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RESEARCH ARTICLE

Qi Wei, He Zhimin

Enzymatic hydrolysis of protein: mechanism and kinetic model

Abstract The bioreaction mechanism and kinetic behavior of protein enzymatic hydrolysis for preparing active peptides were investigated to model and characterize the enzymatic hydrolysis curves. Taking into account single-substrate hydrolysis, enzyme inactivation and substrate or product inhibition, the reaction mechanism could be deduced from a series of experimental results carried out in a stirred tank reactor at different substrate concentrations, enzyme concentrations and temperatures based on M-M equation. An exponential equation $dh/dt = a\exp(-bh)$ was also established, where parameters a and b have different expressions according to different reaction mechanisms, and different values for different reaction systems. For BSA-trypsin model system, the regressive results agree with the experimental data, i.e. the average relative error was only 4.73%, and the reaction constants were determined as K_m = 0.0748 g/L, K_s = 7.961 g/L, k_d = 9.358/min, k_2 = 38.439/min, E_a = 64.826 kJ/mol, E_d = 80.031 kJ/mol in accordance with the proposed kinetic mode. The whole set of exponential kinetic equations can be used to model the bioreaction process of protein enzymatic hydrolysis, to calculate the thermodynamic and kinetic constants, and to optimize the operating parameters for bioreactor design.

Keywords active peptides, bovine serum albumin, enzymatic hydrolysis, kinetic model, trypsin

1 Introduction

Enzymatic hydrolysis of proteins is an important bioprocess to improve the physical, chemical, functional and nutritional properties of original proteins. It is also an effective method to prepare active peptides, which 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 [1]. In recent years, many types of peptides have been applied in food, drug, cosmetic, and some other fields.

However, the enzymatic hydrolysis reaction is highly complicated due to the following factors: (1) diversity of reaction components, i.e. peptide fragments are products as well as reactants of next reaction; (2) multiplicity of reaction types, i.e. a large number of peptide bonds are cleaved in parallel and in sequence simultaneously; (3) complexity of reaction networks, i.e. the existence of substrate inhibition, product diversity and enzyme inactivation during the hydrolysis; (4) multiple exogenous influences including pH, temperature, ion strength and pressure on the reaction rate.

Therefore, it is very difficult to obtain an accurate expression of experimental results via the traditional M-M equation [2].

In order to model the complex kinetic behaviour of enzymatic hydrolysis reaction, BSA-trypsin was selected as a model system, taking the following three factors into account: First, the sequence and structure of BSA have been identified, so it is a valuable standard protein for theoretical research. Second, trypsin possesses a high specificity of cleavage site and its target amino acids are Arg and Lys, which are beneficial to further research of reaction mechanism and kinetic model [3]. Third, there are many active peptides in the hydrolysates of this system such as insulin-stimulating peptide and ileum-contracting peptide [4]. In addition, due to the good solubility and biocompatibility, enzymatic hydrolysates have been used to modify some insoluble medicines and to improve their effects [5]. It is obvious that BSA-trypsin system is of great value not only in theoretical research, but also in practical application.

The aim of the paper is to propose a set of exponential kinetic equations by considering concurrently a number of factors, such as substrate hydrolysis, enzyme inactivation and substrate or product inhibition, based on theoretical analysis and experimental data, which can be widely used

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Qi Wei(✉), He Zhimin
School of Chemical Engineering and Technology,
Tianjin University, Tianjin 300072, China
E-mail: qiwei@tju.edu.cn

to characterize enzymatic hydrolysis of different proteins reaction. Much useful information including reaction mechanism, kinetic and thermodynamic constants can be deduced from the model parameter

s. All these will provide theoretical basis for optimizing reaction conditions, designing novel bioreactors and elucidating reaction mechanism.

2 Materials and methods

2.1 Materials

BSA was obtained from Tianjin Hematology Institute, Chinese Academy of Medical Sciences. Its content was greater than 98%. Trypsin was purchased from Sigma. The maximum activity of the enzyme was 4μ/mg at the optimum conditions (40℃, pH 8). The enzyme can be deactivated by heating to 100℃ for 5 min. All other reagents were of analytical grade.

2.2 Procedure of enzymatic hydrolysis [6,7]

Experiments were carried out in a 0.5 L magnetically stirred batch bioreactor with pH and temperature control (Fig. 1). Substrate solution was prepared by dissolving a given amount of BSA in 400 mL distilled water and adjusted to pH 8.0 with 1 mol/L NaOH, then heated up to the desired temperatures. The reaction was started by addition of trypsin solution. During the hydrolysis, the degree of hydrolysis, i.e. *h* value of reaction process was monitored by pH-stat method, and was expressed as the time-course curves. All experiments were performed in triplicate to obtain the mean values.

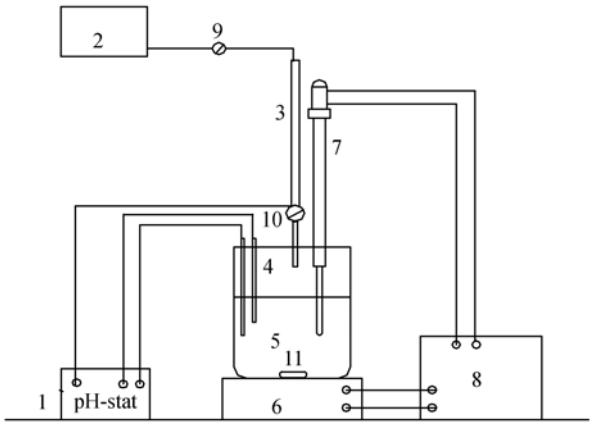

Fig. 1 Schematic diagram of enzymatic hydrolysis of protein

1: pH-stat (pH-controller); 2: alkali; 3: burette; 4: glass electrode; 5: reactor; 6: magnetic stirring heater; 7: conductivity meter; 8: electric relay; 9: valve; 10: magnetic value; 11: magnetic stirrer

3 Theoretical analysis

3.1 Definition and measurement of *h* value

BSA-trypsin system follows the single substrate enzymatic hydrolysis mechanism, and consists of three steps [8]:
Opening of peptide bond:

$$R_1CONHR_2 + H_2O \xrightarrow{Enzyme} R_1COOH + R_2NH_2$$

Proton exchange:

$$R_1COOH + R_2NH_2 \rightarrow R_1COO^- + R_2NH_3^+$$

Titration of amino group:

$$R_2NH_3^+ + OH^- \rightarrow R_2NH_2 + H_2O$$

The degree of hydrolysis (*h*) is defined as the ratio of the broken peptide bonds to the total peptide bonds. Base consumption to maintain constant pH is proportional to the *h* value, and it can be expressed by Eq.(1):

$$h\% = \frac{n}{n_{tot}} \times 100\% = \frac{B \times N_b}{M \times n_{tot} \times \alpha} \times 100\% \quad (1)$$

where B is the volume of base consumption (in L); N_b is the normality of the base (in mol/L); M, the mass of protein (in g); n_{tot}, the total number of peptide bonds (in mol/g); n, the number of hydrolyzed peptide bonds (in mol/g); and α is the average degree of dissociation of α-NH_2 groups.

3.2 Reaction mechanism and kinetic model [9]

The reaction mechanism of protein enzymatic hydrolysis can be simplified as $\mathrm{E+S \Leftrightarrow ES \xrightarrow{k_2} E+P}$, the reaction rate depends on the irreversible step, i.e.

$$r = s_0 \frac{\mathrm{d}h}{\mathrm{d}t} = k_2[\mathrm{ES}] \quad (2)$$

The inactivation mechanism of protease during the reaction can be simplified as $\mathrm{E+ES \xrightarrow{k_3} E_A + E_I + P}$, the inactivating rate is shown as

$$-\frac{\mathrm{d}e}{\mathrm{d}t} = k_3[\mathrm{E}][\mathrm{ES}] \quad (3)$$

Eq. (2) divided by Eq. (3) yields

$$-s_0 \frac{\mathrm{d}h}{\mathrm{d}e} = \frac{k_2}{k_3[\mathrm{E}]} \quad (4)$$

Taking substrate and product inhibitions into account, the enzymatic hydrolysis reaction can be expressed as

$\mathrm{S+ES \xleftrightarrow{K_s} SES}$, $\mathrm{E+P \xleftrightarrow{K_p} EP}$ in which $K_s = \frac{k_{-4}}{k_{+4}}$, $K_p = \frac{k_{-5}}{k_{+5}}$.

The following equations can be deduced in terms of steady-state approach for the balanced reaction:

$$[\mathrm{SES}] = \frac{[\mathrm{S}][\mathrm{ES}]}{K_s} = \frac{[\mathrm{S}]^2[\mathrm{E}]}{K_m K_s} \quad (5)$$

Table 1 Expression of kinetic parameters *a* and *b* for exponential equation

Mechanism	a	b
No inhibition	$\frac{k_2 e_0}{s_0}$	$\frac{k_3 K_m}{k_2}$
Substrate-inhibition	$\frac{k_2 K_s e_0}{s_0 K_s + s_0^2}$	$\frac{k_3 K_m K_s}{k_2 (K_s + s_0)}$
Product-inhibition	$\frac{k_2 e_0 K_p}{s_0 K_p + p K_m}$	$\frac{k_3 K_m K_p s_0}{k_2 (s_0 K_p + p K_m)}$
Substrate and product-inhibitions	$\frac{k_2 e_0 K_s K_p}{K_s K_p s_0 + K_p s_0^2 + K_m K_s p}$	$\frac{k_3 K_m K_s K_p s_0}{k_2 (K_s K_p s_0 + K_p s_0^2 + K_m K_s p)}$

$$[\mathrm{EP}] = \frac{[\mathrm{E}][\mathrm{P}]}{K_p} = \frac{K_m[\mathrm{ES}][\mathrm{P}]}{K_p[\mathrm{S}]} \tag{6}$$

$$[\mathrm{ES}] = \frac{[\mathrm{E}][\mathrm{S}]}{K_m} \tag{7}$$

$$[\mathrm{E}] = \frac{K_m[\mathrm{ES}]}{[\mathrm{S}]} \tag{8}$$

and the total enzyme concentration can be expressed as

$$e = [\mathrm{E}] + [\mathrm{ES}] + [\mathrm{SES}] + [\mathrm{EP}] \tag{9}$$

Substitution of Eqs. (5)–(8) into Eq. (9) yields the following expressions:

$$[\mathrm{E}] = \frac{e}{1 + \frac{[\mathrm{S}]}{K_m} + \frac{[\mathrm{S}]^2}{K_s K_m} + \frac{[\mathrm{P}]}{K_p}} \tag{10}$$

$$[ES] = \frac{e}{\frac{K_m}{[S]} + 1 + \frac{[S]}{K_s} + \frac{K_m[P]}{K_p[S]}} \tag{11}$$

Since $[\mathrm{S}] \approx s_0$, $[\mathrm{P}] \approx p$, Eqs.(10) and (11) can be simplified as

$$[\mathrm{E}] = \frac{e K_m K_s K_p}{K_s K_p s_0 + K_p s_0^2 + K_m K_s p} \tag{12}$$

$$[\mathrm{ES}] = \frac{e s_0 K_s K_p}{K_s K_p s_0 + K_p s_0^2 + K_m K_s p} \tag{13}$$

Substitution of Eq.(12) into Eq.(4) yields

$$-\frac{\mathrm{d}h}{\mathrm{d}e} = \frac{k_2 (K_s K_p s_0 + K_p s_0^2 + K_m K_s p)}{k_3 K_m K_s K_p s_0} \frac{1}{e} \tag{14}$$

Integration of Eq.(14) provides

$$\int_0^h -\mathrm{d}h = \frac{k_2 (K_s K_p s_0 + K_p s_0^2 + K_m K_s p)}{k_3 K_m K_s K_p s_0} \int_{e_0}^{e} \frac{\mathrm{d}e}{e}$$

$$\Rightarrow e = e_0 \exp\left[-\frac{k_3 K_m K_s K_p s_0 h}{k_2 (K_s K_p s_0 + K_p s_0^2 + K_m K_s p)}\right] \tag{15}$$

Furthermore, a set of exponential kinetic equations, which presents a clear relationship between r, s_0 and e_0, is deduced from Eqs.(2), (13) and (15) as follows:

$$r = s_0 \frac{\mathrm{d}h}{\mathrm{d}t} = a s_0 \exp(-bh), \quad \frac{\mathrm{d}h}{\mathrm{d}t} = a \exp(-bh) \tag{16}$$

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

4 Results and discussion

4.1 Experimental verification of the reaction mechanism

Figures 2, 3 and 4 show the time-course relationships of BSA-trypsin model system at different s_0, e_0, and T, respectively. It can be seen that h value increases with time at initial stage then tends to a constant value, which is directly proportional to e_0, but inversely proportional to s_0; the reaction rate ($\mathrm{d}h/\mathrm{d}t$) decreases with time, especially at the middle and late stages of the reaction. Similar to some other enzymatic hydrolysis systems [9], there are probably three factors causing the experimental results: (1) a decrease in the concentration of effective peptide bonds; (2) substrate inhibition or product inhibition; (3) enzyme inactivation.

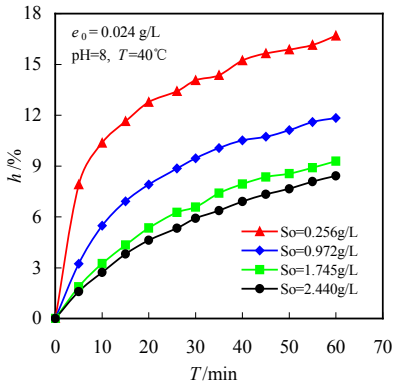

Fig. 2 Influence of substrate concentration on the degree of hydrolysis

Factor 1: Because h values of hydrolysis curves with same s_0 and different e_0 tend to different limit values (Fig. 3) and the addition of extra fresh substrate after 40 min does not cause an evident increase in h (Fig. 5), the concentration

of peptide bonds is not the key factor to the reaction rate.

Factor 2: According to the double Lineweaver-Burk and M-M plots (Fig. 6), it can be seen that when $s_0 < 0.76$ g/L, increasing s_0 accelates reaction rate; but when $s_0 > 0.76$ g·L^{-1}, increasing s_0 has an opposite effect on reaction rate, which proves that s_0 has double effects, i.e. if s_0 exceeds the optimal value, the substrate will be irreversiblely combined with the enzyme and shown as obvious substrate inhibiton.

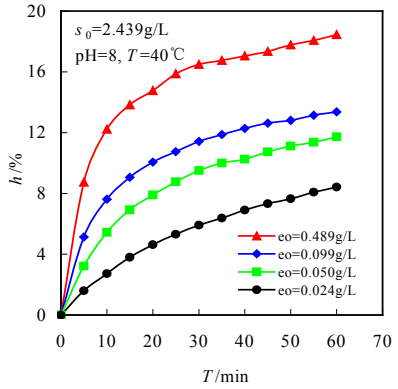

Fig. 3 Influence of enzyme concentration on the degree of hydrolysis

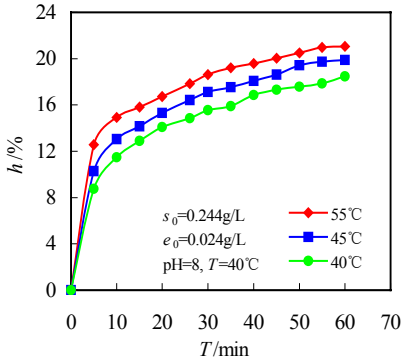

Fig. 4 Influence of temperature on the degree of hydrolysis

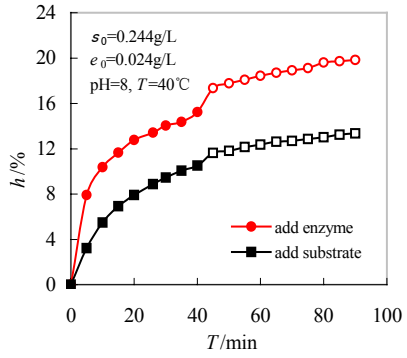

Fig. 5 Effects of addition of fresh BSA and trypsin during the hydrolysis

Factor 3: When the extra fresh trypsin is added into the reaction system, the h value increases suddenly (Fig. 5). This is naturally attributed to the enzyme inactivation, and demonstrated that there are enough substrates in the system. Furthermore, in order to determine the reaction order of enzyme inactivation, it is necessary to examine the relationship between e_0t and h. As shown in Fig. 7, the hydrolysis curves with same s_0 and different e_0 follow the same time-course relationship. There are two possible reasons. One is that trypsin doesn't inactivate, the other is attributed to a second-order inactivation. The former is contray to Fig. 5. Therefore, the inactivation of trpsin is a second-order reaction.

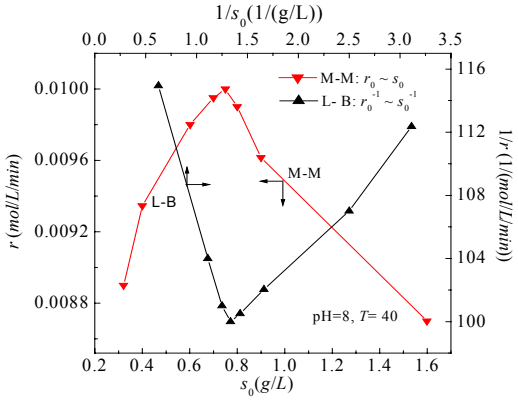

Fig. 6 Lineweaver-Burk plot and Michaelis-Menten plot

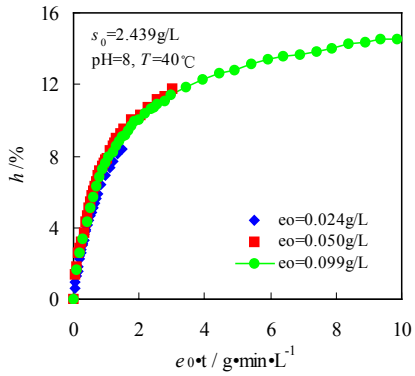

Fig. 7 Influence of initial enzyme concentration on the degree of hydrolysis

On the basis of the above facts, it is demonstrated, like many other systems, that it is the irreversibly competitive inhibition of substrate and the second order inactivation of trypsin rather than the concentration of effective peptide bonds are the most important factors to the rapid decrease in reaction rate and the constant value of h at the end of the reaction. This viewpoint conforms to the proposed mechanism used to establish the kinetic model, so the validity of the exponential kinetic equation can be well verified.

4.2 Determination of the exponential kinetic equation

4.2.1 Influence of s_0, e_0 and T on parameters a and b

In terms of time-course hydrolysis curves in Figs. 2, 3 and 4,

Table 2 Values of the kinetic parameters a and b for tryptic hydrolysis of BSA

s_0/g/L	e_0/g/L	T/(°C)	a	b
2.439	0.489	40	6.068	0.223
	0.099		3.273	0.224
	0.050		1.141	0.221
	0.024		0.375	0.228
0.256	0.024	40	4.674	0.234
0.972			1.162	0.217
1.745			0.527	0.204
2.440			0.371	0.184
0.244	0.024	35	3.602	0.290
		40	4.664	0.237
		45	6.540	0.234
		55	16.493	0.197

the values of kinetic parameters a and b (Table2) corresponding to different experimental conditions are determined through non-linear regression analysis in accordance with the equation $\mathrm{d}h/\mathrm{d}t = a\exp(-bh)$. The influences of s_0, e_0 and temperature on a and b values agree with the substrate inhibition mechanism, i.e. parameter b maintains constant when e_0 is changed (the average value of b is about 0.22), but decreases when s_0 and temperature increase; parameter a increases with e_0 and temperature, but decreases with s_0.

4.2.2 *Calculation of the reaction kinetic constants*

According to a and b expressions derived from substrate in hibition (Table 1), three straight lines, $b^{-1} \sim s_0$, $ab^{-1} \sim e_0\, s_0^{-1}$ and $r^{-1} \sim s_0^{-1}$ can be drawn (Fig. 8). The good linear relationships between dependent and independent variables justify the validity of the proposed reaction mechanism. Furthermore, based on linear regression method, the reaction kinetic constants (K_m, K_s, k_d, k_2) are calculated (Table 3) in accordance with the slope and intercept of these lines. Since K_s and s_0 are of the same order of magnitude, the substrate inhibition to take place. In addition, the optimal substrate concentration corresponding to r_{max} value from Lineweaver-Burk plot (Fig. 6) is s_{opt} = 0.76 g/L, which is very close to the calculated optimum value $s_{opt} = \sqrt{K_m K_s}$ = 0.77 g/L. This demonstrated the reliability of the established kinetic model.

4.2.3 *Calculation of the hydrolysis activation energy and the enzyme inactivation energy*

As shown in Table 1, parameter a has relevance to the reaction rate constant k_2, and ab relates to the enzyme inactivation constant $k_d = k_3 K_m$. So the changes of a and ab caused by temperature will follow the Arrhenius equation $\ln a = -\frac{E_a}{RT} + A_a$; $\ln(ab) = -\frac{E_d}{RT} + A_d$. The values of E_a and E_d (Table 3) can be calculated through the slope of regression straight lines, i.e. $\ln a \sim T^{-1}$ and $\ln (ab) \sim T^{-1}$ (Fig.9).

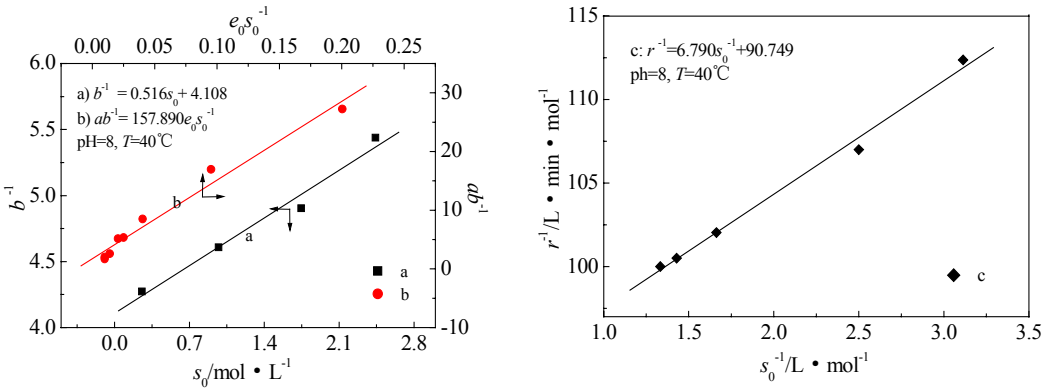

Fig. 8 Determination of kinetic constants

Table 3 Values of kinetic constants and thermodynamics constants for tryptic hydrolysis of BSA

K_m/g/L	K_S/g/L	$k_d = k_3K_m$/(/min)	K_2/(/min)	E_a/kJ/mol	E_d/kJ/mol
0.0748	7.961	9.358	38.439	64.826	80.031

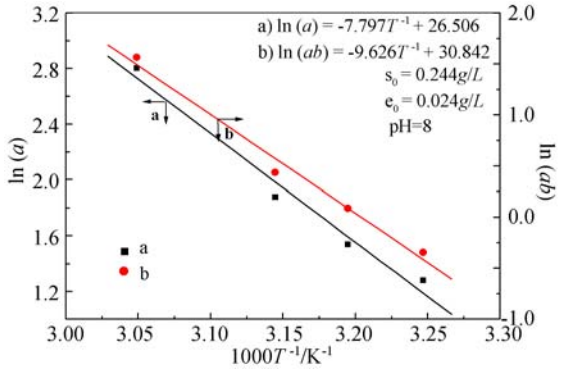

Fig. 9 Determination of thermodynamics constants

4.3 Hydrolysis curve fit and kinetic model application

By integrating Eq.(16), the integral form of kinetic equation characterizing the time-course relationship of BSA-trypsin system can be obtained as follows:

$$h=\frac{1}{b}\cdot\ln(1+abt)=\frac{k_2(K_s+s_0)}{k_3K_mK_s}\ln(1+\frac{k_3K_mK_s^2}{(K_s+s_0)^2}\frac{e_0}{s_0}\times t) \qquad (17)$$

Moreover, the theoretical hydrolysis curves corresponding to different values of s_0 and e_0 are obtained (Fig. 10) by substituting each kinetic constant into Eq. 17. The average of relative error between the calculated values (solid curve) and the experimental data (scatter plot) is 4.74%, which demonstrates again that the proposed reaction mechanism and kinetic model are both reasonable. Meanwhile, the kinetic model can also be uesd to predict the time-course relationships of BSA-trypsin system at different pH and temperature conditons with different s_0 and e_0 values.

5 Conclusions

(1) On the basis of the hydrolysis curves of BSA-trypsin system at different s_0, e_0, and temperature, the reaction mechanism including single substrate hydrolysis, second-order enzyme inactivation and substrate noncompetitive inhibition was proposed. The exponential kinetic equation, $\frac{dh}{dt}=a\exp(-bh)=\frac{1147.5545e_0}{12.8598s_0+s_0^2}\exp\left(-\frac{3.3677h}{12.8598+s_0}\right)$, was also established, and the average relative error of the model is 4.73%.

(2) The whole set of exponential equations is the general kinetic model for protein enzymatic hydrolysis reaction. For different reaction mechanisms, parameters a and b have different expressions (Table 1). So it can be applied to a wide range of operating conditions for various animal and plant protein systems. Furthermore, the functional relationship between s_0, e_0, h and t (Eq. (16) and Table 1) can also be used to model the bioreaction process, calculate the kinetic or thermodynamic constants, and optimize the operating parameters for peptide industrial production or bioreactor design.

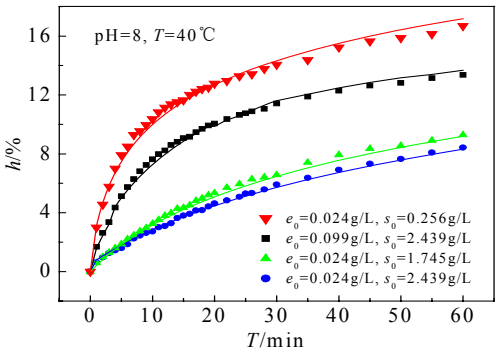

Fig. 10 Fit curves of kinetic model

Nomenclature

A_a, A_d frequency factor, dimensionless
a, b parameters of the exponentially kinetic equation, dimensionless
E_a hydrolysis activation energy(kJ/mol)
E_d enzyme inactivation energy(kJ/mol)
e enzyme concentration(mol/L)
h degree of hydrolysis, dimensionless
K_m M-M constant(g/L)
K_p product inhibition constant(g/L)
K_s substrate inhibition constant(g/L)
k_d inactivation constant of enzyme(/min)
k_2 reaction rate constant of enzymatic hydrolysis(/min)
p product concentration(mol/L)
R gas constant (8.314 J/mol·K)
r reaction rate of enzymatic hydrolysis (mol/L·min)
s substrate concentration (mol/L)
T temperature (K)

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