of general equation for *ES* (or *C*) for the enzyme.

1.2 Derivation of Equations

The second part of this theoretical consideration concerns one of the differential equations in its original form that abound in the literature [1,9] which leads to an equation of rate constant or molar concentration of the enzyme involved in complex formation.

$$\frac{\mathrm{d}[C]}{\mathrm{d}t} = k_1\left(([E_\mathrm{T}] - [C])[S] - K_\mathrm{m}[C]\right) \quad (7)$$

Next, the mass conservation equation in its original form in literature is as in the text ([$\hat{S}$] = [*S*] + [*C*]). According to Tzafriri [1] who omitted some steps, substitution of equation [$\hat{S}$]–[*P*] into Eq. (7) Gives an equation for the concentration of bound enzyme. Before proceeding further, $\frac{\mathrm{d}[C]}{\mathrm{d}t}$ has its unit as mol/L.min, because it is often the case that the concentration of the enzyme involved in complex formation like the total enzyme is mol/L. If so, the unit of concentration of the free substrate and Michaelis-Menten constant must be mol/L and consequently, the unit of second order rate constant, k_1 must also be L/mol.min. But dimensionally, it is immaterial if the unit of k_1 is L/g.min so long as the unit of [*S*] and K_m is g/L.

However, one may need to know what happens when [$\hat{S}$] – [*C*], *i.e*. [*S*] is substituted into Eq. (7), because, *ab initio* the unit of [*S*] is g/L for polysaccharide of unknown molar mass. Since substrate mass conservation equation is Eq. (3) or Eq. (5), [*C*] can no longer represent mass concentration or molar concentration of the enzyme involved in complex formation considering the equation ([$\hat{S}$] = [*S*] + [*C*] = [S_T] – [*P*] as in literature) or $\hat{S}_{mc} = S_{mc} + C_{mc} = S_{Tmc} - P_{mc}$ in this research is strictly substrate mass conservation equation. The parameter [*C*] cannot represent the mass concentration of the enzyme and substrate at the same time because they are different chemical species. According to Tzafriri [1], [$\hat{S}$] ≈ [S_T] at the initial transient; this implies that [*P*] ≈ 0 and [$\hat{S}$] – [*C*] ≈ [S_T]– [*C*].

However, if for simplicity sake δ is taken as the number of mol/L of the enzyme that formed a complex with the substrate, the concentration of the substrate cannot be > δ as earlier suggested in a submitted manuscript. The concentration of enzyme-substrate complex is δ'. The concentration of product (reducing sugar), fragment, and enzyme involved in the complex formation and released after the hydrolytic action is δ close to the initial transient. If this is the case, the mass of the product should be δM_p (or going back to the usual symbol, [*C*]M_p where M_p is the molar mass of maltose). Therefore, [$\hat{S}$] – [*C*]M_p = [*S*]. On account of this case, Eq. (7) can be re-written as:

$$\frac{\mathrm{d}[C]}{\mathrm{d}t} = k_1\left(([E_\mathrm{T}] - [C])([\hat{S}] - [C]M_\mathrm{p}) - K_\mathrm{m}[C]\right) \quad (8)$$

Equation (8) implies that the unit of [*C*] left and right and [E_T] remains mol/L so long as the unit of other parameters remains g/L while the unit of k_1 is L/g.min. On the other hand in line with Tzafriri's [1] proposal, [$\hat{S}$] ≈ [S_T] at the initial transient such that [S_T] can be substituted into Eq. (8) to give:

$$\frac{\mathrm{d}[C]}{\mathrm{d}t} = k_1\left(([E_\mathrm{T}] - [C])([S_\mathrm{T}] - [C]M_\mathrm{p}) - K_\mathrm{m}[C]\right) \quad (9)$$

Further algebraic manipulation of Eq. (8) is as good as doing same to Eq. (9). Expansion and rearrangement of Eq. (8) gives:

$$\frac{d[C]}{k_1 dt} = [E_\mathrm{T}][\hat{S}] - [C][\hat{S}] - [E_\mathrm{T}]\,[C]M_\mathrm{p} + [C]^2 M_\mathrm{p} - K_\mathrm{m}[C] \quad (10)$$

$$= [C]^2 M_\mathrm{p} - [C]\left(K_\mathrm{m} + [E_\mathrm{T}]M_\mathrm{p} + [\hat{S}]\right) + [E_\mathrm{T}][\hat{S}] \quad (11)$$

If the left-hand side of Eq. (11) is quadratic as suggested by Tzafriri [1], then the implication is that,

$$\frac{d[C]}{k_1 dt} = 0 \quad (12)$$

However, Eq. (12) is superior to an approximation such as,

$$\frac{d[C]}{k_1 dt} \approx 0 \quad (13)$$

Hence there is a clear direction towards sQSSA when $[S_T]$ is very high as to be » $[E_T]$ on mole – mole basis. But this research is solely interested in very high $[E_T]$ as to be > $[S_T]$ when the latter is very low, < K_m for instance. Nonetheless, given that Eq. (11) = 0, then,

$$[C_{\pm}] = \frac{(K_m+[E_T]M_P+[\hat{S}])\pm\sqrt{(K_m+[E_T]M_p+[\hat{S}])^2-4M_p[E_T][\hat{S}]}}{2M_p} \quad (14)$$

At the initial transient when $[\hat{S}] \approx [S_T]$,

$$[C_{\pm}] = \frac{(K_m+[E_T]M_P+[S_T])\pm\sqrt{(K_m+[E_T]M_p+[S_T])^2-4M_p[E_T][S_T]}}{2M_p} \quad 15)$$

Next, another equation of $[\hat{S}]$ is derived starting with,

$$[C]_- = \frac{(K_m+[E_T]M_P+[\hat{S}])-\sqrt{(K_m+[E_T]M_p+[\hat{S}])^2-4M_p[E_T][\hat{S}]}}{2M_p} \quad (16)$$

Equations (14), (15), and (16) are expressions of rate constant where [C] is v/k_2. All equations address the contentious issue of dimensional accuracy.

Expansion of Eq. (16) gives:

$$\frac{2vM_p}{k_2} - \chi = -\sqrt{\chi^2 - 4M_p[E_T][\hat{S}]} \quad (17)$$

where $[C]_-$ and χ are v/k_2 and $(K_m + [E_T]M_p + [\hat{S}]$ respectively. Squaring of Eq. (17), expansion, elimination, rearrangement, and factorization gives:

$$[\hat{S}]\left([E_T] - \frac{v}{k_2}\right) = \frac{v}{k_2}\left(K_m + [E_T]M_p - \frac{M_p v}{k_2}\right) \quad (18)$$

Then,

$$[\hat{S}] = \frac{v\left(K_m+[E_T]M_p - \frac{M_p v}{k_2}\right)}{k_2\left([E_T] - \frac{v}{k_2}\right)} \quad (19)$$

Alternative form of Eq. (19) is derived as follows. Squaring of Eq. (17), expansion, elimination, rearrangement, and factorization gives first:

$$\frac{4vM_p}{k_2}\left(K_m + [E_T]M_p + [\hat{S}]\right) = 4[\hat{S}][E_T]M_{\mathbf{p}} + \frac{4v^2M_p^2}{k_2^2} \quad (20)$$

Then further expansion, elimination, and rearrangement gives:

$$\frac{vM_p^2[E_T]}{k_2} - \frac{v^2M_p^2}{k_2^2} = [\hat{S}]M_p[E_T] - \frac{vM_pK_m}{k_2} - \frac{vM_p[\hat{S}]}{k_2} \quad (21)$$

Rearrangement of Eq. (21) gives

$$\frac{vM_p^2[E_T]}{k_2} - \frac{v^2M_p^2}{k_2^2} + \frac{vM_pK_m}{k_2} = [\hat{S}]M_p[E_T] - \frac{vM_p[\hat{S}]}{k_2} \quad (22)$$

The parameter $[\hat{S}]$ is made subject of the formula after rearrangement of Eq. (22) to give:

$$[\hat{S}] = \frac{\left(\frac{vM_p^2[E_T]}{k_2} - \frac{v^2M_p^2}{k_2^2} + \frac{vM_pK_m}{k_2}\right)}{M_p[E_T] - \frac{vM_p}{k_2}} \quad (23)$$

Equation (23) appears to be equivalent to Eq. (19) as long as v/k_2 is taken generally as being ≡ Eq. (16).

Beginning from Eq. (19) one can derive an equation for the determination of k_2 at the initial transient ($[\hat{S}] \approx [S_T]$). Rearrangement gives:

$$[S_T][E_T]k_2^2 - [S_T]k_2v = vK_mk_2 + [E_T]M_pv\,k_2 - M_pv^2 \quad (24)$$

The quadratic equation arising from Eq. (24) is:

$$k_{2\pm} = \frac{([S_T]v+[E_T]M_pv+vK_m)\pm\sqrt{([S_T]v+[E_T]M_pv+vK_m)^2 - 4[S_T][E_T]M_pv^2}}{2[S_T][E_T]} \quad (25)$$

k_{2+} appears to be more appropriate being »1unlike k_{2-}. Thus,

$$k_{2+} = \frac{([S_T]v+[E_T]M_pv+vK_m)+\sqrt{([S_T]v+[E_T]M_pv+vK_m)^2 - 4[S_T][E_T]M_pv^2}}{2[S_T][E_T]} \quad (26)$$

One can re-derive MM-like equation in terms of [S] when $\frac{d[C]}{k_1 dt} \approx 0$. Substituting [S]+[C] for $[\hat{S}]$ in Eq. (11) gives after expansion,

$$C^2M_p - [C][S] - C^2M_p - [C][E_T]M_p - [C]K_m + [E_T][S] + [E_T][C]M_p = 0 \quad (27)$$

After elimination and rearrangement, [C] is given as:

$$[C] = \frac{[E_T][S]}{[S]+K_m} \quad (28)$$

Schnell and Maini [10] derived the MM equation by replacing $[\hat{S}]$ with $[S_T] - [P]$ ($[S_T] - [P] \approx [S_T]$) leading to what appears to be initial transient condition, $[\hat{S}] \approx [S_T]$.

$$[C] = \frac{[E_T][S_T]}{[S_T]+K_m} \quad (29)$$

It is very obvious that when $[S_T] - [P]$ is used the result obtainable must be different from the result from Eq. (28). But Cornish-Bowden presents MM equation in another way as follows:

$$[C] = \frac{[E_T]([S_T]-[P])}{K_m + [S_T]-[P]} \quad (30)$$

It should be stated that $[S]$(or $[\hat{S}] - [C]$) $\neq [S_T] - [P]$ when, in particular, t » 0. Thus having established the value of $[C]$, Eq. (16), there may be need to determine the value of $[S]$ as in Eq. (28), and thereafter examine whether $[\hat{S}]$ (Eq. (19 or 23)) gives the same results as $M_p [C]$ (*i.e.* Eq. (16)) $M_p + [S]$. In the first place from Eq. (28),

$$[S] = \frac{[C]K_m}{[E_T]-[C]} \quad (31)$$

This is to say that,

$$[\hat{S}] = \frac{[C]K_m}{[E_T]-[C]} + [C]M_p \quad (32a)$$

$$= [C]\left(\frac{K_m}{[E_T]-[C]} + M_p\right) \quad (32b)$$

2. MATERIALS AND METHODS

2.1 Materials

Aspergillus oryzea alpha-amylase (EC 3.2.1.1) and 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 Mumbai India, and 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. The water bath was purchased from Hospibrand, USA.

2.3 Method

The method here is as previously described in the submitted manuscript but restated here for quick reference. Twenty grams of potato starch was mixed in 100mL of distilled water and boiled at 100ºC for 3 minutes, cooled to room temperature, and a decrease in volume was corrected by topping the volume with distilled water to 100 mL to give 20 g/L as stock. Dilution of the stock was made to give different concentrations ranging from 10-20 g/L and also 2 – 4 g/L. A stock solution of the enzyme was prepared by dissolving 0.01 g of the enzyme in a buffer tablet solution (*p*H=7) to which is added 15 mL NaCl (aq) (0.9 g/100 mL) and 15 mL $CaCl_2$ (5 mmol/L) to a final volume of 100 mL. The concentrations are stock solution concentration and diluted stock solution giving a final concentration of 0.04 g/L. Two different concentrations, the stock, and diluted solution were assayed for different reasons. A capsule of chloramphenicol was added to the solution of the enzyme to prevent any trace of microbial attack. The assay was carried out according to the method described by Bernfeld [11]. Spectrophotometer readings were taken at a wave length of 540 nm. The extinction coefficient was ~ 181 L/mol.cm. Kinetic parameters were determined by the alternative direct linear plot (ADLP) [12]. Microsoft Excel was used to draw the lines linking the x any y points (observations) on the x and y-axis respectively. To retain the lines drawn, the highest data point on the side representing the y-axis in the table of the variable (the points or observation) is left while the lower data points are deleted.

2.4 Statistics

Unpaired *t*-test for significant difference is carried out using internet-based graph pad (www.graphpad. com/quick calcs /*t*-test). Micro-soft Excel was used to determine standard deviation (n=6).

3. RESULTS AND DISCUSSION

The change from free substrate to the total substrate as reported in the literature [1,2] raises a question about what may constitute the difference between substrate concentration $[S_T]$ at the time, t = 0 and substrate concentration when $t > 0$ (or when t » $1/k_2$) or the free substrate concentration. Nonetheless, this question motivated the need to derive an equation with

which to evaluate the exact value of total substrate concentration distinct from [S_T] as implied in the derived Eq. (19) and (23). The simplest form of total concentration equation is: $[\hat{S}] = [S_T] - [P]\ (\text{or}\ [S] + [C])$ as to be in line with mass conservation law [4, 5]. The result of the latter has to be compared with the result from Eq. (23) as shown in Tables 1, 2, and 3. In all cases, using low substrate concentration regime (2 – 4 g/L.), high substrate concentration regime (10 – 20 g/L) with the same concentration of enzyme (Table 1), and assay in one minute and in five minutes (Table 2), the values from [S_T] – [P] and Eq. (23) were very similar and thus there was no significant difference (P > 0.05). Table 3 also showed similar results from [S_T] – [P] and Eq. (23) as to imply that there was no significant difference (P > 0.05) when much lower concentration of the substrate (0.5 – 4 g/L) and very high concentration of the enzyme (1.9231 exp (-6) mol/L) were used for assay. [$\hat{S}_{mc}$] = [S_{mc}]+ M_p[C]. as shown in Tables 1, 2, and 3. The results in all cases are similar to results from $[\hat{S}] = [S_T] - [P]$.

Since the general rate equation has been implicated to be part of the inequality that determines the sufficient condition for the validity of the initial transient approximation as originally advanced by Borghans et al. [2] and cited by Tzafriri [1] it has become very imperative to examine the equation so as to ascertain its dimensional consistency. This has been done as analysis of scheme 6 in particular shows. Briefly, if δ mol/L of enzyme substrate complex, ES undergoes catalytic cycle, breaking into maltose for instance where a polysaccharide is a substrate, free enzyme and fragments, the total mass of the right hand side of the equation includes the mass of the free protein involved in complex formation, the fragment and reducing sugar given as δ (M_2 + M_F + M_p) where M_2 and M_F are the molar masses of the protein, enzyme, and the polysaccharide fragment. By this in part, M_p appears in Eq. (16) and Eq. (26). In this respect, it is suggested that direct use of the original general rate equation as found in literature may not give dimensionally and quantitatively consistent parameter. If the substrate concentration regime is appropriate for a given enzyme then the value of the conventional equation, $k_2 = v_{max}/[E_T]$ should not be widely different from the values obtainable from Eq. (26).The corollary is that v/k_2 – generated data should also not be widely different from results obtainable from Eq. (16).

Indeed, it has been shown in this research that the results obtained from Eq. (16) were not significantly different (P > 0.05) from results obtained from v/k_2 as shown in Table 1 at higher substrate concentration (10 – 20 g/L) and in Tables 2 and 3. This was not to be where the substrate concentration range was 2 – 4 g/L. The mean of calculated k_2 values using Eq. (26) where substrate concentration was 2 – 4 g/L was ~1.7-fold higher than the value from $v_{max}/[E_T]$ where [E_T] = 0.769 μmol/L; where substrate concentration is 10 – 20 g/L, the mean of calculated k_2 values using Eq. (26) was 1.08-fold higher than the value from $v_{max}/[E_T]$; with the same substrate concentration range, 10 – 20 g/L, but with a much higher concentration of the enzyme (1.923 μmol/L) the mean of calculated k_2 values using Eq. (26) was ~ 1.04-fold higher than the value from $v_{max}/[E_T]$. The reason as to the wide disparity in the result for k_2 from different approaches where substrate concentration range is 2 – 4 g/L, unlike other higher concentration regime and much higher concentration of enzyme seems to be very difficult. However, one may resort to one of quasi-steady state assumption (QSSA) for the explanation. The closest, where k_2 values using Eq. (26) was ~ 1.04-fold higher than value from $v_{max}/[E_T]$ can be explained on the basis of the fact that when [E_T] » [S_T] or as proposed by Schnell [13] when $\frac{k_2 K_m}{(k_{-1}+k_2)[E_{T(mol)}]} \ll 1$ the condition for validity of reverse QSSA (rQSSA) in which $\frac{d[S_T]}{dt} \approx 0$ [9] is fulfilled. This seems to apply to a situation where the k_2 values using Eq. (26) was ~ 1.07-fold higher than the value from $v_{max}/[E_T]$ but to a lesser extend since perhaps there might have been a slight tendency to standard QSSA (sQSSA) with much higher substrate concentration regime. It seems where the value of k_2 by Eq. (26) was 1.7-fold higher than the value from $v_{max}/[E_T]$ is a reflection of the fact that neither the condition of validity of rQSSA nor sQSSA is fulfilled. Besides, the substrate concentration regime used in the latter case may be bellow the real Michaelis – Menten constant, K_m. It is possible too that the lower substrate concentration regime (2 – 4 g/L) may be approaching the value of the molar concentration of the enzyme (in this case [E_T] = 0.79 μmol/L). It must be made clear, however, that with high enzyme concentration, the substrate concentration regime may not be sufficient to saturate the enzyme and consequently, the K_m

may not be attained. The implication is that while K_m may appear in most of the inequalities or equations, it may not be exactly what it stands for rather, it may be enzyme substrate dissociation constant, K_s.

In the light of the need to justify the requirement for dimensional consistency, the quadratic equation for the determination of the concentration of bound enzyme is stated below, to examine the dimension of the equation [1].

$$C_{\pm} = \frac{(E_T+K_m+\hat{S}) \pm \sqrt{(E_T+K_m+\hat{S})^2 - 4E_T\hat{S}}}{2} \quad (33)$$

$$C_{-} = \frac{(E_T+K_m+\hat{S}) - \sqrt{(E_T+K_m+\hat{S})^2 - 4E_T\hat{S}}}{2} \quad (34)$$

Upon substitution of $[S_T] - 324P]$ or Eq. (32) into Eq. (33), the result after calculation gives a mass concentration of the bound enzyme which when divided by the molar mass of the enzyme yields results similar to the results reported in Tables 1, 2, and 3. Indeed results from Eq. (16) $\cong$ results from Eq. (34)/M_2. For instance, using Eq. (34) with lower substrate concentration regime, 2 – 4 g/L, the molar concentration of bound enzyme is $\approx$ 2.48 exp (-7) mol/L when $[S_{Tmc}]-[P_{mc}]$ = 1.911 g/L (Table 1). Further to the issue of dimensional consistency is what, Maini and Schnell [9] called the generalized rate equation for the formation of product given as:

$$v = \frac{k_2\left\{(E_T+K_m+\hat{S}) - \sqrt{(E_T+K_m+\hat{S})^2 - 4E_T\hat{S}}\right\}}{2} \quad (35)$$

The issue is that as long as v is the velocity of hydrolysis of substrate yielding reducing sugar, and its unit is mol/L, then all the parameters in the curled bracket must be in mol/L. If this should be the case, then two different chemical species, the enzyme and substrate with different molar masses, should be in mol/L. If this is not the case, then the concentration of the parameters in the curled bracket must be in g/L, and, as such, it should be the mass concentration of the enzyme bound in the enzyme-substrate complex, *ES* as reflected in Eq. (34). The fact that an idea is published in the journal of high repute (indexed) does not preclude a mistake and as such Eq. (35) may be in error that can be corrected by dividing the right-hand side by the molar mass of the enzyme. Doing so gives $\approx$ 2.48 exp (-7) mol/L k_2 when $[S_{Tmc}]-[P_{mc}]$ = 1.911 g/L (Table 1). Equation (35) may, therefore, be re-written as:

$$v = \frac{k_2\left\{(E_T+K_m+\hat{S}) - \sqrt{(E_T+K_m+\hat{S})^2 - 4E_T\hat{S}}\right\}}{2M_2} \quad (36)$$

The validity of the equations, Eq. (36) in particular, as to be more appropriate, has been questioned on the grounds that the equation has been developed by the sQSSA for the complex *C* without examining if the sQSSA holds for this case [Main and Schnell, 2000]. However, in this research, relatively high and much higher concentration of the enzyme were assayed as to imply that either $[S_T] \approx [E_T]$ or $[S_T] \ll [E_T]$. In the latter case, rQSSA should be applicable, and as such, the enzyme – substrate dissociation constant may be the case instead of K_m.

Table 1. Results arising from general kinetic equation and other equations after three minutes duration of assay

$v_{max}-v$ (μM/mL.min)	$[S_{Tmc}]-[P_{mc}]$(g/L)	$[\hat{S}_{mc}]$ (g/L)	$[C]_-$exp(–7) (mol/L)	v/k_2exp(–7) (mol/L)	$[S_{mc}]$ (g/L)	$[S_{mc}]+M_p[C]_-$(g/L)
Lower substrate concentration regime						
284.3	1.911	1.913	2.488760	3.787088	1.913126	1.913206
227.0	2.292	2.295	2.804030	4.574176	2.294493	2.294584
182.3	2.678	2.681	3.086755	5.188187	2.680898	2.680999
159.2	2.870	2.873	3.215570	5.505495	2.873137	2.873241
139.7	3.064	3.067	3.338590	5.773352	3.067346	3.067455
113.9	3.255	3.259	3.453280	6.127747	3.258558	3.258670
65.7	3.440	3.444	3.558750	6.789835	3.443765	3.443880
32.2	3.829	3.833	3.764250	7.250000	3.833192	3.833314

$v_{max} - v$ (μM/mL.min)	$[S_{Tmc}] - [P_{mc}]$ (g/L)	$[\hat{S}_{mc}]$ (g/L)	$[C]_{-}\exp(-7)$ (mol/L)	$v/k_2\exp(-7)$ (mol/L)	$[S_{mc}]$ (g/L)	$[S_{mc}] + M_p[C]_{-}$ (g/L)
Higher substrate concentration regime						
1094.3	9.891	9.891	1.841286	1.805810	9.8908570	9.890917
1076.3	11.885	11.885	2.110650	1.902636	11.884946	11.885000
1045.2	13.875	13.885	2.355819	2.069930	13.874924	13.875001
1019.3	14.867	14.867	2.470163	2.209252	14.866900	14.867000
998.8	15.860	15.860	2.579806	2.319527	15.859800	15.859890
961.0	16.848	16.848	2.684444	2.522862	16.847913	16.848000
935.2	17.840	17.840	2.785260	2.661646	17.839790	17.839880
886.2	19.824	19.824	2.975189	2.925229	19.823583	19.823679

As described in the text, v_{max}, v, $[S_{Tmc}]$, and $[P_{mc}]$ are respectively, maximum velocity of hydrolysis, velocity of hydrolysis, substrate mass concentration at time, $t = 0$, and mass concentration of product, reducing sugar, maltose to be specific;$[\hat{S}_{mc}]$, $[C]_{-}$, $[S_{mc}]$, and M_p are "total substrate concentration" as implied in mass conservation equation, molar concentration of enzyme involved in complex formation with the substrate, free substrate mass concentration, and molar mass of product –Values of $[C]_{-}$, $[S_{mc}]$, and $([S_{mc}]+M_p[C]_{-})$ are approximations to six decimal places to reveal differences between relevant parameters. The lower substrate concentration ranges from 2 – 4 g/L. The higher substrate concentration regime ranges from 10 – 20 g/L.

Table 2. Results arising from general kinetic equation and other equations after 1 min and 5 min duration of assay

$v_{max} - v$ (μM/mL.min)	$[S_{Tmc}] - [P_{mc}]$ (g/L)	$[\hat{S}_{mc}]$ (g/L)	$[C]_{-}\exp(-7)$ (mol/L)	$v/k_2\exp(-7)$ (mol/L)	$[S_{mc}]$ (g/L)	$[S_{mc}] + M_p[C]_{-}$ (g/L)
Where the duration of the assay is 1 min						
1560.3	9.816	9.816	1.958410	2.053282	9.814906	9.814967
1508.2	11.799	11.800	2.239051	2.241718	11.798879	11.798951
1445.7	13.779	13.780	2.492985	2.467559	13.778605	13.778685
1439.9	14.777	14.779	2.612248	2.488521	14.776717	14.776802
1385.0	15.759	15.761	2.724386	2.687040	15.758911	15.75900
1349.6	16.748	16.750	2.832358	2.814724	16.747456	16.747548
1317.9	17.737	17.739	2.935857	2.929434	17.737164	17.737250
1220.1	19.706	19.708	3.129121	3.282996	19.705451	19.705552
Where the duration of the assay is 5 min						
375.4	9.312	9.312	3.633844	4.083173	9.311892	9.312009
364.3	11.294	11.297	4.004634	4.189846	11.293895	11.294025
347.2	13.266	13.266	4.311970	4.353654	13.561894	13.266274
337.1	14.250	14.250	4.446820	4.451090	14.249612	14.249756
334.9	15.849	15.847	4.644238	4.472519	15.846117	15.846268
326.0	16.232	16.232	4.688380	4.558077	16.231798	16.231798
299.9	17.190	17.190	4.792653	4.809096	17.189495	17.189651
263.0	19.826	19.826	5.045567	5.163269	19.825855	19.825986

As described in the text, v_{max}, v, $[S_{Tmc}]$, and $[P_{mc}]$ are respectively, maximum velocity of hydrolysis, velocity of hydrolysis, substrate mass concentration at time, $t = 0$, and mass concentration of product, reducing sugar, maltose to be specific; $[\hat{S}_{mc}]$, $[C]_{-}$, $[S_{mc}]$, and M_p are "total substrate concentration" as implied in mass conservation equation, molar concentration of enzyme involved in complex formation with the substrate, free substrate mass concentration, and molar mass of the product. Values of $[C]_{-}$, $[S_{mc}]$, and $([S_{mc}]+M_p[C]_{-})$ are approximations to six decimal places to reveal differences between relevant parameters. The higher substrate concentration regime ranges from 10 – 20 g/L.

Table 3. Results are arising from the general kinetic equation and other equations in 3 min assay with low substrate concentration and much higher enzyme concentration

$v_{max} - v$ (μM/ml.min)	$[S_{Tmc}]-[P_{mc}]$ (g/L)	$[\hat{S}_{mc}]$ (g/L)	$[C]_{-}$exp (–7)(mol/L)	v/k_2 exp(–7) (mol/L)	$[S_{mc}]$ (g/L)	$[S_{mc}]+ M_p[C]_{-}$ (g/L)
818.0	0.441	0.441	2.984765	3.500000	0.440935	0.441032
731.0	0.912	0.913	5.298259	5.173077	0.912673	0.912844
613.0	1.375	1.375	7.002594	7.442308	1.374385	1.374612
552.0	1.855	1.986	8.705864	8.615385	1.985203	1.985485
512.5	2.342	2.342	9.497250	9.375000	2.341743	2.341743
487.3	2.834	2.834	10.411926	9.859615	2.833549	2.833549
439.3	3.318	3.319	11.159054	10.782692	3.317972	3.317972
413.2	3.810	3.810	11.797983	11.284615	3.809495	3.809496

As described in the text, v_{max}, v, $[S_{Tmc}]$, and $[P_{mc}]$ are respectively, maximum velocity of hydrolysis, velocity of hydrolysis, substrate mass concentration at time, t = 0, and mass concentration of product, reducing sugar, maltose to be specific;; $[\hat{S}_{mc}]$, $[C]_{-}$, $[S_{mc}]$, and M_p are "total substrate concentration" as implied in mass conservation equation, molar concentration of enzyme involved in complex formation with the substrate, free substrate mass concentration, and molar mass of product. Values of $[C]_{-}$, $[S_{mc}]$, and $([S_{mc}]+M_p[C]_{-})$ are approximations to six decimal places to reveal differences between relevant parameters. Much lower substrate concentration regime ranges from 0.5 – 4 g/L. The high enzyme concentration is 0.1 g/L (~1.923 μmol/L).

4. CONCLUSION

In this research, both scheme illustrating the complex formation and product formation and various equations were examined to establish their dimensional consistency. Hence it is confirmed that when δ enzyme-substrate complex breaks up in the forward direction, δ moles of reducing sugar, fragments (if polysaccharide is a substrate), and free enzyme are formed. Based on the analysis of the reaction scheme in the theoretical section, the molar concentration of bound enzyme can be calculated (Eq. 16). Equation (34) expresses the mass concentration of bound enzyme such that its division by molar mass gives a similar result as Eq. (16). The general rate equation needs the molar mass as denominator to be dimensionally consistent (Eq. (36)). The shift from the free substrate to "total substrate" seems valid going by the similarity (regarding result) between $[S_T]$ - $[P]$ M_P and Eq. (19), Eq. (23), and Eq. (32b). The kinetic constants seem to satisfy mainly the condition for validity of rQSSA.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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