

# Why substrate depletion has apparent first-order kinetics in enzymatic digestion

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## Abstract

A number of enzyme digestion assays show apparent first-order kinetics of reactant depletion. There are four possible explanations of this phenomenon: (i) the reaction is dominated by a first-order limiting step, (ii) the digestion follows a pseudo-first-order kinetics under the excess of a reactant species, (iii) the first-order kinetics is only applicable to the slow transient of the reaction, or (iv) the aggregate behavior of the reaction pathway produces behavior indistinguishable from the first-order kinetics. In this paper, we investigate the kinetics for protein digestion by formulating rate equations for two proposed mechanisms, namely the one-by-one mechanism and the zipper mechanism. Our analysis shows that the kinetics of protein digestion follows apparent first-order kinetics irrespective of the mechanism for low initial substrate concentration compared to the initial enzyme concentration. Also, our results provide an explanation for experimental observations and suggest a new experimental protocol that could reveal information on the mechanism of digestion.

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## 1. Introduction

Determining the substrate decay is a common objective in many agricultural (Herman and Scherer, 2003) and enzyme digestion studies (Herman et al., 2003; Noda et al., 1994). Enzyme digestion is used to evaluate the potential of novel proteins to act as food allergens. No single protein property is known to correlate highly with the allergenic potential of a protein, so a weight-of-evidence approach is employed. This is where several factors are considered together to predict whether a novel protein will act as an allergen (Codex, 2003; Metcalf et al., 1996).

Enzyme digestion involves complex pathways and multiple mechanisms. For example, 14 peptides and a number of intermediates have been identified as products of trypsin digestion of reduced propylated lysozyme (Noda et al., 1994). Pepsin digestion of Cry34Ab1 and Cry35Ab1 shows the appearance of multiple smaller fragments of approximately 15–40 kDa as intermediates and products (Herman et al., 2003). The kinetics of these proteases is commonly believed to follow either

the one-by-one scheme or the zipper scheme (Bull and Currie, 1949; Hollands and Fruton, 1968; Sachdev and Fruton, 1975). In spite of the complicated reaction pathways, kinetics of protein digestion and several intermediate products show simple decay curves with apparent first-order behavior. This pattern can often be described by a single exponential decay, at least during the terminal phase of degradation, and appears to describe the digestion of both parent proteins and digestion fragments (Baderschnider et al., 2002; Garrett et al., 2004; Herman and Scherer, 2003; Herman et al., 2005; Noda et al., 1994). When a multiphasic pattern of decay is seen, the terminal phase has been observed to be slower than the initial phase (Anson, 1938; Herman et al., 2003, 2005; Takagi et al., 2003).

This apparent first-order reaction kinetics of the reactant disappearance is not exclusive to the proteases. A number of complex reaction pathways with several intermediate products show this behavior as well (Herman and Scherer, 2003; Bandstra and Tratnyek, 2005). In past two decades, Bardsley et al. (see, for example, Bardsley et al., 1980; Bardsley, 1983) have shown that enzyme kinetics mechanisms with several steps can give rise to simple rate profiles and double reciprocal plots, if the rate constants chosen are subject to an arbitrary upper limit. Bardsley has shown that complex mechanisms can exhibit curves similar to

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that of simple mechanisms. Could the time-course of a complex reaction mechanism show the same behavior as a simple reaction mechanism? The experimental evidence seems to suggest that this is the case. It is not clear why complex reactions can exhibit simple behavior, or what the conditions are for the validity of this phenomenon. There are four proposed explanations of this phenomenon:

- Reactions, such as protein digestion, are multi-step processes, and a small number of rate-determining steps usually determine the kinetics of complex reaction (Hinch and Schnell, 2004; Kluger, 1990).
- The time evolution of enzyme-catalyzed reactions follows a pseudo-first-order kinetics under conditions of low substrate concentration with respect to the Michaelis–Menten constant (Schnell and Mendoza, 2004).
- The first-order kinetics is only applicable to the slow transient of the reaction. It has been shown that enzyme-catalyzed reactions can be described by a first-order kinetics after an initial transient if the rapid-equilibrium approximation is valid (Schnell and Maini, 2000).
- The aggregate behavior of the complex pathway produces a behavior indistinguishable from the first-order kinetics. A recent computational study (Bandstra and Tratnyek, 2005) of parallel irreversible zero-, first-, or second-order reactions demonstrate that the reactant disappearance converges to an apparent first-order kinetics.

Caution is warranted in interpreting results and comparing the rates of pepsin digestion determined using the kinetic approach which relies on the apparent first-order substrate disappearance. Many other factors can also affect the digestion rates such as enzyme activity, purity of protein substrate, concentrations of pepsin and substrate, pH, temperature, or the homogeneity of the reaction medium. Moreover, enzyme digestion is a multi-step reaction process, and the kinetics may not be simple to predict. For example, pepsin has been shown to hydrolyze proteins using different mechanisms depending on reaction conditions (Choisnard et al., 2002), or depending on the protein substrate. One pepsin molecule cleaves the protein substrate once, and then dissociates (the one-by-one mechanism). Alternatively, a pepsin molecule cleaves the protein substrate multiples times without dissociating from it, generating intermediate peptide products as it goes (the zipper mechanism). It seems also possible that pepsin digestion can be somewhere between these two extremes. Choisnard et al. (2002) have shown that at pH 4.5, pepsin hydrolyzed native hemoglobin uses the one-by-one mechanism, while pepsin hydrolysis of denatured hemoglobin is controlled by the zipper mechanism. However, it is unclear whether the rate of decay of the starting substrate would follow first-order kinetics regardless of the mechanism.

The primary objective of this study is to find an explanation for the apparent first-order behavior observed in the digestion of proteins. Secondly, we consider how this behavior relates to the reaction mechanism. We analyze the enzyme–substrate kinetics of both the one-by-one and zipper mechanism and compare the behavior with the kinetics of proteases. We simulate

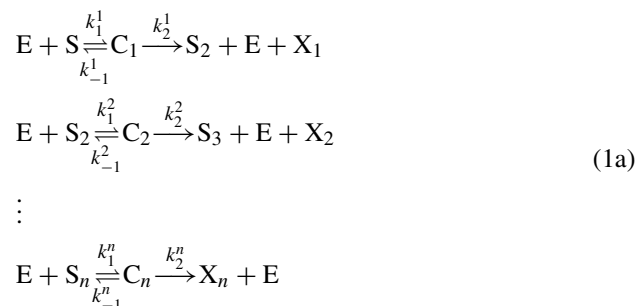
the kinetics of the protein–enzyme interaction by these two mechanisms with large number of intermediates. The important aspect of our study is that the rate constants are chosen from a uniform random distribution with varying ranges. This is in order to account for a potentially large number of intermediates in the digestion process whose kinetics are unknown. The results of the simulation are compared and characterized to a first-order linear fit. Our study has an important result in understanding the applicability of the first-order models often used to profile protein digestions in simulated gastric fluid in the agricultural, food chemistry and pharmaceutical community (England et al., 1997; Win et al., 2005). Also, we suggest a new perspective of experimental investigations that could reveal more about the actual mechanism of the digestive process.

## 2. Materials and methods

The two possible mechanisms proposed for protein hydrolysis are the one-by-one and the zipper mechanism.

### (i) One-by-one mechanism

In this mechanism, the digestion enzyme breaks one protein molecule at a time, and then the enzyme dissociates from the substrate. This process can be represented schematically:



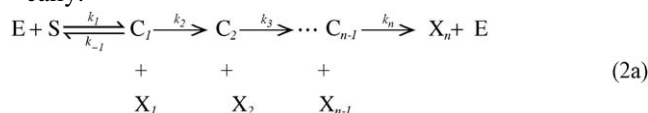
Here, the primary substrate  $S$  (the target protein) combines with an enzyme  $E$  to form a complex  $C_1$ . The complex is then transformed into another substrate  $S_2$  and  $X_1$ .  $X_1$  may be a low molecular weight (MW) peptide.  $S_2$  combines with the same enzyme  $E$  to form another complex  $C_2$  and is then converted into  $S_3$ . This process continues until the final fragment  $X_n$  is produced. Here,  $n$  is the number of intermediate reactions before the final fragment  $X_n$ .

The rate equations for one-by-one mechanism are as follows:

$$\begin{aligned}
 \frac{dS}{dt} &= k_{-1}^1 C_1 - k_1^1 S E \\
 \frac{dS_i}{dt} &= k_{-1}^i C_i - k_1^i S_i E + k_2^{i-1} C_{i-1} \quad \text{for } i = 2, 3, \dots, n \\
 \frac{dC_i}{dt} &= k_1^i S E + k_1^i S_i E - (k_{-1}^i + k_2^i) C_i \quad \text{for } i = 2, \dots, n \\
 \frac{dE}{dt} &= -\sum_{i=1}^n \frac{dC_i}{dt}
 \end{aligned} \tag{1b}$$

(ii) Zipper mechanism

In this mechanism, the digestion enzyme binds to the substrate and remains as a complex until the protein is completely digested. This process is represented schematically:



The enzyme E binds to the primary substrate S and forms a complex C<sub>1</sub> and a low MW fragment X<sub>1</sub>. At the first cleavage, the enzyme is irreversibly bound, forming another complex C<sub>2</sub> and small peptide X<sub>2</sub>. This process then continues until the final fragment X<sub>n</sub> is produced.

The rate equations describing the zipper mechanism are as follows:

$$\begin{aligned} \frac{dS}{dt} &= -k_1SE + k_{-1}C_1 \\ \frac{dC_1}{dt} &= k_1SE - k_{-1}C_1 - k_2C_1 \\ \frac{dC_i}{dt} &= k_iC_{i-1} - k_{i+1}C_i \quad \text{for } i = 2, 3, \dots, n \\ \frac{dE}{dt} &= -k_1SE + k_{-1}C_1 + k_nC_{n-1} \end{aligned} \quad (2b)$$

2.1. Simulation procedure

We study the general behavior of these reaction mechanisms using a numerical approach. We consider a broad range of parameter values and initial conditions, and we examine the behavior of the time-course of the substrate depletion in the proteolysis. The simulations were carried out using Matlab 7.0. One experimental run consists of the following: we choose *n*, the number of intermediate steps, the initial substrate S<sub>0</sub> and enzyme E<sub>0</sub> concentrations, and *k*<sub>min</sub> and *k*<sub>max</sub>. We randomly choose the *k*<sub>*i*</sub>s, from a uniform distribution within this range. Then, we run the simulation for both reaction mechanisms. We repeat this for 50 sets of *k*<sub>*i*</sub> and take the average of all these results. We study the primary substrate (S) time-course since this is what is measured in experiments (Anson, 1938; Herman et al., 2003, 2005; Takagi et al., 2003). We examine the logarithmic plot of the time-course of the primary substrate and characterize this using linear regression. This is in order to compare our results qualitatively with that of experimental observations. The goodness of fit is estimated using the linear correlation coefficient *r*. This correlation coefficient *r* is defined as follows:

$$r = \frac{N \sum t \log S - \sum t \sum \log S}{\sqrt{[N \sum t^2 - (\sum t)^2] [N \sum (\log S)^2 - (\sum \log S)^2]}} \quad (3)$$

where *N* is the number of points in the curve.

We vary all the parameters, *n*, S<sub>0</sub>, E<sub>0</sub>, *k*<sub>min</sub>, and *k*<sub>max</sub>, to find the range of validity of this apparent first-order behavior. We look, in particular, at the magnitude of the range of the rate constants, Δ*k* = *k*<sub>max</sub> - *k*<sub>min</sub>.

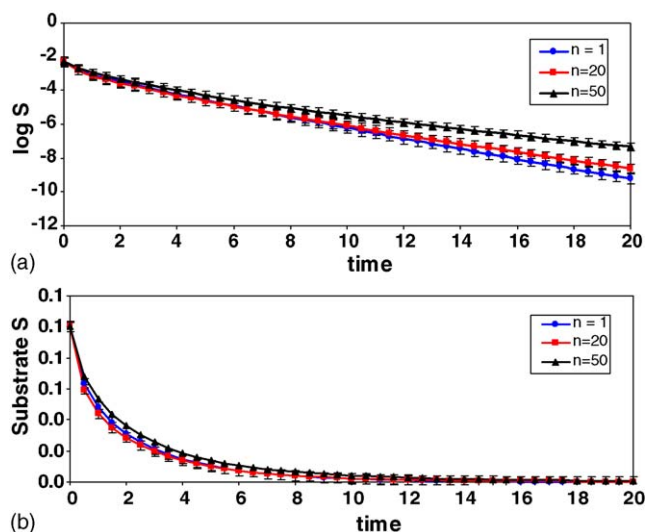


Fig. 1. Substrate depletion for the one-by-one mechanism: (a) log S and (b) S against time, averaged over 50 samples, showing standard deviation. The plots show apparent first-order kinetics; this is particularly clear in (a). Parameter values are: E<sub>0</sub> = 1, S<sub>0</sub> = 0.1, *k*<sub>min</sub> = 0.5, and *k*<sub>max</sub> = 2.0.

3. The one-by-one mechanism behavior

We find that the logarithmic curve of the primary substrate S depletion follows apparent first-order kinetics for the one-by-one mechanism. Fig. 1 shows the substrate depletion. In Fig. 1(a), we plot log S against time, and in Fig. 1(b), we show S against time. In both the figures, we are showing curves which are an average of 50 samples with their standard deviations. The logarithmic plot shows apparent first-order kinetics. Simulation for large number of intermediates *n* show no significant deviations from *n* = 1. The parameter values in this instance are E<sub>0</sub> = 1, S<sub>0</sub> = 0.1, *k*<sub>min</sub> = 0.5, and *k*<sub>max</sub> = 2.0.

3.1. Effects of the initial substrate concentration

In Fig. 2, we illustrate the effects of the variation of S<sub>0</sub> on the apparent first-order kinetics for the one-by-one mechanism. The correlation coefficient *r* does not change significantly with S<sub>0</sub>. We also see that variation of Δ*k* has no significant impact on *r*. Apparent first-order kinetics is observed. The initial enzyme concentration is equal to the unity in all the simulations (E<sub>0</sub> = 1). Note that that the correlation coefficient *r* is maximum for Δ*k* = 0.1. As the magnitude of Δ*k* increases there is a deviation from the linear behavior indicated by the decrease in *r*. Also, we observe that an increase in the number of intermediate steps does not noticeably detract from the apparent first-order behavior.

3.2. Varying the range of the rate constants

We find that the slope of the linear fit depends on both the magnitude of *k*<sub>min</sub> and *k*<sub>max</sub>. However, the qualitative behavior is dependent only on Δ*k*. For example, the behavior of the system is essentially the same for (*k*<sub>min</sub>, *k*<sub>max</sub>) = (0.5, 0.6) and (2.1, 2.2) since the Δ*k* = 0.1 in both cases. However, the mean of the chosen

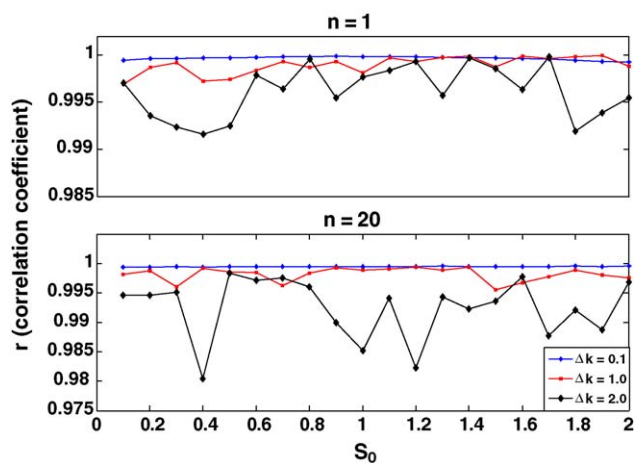


Fig. 2. Effects of the variation of  $S_0$  on the apparent first-order kinetics for the one-by-one mechanism. The correlation coefficient  $r$  does not change significantly with  $S_0$ . We also see that variation of  $\Delta k$  has no significant impact on  $r$ . Apparent first-order kinetics is observed. Parameters values:  $E_0 = 1$  and  $n = 1$  and 20.

rate constants follow a central limit and are distributed normally irrespective of the nature of the parent distribution (results not shown). So the behavior of the system is invariant to the nature of parent distribution.

In Fig. 3, we vary the magnitude of the range of the rate constants,  $\Delta k = k_{\max} - k_{\min}$  in the one-by-one mechanism. We find that the correlation coefficient  $r$  slowly decreases with the magnitude of  $\Delta k$  as the number of intermediate step  $n$  increases. The behavior is apparent first-order for small  $\Delta k$  and continues to be even for large  $\Delta k$ . The coefficient  $r$  is calculated by averaging the simulation results for a range of  $S_0 = \{0.1, 0.2, \dots, 2\}$  with  $E_0 = 1$ . The standard deviation of  $r$  is negligible, less than 0.05 (results not shown). It is to be noted that even the minimum value of  $r$  is well within the experimentally accepted correlation coefficient for a linear fit. Therefore, the above result, for all experimental purposes, may not be considered as a noticeable variation from the apparent first-order kinetics.

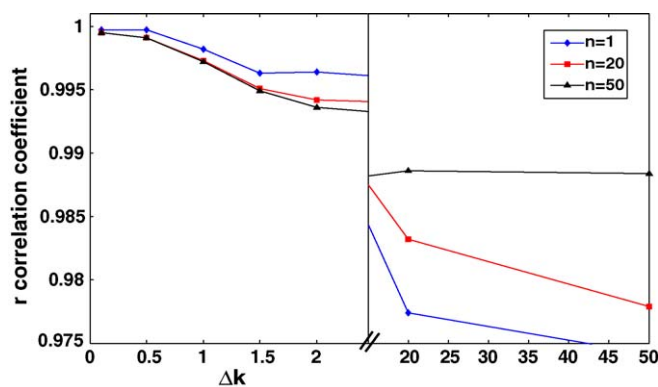


Fig. 3. Effects of the variation of rate constants on the apparent first-order kinetics for the one-by-one mechanism. The magnitude of the range of the rate constants,  $\Delta k = k_{\max} - k_{\min}$ , is varied. The correlation coefficient  $r$  slowly decreases with the magnitude of  $\Delta k$  as the number of intermediate step  $n$  increases. The behavior is apparent first-order for small  $\Delta k$  and continues to be even for large  $\Delta k$ . The coefficient  $r$  is calculated by averaging the simulation results for a range of  $S_0 = \{0.1, 0.2, \dots, 2\}$  with  $E_0 = 1$ .

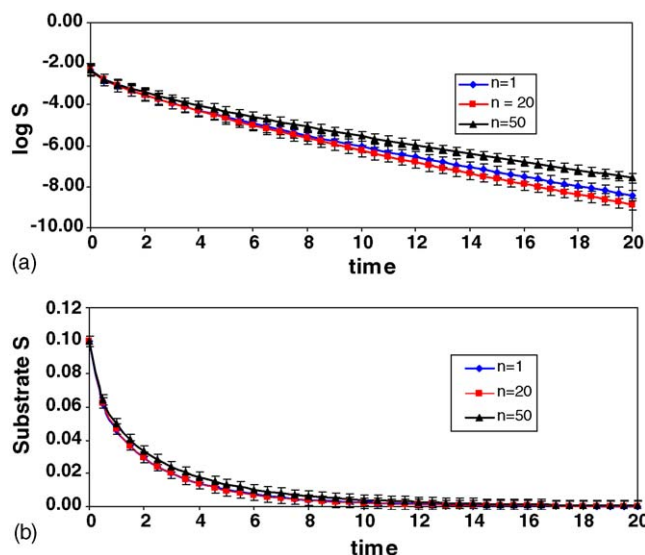


Fig. 4. Substrate depletion for the zipper mechanism with apparent first-order kinetics: (a)  $\log S$  and (b)  $S$  against time, averaged over 50 samples, showing standard deviation. Note that there is no significant deviation from the apparent first-order behavior for higher  $n$  values. Parameter values are:  $E_0 = 1$ ,  $S_0 = 0.1$ , and  $\Delta k = 1.5$ .

#### 4. The zipper mechanism behavior

Our result shows that the time-course of substrate depletion can follow apparent first-order behavior in the zipper mechanism. However, the time-course of the substrate depends on the intermediate steps of the proteolysis. In Fig. 4, we show apparent first-order kinetics for the time-course of the primary substrate for  $n = 1, 20$ , and 50. Here, parameter values are:  $E_0 = 1$ ,  $S_0 = 0.1$ , and  $\Delta k = 1.5$ . This behavior is not affected by higher  $n$  values. However, this is not generally true. It seems that we can only have apparent first-order kinetics for  $S_0/E_0 < 1$ .

This is clearly illustrated in Fig. 5, where we study the variation of initial enzyme and substrate concentration ratio ( $S_0/E_0$ ) on the apparent first-order kinetics. The correlation coefficient  $r$  varies drastically as the number of intermediate complexes increase for  $S_0/E_0 > 1$ . This indicates that the kinetics of the pri-

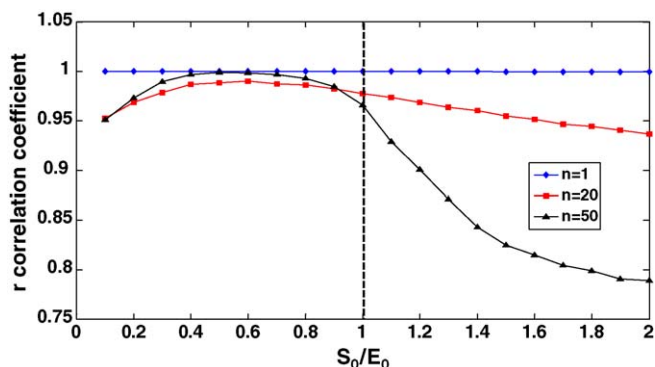


Fig. 5. Effects of the variation of initial concentrations on the apparent first-order kinetics for the zipper mechanism. The correlation coefficient  $r$  varies drastically as the number of intermediate complexes increase indicating that the kinetics of the primary substrate  $S$  does not follow first-order kinetics for  $S_0/E_0 > 1$ . The parameter value is:  $\Delta k = 0.1$ .

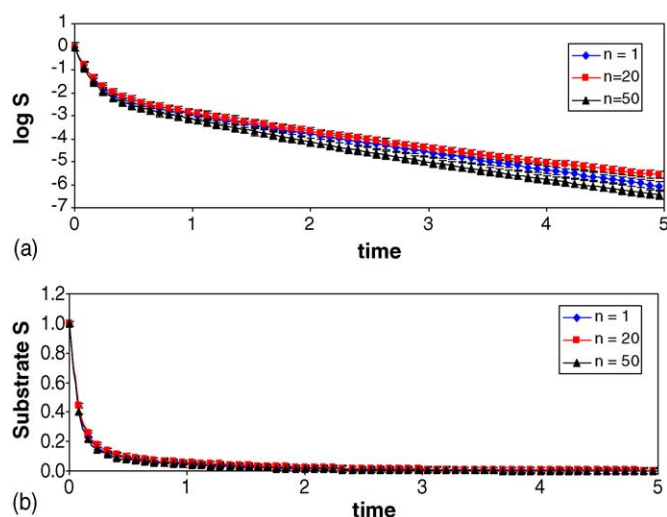


Fig. 6. Substrate depletion with apparent first-order kinetics after an initial transient: (a)  $\log S$  and (b)  $S$  against time, averaged over 50 samples, showing standard deviation. The presence of two time-scales can be appreciated in (a). Parameter values:  $E_0 = 10$ ,  $S_0 = 1$ , and  $\Delta k = 1.5$ .

mary substrate  $S$  does not follow first-order kinetics. The same trend is maintained even for higher values of  $\Delta k$ .

## 5. Biphasic behavior in enzyme digestion

We observe an initial transient in the substrate depletion which does not follow apparent first-order kinetics. After this initial transient, the reaction shows apparent first-order behavior in the substrate depletion (Fig. 6). The presence of two time-scales can be appreciated in the logarithmic plot (Fig. 6(a)). We observe this strongly in the case of  $E_0 = 10$  and  $S_0 = 1$ . We see a steep initial decrease in the substrate time-course, followed by a slow and steady decay in both the mechanisms. However, if the initial fast transient is neglected, the substrate decay follows an apparent first-order kinetics. This biphasic behavior is always present in these mechanisms. However, it is not always observable. This type of behavior is dealt with in detail by Schnell et al. (Schnell and Maini, 2000; Schnell and Mendoza, 2004), who have derived a closed form solution for single enzyme, single substrate reaction with  $E_0 \gg S_0$  or  $S_0 \gg K_M$ , where  $K_M$  is the Michaelis–Menten constant.

## 6. Discussion

The analysis carried out in this paper shows that the kinetics of protein digestion follows apparent first-order kinetics in the one-by-one mechanism. We observe apparent first-order behavior for the substrate depletion in the zipper mechanism for low initial substrate concentration with respect to the initial enzyme concentration ( $S_0/E_0 < 1$ ).

In both mechanisms, the overall dynamics is invariant to the number of intermediate steps  $n$  when it follows apparent first-order kinetics. In an experimental situation, if we find a substrate disappearance following apparent first-order kinetics, we can say very little about the reaction mechanism. For  $S_0/E_0 < 1$ , we

cannot even differentiate between the one-by-one mechanism and the zipper mechanism. In the other case,  $S_0/E_0 > 1$ , we must then have the one-by-one mechanism. In either case, there is no way to determine the number of intermediates by studying the substrate depletion. As for the rate constants, they could vary significantly and yet produce the same substrate depletion curve. Therefore, as long as we look into the dynamics of  $S$ , it is not possible to get any information about the complexity or about the intermediate steps in the mechanisms.

The experimental results (Herman and Scherer, 2003; Herman et al., 2005), discussed in Section 1, show that protein digestion by pepsin follows apparent first-order kinetics. These experiments were carried out with  $S_0/E_0 < 1$ . Although it is not possible to distinguish the reaction mechanism under this condition, this corresponds with our expectation of a substrate depletion following apparent first-order kinetics.

We present an experimentally testable proposal to examine the kinetics when  $S_0 > E_0$  to extract more information about the mechanism. If apparent first-order kinetics are found in the substrate depletion then we have the one-by-one mechanism. Otherwise, the digestion could follow the zipper mechanism.

Following the kinetics of the free enzyme could reveal more information about the mechanism since  $E$  is strongly coupled to all intermediates in both one-by-one and zipper mechanism. Thomas et al. (2004) have discussed a multi-laboratory evaluation of pepsin digestion assay used in assessing the safety of novel proteins. The experiments described in this work provide some insight about how to follow the enzyme concentration profile with time. As described in their experiments, enzyme–substrate sample aliquots can be periodically removed from the reaction pool and quenched and run on SDS-PAGE electrophoresis to determine the enzyme time-course. It may be worthwhile to explore possibilities to implement it in other ways also. The time-course of enzyme, if followed, may reveal other ways of evaluating the efficiency of enzyme digestion with different proteins. An analysis of the enzyme kinetics in a similar vein will follow.

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