1 ***Original Research Article***

2

3 **Title:** The key to effective catalytic action is pre-
4 catalytic site activity preceding enzyme-substrate
5 complex formation.

6 **ABSTRACT**

Aims: 1) To show that attractive electrostatic interaction is essential to stable enzyme-substrate formation, ii) to determine the minimum interparticle distance for maximum attractive interaction, iii) to determine the duration and the velocity of transit before enzyme substrate collision, and iv) to determine and show that the translational diffusion coefficient as time tends to infinity is much lower than at the beginning outside the influence of electrostatic interaction.

Study design: Theoretical and Experimental

Place and Duration of Study: Department of Chemistry and Biochemistry, Research Division, 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 lasted between June, 2016 and March, 2017.

Methodology: Bernfeld method of enzyme assay was used. Assays were carried out on *Aspergillus oryzea* salivary alpha amylase. Data obtained for the velocity of hydrolysis of starch were used to determine concentration of enzyme involved in catalytic activity. Each concentration and the concentration of substrate were used to calculate the maximum interparticle distance between the enzyme and substrate in a reaction mixture volume equal to 2mL.

Results: The terminal diffusion coefficient was 1.23±0.12 exp (**-**13) m^2 /s. The duration of transit through the shortest interparticle distance and the velocity were 78.9±5.5 μs and 91.0±1.5 μm/s respectively.

Conclusion: The electrostatic interaction model is suitable for the description of the binding of the enzyme to the substrate. The diffusion coefficient was expectedly « bulk diffusion coefficient. The work done (a function of hydrodynamic radius) by the advancing enzyme per unit time is unique to the nature of the bullet molecule**.** Diffusion coupled with attractive electrostatic interaction between combining particles could enhance the frequency of effective collision of the particles.

Keywords: Terminal diffusion coefficient; effective collision; electrostatic interaction*; Aspergillus oryzea salivary amylase; translational velocity.*

1. INTRODUCTION

It is a well known fact that one of the factors affecting rate of reaction is the concentration of reactants. Hence in medical practice, effectiveness of drug is predetermined by appropriate dosage. Also concentration and implicit interparticle distance can also influence the rate of intestinal digestion of food if excess water is taken in the course of ingestion of food. It is important for individual to take small volume of warm water during ingestion of food and larger volume about ≥ 20 min latter after consumption of food. Issues concerning concentration can be analyzed in terms of interparticle distance and random motion that may increase the time spent before collision takes place, the random distribution of interacting particles notwithstanding. The question is, can collision take place if there is repulsive interaction between the reactants, substrate and enzyme, and drug and poison to be specific? In order to destroy a poison the drug must bind to the poison molecule or pathogen, just as transformation of substrate begins as soon as enzyme-substrate complex is formed. This is unlikely if weak electrostatic repulsion occurs between the molecules. Yet there is a claim to the effect that for some enzyme catalyzed reaction in the presence of enabling factor, defined ionic strength, the rate of catalysis is higher when the substrate and the enzyme possess the same charge according to the equation, $\log k = \log k_0 + Z_A Z_B I^{1/2}$ where k is the measured rate constant, k_0 is the zero ionic strength rate constant, Z_A and Z_B are the electrostatic charges of the reacting species, and I is the ionic strength of the solution [1]. But it seems the model may be more suitable for product-active site catalytic group repulsive interaction that can enhance product departure that can create room for another catalytic round in a way that can minimize product inhibition. The important issue is that there are events or pre-catalytic activities of both enzyme and substrate. Such include translational diffusion, translational velocity occasioned by attractive interaction between the enzyme and substrate. There could be ineffective collision before effective collision takes place for binding of enzyme to substrate. Diffusion of enzyme to the surface of starch granule and ultimate binding to the surface of the granule has been observed [2]**.** This could have been impossible if there is repulsive interaction between the starch granule and the enzyme.

The importance of diffusion in any reaction, biological reaction in particular, as in this research for instance, cannot be overemphasized. Effect of diffusion on free enzyme and in particular,

immobilized enzyme, has been studied [3]. The authors showed that intraparticle diffusion resistance has a significant effect on the Congo red biodegradation rate. However, enzymes *in vivo* as well as in most *in vitro* studies are not immobilized. According to Berzzani *et al*. [4] alpha amylase hydrolysis is carried out by a side-by-side digestion mechanism but only after the enzyme diffuses and binds to the substrate. The authors observed that the rate of reaction is influenced by the structure of the substrate because it affects the rate of diffusion of the enzyme.

Furthermore, research by Butterworth *et al*. [2] shows that the diffusion coefficient of the amylase is derivable from the equation, $k = X^2/6D$, where X^2, k, and D are the surface area of the granule based on assumption of sphericity, apparent 1st order rate constant for the utilization of substrate, and diffusion coefficient of the enzyme. It is obvious that, the more finely divided or granular with smaller particle diameter, the greater the surface area exposed to collision and catalytic action: But how enhanced surface area can determine the magnitude of D, a parameter solely dependent on relative molecular mass, temperature, and consequently the prevailing coefficient of viscousity is not clear. It is however, instructive to note, according to Butterworth *et al*. [2], that "binding involves collision with the granule and then capture" and the granule–amylase collision rate in water is probably at least as high as 10^8/s. Besides, the value of D obtained using $k = X^2/6D$ is 1×10^{-10} cm^2/s (1×10^{-14}/m^2) [2].

While some reactions are diffusion controlled, others such as enzyme catalyzed reaction may not be. But no reaction can proceed without diffusion-dependent encounter complex formation and ultimately enzyme-substrate complex formation. The presence of substrate in the reaction mixture may constitute a crowding agent despite its presence as substrate. In this regard, effect of crowding on the rate of diffusion had been investigated [5-7]**.** The objectives in this research are: i) to show that attractive electrostatic interaction is essential to stable enzyme-substrate formation, ii) to determine the minimum interparticle distance for maximum attractive interaction, iii) to determine the duration and the velocity of transit before enzyme substrate collision, and iv) to determine and show that the translational diffusion coefficient as time tends to infinity is much lower than at the beginning outside the influence of electrostatic interaction.

1.1 Theory

Let the attractive electrostatic energy (w) be expressed according to Coulomb's law as:

$$w = \phi\, e^2/4\pi\varepsilon_0\varepsilon_r R_0 \qquad (1)$$

where e, ε_0, ε_r, and R_0 are charge of an electron, permittivity in a vacuum, relative permittivity of water, and interparticle distance between enzyme and substrate. The interparticle distance is of two kinds, the maximum interparticle distance before strong encounter complex formation and the minimum interparticle distance reached when the particles close the gap or distance between them. If Z_+ and Z_- are the charges (which may not be known, without separate experimental determination) of the interacting particles, starch and enzyme molecules, the factor ϕ is introduced to serve as variable which depends on the magnitude of R_0. Whichever is the charge of the substrate, it should not be a full charge because it merely contains polar group such as - $O^{\delta-}$ — $H^{\delta+}$.

In the first place, attractive interaction between the substrate and enzyme is proposed because it may be impossible to achieve encounter complex formation and stability let alone the enzyme-substrate complex formation if there is mutual repulsive interaction. Yet, if the net charge of the enzyme is known at a given *p*H, the partial charge of the substrate may not be known. Therefore, the factor ϕ is taken to be a multiple of energy based on Coulomb law. Since the partial and full charges are not known except by separate experimentation, there should be a way of eliminating them as may be shown shortly. Although enzyme and substrate are stated, the model formulation is a general one as it may be applicable to "missile" (or preferably bullet)-target relationship. Thus it could be applied to soluble drug-pathogen/poison interaction.

As the bullet molecule moves under electrostatic influence towards the target molecule/cell, it reaches a terminal velocity (u_b). Therefore, with Stokes-Einstein model, the electrostatic force (f_{es}) is given as:

$$f_{es} = \phi\, e^2/4\pi\varepsilon_0\varepsilon_r R_0^{\,2} = 6\pi\eta r_b u_b \qquad (2)$$

It is assumed that so long as activity of the enzyme for instance occurs with a given concentration, there may have been attractive interaction achieved when the enzyme reaches a point at which there could be attractive influence. At this juncture it is important to state Einstein model $\hat{l}^2/2D$ (where l and D are the average distance and diffusion coefficient respectively) is very much applicable strictly to defined average distance covered in which large number of molecules undergoing random motion are involved. Where random motion ends, directional motion made possible by electrostatic influence begins. Thus the initial random motion which may increase the distance covered before electrostatic influence assumes preeminence is not taken into account. Rather Coulomb's law is allowed to be the decisive factor that determines the magnitude of f_{es}. It cannot be overemphasized that if $R_0 \rightarrow \infty$, f_{es}

and $u_b \rightarrow$ zero. With time, the substrate and enzyme come to rest when the enzyme-substrate complex is formed so that $u_b \rightarrow$ zero. This is not to suggest that there is no more motion, rather whatever motion, it should be the motion of the complex due to thermal energy and not as separate molecular motion.

Making u_b subject of the formula in Eq. (2) gives:

$$u_b = (\phi\, e^2/4\pi\varepsilon_0\varepsilon_r R_0^2)/6\pi\eta r_b \quad (3)$$

If the work per unit time of the bullet molecule in overcoming random motion and solvent resistance is P, then, w is given as:

$$w = Pt_e \quad (4a)$$

where t_e is the time spent in covering a distance of r_{ts} (this is, according to Newtonian mechanics = t_e $u_b/2$). In the light of Eq. (4a),

$$Pt_e = \phi\, e^2/4\pi\varepsilon_0\varepsilon_r R_0 \quad (4b)$$

Meanwhile,

$$t_e = 2r_{ts}/u_b \quad (5)$$

Replacing t_e in Eq. (4b) with Eq. (5) gives:

$$2Pr_{ts}/u_b = \phi\, e^2/4\pi\varepsilon_0\varepsilon_r R_0 \quad (6)$$

Substituting Eq. (3) for u_b in Eq. (6) gives:

$$\phi\, e^2/4\pi\varepsilon_0\varepsilon_r R_0 = (2Pr_{ts}/\phi\, e^2)\, 4\pi\varepsilon_0\varepsilon_r R_0^2.\, 6\pi\eta r_b \quad (7)$$

Making ϕ^2 subject of the formula in Eq. (7) yields:

$$\phi^2 = 12\pi\eta r_b P\, (4\pi\varepsilon_0\varepsilon_r/e^2)^2 R_0^3 r_{ts} \quad (8)$$

Meanwhile let,

$$r_{ts} = \Upsilon_b(R_0 - \acute{R}) \quad (9a)$$

where $\acute{R}$ is the distance between the centres of the bullet and the target or the sum of their hydrodynamic radii (the enzyme and gelatinized starch molecule, for instance, as in this study) and is a fraction which takes into account the fact that the distance travelled by the enzyme is a fraction of the total distance between the particles. The parameter is defined as:

$$\Upsilon_b = (M_3/M_2)^{1/2}/((M_3/M_2)^{1/2}+1) \quad (9b)$$

where M_3 and M_2 are the molar masses of the starch molecule and the enzyme molecule respectively, such that, $M_3 > M_2$. Substitution of Eq. (9a) into Eq. (8) and upon simplification gives:

$$\phi = \Upsilon_b^{1/2}\, 13.8564\, (\pi\eta r_b P)^{1/2}\, \pi\varepsilon_0\varepsilon_r\, [R_0^3(R_0 - \acute{R})]^{1/2}/e^2 \quad (10a)$$

Substituting Eq. (10a) for ϕ in Eq. (3) gives:

$$u_b = (\tau_b)^{1/2}\, 13.8564\, (\pi\eta r_b P)^{1/2}\, \pi\varepsilon_0\varepsilon_r\, [R_0^3(R_0 - \acute{R})]^{1/2}\, e^2/e^2.4\pi\varepsilon_0\varepsilon_r\, R_0^2.6\pi\eta r_b \quad (10b)$$

Simplification of Eq. (10b) yields:

$$u_b = 13.8564(P\tau_b/\pi\eta r_b)^{1/2}\, [R_0^3(R_0 - \acute{R})]^{1/2}/24R_0^2 \quad (11)$$

$$u_b = 0.57735(P\tau_b/\pi\eta r_b)^{1/2}\, [(R_0 - \acute{R})/R_0]^{1/2} \quad (12)$$

Meanwhile,

$$u_b = 2\nu(R_0 - \acute{R})\, \tau_b \quad (13a)$$

where ν (which can be expressed as Smolucowski's equation [8] below) is the frequency of collision of the lighter enzyme with the larger molecular mass substrate that may be less soluble.

$$\nu = 2\pi\, \acute{R}\, D_b\, C_\infty \quad (13b)$$

where D_b and C_∞ are the diffusion coefficient and concentration of colliding molecules (enzyme as the bullet molecule) per cubic metre respectively.

$$C_\infty = 10^3 N_A v/k_2 \quad (13c)$$

where v and k_2 are the velocity of transformation of substrate and rate constant for product formation; 10^3 is the conversion factor from litres to cubic metre. The expression v/k_2 is the concentration of the enzyme in mol/L involved in the hydrolysis of starch.

Combining Eq. (12) and Eq. (13a) gives:

$$2\nu\, (R_0 - \acute{R})\, \tau_b = 0.57735(P\tau_b/\pi\eta r_b)^{1/2}\, [(R_0 - \acute{R})/R_0]^{1/2} \quad (14a)$$

Squaring both sides of Eq. (14a) gives:

$$[2\, \nu\, (R_0 - \acute{R})\, \tau_b]^2 = 0.57735^2\, (P\tau_b/\pi\eta r_b)((R_0 - \acute{R})/R_0) \quad (14b)$$

Simplification and rearrangement gives:

$$\nu^2 = (0.57735/2)^2\, P/\tau_b\pi\eta r_b\, R_0\, (R_0 - \acute{R}) \quad (15a)$$

Simplification gives:

$$\nu^2 = 0.083333255P/\tau_b\pi\eta r_b\, R_0\, (R_0 - \acute{R}) \quad (15b)$$

Taking the square root of Eq. (15b) gives:

$$\nu = 0.288675\, \{(P/\tau_b\pi\eta r_b\, R_0\, (R_0 - \acute{R})\}^{1/2} \quad (15c)$$

Looking at Eq. (15c), ν is clearly inversely proportional to R_0 if $R_0 \gg \acute{R}$. The capacity of the enzyme to attract or to be catalytically attracted to the substrate should influence the frequency of effective collision for complex formation. The coefficient of viscousity is temperature dependent,

157 decreasing with increasing temperature, that may result in the increase and decrease in ν and t_e
158 respectively.

159 For the mesophiles and thermophiles, the activity increases with an increase in
160 temperature at temperatures below the melting point. The increasing temperature increases the
161 conformational flexibility needed for function apart from increase in collision rate with directionality
162 made possible by attractive interaction between the enzyme and substrate. This is also applicable to
163 drug-pathogen/poison/inhibitor interaction. The psychrophiles, unlike mesophiles and thermophiles,
164 which are already in a state of conformational flexibility [9] is mainly controlled by the lower rate of
165 collision due to lower temperature but largely compensated for by the high conformational flexibility of
166 the enzyme's active site.

167 The work down can also be stated as:

168 $$w = 6\pi\eta r_b u_b R_0 \quad (15d)$$

169 Therefore,

170 $$P = 6\pi\eta r_b u_b R_0 \nu \quad (16a)$$

171 **1.1.1 Alternative expression for work per unit time.**

172 To obtain another expression for P there is need to start from the known to the unknown. The
173 maximum value of R_0 is known and it is given as: $10^{-6} V/(n_S + n_E)N_A\}^{1/3}$ where $n_E = 1000.V_E v/k_2$ where
174 V_E is the volume of enzyme used, and n_S is the number of moles of the substrate used. The volume of
175 substrate (V_S) used *is* $= V_E = 1$ *mL. 1000* is the conversion from mL to liter while 10^{-6} is the
176 conversion factor from mL to m^3; $V = V_E + V_S$. To avoid confusion it is hereby restated that there are
177 two forms of R_o, maximum interparticle distance (Max.R_0) before attractive interaction between
178 particles begins and the minimum interparticle distance (Mini.R_0) at which attractive interaction
179 begins. Therefore, from the plot of ν^2 versus $1/R_o(R_o - \acute{R})$, using Eq. (15b) where R_0 (R_0 = Max.R_0) is
180 used, the resulting first slope ($S_{lope\text{-}1}$) is: $0.083333255P/\tau_b\pi\eta r_b$. Hence,

181 $$P = S_{lope\text{-}1}\tau_b\pi\eta r_b/0.083333255$$

182 $$= 12\, S_{lope\text{-}1}\tau_b\pi\eta r_b$$

183 (16b)

184 **1.1.2 Determination of translational velocity of the enzyme in terms of different slopes**

185 Equations (16a) and Eq. (16b) are similar. Thus,

186 $$6\pi\eta r_b u_b R_0 \nu = 12\, S_{lope\text{-}1}\tau_b\pi\eta r_b \quad (16c)$$

Simplification of Eq. (16c) gives after rearrangement:

$$\nu = 2\, S_{\text{lope-1}} \mathfrak{r}_b / u_b\, R_0 \tag{17}$$

A plot of ν versus $1/R_0$ (recall once again that for this purpose R_0 = Max. R_0) gives a second slope ($S_{\text{lope-2}}$) given as:

$$S_{\text{lope-2}} = 2S_{\text{lope-1}} \mathfrak{r}_b / u_b \tag{18}$$

Thus,

$$u_b = 2S_{\text{lope-1}} \mathfrak{r}_b / S_{\text{lope-2}} \tag{19}$$

1.1.3 Determination of minimum R_0 values

Meanwhile, substituting Eq. (16b) into Eq. (12) gives:

$$u_b = 0.57735(12S_{\text{lope-1}})^{1/2} \mathfrak{r}_b\, [(R_0 - \acute{R})/R_0]^{1/2} \tag{20a}$$

Since Eq. (19) expresses translational velocity derivable from two different constants (different slopes), then when Eq. (19) and Eq. (20a) (or the simplified form) are combined, the value of R_0 in Eq. (20a) becomes the initial/starting minimum interparticle distance (Mini.R_0) at which electrostatic interaction begins under the given condition. Therefore, R_0 is redesignated as Mini.R_0 in subsequent equations.

Taking the square of Eq. (20a) gives:

$$u_b^2 = 0.57735^2.12S_{\text{lope-1}} \mathfrak{r}_b^2\, (\text{Mini.}R_0 - \acute{R})/\text{Mini.}R_0 \tag{20b}$$

Simplification gives:

$$u_b^2 = 4\, S_{\text{lope-1}}\, \mathfrak{r}_b^2\, (\text{Mini.}R_o - \acute{R})/\text{Mini.}R_0 \tag{20c}$$

Taking the square root of Eq. (20c) gives:

$$u_b = 2\mathfrak{r}_b \{S_{\text{lope-1}}\, (\text{Mini.}R_0 - \acute{R})/\text{Mini.}R_0\}^{1/2} \tag{20d}$$

There should be a value of Mini.R_0 in Eq. (20d) which gives result similar to the result using Eq. (19). Combining Eq. (19) and Eq. (20d) enables one to determine the very interparticle distance where electrostatic influence begins practically. Thus,

$$2S_{\text{lope-1}} \mathfrak{r}_b / S_{\text{lope-2}} = 2\mathfrak{r}_b \{S_{\text{lope-1}}\, (\text{Mini.}R_0 - \acute{R})/\text{Mini.}R_0\}^{1/2} \tag{21a}$$

Simplification and squaring of Eq. (21a) gives:

$$S_{\text{lope-1}}\, (\text{Mini.}R_0 - \acute{R})/\text{Mini.}R_0 = (S_{\text{lope-1}} / S_{\text{lope-2}})^2 \tag{21b}$$

Simplification and rearrangement of Eq. (21b) yields:

$$\text{Mini.}R_0 - \acute{R} = \text{Mini.}R_0\, S_{\text{lope-1}} / S_{\text{lope-2}}^2 \tag{21c}$$

Making Min.R_0 subject of the formula gives:

$$\text{Mini.}R_0 = \acute{R} / \{1 - (S_{\text{lope-1}} / S_{\text{lope-2}}{}^2)\} \quad (21d)$$

In order to determine the Bjerrum length, ϕ can be substituted into Bjerrum equation below.

$$\lambda_B = \phi\, e^2 / 4\pi\varepsilon_0\varepsilon_r\, k_B T \quad (22)$$

Replacing ϕ in Eq. (22) with its expression (Eq. 10a), gives after simplification,

$$\lambda_B = (12\pi\eta r_b P)^{1/2}[R_0{}^3 \boldsymbol{\tau}_b (R_0 - \acute{R})]^{1/2} / k_B T \quad (23)$$

$$\lambda_B = 12\pi\eta r_b \boldsymbol{\tau}_b [S_{\text{lope-1}} R_0{}^3 (R_0 - \acute{R})]^{1/2} / k_B T \quad (24)$$

The implication of Eqs (23), (24) and (10a) is that λ_B and ϕ respectively may vary according to the value of R_0, either Max. R_0 or Min. R_0 as the case may be.

2. MATERIALS AND METHODS

2.1 Chemicals

Aspergillus oryzea alpha amylase (EC 3.2.1.1) and soluble potato starch (molar mass = 1000 kg/mol [10]) 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 [11, 12].

2.2 Equipment

Electronic weighing machine was purchased from Wenser Weighing Scale Limited and 721/722 visible spectrophotometer was purchased from Spectrum Instruments, China. *p*H meter was purchased from Hanna Instruments, Italy.

2.3 Method

The enzyme was assay according to Bernfeld method [13] using gelatinized potato starch whose concentration ranges from 3-24g/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/10mL of potato starch was gelatinized 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 0.01g/100mL of *Aspergillus oryzea* alpha amylase was prepared by dissolving 0.01g of the enzyme in 100 mL of Tris HCl buffer at *p*H = 7. Concentration equal to 0.02 g/L was then prepared by appropriate dilution of the stock solution of the enzyme. The rest was stored in a freezer. The kinetic parameters and subsequently rate constant for product formation and release in particular, were first

247 determined according to Lineweaver-Burk method [14]. The work done per unit time by advancing
248 enzyme molecule, its translational velocity, Min.R_0, and the Bjerrum length were calculated using
249 equations (16b), (19), (21d), and (23/24) respectively.

250 **2.4 Statistical Analysis**

251 All values obtained are expressed as mean ± SD. Each parameter is an average of values from four
252 determinations.

253 **3. RESULTS AND DISCUSSION**

254 The velocities of hydrolysis of different concentration of gelatinized starch are shown in Table
255 1. From the molar concentrations of the combining enzyme and substrate the average (maximum)
256 interparticle distances (Max.R_0) was calculated and results are shown in Table 1. In order to
257 determine the work per unit time against solvent resistance, the square of ν of effective collision was
258 plotted versus the reciprocal of the product of Max.R_0, and the difference between the latter and the
259 sum of the radii of the combining enzyme and substrate (Fig.1). Figure 2 shows the plot of ν versus
260 $1/R_0$ (R_0 = Max.R_0) for the determination of translational velocity of the advancing enzyme.

261 The results (Table 2) show clearly the minimum interparticle distance where the maximum
262 attractive effect occurred, the translational velocity, and translational diffusion coefficient which, is
263 lower than single solution-component diffusion coefficient of the enzyme in the absence of the
264 substrate. Specifically the diffusion coefficient obtained in this research (Table 2) is about 10-fold
265 higher than the value advanced by Butterworth *et al* [2]. It seems papers on diffusion of whatever kind
266 expresses the effect of interparticle distance but not in a manner that is reflective of such minimum
267 distance between particles that can result in initial mutual electrostatic interaction. Reduction in
268 translational diffusion coefficient has been attributed to the effect of molecular crowding whereby the
269 resulting hydrodynamic interaction reduces the “dilute-state” translational diffusion coefficient. “The
270 reduction factors found for bovine serum albumin (BSA) in the study, 0.2 at 25% volume fraction and
271 0.4 at 13% volume fraction, occur already at nanoseconds time scale and are attributed solely to
272 hydrodynamic interactions, *i.e.*, an increased effective viscosity of the cellular medium, but not to
273 hindrance due to obstacles” [7]. In the present study, the decrease in the translational velocity of the
274 protein - the enzyme – is due to binding after initial terminal velocity resulting from “semi-electrostatic”
275 attraction between the enzyme and substrate but resisted by the solvent medium. This is important in
276 the light of the need to stabilize the enzyme-substrate complex. The substrate merely possess “partial

charge" because it is not ionic unlike the enzyme that may possess net charge determined by a given *p*H status. The implication in the light of Coulomb law is that the value of Z for the substrate in the equation $Z.Z_{+}e^{2}/4\pi\varepsilon_{0}\varepsilon_{r}R_{0}$, is not up to a unit charge, be it either negative or positive. Experimental studies have shown that decreases in the diffusion coefficient of positively and negatively charged nanoparticles (up to three orders of magnitude) in reconstituted extracellular matrix (ECM) hydrogels are due to electrostatic attraction and binding [15, 16].

Results in the past have shown that neutral particles diffuse faster than charged particles [17]. For uncharged particles, like the starch molecules, the diffusion coefficient decreases as a result of steric and hydrodynamic interactions [17]. The same authors report that for charged particles like the enzyme electrostatic forces cause an almost uniform decrease in the diffusivity of the particles. Therefore, the decrease in translational diffusion coefficient of the enzyme which, has a net charge under a given *p*H, and expected decrease in translational velocity (u_b) as binding occurs as observed in this research (Table 2) cannot be an exception. The conclusion by the authors [17] to the effect that optimal particles for delivery to tumors should be initially cationic to target the tumor vessels and then change to neutral charge after exiting the blood vessels is similar to the proposition in this research that product departure from active site is better enhanced if repulsive term takes preeminence while attractive interaction should be the case for approaching bullet and target, enzyme and substrate or drug and poison/pathogen /cancer cell, as the case may be. This is also similar to the view that the interaction between protein and starch is mainly electrostatic in nature, between the anionic groups of the starch and the positively charged groups of the protein [18]**.** However, the anionic groups referred to by the authors may not necessarily imply negative charge as applicable to a protein; but rather, it may be polar as applicable to chemically unmodified gelatinized starch.

The minimum interparticle distance between the substrate and the enzyme was determined. It was expectedly shorter than the average interparticle distance referred to as maximum interparticle distance which depends on reaction mixture concentration. This ultimately influences the rate of enzymatic hydrolysis of the starch which is under the effect of the magnitude of translational velocity, translational diffusion coefficient, and ultimately collision frequency. Increasing concentration of the substrate in the presence of fixed concentration of the enzyme justifies this claim (Table 1). However, it has been suggested that in the diffusion-controlled limit where every encounter between reactants results in a reaction, the reduction of diffusion in the crowded environment will lead to the reduced

307 reaction rate [5, 7]. But the presence of some additives (though in this research gelatinized starch is
308 seen both as a crowding agent and a substrate) is known to enhance enzymatic activity, though such
309 additives may be much smaller than the enzyme. Most stabilizing agents are organic in nature.
310 Therefore, starch and protein such as albumin may cause the enzyme's stability and consequently,
311 enhance its activity. Besides it is known that crowding is a regular event in gastrointestinal tract during
312 meal as well as in the mouth where the first digestion of starch/glycogen begins quickly despite the
313 fact that saliva is a multi-component fluid containing other proteins. While the rate of collision may be
314 high, for whatever reason, not all collisions result to effective enzyme-substrate or drug pathogen
315 complex formation. This is similar to the claim that in the biophysics of association reactions, not
316 every encounter will result in a reaction [5]**.** Specific binding occurs through sites (active sites for
317 instance) that must be properly aligned for the reaction to occur, and this is referred to as anisotropic
318 reactivity [5, 7]. It may not be wrong to suggest that the presence of improperly oriented substrate
319 molecules (starch) as free substrate may constitute crowding agent. This may promote what has been
320 called caging effects (which keep reactants, the enzyme and substrate in proximity) that could
321 increase the reaction rate by increasing the probability of reorientation and recollision [5]. This may be
322 in line with the proposition that unbinding of substrate from the active site enhances the rate of
323 hydrolysis [19]. In line with anisotropy is the explanation offered by Berzzani *et al*. [4]**,** to the effect that
324 a side-by-side digestion mechanism is employed by the enzyme. This presupposes a unidirectional
325 enzyme-substrate catalytic orientation accounting for anisotropy. It is not unlikely that effective
326 electrostatic attraction, higher substrate concentration that can promote cage effect and high mobility
327 of the enzyme can reduce the effect of anisotropy.

328 Although it has been pointed out that the rate of formation of encounter complex and
329 ultimately, enzyme substrate complex can be hindered due to increased viscosity, hindrance due to
330 obstacles, and transient adsorption at larger obstacles [7] there is always increasing velocity of
331 hydrolysis (Table 1) with increasing concentration of substrate. This is not unexpected because
332 viscousity is temperature dependent while obstacles cannot be everywhere at the same time under a
333 given temperature an index of thermal energy that can always perturb any non-catalytic binding.
334 Coupled with stronger electrostatic attraction between the substrate and enzyme there should be
335 continuous enzymatic action so long as there is no substrate exhaustion. Force of attraction imposes
336 directionality thereby reducing randomness or precisely the entropic factor [20]. The importance of

electrostatic interaction between the active site and the substrate is better appreciated if consideration is given to tendency to thermally induced disorder that can dislodge the substrate from the catalytic site. Thus the electrostatic energy must not be less than the thermal energy $k_B T$ when the interparticle distance approaches zero. If the contrary is the case, then unfolding of the enzyme, which may have lost its capacity to bind the enzyme, may have occurred. This is where Bjerrum length given as $\lambda_B = e^2/4\pi\varepsilon_0\varepsilon_r k_B T$ (≈ 7.155 Å at 298.15 K, for instance) becomes very useful so long as it can be applied to charge-polar interaction involving either multivalent or univalent protein. Indeed it may be applicable given the fact that without information about the partial charge of the substrate and the charge of the enzyme, let alone where such information is known, the Bjerrum length can be determined according to model formulated in this research.

Given the value of ϕ (≈ 0.281), or using Eq.(23), at a shorter/minimum value of R_0, short interparticle distance at which electrostatic energy is equal to thermal energy is ≈ 2.03±0.06 Å. This value compared to 7.155 Å should not be unexpected considering the fact that, two electrostatically interacting univalent/multivalent charged particles has greater attractive force than charge-polar electrostatic attraction applicable to gelatinized starch and the enzyme for instance. This is to say that under the influence of the full charge of the interacting particle, the value of R_0 should be longer. The importance of Bjerrum length is better appreciated if one realizes the fact that the catalytic activity or rate constant may be higher at higher temperature which should however, be lower than melting temperature. Therefore, at such length, the substrate and enzyme are at their shortest interparticle distance with sufficiently strong electrostatic attraction that can bind the substrate against thermal destabilization. This is in line with the observation that, while the driving force for ligand binding is often ascribed to the hydrophobic effect, electrostatic interactions also influence the binding process of both charged and nonpolar ligands [21]. Enhancement of the diffusional association rates can be achieved by attractive electrostatic interactions between the substrate and the protein binding site. Therefore**,** the localized potentials at the binding site are sufficient for efficient electrostatic steering of the substrate into the binding site [21]**.** This discussion can be brought to an end, by stating that the model should be a very good guide to ingestion of food without much gastrointestinal dilution through the ingestion of much water (which must be warm when taken in small volume, < half a glass) just as pharmacodynamic defined as the observed effect resulting from a certain drug concentration [22] is best achieved with adequate and safe drug dose.

Table 1. Results of assay showing velocity of hydrolysis of gelatinized starch, and calculated maximum interparticle distance, maximum distance likely to be covered, and their product.

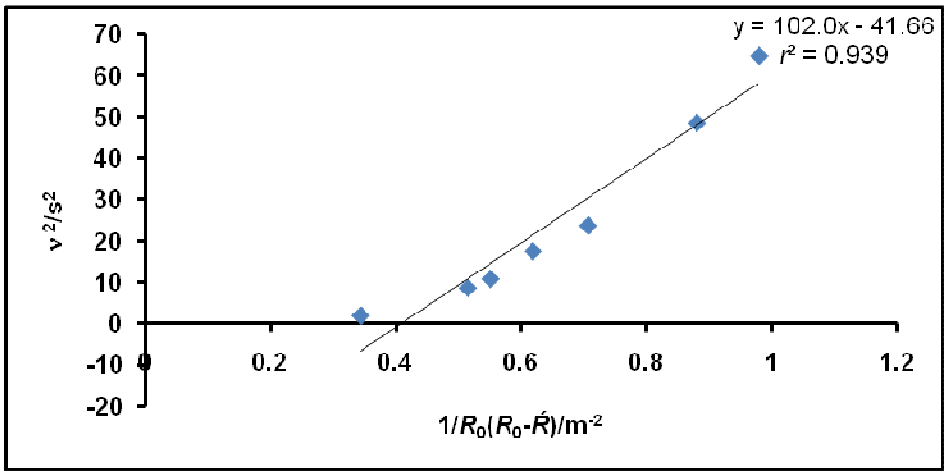
v / U/mL	Max. R_0 exp(-7)/m	Max. (R_0 - $\acute{R}$) exp(-7)/m	Max. R_0(R_0 - $\acute{R}$) exp(-14)/m^2
1.15±0.15	1.80±0.06	1.70±0.06	3.06±0.11
2.37±0.29	1.45±0.05	1.35±0.05	1.96±0.07
2.66±0.08	1.39±0.01	1.29±0.01	1.79±0.01
3.35±0.66	1.30±0.08	1.20±0.11	1.56±0.14
3.93±0.00	1.24±0.00	1.14±0.00	1.41±0.00
5.61±0.00	1.12±0.00	1.02±0.00	1.14±0.00
6.54±0.00	1.06±0.00	0.96±0.00	1.02±0.00

First slope, S_{LOPE-1} = 102.00±2.76 exp (-10) / $(m/s)^2$; second slope, S_{LOPE-2} =182.60±7.92 exp(-6). The range of the concentration of gelatinized potato starch is 3-24g/l. The first slope is from the plot of square of frequency of collision (ν) versus the reciprocal of the product of maximum interparticle distance (Max.R_0) and the difference between Max.R_0, and the sum ($\acute{R}$) of the radii of colliding particles, the substrate and enzyme to be specific. The values of Max.R_0 were calculated from $\{10^{-6}V/(n_S + n_E)N_A\}^{1/3}$ were $n_E = 1000.V_E\nu/k_2$ where V_E is the volume of enzyme used, and n_S is the number of moles of the substrate used. The volume of substrate (V_S) used is = V_E = 1 mL. 1000 is the conversion from mL to liter while 10^{-6} is the conversion factor from mL to m^3; $V = V_E + V_S$. The second slope is from the plot of ν versus 1/Max.R_0. The hydrodynamic radii of gelatinized potato starch and Aspergillus oryzea alpha amylase are 7.37nm and 2.61nm respectively. Bulk diffusion coefficient (D_b) = 9.395 exp (-11) m^2/s; k_2 = 26437.97±263.09/min at 298.15K and pH = 7. Results were approximated to two decimal places.

Table 2. Results showing minimum interparticle distance for electrostatic attraction, minimum distance before collision, power of attractive interaction and other physico-chemical parameters.

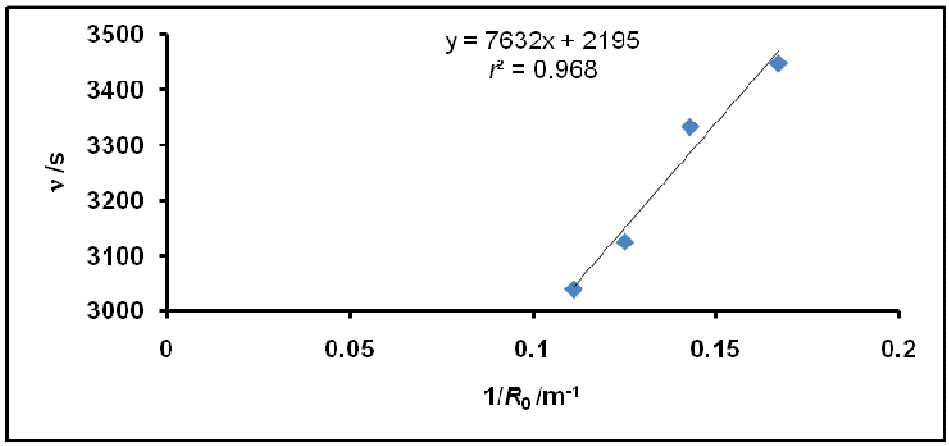
Mini.R_0/nm	Mini. (R_0 - $\acute{R}$)/nm	P/ exp(-19)/J/s	u_{es} /exp(-5)/m/s	t_e /exp(-5)/s	D_∞/exp(-13)/ m^2/s
14.39±0.38	4.41±0.38	7.29±0.20	9.10±0.15	7.89±0.55	1.23±0.12

Min.R_0, Min.(R_0 - $\acute{R}$), P, u_{es}, t_e, and D_∞ are the minimum interparticle distance, minimum distance covered before the enzyme comes to rest during complex formation, power of attractive interaction, translational velocity, duration of transit, and translational diffusion coefficient respectively as the enzyme comes to rest upon binding to the substrate after existence as free molecular entity.



389

390 **Fig. 1. A plot of ν^2 versus $1/R_0\,(R_0\text{-}\acute{R})$, for the determination of work done per unit time against**
391 **solvent resistance by the advancing enzyme molecule.** R_0 in this case is the maximum
392 interparticle distance, Max.R_0 and $\acute{R}$ is the sum of the radii of colliding particles; ν is the frequency of
393 collision.



394

395 **Fig. 2. A plot of ν versus $1/R_0$, for the determination of translational velocity under the**
396 **influence of electrostatic attraction.** R_0, in this case, is the maximum interparticle distance and ν is
397 the frequency of collision.

398 **4. CONCLUSION**

399 In conclusion, the electrostatic interaction model is most suitable for the description of the
400 binding of the enzyme to the substrate. The minimum interparticle distance is expectedly shorter than
401 the average interparticle distance determined from dissolved solute (enzyme and starch) per unit
402 volume. The diffusion coefficient is 1.23±0.12 exp (-13) m^2/s which is expectedly « bulk diffusion
403 coefficient. The duration of transit through the shortest (minimum) interparticle distance and the

velocity are 78.9±5.5 μs and 91.0±1.5 μm/s respectively. Although electrostatic attraction becomes stronger as interparticle distance decreases, both hydrodynamic interaction over a short distance and binding effect reduce the rate of translational motion. The work done by the advancing enzyme (or bullet in general, enzyme or drug) per unit time is unique to the nature of the bullet molecule. If the bullet molecule is made more mobile duration of transit should be shorter. Ultimately, it is important to ingest small quantity of warm water during meal to avoid dilution.

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