A Novel Exponential Kinetic Model for Casein Tryptic Hydrolysis to Prepare Active Peptides*

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Abstract The kinetics of casein tryptic hydrolysis to prepare active peptides was investigated. Taking into account the reaction mechanism including single substrate hydrolysis, irreversible enzyme inactivation, and substrate inhibition, a set of exponential equations was established to characterize the enzymatic hydrolysis curves. The verification was carried out by a series of experimental results and indicated that the average regressive error was less than 5%. According to the proposed kinetic model, the kinetic constants and thermodynamic constants of the reaction system were also calculated.
Keywords active peptide, casein, hydrolysis, kinetics, trypsin

1 INTRODUCTION

Casein is one of the most valuable food proteins. Tryptic hydrolysis of casein is an important bioreaction that can be used to improve the physical, chemical, functional and nutritional properties of original proteins. It is also a significant way to prepare many biological active peptides such as casoxins, casomorphins, immunopeptides and caseinophosphopeptides (CPPs)[1,2]. These active peptides from casein source possess many physiological properties including mineral binding, opioid activity, growth enhancer for bifidobacteria, anticancer activity and regulation of the blood pressure or the immune system[3–6]. In recent years, many types of them have been applied in food, drug, cosmetics, and other fields[7,8].

The kinetic study of protein enzymatic hydrolysis is helpful to optimizing the operating parameters for the reactor design, and to providing the theoretical predication for target peptide production. However, the hydrolysis reaction is highly complicated due to the following factors:

- A large number of peptide bonds which are cleaved in parallel and in sequence simultaneously;

- Different accessibility of various peptide bonds to enzymatic attack;

- The existence of substrate inhibition, product diversity and enzyme inactivation during the hydrolysis;

- Multiply exogenous influences including pH, temperature, ionic strength or pressure on the reaction rate.

So it is very difficult to obtain an accurate expression of experimental results *via* the instinctive kinetic model.

The aim of the present work is to propose a set of exponential kinetic equations that can be widely used to characterize enzymatic reaction of protein hydrolysis. The kinetic equations are described by means of casein tryptic hydrolysis. Much useful information including reaction mechanism, kinetic constants and thermodynamic constants can be deduced from the model parameters. The results of fitting hydrolysis curves and the application of kinteic models are also presented.

2 MATERIALS AND METHODS
2.1 Materials

Casein was obtained from Tianjin Hematology Institute, Chinese Academy of Medical Sciences. Its content was 86% of protein weight determined by the Kjeldahl's method. Prior to use, casein was purified by precipitation *via* the addition of HCl to pH=4.6 and filtration through a 0.45 μm cellulose acetate millipore film.

Trypsin was purchased from Life Technologies Inc., USA. The maximum activity of enzyme was $4\,\mathrm{IU\cdot mg^{-1}}$ at the optimum conditions (40℃, pH 8). It was stable from 20 to 45℃ and at pH 7 to 9. The enzyme can be deactivated by heating at 100℃ for 5 min.

All other reagents were of analytical grade.
2.2 Definition and measurement of *DH*

The degree of hydrolysis (*DH*) is defined as the ratio of the broken peptide bonds to the total peptide bonds. Alkali consumption to maintain constant pH is proportional to the *DH* value, and it can be expressed by the following equation[9]: $DH = \dfrac{h}{h_{\mathrm{tot}}} \times 100\% = \dfrac{B \times N_{\mathrm{b}} \times 1000}{MP \times h_{\mathrm{tot}} \times \alpha} \times 100\%$, where B is the volume of alkali consumption (L); N_{b} is the molarity of the alkali used (mol·L^{-1}); MP is the mass of protein (g); h_{tot} is the total number of peptide bonds (mmol·g^{-1}); α is

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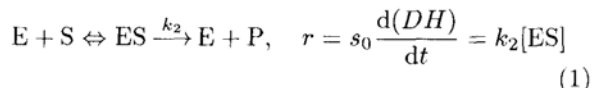the average degree of dissociation of α-NH$_2$ groups.

2.3 Procedure used in enzymatic hydrolysis

Experiments were carried out in a 0.5 L magnetically stirred batch bioreactor with pH and temperature control. Substrate solution was prepared by dissolving a given amount of casein in 400 ml distilled water and adjusted to pH 8.0 with 1 mol·L^{-1} NaOH, then heated up to the desired temperature. The reaction was started by addition of trypsin solution. During the hydrolysis, the DH of the reaction process was monitored by the pH-stat method, and was expressed as the time-course curves. All experiments were performed in triplicate to obtain the mean values.
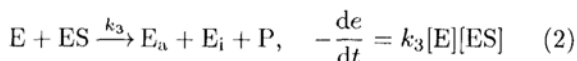
3 THEORETICAL ANALYSIS

Trypsin is a proteolytic enzyme which specifically targets amino acids Arg and Lys. Its ability to cleave a substrate mainly depends on the DH value, initial substrate concentration (s_0) and initial enzyme concentration (e_0). By taking into account
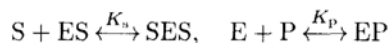
- Single substrate enzymatic hydrolysis

$$\text{E} + \text{S} \Leftrightarrow \text{ES} \xrightarrow{k_2} \text{E} + \text{P}, \quad r = s_0 \frac{\mathrm{d}(DH)}{\mathrm{d}t} = k_2[\text{ES}] \tag{1}$$

- Enzyme inactivation

$$\text{E} + \text{ES} \xrightarrow{k_3} \text{E}_a + \text{E}_i + \text{P}, \quad -\frac{\mathrm{d}e}{\mathrm{d}t} = k_3[\text{E}][\text{ES}] \tag{2}$$

- Substrate or product inhibiton

$$\text{S} + \text{ES} \xleftrightarrow{K_s} \text{SES}, \quad \text{E} + \text{P} \xleftrightarrow{K_p} \text{EP}$$

- Total enzyme concentration expression

$$e = [\text{E}] + [\text{ES}] + [\text{SES}] + [\text{EP}] \tag{3}$$

The following relationships can be deduced in terms of the steady-state approach

$$[\text{E}] = \frac{e}{1 + \dfrac{[\text{S}]}{K_m} + \dfrac{[\text{S}]^2}{K_s K_m} + \dfrac{p}{K_p}} \tag{4}$$

$$[\text{ES}] = \frac{e}{\dfrac{K_m}{[\text{S}]} + 1 + \dfrac{[\text{S}]}{K_s} + \dfrac{K_m p}{K_p[\text{S}]}} \tag{5}$$

Furthermore, a set of exponential kinetic equations, which presents a clear dependence on s_0 and e_0, is deduced from Eqs. (1)—(5) as follows

$$r = s_0 \frac{\mathrm{d}(DH)}{\mathrm{d}t} = a s_0 \exp\left[-b(DH)\right]$$

$$\frac{\mathrm{d}(DH)}{\mathrm{d}t} = a \exp\left[-b(DH)\right] \tag{6}$$

where parameters a and b have different expressions according to different reaction mechanisms (Table 1), and have different values for different reaction systems.

4 RESULTS AND DISCUSSION

4.1 Experimental verification of the reaction mechanism

As shown in Figs. 1 and 2, the casein tryptic hydrolysis indicates that:

- DH increases with time and the curves vary with different conditions;
- DH is directly proportional to e_0, but inversely proportional to s_0;
- The reaction rate ($\mathrm{d}DH/\mathrm{d}t$) decreases with time, especially after the initial stage of reaction.

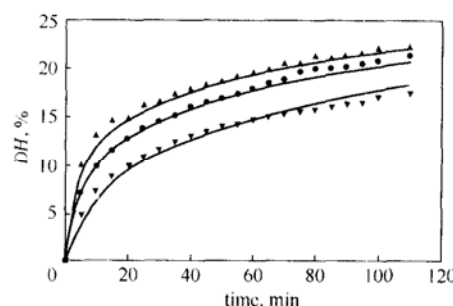**Figure 1** Time-course relationship of casein tryptic hydrolysis at different substrate concentrations
($e_0 = 0.13\,\text{g·L}^{-1}$, pH=8, $T = 40°\text{C}$)
▲ ● ▼ experimental data;
—— kinetic model fit curves
s_0, g·L^{-1}: ▲ 1.58; ● 2.72; ▼ 6.29

Table 1 Expressions of kinetic parameters a and b^*

Mechanism	a	b
no inhibition	$\dfrac{k_2 e_0}{s_0}$	$\dfrac{k_3 K_m}{k_2}$
substrate-inhibition	$\dfrac{k_2 K_s e_0}{s_0 K_s + s_0^2}$	$\dfrac{k_3 K_m K_s}{k_2(K_s + s_0)}$
product-inhibition	$\dfrac{k_2 e_0 K_p}{s_0 K_p + p K_m}$	$\dfrac{k_3 K_m K_p s_0}{k_2(s_0 K_p + p K_m)}$
substrate and product-inhibition	$\dfrac{k_2 e_0 K_s K_p}{K_s K_p s_0 + K_p s_0^2 + K_m K_s p}$	$\dfrac{k_3 K_m K_s K_p s_0}{k_2(K_s K_p s_0 + K_p s_0^2 + K_m K_s p)}$

* a and b are kinetic parameters in the exponential equation: $\dfrac{\mathrm{d}(DH)}{\mathrm{d}t} = a\exp\left[-b(DH)\right]$ for protein enzymatic hydrolysis.
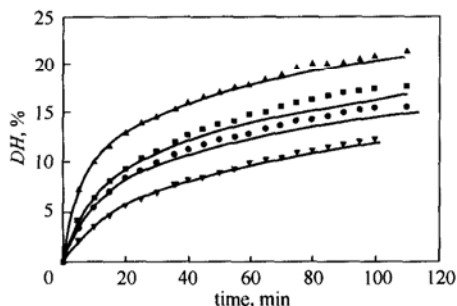
Figure 2   Time-course relationship of casein tryptic hydrolysis at different enzyme concentrations
($s_0 = 2.50\,\text{g·L}^{-1}$, pH=8, $T = 40°C$)
▲ ■ ● ▼ experimental data;
—— kinetic model fit curves
e_0, g·L^{-1}: ▲ 0.13; ■ 0.05; ● 0.03; ▼ 0.02

Similar to some other enzymatic hydrolysis systems[10], there are probably three reasons for the experimental results: (1) Decrease of the concentration of effective peptide bonds; (2) Enzyme inactivation and (3) Substrate inhibition or product inhibition.

In order to study the influences of above three factors, correspondingly, a series of experiments are designed and several conclusions are summarized as follows.

Factor 1 Since the addition of extra fresh casein doesn't cause an evident increase in DH (Fig. 3), it is obvious that the concentration of peptide bonds is not the key to the reaction rate;

Factor 2 When the extra fresh trypsin is added into the reaction system, the DH increases suddenly (Fig. 3). This is naturally attributed to the enzyme inactivation;

Factor 3 According to the double Lineweaver-Burk plot (Fig. 4), it is shown clearly that the increase of s_0 ($s_0 > 5\,\text{g·L}^{-1}$) has opposite effect on r_0 value, which proves the presence of substrate inhibiton.

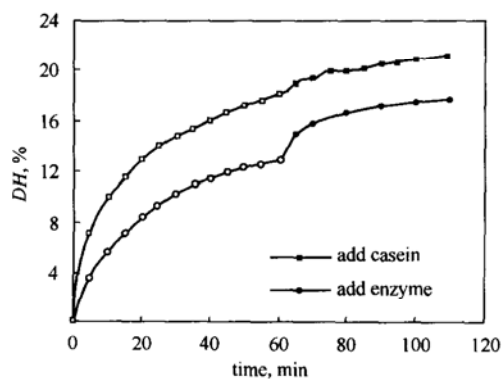**Figure 3   Influence of adding extra fresh casein or trypsin on DH during the hydrolysis**
[Reaction conditions: $s_0 = 2.72\,\text{g·L}^{-1}$, $e_0 = 0.13\,\text{g·L}^{-1}$, pH=8, 40°C (addition of casein); $s_0 = 2.50\,\text{g·L}^{-1}$, $e_0 = 0.03\,\text{g·L}^{-1}$, pH=8, 40°C (addition of trypsin)]

Based on the above facts, it is demonstrated, like many other systems, that it is the competitive inhibition of casein[11] and the irreversible inactivation of trypsin[12] rather than the concentration of effective peptide bonds are the most important factors to the rapid decrease of reaction rate. This viewpoint is conformed to the proposed mechanism that is used to establish the kinetic model.
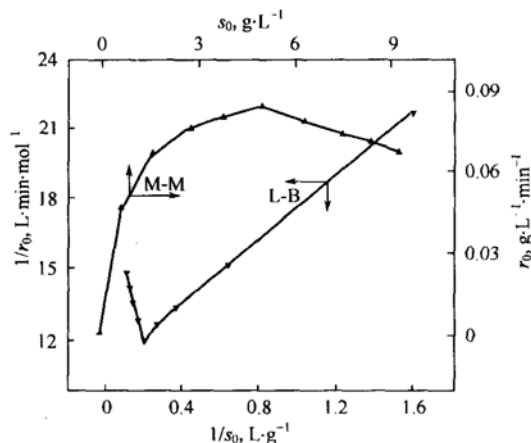

Figure 4   The Lineweaver-Burk double reciprocal plot and the Michaelis-Menten plot of casein-trypsin reaction system(pH=8, $T = 40°C$)
—▲— M-M: r_0-s_0; —▼— L-B: r_0^{-1}-s_0^{-1}

4.2   Determination of the exponential kinetic equation

In terms of time-course hydrolysis curves, the values of model parameters a and b (Table 2) corresponding to the different experimental conditions are determined through the non-linear regression analysis in accordance with the exponential equation [Eq. (6)] The influences of s_0, e_0 and temperature on a and b values agree well with the substrate inhibition mechanism, *i.e.* the parameter b maintains constant when e_0 is changed, but decreases when s_0 and temperature increase while parameter a increases with e_0 and temperature, but decreases with s_0.

Table 2   Values of kinetic parameters a and b for tryptic hydrolysis of casein (pH=8)

| s_0, g·L^{-1}	e_0, g·L^{-1}	T, °C	a	b
2.50	0.02	40	0.568	0.207
2.50	0.03	40	1.259	0.206
2.50	0.05	40	1.264	0.205
2.50	0.13	40	3.501	0.203
1.58	0.13	40	5.755	0.228
2.72	0.13	40	3.540	0.208
3.75	0.13	40	3.073	0.193
6.29	0.13	40	1.556	0.171
1.50	0.02	35	0.526	0.196
1.50	0.02	40	0.694	0.176
1.50	0.02	45	0.905	0.169
1.50	0.02	50	1.180	0.157

Furthermore, based on a and b expressions derived from theoretical analysis (Table 1) and linear regression method, the reaction kinetic constants (K_m, K_s,

k_{d}, k_2) are calculated (Table 3) in accordance with the slopes and intercepts of three straight lines (Fig. 5). The good linear relationships between dependent and independent variables justify the validity of the proposed reaction mechanism. Therefore, the kinetic model for tryptic hydrolysis of casein is determined as

$$
\frac{\mathrm{d}(DH)}{\mathrm{d}t} = a\exp\left[-b(DH)\right] = \frac{k_2 K_{\mathrm{s}} e_0}{K_{\mathrm{s}} s_0 + s_0^2} \cdot
$$
$$
\exp\left[-\frac{k_3 K_{\mathrm{m}} K_{\mathrm{s}}}{k_2(K_{\mathrm{s}} + s_0)}(DH)\right] \quad (7)
$$

Table 3 Values of kinetic and thermodynamic constants for casein tryptic hydrolysis

K_{m} $g\cdot L^{-1}$	K_{s} $g\cdot L^{-1}$	k_2 min^{-1}	k_{d} min^{-1}	E_{A} $kJ\cdot mol^{-1}$	E_{D} $kJ\cdot mol^{-1}$
0.675	12.860	89.236	23.369	40.932	46.051
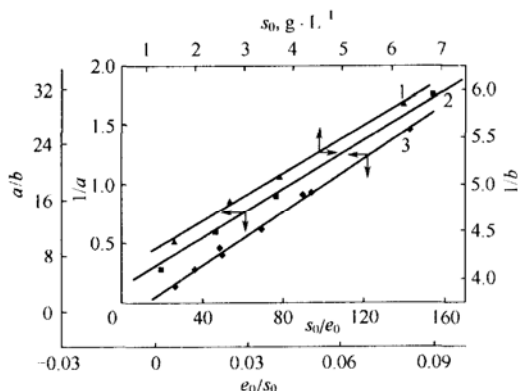
Figure 5 Calculated plots for kinetic constants of casein tryptic hydrolysis (pH=8, $T = 40$℃)
1—$1/b = 0.308 s_0 + 3.954$; 2—$1/a = 0.012 s_0/e_0$;
3—$a/b = 340.750 e_0/s_0$

The influence of temperature on the DH value is studied when other hydrolysis conditions are constant (Table 2). Since parameter a has relevance to the reaction rate constant k_2, and ab relates to the enzyme inactivation constant k_{d}, the values of E_{A} and E_{D} can be calculated (Table 3) through the slopes of regression straight lines (Fig. 6) in accordance with the Arrhenius equation:

$$
\ln a = -\frac{E_{\mathrm{A}}}{RT} + A_{\mathrm{A}}, \quad \ln(ab) = -\frac{E_{\mathrm{D}}}{RT} + A_{\mathrm{D}}
$$

where A_{A} and A_{D} are frequency factors; R is gas constant, $8.314\,\mathrm{J\cdot mol^{-1}\cdot K^{-1}}$.

4.3 Hydrolysis curve simulation and kinetic model application

Inserting the kinetic constants into the integral form of time-course equation

$$
DH = \frac{1}{b}\cdot\ln(1 + ab\cdot t) = \frac{k_2(K_{\mathrm{s}} + s_0)}{k_3 K_{\mathrm{m}} K_{\mathrm{s}}} \cdot
$$
$$
\ln\left[1 + \frac{k_3 K_{\mathrm{m}} K_{\mathrm{s}}^2}{(K_{\mathrm{s}} + s_0)^2}\frac{e_0}{s_0} \times t\right] \quad (8)
$$

the theoretical hydrolysis curves corresponding to different s_0 and e_0 are obtained (Figs. 1 and 2). The average relative error between the calculated value (solid curves) and the experimental data (scatter plot) is less than 5% (Tables 4 and 5). It demonstrates again that the reaction mechanism and kinetic model proposed are both reasonable.
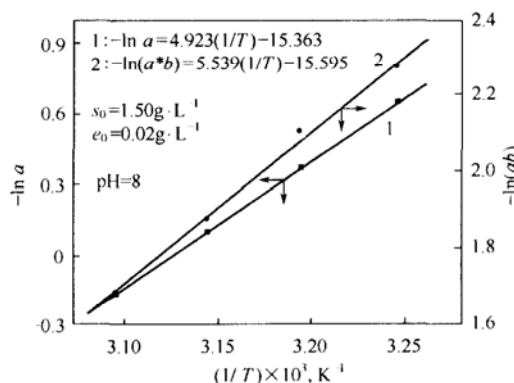
Figure 6 Calculated plots for thermodynamic constants of casein hydrolysis by trypsin

5 CONCLUSIONS

(1) Based on the hydrolysis curves of casein-trypsin system at different s_0, e_0 and temperature, the reaction mechanism was proposed including single substrate hydrolysis, second-order enzyme inactivation and substrate noncompetitive inhibition.

(2) The whole set of exponential kinetic equations is deduced from different reaction mechanisms, in which the substrate inhibition mechanism is suited for the casein-trypsin system and can be applied to a wide range of operating conditions. Furthermore, the functional relationship between s_0, e_0, DH and time [Eqs. (7) and (8)] can be used to model and predict the bioreaction process of casein tryptic hydrolysis, and to optimize the operating parameters for bioreactor design or for the industrial production of the peptide.

NOMENCLATURE

E	free enzyme
E_{A}	casein hydrolysis activation energy, $kJ\cdot mol^{-1}$
E_{D}	trypsin inactivation energy, $kJ\cdot mol^{-1}$
E_{a}	active enzyme
E_{i}	inactive enzyme
EP	enzyme product complex
ES	enzyme substrate complex
e	enzyme concentration, $g\cdot L^{-1}$
K_{m}	M-M constant, $g\cdot L^{-1}$
K_{P}	product inhibition constant, $g\cdot L^{-1}$
K_{s}	substrate inhibition constant, $g\cdot L^{-1}$
k_{d}	enzyme inactivation reaction rate constant, $(k_{\mathrm{d}} = k_3 K_{\mathrm{m}})$, min^{-1}
k_2	prodrolysis reaction rate constant, min^{-1}
k_3	enzyme inactivation reaction rate constant, $L\cdot g\cdot min^{-1}$
P	product
p	product concentration, $g\cdot L^{-1}$
r	reaction rate, $g\cdot L^{-1}\cdot min^{-1}$
S	free substrate
s	substrate concentration, $g\cdot L^{-1}$

Table 4   Hydrolysis curve fitted by kinetic model at different substrate concentrations
$(\text{pH}=8,\ T = 40^\circ\text{C},\ e_0 = 0.13\,\text{g}\cdot\text{L}^{-1})$

| Reaction time min | DH, % | | | | | |
| | $s_0 = 1.58\,\text{g}\cdot\text{L}^{-1}$ | | $s_0 = 2.72\,\text{g}\cdot\text{L}^{-1}$ | | $s_0 = 6.29\,\text{g}\cdot\text{L}^{-1}$ | |
	exp.	cal.*	exp.	cal.*	exp.	cal.*
0	0	0	0	0	0	0
10	12.628	11.947	10.080	9.962	7.000	6.578
20	14.936	14.785	12.938	12.892	10.128	9.546
30	16.615	16.478	14.643	14.671	11.808	11.486
40	17.863	17.689	16.073	15.953	13.185	12.930
50	18.724	18.632	17.000	16.956	14.064	14.080
60	19.541	19.405	17.953	17.780	14.823	15.037
70	20.488	20.059	18.956	18.479	15.452	15.855
80	21.220	20.627	20.034	19.086	15.810	16.570
90	21.223	21.128	20.210	19.622	16.276	17.206
100	21.952	21.577	20.762	20.103	16.991	17.777
110	22.080	21.983	21.338	20.538	17.414	18.296
average relative error	1.41%		1.73%		3.40%	

* Calculated by Eq. (8)

Table 5   Hydrolysis curve fitted by kinetic model at different enzyme concentrations
$(\text{pH}=8,\ T = 40^\circ\text{C},\ s_0 = 2.50\,\text{g}\cdot\text{L}^{-1})$

| Reaction time min | DH, % | | | | | | | |
| | $e_0 = 0.02\,\text{g}\cdot\text{L}^{-1}$ | | $e_0 = 0.03\,\text{g}\cdot\text{L}^{-1}$ | | $e_0 = 0.05\,\text{g}\cdot\text{L}^{-1}$ | | $e_0 = 0.13\,\text{g}\cdot\text{L}^{-1}$ | |
	exp.	cal.*	exp.	cal.*	exp.	cal.*	exp.	cal.*
0	0	0	0	0	0	0	0	0
10	3.237	3.307	5.271	5.203	6.608	6.849	10.080	10.165
20	5.068	5.203	8.007	7.571	9.416	9.472	12.938	13.074
30	6.462	6.539	9.644	9.121	11.233	11.126	14.643	14.836
40	7.690	7.571	10.966	10.277	12.814	12.338	16.073	16.104
50	8.469	8.412	11.887	11.197	13.923	13.294	17.000	17.095
60	9.390	9.121	12.525	11.963	14.867	14.084	17.953	17.909
70	10.010	9.735	13.252	12.619	15.493	14.758	18.956	18.599
80	10.518	10.277	13.943	13.192	16.201	15.344	20.034	19.198
90	11.085	10.760	14.505	13.701	16.786	15.864	20.210	19.728
100	11.452	11.197	14.777	14.159	17.140	16.330	20.762	20.202
110			15.050	14.575	17.476	16.753	21.338	20.632
average relative error	1.94%		4.31%		3.59%		1.55%	

* Calculated by Eq. (8)

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