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Total Enzyme-substrate Complex Which Includes Product-destined Enzyme-substrate Complex

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Author's contribution

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

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ABSTRACT

Background: There is no much interest in the determination of total enzyme-substrate complex concentration ($[ES]_T$) which includes undissociated *ES* that is unaccounted for unlike the usual *ES* destined for transformation into free enzyme and product or substrate. The reason is speculatively as a result of the lack of awareness of such possibility via sequestration.
Objectives: 1) To derive on the basis of both reverse – and standard – quasi-steady – state assumptions equations for the determination of $[ES]_T$ which is not restricted to the complex which dissociates to product/substrate and free enzyme and 2) quantitate the value of $[ES]_T$.
Methods: A theoretical research and experimentation using Bernfeld method to determine velocities of amylolysis with which to calculate relevant parameters.
Results: The $[ES_T]$ is < $[E]$ (*i. e*. $[E_T] - [ES]$); $[ES_T]$ decreased with increasing $[S_T]$ and increased with increasing concentration of enzyme $[E_T]$ while the velocity of amylolysis, v and maximum velocity of amylolysis, v_{max} expectedly increased with increasing $[E_T]$ and $[S_T]$.
Conclusion: The equations for the determination of the total enzyme-substrate complex, free enzyme without any complex formation before and after dissociation of enzyme-complex into product and/or substrate and free enzyme were derived. The difference, $[E_T] - [ES]$ is a heterogeneous mixture of undissociated *ES* and free enzyme without any complex formation. This is the case because $[ES]$ which dissociates into product is only a part of the total enzyme-substrate complex. There is a continuous formation of *ES* during and at the expiry of the duration of assay as long as there is no total substrate depletion.

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1. INTRODUCTION

Alpha-Amylase (E.C.3.2.1.1) is an enzyme that catalyses the hydrolysis of internal α-1,4-glycosidic linkages in starch into low molecular weight products, such as glucose, maltose and maltotriose units [1-3]. Amylases (including *Aspergillus oryzea* amylase in this research) are important industrial enzymes and are of great significance for biotechnology [3,4]. The enzyme, alpha-amylase as biocatalyst, is very important for processes and reactions peculiar to organic synthesis, pharmaceutical, food, cosmetics, and beverage preparations [5,6]. Unfortunately not all enzyme-substrate complex molecules dissociate into product. It is therefore, postulated that only a part of the total complex dissociates. If all the molecules of the *ES* dissociates into free enzyme and product at the same time, higher yield of the product could be the case.

According to Sharmila et al. [7], enzyme reactions do not follow the law of mass action directly. The rate of the reaction only increases to a certain extent as the concentration of the substrate increases. The maximum reaction rate is reached at high substrate concentration due to enzyme's saturation, described as being in contrast to the law of mass action [7]. This seems to precipitate researches into various concepts with which to validate kinetic parameters: In this regard a lot of research activities are devoted to the determination of kinetic parameters. The validity of such kinetic parameters are assessed on the basis of various quasi-steady-state assumptions [8-12] necessitating in some cases the concept of post-catalytic action total substrate concentration (POSTSC) [13-15]. This is in contrast to pre-catalytic action total substrate concentration (PRESC), at time $t = 0$. There is no much interest in the determination of total enzyme-substrate complex (*ES*) concentration which includes some complexes unaccounted for unlike the usual *ES* destined for transformation into free enzyme and product. The reason is speculatively as a result of the lack of awareness of the possibility of sequestration. The equations of mass conservation for the substrate and enzyme are known [13,14]; such equations often encountered are as follows:

$$[S_T] = [S]_{(t)} + [C]_{(t)} + [P]_{(t)} \quad (1)$$

Where $[S_T]$, $[S]_{(t)}$, $[C]_{(t)}$ and $[P]_{(t)}$ are the PRESC at time, $t = 0$, substrate concentration in time > 0, concentration of the substrate forming complex with the enzyme, and mass concentration of the product, maltose in this research respectively.

The POSTSC ($[\hat{S}]$) equation is

$$[\hat{S}] = [S]_{(t)} + [C]_{(t)} = [S_T] - [P]_{(t)} \quad (2)$$

Also in literature [16] there is another substrate mass- conservation equation given as

$$[S_T]_{(t=0)} = [S_f]_{(t)} + [C]_{(t)} + [S_{fr}]_{(t)} + [P]_{(t)} \quad (3)$$

Where, $[S_f]_{(t)}$ and $[S_{fr}]_{(t)}$ are the free substrate concentration and concentration of the fragment for a polysaccharide respectively. The mass conservation equation for the enzyme is given as

$$[E_T] = [E]_{(t)} + [C]_{(t)} \quad (4)$$

Where, in this case $[E_T]$, $[E]_{(t)}$ and $[C]_{(t)}$ are the total enzyme concentration in time, $t = 0$, free enzyme concentration in time $t > 0$ but « ∞ and enzyme-substrate concentration respectively (this is ≡ the concentration of enzyme involved in complex formation), within the same duration of assay. The proposition in this research is that only a part of the usual $[E]_{(t)}$ is in practical term free because complexes are always formed even at the end of a chosen duration of assay.

There is another method (derived equation) in literature for the determination of the concentration of substrate, enzyme-substrate complex, free enzyme, and product with non-mechanism based enzyme inactivation, in terms of dimensionless reaction diffusion parameters using unfamiliar homotopy perturbation method (HPM). The HPM is a technique for finding solutions of nonlinear equations without the linearisation process [7]. The objectives in this research are 1) to derive on the basis of rQSSA and sQSSA, the equations for the determination of total enzyme-substrate complex which is not restricted to the complex which dissociates to product and 2) quantitate the value of the total *ES*.

2. THEORY

In time t_1 before the end of the chosen duration of assay, t_∞ ($0 < t_1 < t_\infty$) the total complex is taken

as $[ES]_T$. Therefore, the free enzyme is $[E_T] - [ES]_T = [E_F]_{(1)}$. When an enzyme-substrate complex concentration equal to $[ES]$ breaks into free enzyme and either product or free substrate, the complex concentration left is $[ES]_T - [ES]$; in time t_∞ the free enzyme concentration is therefore, given as $[E_F]_{(2)} = [E_T] - ([ES]_T - [ES])$. It is obvious that $[E_F]_{(2)} > [E_F]_{(1)}$. Based on the preceding analysis one can derive the equation for the determination of the total concentration of enzyme-substrate complex which includes all kinds of complexes.

From the extant population of all normal enzyme-substrate complexes, *ES* complexes (*i.e*. active site and associated carbohydrate binding module [17,18] mediated complex formation), a subpopulation dissociates into product, free substrate, and free enzyme. Hence,

$$-\partial [ES]/\partial t = (k_{-1} + k_2)[ES] = k_{-1}[ES] + k_2[ES] \quad (5)$$

Where k_{-1}, k_2 and t are the reverse rate constant for the dissociation of *ES* into substrate and free enzyme, rate constant for dissociation into product and free enzyme, and time « 1 s respectively. The implication is that as $[ES]$ is decreasing, the free enzyme, $[E_F]$ is increasing. Therefore,

$$-\partial [ES]/\partial t = \partial[E_F]/\partial t = k_{-1}[ES] + k_2[ES] \quad (6)$$

But $[ES] = [E_T] - [E_F]$. Substitution of the latter into Eq. (6) gives

$$\partial [E_F]/\partial t = (k_{-1} + k_2)([E_T] - [E_F]) \quad (7)$$

Rearrangement gives

$$\frac{\partial [E_F]}{([E_T] - [E_F])} = (k_{-1} + k_2)\partial t \quad (8)$$

Next is integration as follows.

$$\int_{t_1}^{t_\infty} \frac{\partial [E_F]}{([E_T] - [E_F])} = (k_{-1} + k_2)\partial t \quad (9)$$

This gives

$$-\ln([E_T] - [E]) = (k_{-1} + k_2)\, t + c \quad (10a)$$

Where, as usual c is a constant. It needs to be emphasised that $[E_F]$ is hardly = 0, though a trend to zero is not unlikely as $[ES] \rightarrow [E_T]$. A parameter whose magnitude may be = 0 is $[ES]$ at $t = 0$. But $[E_F] = K_s\,[ES]/[S]$. Substitution of the latter into Eq. (10a) gives

$$-\ln\left([E_T] - \frac{K_s\,[ES]}{[S]}\right) = (k_{-1} + k_2)t + c \quad (10b)$$

If $t = 0$, then $c = -\ln [E_T]$ because $[ES] = 0$. Substitution of $-\ln [E_T]$ into Eq. (10b) gives

$$-\ln\left([E_T] - \frac{K_s\,[ES]}{[S]}\right) = (k_{-1} + k_2)t - \ln [E_T] \quad (11)$$

Rearrangement gives

$$\frac{1}{(k_{-1}+k_2)} \ln \frac{[E_T]}{[E_T] - \frac{K_S[ES]}{[S]}} = t \quad (12)$$

If the result of an enzyme catalysed reaction shows that $k_{-1} \ll k_2$, then,

$$\frac{1}{(k_2)} \ln \frac{[E_T]}{[E_T] - \frac{K_S\,[ES]}{[S]}} \approx t \quad (13)$$

On the other hand if $k_2 \ll k_{-1}$, then,

$$\frac{1}{(k_{-1})} \ln \frac{[E_T]}{[E_T] - \frac{K_S[ES]}{[S]}} \approx t \quad (14)$$

It needs to be stated categorically that the time t is not the chosen duration of an assay but belongs to a time scale « unity peculiar to events within and around the active site domain.

Next the total *ES* complex (this includes the *ES* formed outside the active site and the binding domains associated with the active site) is applied as follows. This is with the understanding that the total free enzyme resulting from dissociation of a part of $[ES]_T$ into existing *E* is the case.

First,

$$\partial [E]/\partial t = (k_2 + k_{-1})[ES]_T \quad (15)$$

Where, $[ES]_T$, the total *ES* complex = $[E][S_T]/K_s$. Therefore,

$$\int_{t_1}^{t_\infty} \frac{\partial [E]}{[E]} = (k_{-1} + k_2)\frac{[S_T]}{K_s}\partial t \quad (16)$$

Where, $[S_T]$ is the concentration of the substrate.

$$\ln \frac{[E_F]_{(2)}}{[E_F]_{(1)}} = (k_{-1} + k_2)\frac{[S_T]}{K_s} t \quad (17)$$

Both $[E_F]_{(2)}$ and $[E_F]_{(1)}$ have been defined earlier; substituting them into Eq. (17) gives

$$\ln \frac{[E_T] - [ES]_T + [ES]}{[E_T] - [ES]_T} = (k_{-1} + k_2)\frac{[S_T]}{K_s} t \quad (18)$$

As usual if $k_{-1} \ll k_2$

$$\ln \frac{[E_T] - [ES]_T + [ES]}{[E_T] - [ES]_T} \approx k_2 \frac{[S_T]}{K_s} t \quad (19)$$

And if $k_2 \ll k_{-1}$, then

$$\mathrm{I\,n}\frac{[E_\mathrm{T}]-[ES]_\mathrm{T}+[ES]}{[E_\mathrm{T}]-[ES]_\mathrm{T}} \approx k_{-1}\frac{[S_\mathrm{T}]}{K_\mathrm{s}}t \quad (20)$$

For the purpose of generalisation, (k_{-1} + k_2) is redesignated as k_x so that

$$\frac{K_\mathrm{s}}{[S_\mathrm{T}]k_\mathrm{x}}\mathrm{I\,n}\frac{[E_\mathrm{T}]-[ES]_\mathrm{T}+[ES]}{[E_\mathrm{T}]-[ES]_\mathrm{T}} = t \quad (21)$$

Pulling Eq. (12) and Eq. (21) together gives

$$\frac{1}{(k_\mathrm{x})}\mathrm{I\,n}\frac{[E_\mathrm{T}]}{[E_\mathrm{T}]-\frac{K_\mathrm{S}\,[ES]}{[S]}} = \frac{K_\mathrm{s}}{[S_\mathrm{T}]k_\mathrm{x}}\mathrm{I\,n}\frac{[E_\mathrm{T}]-[ES]_\mathrm{T}+[ES]}{[E_\mathrm{T}]-[ES]_\mathrm{T}} \quad (22)$$

Simplification and rearrangement of Eq. (22) gives first,

$$[E_\mathrm{T}] - [ES]_\mathrm{T} = \frac{[ES]}{\left(\frac{[E_\mathrm{T}]}{[E_\mathrm{T}]-K_\mathrm{S}\,[ES]/[S_\mathrm{T}]}\right)^{[S_\mathrm{T}]/K_\mathrm{S}}-1} \quad (23)$$

Thus,

$$[ES]_\mathrm{T} = [E_\mathrm{T}] - \frac{[ES]}{\left(\frac{[E_\mathrm{T}]}{[E_\mathrm{T}]-K_\mathrm{S}\,[ES]/[S_\mathrm{T}]}\right)^{[S_\mathrm{T}]/K_\mathrm{S}}-1} \quad (24)$$

The values of $[E_\mathrm{F}]_{(1)}$ and $[E_\mathrm{F}]_{(2)}$ can be restated as:

$$[E_\mathrm{F}]_{(1)} = \frac{[ES]}{\left(\frac{[E_\mathrm{T}]}{[E_\mathrm{T}]-K_\mathrm{S}\,[ES]/[S_\mathrm{T}]}\right)^{[S_\mathrm{T}]/K_\mathrm{S}}-1} \quad (25)$$

$$[E_\mathrm{F}]_{(2)} = \frac{[ES]}{\left(\frac{[E_\mathrm{T}]}{[E_\mathrm{T}]-K_\mathrm{S}\,[ES]/[S_\mathrm{T}]}\right)^{[S_\mathrm{T}]/K_\mathrm{S}}-1} + [ES] \quad (26)$$

3. MATERIALS AND METHODS

3.1 Chemicals

Aspergillus oryzea alpha-amylase (EC 3.2.1.1) and soluble potato starch were purchased from Sigma – Aldrich, USA. Tris 3, 5 – dinitrosalicylic acid, maltose, and sodium potassium tartrate tetrahydrate were purchased from Kem light laboratories Mumbia, India. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd, Poole England. Distilled water was purchased from local market. The molar mass of the enzyme is ~ 52 k Da [19,20].

3.2 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.

3.3 Methods

The enzyme was assayed according to Bernfeld method [21] using gelatinised potato starch whose concentration ranges from 5-10 g/L. Reducing sugar produced upon hydrolysis of the substrate using maltose as standard was determined at 540 nm with extinction coefficient equal to ~ 181 L/mol.cm. Concentration equal to 1g/100mL of potato starch was gelatinised at 100^{o}C for 3 min and subjected to serial dilution after making up for the loss of moisture due to evaporation. Concentration equal to 0.01 g/100mL of *Aspergillus oryzea* alpha-amylase was prepared by dissolving 0.01g of the enzyme in 100 mL of Tris HCl buffer at pH = 6, subjected first to a 2-fold dilution before concentrations equal to between 40 - and 60 - fold dilution of the diluted stock was assayed. The rest was stored in a freezer.

The kinetic parameters and subsequently rate constant for product formation and release in particular, were first determined according to Lineweaver-Burk method [22]. The total *ES* concentration, which includes the *ES* that is often unaccounted for, the *E* concentrations before the end of assay and at the end of assay were calculated using Eq. (24), Eq. (25), and Eq. (26) respectively. The rate constant, k_2 and the *ES* destined for product formation were calculated with $v_{max}/[E_T]$ and v/k_2 (where v is the velocity of amylolysis) respectively.

3.4 Statistical Analysis

All values of velocities of hydrolysis of starch obtained are expressed as mean ± SD; The SD was determined according to the method described by Hozo et al. [23]. The mean values of 4 determinations were used to determine other parameters.

4. RESULTS AND DISCUSSION

The issue of substrate mass balance or conservation abounds in literature [12-15,24]. There was a need to introduce the concept of total substrate concentration. This may be terminologically confused with the total substrate concentration as measured before the commencement of an assay or at the initial transient when $t \approx 0$. The concept suggests that at the expiry of the duration of assay, the free substrate calculated as either $[S_t] - [P]$ or $[S_t]/\exp(k\,t)$, where k and t are the pseudo-first order rate constant for the hydrolysis of starch and duration of assay respectively is composed

of substrate molecules (or granules) which are still in complex formation with the enzyme correctly with active sites and incorrectly with sites other than active site. Otherwise when a complex dissociates into product/substrate, it ceases to exist. But this does not stop complex formation at other locations within the system as dissociation takes place. This being clearly applicable to the enzyme called for the assays with different concentration of the enzyme in order to practically evaluate the formulated equations illustrating the phenomenon of sequestration [15,25] and the concept of post-catalytic action total enzyme concentration (POSTEC) as well as pre-catalytic action total enzyme concentration (PREEC).

Equation (24) describes quantitatively the total enzyme-substrate complex part of which proceeds to product formation and release and/or release of free substrate and enzyme in different locations in the reaction mixture. Equation (25) describes quantitatively the free enzyme before *ES* dissociation while Eq. (26) is the case after the dissociation of *ES*. Experimental results show as usual that the velocity of amylolysis and concentration of *ES* transformed to product increased with increasing concentration of the substrate as shown in Table 1 and Table 2 for lower and higher enzyme concentrations respectively. However, the issue is that not all the *ES* molecules dissociated into free enzyme, product, and substrate. This is the focus of this research. The concentration of the enzyme free from any complex formation before ES dissociation appeared to exhibit increasing trend in a more regular pattern with lower concentration of the enzyme (Table 1) unlike with higher concentration of the enzyme due perhaps to experimental error in the latter (Table 2).

Unexpectedly the calculated "heterogeneous" total [*E*] showed a decreasing trend with both lower (Table 1) and higher (Table 2) concentration of the enzyme; the adjective "heterogeneous" suggests the fact that [E_T] – [*ES*] gives result which does not imply that the

Table 1. Results of assay of ~ 1.60 exp (–8) mol/L enzyme showing velocity, *v* of amylolysis of gelatinised starch and the molar concentrations of reaction mixture chemical species

v (μM/min)	[*ES*] (exp (–9) M)	[*E*] (exp(–9) M)	$[ES]_T$ (exp(–9) M)	$[E_f]_{(1)}$ (exp (–9) M)	$[E_f]_{(2)}$ (exp (–9) M)
104.0 ± 2.2	2.6	13.4	10.2	5.8	8.5
118.2 ± 2.7	~ 3.0	13.0	9.6	6.4	9.4
135.1 ± 3.9	3.4	12.6	9.6	~6.5	9.9
151.0 ± 6.0	3.8	12.2	9.5	6.5	10.3
163.8 ± 1.5	4.1	11.9	9.3	6.8	10.9
174.9 ± 4.5	4.4	11.6	~9.0	7.0	11.4

The concentration of the gelatinised starch ranges between 5 -10 g/L. The adjective "heterogeneous" qualifying [E] suggests the fact that [E_f]– [ES] gives result which does not imply that the enzyme is totally free; there may be sequestrated enzyme that did not proceed to product regardless of the sites where the complex is formed. The parameters determined are: the velocity of amylolysis of gelatinised starch, v, enzyme-substrate complex, [ES] prepared for product release, "heterogeneous" concentration of free enzyme, [E], "total" enzyme-substrate complex, $[ES]_t$, free [E] before the end of assay, $[E_f]_{(1)}$, and at the end of assay, $[E_f]_{(2)}$

Table 2. Results of assay of ~ 2.40 exp (–8) mol/L enzyme showing velocity, *v* of amylolysis of gelatinised starch and the molar concentrations of reaction mixture chemical species

v (μM/min)	[*ES*] (exp (–9) M)	[*E*] (exp (–9) M)	$[ES]_T$ (exp (–9) M)	$[E_f]_{(1)}$ (exp (–9) M)	$[E_f]_{(2)}$ (exp (–9) M)
176.7 ± 9.0	4.7	19.3	14.7	9.3	14.0
195.1 ± 1.2	5.2	18.8	13.6	~10.5	15.7
222.7 ± 1.7	5.9	18.1	~13.6	10.5	16.4
254.0 ± 6.0	6.8	17.3	13.9	10.2	16.9
261.4 ± 4.5	7.1	16.9	13.1	10.9	18.1
294.5 ± 6.0	7.9	16.2	13.3	10.7	18.6

The concentration of the gelatinised starch ranges between 5 -10 g/L. The adjective "heterogeneous" qualifying [E] is as explained earlier under Table 1. The parameters determined are: the velocity of amylolysis of gelatinised starch, v, enzyme-substrate complex, [ES] prepared for product release, "heterogeneous" concentration of free enzyme, [E], "total" enzyme-substrate complex, $[ES]_t$, free [E] before the end of assay, $[E_f]_{(1)}$, and at the end of assay, $[E_f]_{(2)}$

POSTEC enzyme is totally free; there may be sequestrated enzyme that did not proceed to product formation regardless of the sites where the complex is formed. Opinion in this regard had been expressed over 49 years ago to the effect that over-all rates cannot be increased and will in general decrease by adsorbing the enzyme to large particulate structures (this, perhaps in this case are non-substrate particles) [26]. Against expectation is the observed decreasing trend in the total *ES* with the increasing concentration of the substrate; one should have expected greater adsorption or greater sequestration due to higher concentration of the substrate.

Since the concentration of *ES* prepared for transformation to product, for a given concentration of an enzyme increases with the increasing concentration of the substrate, it is not out of place to opine that the percentage of non-catalytic oriented *ES* formed at sites other than the catalytic active site and associated binding modules, is decreasing; this may mean that more enzyme molecules are left free even with the increasing $[S_T]$. This may be as a result of "cage" effect [27,28] associated with the highest concentration of substrate per a molecule of the enzyme. A cage effect may include a reduction of randomness of the molecules of enzyme that become within reach of the substrate and reduces tendency to incorrect binding to sites other than active sites. When higher concentration of catalytically oriented *ES* dissociates into product, equivalent high concentration of free enzyme is released into a pool of existing free enzyme molecules that are not bound in any complex thereby accounting for increasing free enzyme with increasing $[S_T]$. Sequestration or adsorption of enzyme may play a regulatory role in the metabolism of nutritional material and drug. This should be worthy of a feature investigation. However, sequestration does not imply that there is no possibility of dissociation. But binding of enzyme to the wrong target due to polarity and hydrophilicity may also reduce rate of catalytic function and in the case of a drug, the therapeutic potential may be compromised. The latter has often encouraged drug targeting approaches via nanoparticle/microparticle delivery systems which are under investigation and have a lot of applications [29]. There is also nanocaged enzyme with enhanced catalytic activity [27].

5. CONCLUSION

The equations for the determination of the total enzyme-substrate complex, free enzyme without any complex formation before and after dissociation of enzyme-complex into product and/or substrate and free enzyme were derived. The total enzyme-substrate complex values were lower than the usual $[E_T]$–$[ES]$, the so-called free enzyme. This means that the latter is a heterogeneous mixture of undissociated *ES* and free enzyme without any complex formation. This is the case because $[ES]$ which dissociates into product is only a part of the total enzyme-substrate complex. Thus, $[ES]_T + [E_f]_{(1)} = [E_T]$; $[ES] < [ES]_T$ and $[E] > [ES]_T$. The application of this finding may entail the need for a means of eliminating the product and the use of immobilised (or nanocaged) enzymes whose catalytic and carbohydrate binding domains are freely exposed to the free substrate molecules. Perhaps Le Chatelier's principle could be applicable in such cases.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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