Kinetics of Hydrolysis of Egg White Protein by Pepsin

Chang-Qing RUAN[1,2], Yu-Jie CHI[1] and Rui-Dong ZHANG[1]

[1]College of Food Science, Northeast Agricultural University, Harbin, Heilongjiang, P. R. China;
[2]College of Food Science, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang, P. R. China

Abstract

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Taking into account the enzyme inactivation and substrate inhibition, the bioreaction mechanism and kinetics characteristic of egg white protein (EWP) enzymatic hydrolysis by pepsin were investigated. A logarithmic equation $h = (1/b) \ln (1 + abt)$ indicating the relationship between the degree of hydrolysis (DH) and time was established. For EWP-pepsin system, the reaction mechanism could be deduced from a series of experimental results at different temperatures, pH values, substrate concentrations, and enzyme concentrations. The reaction kinetics and thermodynamic constants (K_S = 3916.5 g/l, k_2 = 17 202.86 min^{-1}, k_d = 21 962.03, E_a = 56.89 kJ/mol, E_d = 51.99 kJ/mol) were responsible for the empirical equations. The results of nonlinear regression of the proposed kinetic model agreed with the experimental data, i.e. the average relative error was less than 5%. As a conclusion, the kinetic equations can be used to fit the enzymatic hydrolysis process of egg white protein and to optimise the operating parameters of bioactive peptides preparation for the bioreactor design.

Keywords: egg white protein; pepsin; enzymatic hydrolysis; kinetics; bioactive peptides

Egg white proteins are broadly recognised as a valuable source of dietary nitrogen and as containing much more biological functional substances (Li Chan & Nakai 1989). Recently, egg white protein hydrolysates showed many functional properties as a readily available source of protein in the processing technologies of food industry. Some recent works report studies on the bioactivity of peptides possessing antihypertensive, antioxidant, and antibacterial activities which were derived from egg white protein (Dávalos *et al*. 2004; Miguel *et al*. 2004; Pellegrini *et al*. 2004). Different proteases, such as pepsin, trypsin, of chymotrypsin, have been used to hydrolyse protein to produce peptides possessing special bioactivities. Among the bioactive peptides, those with antihypertensive effects are receiving special attention due to the prevalence and importance of hypertension in the western population.

On the other hand, there is a distinct relationship between the degree of hydrolysis (DH) and functional properties such as the distribution of molecular weights, surface hydrophobicity, solubility, foaming and emulsifying properties (Campbell *et al*. 2003; Cigić & Zelenik-Blatnik 2004; Behnke *et al*. 2006). However, the relationship between the DH and bioactivity of the peptides derived from egg white protein is not clear. Low DH could sometimes provide a high angiotensin I- converting enzyme inhibitory activity, antihypertensive effect, and antioxidant activity (Dávalos *et al*. 2004; Miguel *et al*. 2007).

The hydrolysis of short-chain peptides follows a simple kinetic model. However, the process of

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enzymatic hydrolysis of a single protein or a native protein with high molecular weights is very complicated, so the empirical kinetic models are usually applied to describe their hydrolytic behaviour. Some empirical kinetic models in different enzyme-proteins systems of chickpea flour-trypsin (Moreno & Fernandez Cuadrado 1993), milk whey protein-trypsin (Margot *et al*. 1997), bovine haemoglobin protein-alcalase (Márquez & Vázquez 1999), and casein-trypsin (He *et al*. 2002) have been established. In the study of the heat-induced and pressure-induced changes in the susceptibility of egg white proteins to tryptic hydrolysis. Van Der Plancken *et al*. (2003, 2004) found that the enzymatic processing could be described by a modified first-order kinetic model. Up to now, the kinetic model of hydrolysis with regard to EWP-pepsin system has not been reported.

The purpose of this work is to determine the hydrolysis mechanism of egg white protein based on the principle of enzyme kinetics, and to evaluate the kinetics parameters as well as the equation for the operating conditions closer to those used in industry.

MATERIALS AND METHODS

Materials. Pepsin (EC 3.4.23.1, 424 U/mg from porcine gastric mucosa) was purchased from Sigma-Aldrich Chemical Co. (Beijing, China). Crude egg white was obtained from fresh chicken eggs bought from a local supermarket. All other chemicals used in this research were of analytical grade.

Enzymatic hydrolysis of egg white protein. Egg white was dissolved in distilled water at different concentrations, and thermally denatured at 90°C in a water bath for 15 min (Adler-Nissen 1986), then the pH of the denatured solution aliquots was adjusted to 1.5, 2.0, 2.5, and 3.0 with 1.0 mol/l HCl aqueous solution, respectively. The hydrolysis reaction was performed by adding 0.1 g/l, 0.3 g/l, 0.5 g/l, and 0.8 g/l pepsin, and at 30°C, 35°C, 40°C, and 45°C in a batch stirred tank reactor, and pH was kept stable by adding 1 mol/l HCl solution using automatic potentiometric tirator. The hydrolysates were sampled at different times for the DH value determination. Inactivation of pepsin was achieved by increasing the pH to 7.0 with 1M NaOH. The hydrolysates were then centrifuged at 4000 × g for 15 minutes.

DH determination. DH is defined as the ratio of the number of peptide bonds cleaved (number of free amino groups formed during proteolysis) expressed as hydrolysis equivalents (h), in relation to the total number of peptide bonds before hydrolysis (h_{tot}).

$$\mathrm{DH}\ (\%) = \frac{h}{h_{tot}} \times 100$$

The DH during enzymatic reactions of egg white with pepsin was measured by the spectrophotometric ninhydrin method as described by Moore & Stein (1948) with some modifications by Schwartz & Engel (1950). The percentage of DH was calculated according to the folowing formula:

$$\mathrm{DH}\ (\%) = \frac{N - N_0}{h_{tot}} \times 100$$

where:

N – amount in the substrate of liberated amino-groups of proteolytic products (mmol/g)

N_0 – amount of original amino-groups in the substrate (mmol/g)

h_{tot} – calculated from amino acid analysis by summing the mmoles of each individual amino acid per gram of egg white protein (Jones 1931; Lunven *et al*. 1973)

Modelling of protein enzymatic hydrolysis. The reaction mechanism of protein enzymatic hydrolysis for the substrate-inhibition and enzyme inactivation can be modelled as:

$$\mathrm{S} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{SES}$$
$$+$$
$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P_1}$$
$$+$$
$$\mathrm{E} \xrightarrow{k_4} \mathrm{E_A} + \mathrm{E_B} + \mathrm{P_2}$$

where:

E, S – free enzyme and substrate

ES, SES – intermediate enzyme-substrate complexes

P_1, P_2 – end products of the enzymatic reaction

k_1, k_{-1}, k_2, k_3, k_{-3}, k_4 – reaction rate constants

The corresponding reaction rate depends on the irreversible step:

$$v = s_0 \frac{\mathrm{d}h}{\mathrm{d}t} = k_2[\mathrm{ES}] \qquad (1)$$

It is assumed that the balanced reaction is at a steady state, the following mass balances for ES and SES complexes can be written as

$$\frac{d[ES]}{dt} = k_1[E][S] + k_{-3}[SES] - [E][S] - (k_{-1}[ES] + k_2[ES] + k_3[S][ES]) = 0 \quad (2)$$

$$\frac{d[SES]}{dt} = k_3[S][ES] + k_{-3}[SES] = 0 \quad (3)$$

The combinations of Eqs (2) and (3) leading to the kinetic equation for the inactivation process are given by

$$[ES] = \frac{[E][S]}{K_M} \quad (4)$$

$$[SES] = \frac{[E][S]^2}{K_M K_S} \quad (5)$$

where

K_M – Michaelis-Menten coefficient

K_S – substrate inhibition coefficient

$$K_M = \frac{k_{-1} + k_2}{k_1} \quad (6)$$

$$K_S = \frac{k_{-1}}{k_3} \quad (7)$$

The total enzyme concentration (e) at a given moment is expressed as

$$e = [E] + [ES] + [SES] \quad (8)$$

The substitution of Eqs (4) and (5) into Eq. (8) yields the expression for the free enzyme concentration ($[S] \approx s_0$):

$$[E] = \frac{K_M K_S e}{K_M K_S + K_M[S] + [S]^2} = \frac{K_M e}{K_M + s_0 + s_0^2/K_S} \quad (9)$$

If the process of inhibition by the substrate is controlling:

$$K_M \leq s_0 + s_0^2/K_S$$

Eq. (9) is reduced to:

$$[E] = \frac{K_M K_S e}{K_S s_0 + s_0^2} \quad (10)$$

Eq. (4) is reduced to:

$$[E] = \frac{K_S e}{K_S + s_0} \quad (11)$$

The kinetic equation for the enzymatic deactivation process given by the reaction mechanism will be:

$$-\frac{de}{dt} = k_4[E][ES] \quad (12)$$

The result of Eq. (1) divided by Eq. (12) is:

$$-\frac{dh}{de} = \frac{k_2}{k_4 s_0[E]} \quad (13)$$

The substitution of Eq. (10) into Eq. (13)

$$-\frac{dh}{de} = \frac{k_2(K_S s_0 + s_0^2)}{k_4 K_M K_S s_0} \frac{1}{e} \quad (14)$$

Integration of Eq. (14) provides (h: 0 to h, e: e_0 to e)

$$e = e_0 \exp\left(-\frac{k_4 K_M K_S s_0}{k_2(K_S s_0 + s_0^2)} \times h\right) \quad (15)$$

From here, the relationship between Eqs (1), (11) and (15) makes it possible to obtain the following equation for the reaction rate:

$$v = s_0 \frac{dh}{dt} = \frac{k_2 K_S e_0}{K_S + s_0} \exp\left(-\frac{k_4 K_M K_S}{k_2(K_S + s_0)} \times h\right) \quad (16)$$

If :

$$a = \frac{k_2 K_S e_0}{K_S s_0 + s_0^2}, \quad b = \frac{k_4 K_M K_S}{k_2(K_S + s_0)} \quad (17)$$

Then:

$$v = a s_0 \exp(-bh) \quad (18)$$

$$\frac{dh}{dt} = a \exp(-bh) \quad (19)$$

$$h = \frac{1}{b} \ln(1 + abt) \quad (20)$$

Statistical analysis. All the tests of DH determination were conducted in triplicates. Nonlinear regression analysis was performed using the CFTool command in MatLab 6.5 (program omitted). The mean, linear regression analysis, coefficient of determination (r^2) as well as significant difference of tests within the 95% and 90% confidence interval were determined by SAS 6.12 statistical function.

RESULTS AND DISCUSSION

DH factors influencing

Effect of temperature on DH. The process of hydrolysis at different temperatures is shown in

Figure 1. The DH values increased rapidly from 0 to 6.24 within 10 min, and increased slowly from the 10th to the 180th minutes. The highest DH value was obtained at 45°C, the lowest one was shown at 30°C. The temperature-activity profile of native porcine pepsin could be retained from 30°C to 45°C during the enzymatic process with EWP. Generally, the DH increases with the temperature increasing at the same reaction time because a higher temperature supports protein unfolding, enzymatic activity increasing, and lowering the activation energy for the substrate to product conversion (Whitaker 2000). However, each protease has a suitable temperature range for maintaining the enzymatic activity (Smith *et al*. 1991). Free porcine pepsin showed a high stability at 40°C in using 10 g/l haemoglobin solution in 0.01 mol/l HCl for 5 h, but the activity was reduced by 40% at 50°C (Altun & Cetinus 2007). This result indicates that a higher temperature can result in conformational transition and deactivation of pepsin (Kozlov *et al*. 1979).

Effect of pH on DH. The DH of egg white protein hydrolysed by pepsin under different pH values is shown in Figure 2. The results showed that the hydrolysis rates increased with pH value, and that the DH is the highest at pH 2.0. The reaction rates decreased more rapidly with time at pH 3.0. Each enzyme has an appropriate interval of pH that helps to maintain its three-dimensional structure in the active site and provide essential ionisable groups (Whitaker 2000). If pH value is above 5.0, pepsin can be denatured which can even result in inactivation (Kozlov *et al*. 1979; Pohl & Dunn 1988). This is in agreement with the work by (Christensen 1955; Schlamowitz & Peterson 1959) who reported that pepsin had optimal activity with native proteins at pH approximately 1.0, and at pH 1.5–3.5 with some denatured proteins.

Effect of substrate concentration on DH. The DH curves of egg white protein at various initial substrate concentrations (105.0 g/l, 175.0 g/l, 262.5 g/l, and 350.0 g/l) are shown in Figure 3. The DH decreased with the substrate concentration increasing while the enzymatic reactions at the lower substrate concentration (s_0 = 105.0 g/l) showed a higher reaction rate with DH reaching 6.93 at 180th minutes. For this reason, at a constant enzyme concentration and a lower concentration of the substrate, the substrate concentration is the limiting factor, thus the enzyme reaction rate will increase with the increasing substrate concentration. However, at higher concentrations, the substrate will often act as a dead-end inhibitor, particularly when the reaction is studied in the nonphysiological direction (Leskovacs 2004). Briefly, the substrate inhibition can not be ignored in the EWP-pepsin hydrolysis system because the inactive intermediate complexes of the enzyme and excessive substrate cannot decompose to yield hydrolysates (Yasnoff & Bull 1953; Humphreys & Fruton 1968; Deisseroth & Dounce 1970).

Effect of the enzyme concentration on DH. Higher DH values were observed in Figure 4 at ascending pepsin concentrations and at other conditions being constant (s_0 = 87.5 g/l, pH = 2.0, T = 35°C). This means that when a sufficient concentration of the substrate is available, the increasing enzyme concentration will increase the enzymatic reaction rate. The results demonstrated that the reaction rate was in the direct proportion to the rate of the yield of the intermediate complexes,

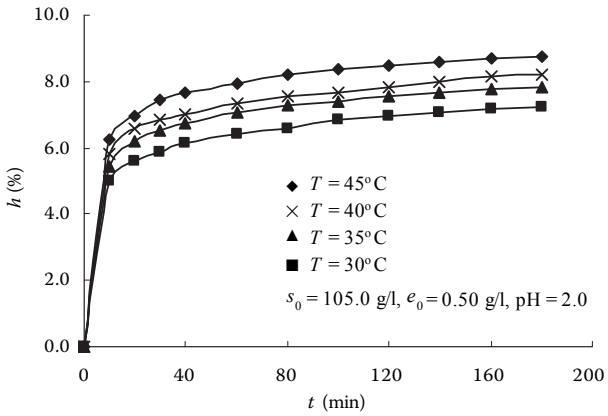

Figure 1. Hydrolysis curves for different temperature

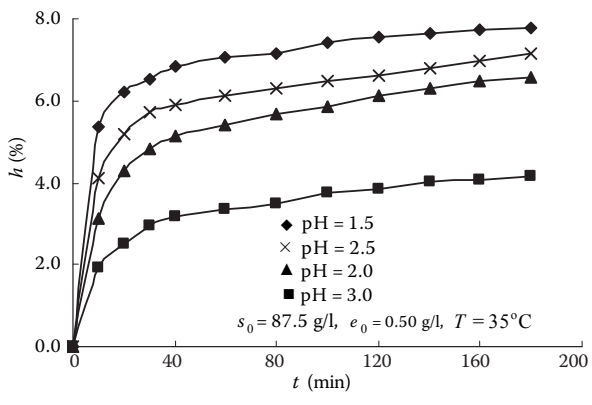

Figure 2. Hydrolysis curves for different pH value

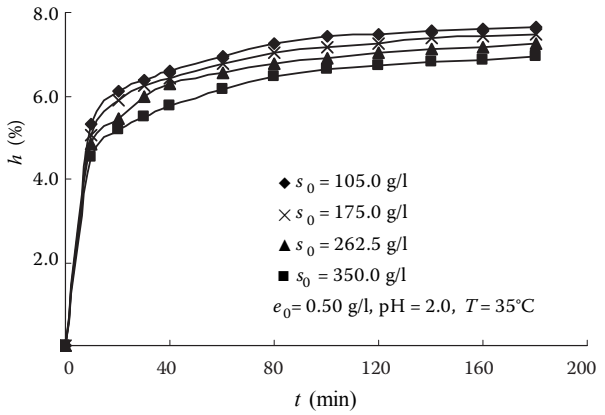

Figure 3. Hydrolysis curves for different substrate concentration

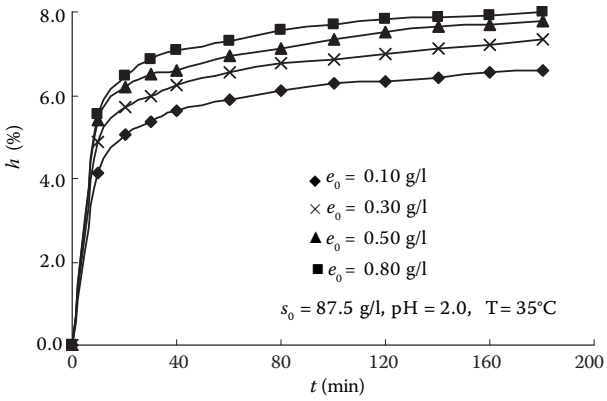

Figure 4. Hydrolysis curves for different enzyme concentration

which was dependent on the amount of enzyme (Davies 1990); while the substrate depletion becomes significant, further increases in the enzyme concentration will no longer demonstrate as a steep change in the reaction velocity as a function of the enzyme concentration (Copeland 2000). Furthermore, potent peptides of egg white proteins are generated by porcine pepsin having a cleavage site specificity, cleaving preferentially at the carboxyl termini of phenylalanine and leucine residues of the substrate (NC-IUBMB 1992–1999). These results indicate that a high concentration of pepsin is not suitable for the hydrolysis reaction.

Experimental verification of the reaction process

As shown in Figures 1–4, the time-course relationships of EWP-pepsin model system are characterised by a high initial reaction rate, followed by a rapid decrease in the rate tending towards a constant value with the time increasing. The downward trend of the hydrolysis curves is attributed to the decreasing concentration of the effective peptide bonds, substrate or product inhibition, and enzyme inhibition or inactivation.

In order to study the changes of DH at higher enzyme and substrate concentrations, a series of experiments were carried out and the results are summarised as follows.

The effect of the changing substrate concentration on DH was followed in the course of hydrolysis (s_0 = 87.5 g/l, e_0 = 0.50 g/l, pH = 2.0 and T = 35°C). The DH increased obviously with the increasing substrate concentration at the beginning of the reaction (from 0 min to 60 min); however, with the addition of extra substrate (20.0 g/l) no obvious increase of DH occurred from 60 min to 180 min ($P > 0.1$, Figure omitted). It is possible that the results were not due to the decrease on the substrate concentration, and that the concentration of peptide bonds is not the key to the reaction rate (Moreno & Fernandez Cuadrado 1993; He *et al*. 2002).

To investigate the possibility of enzymatic inhibition, the ratio of enzymatic reaction $\Delta h/\Delta t$ was plotted versus the substrate concentration s_0 (Figure 5). The results showed that the $\Delta h/\Delta t$ rapidly increased at lower substrate concentrations, maximum value being 0.0431 min^{-1} at the substrate concentration of 87.5 g/l, and then it slowly recreased to 0.0385 min^{-1} at the substrate concentration of 350 g/l.

In some cases, the occurrence of excess-substrate inhibition significantly reduced the enzymatic reaction rate (Bailey & Ollis 1986). The hydrolysis curve can be explained as a result of the competition between the substrate and inhibitory peptides, which are continuously solubilised in the process of hydrolysis. Since the reaction between the enzyme and inhibitory peptides proceeds with no net formation of free amino groups, its contribution to the overall reaction rate measured will be zero. However, since a certain fraction of the enzyme will be engaged in carrying out the reaction with the inhibitor, the effect will be an overall decrease in the reaction rate as compared to the reaction where no inhibitor is present (Moreno & Fernandez Cuadrado 1993).

To verify whether or not the enzyme inactivation existed or not, the concentration of pepsin was increased twofold after 60 min in the reaction

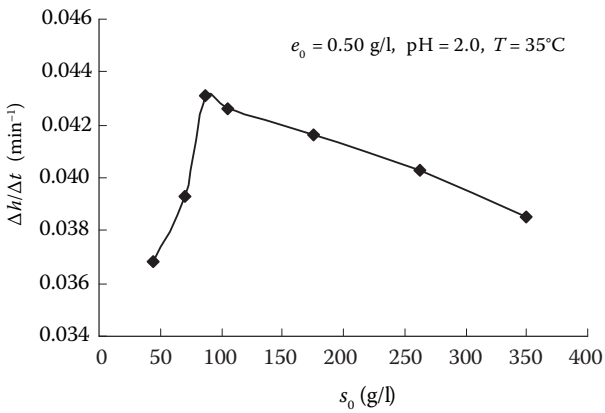

Figure 5. Effect of substrate concentration on Δh/Δt

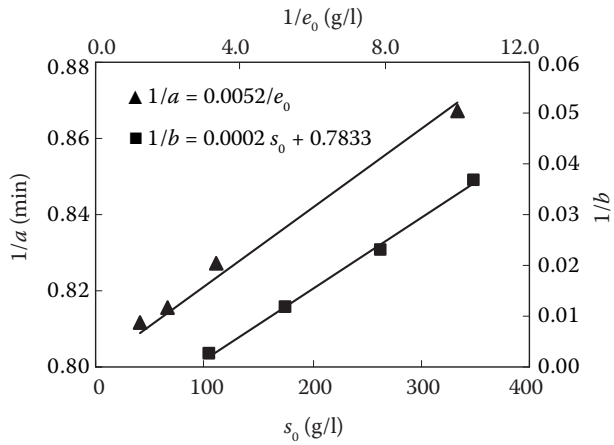

Figure 6. Determination of kinetic constants a and b

system (s_0 = 87.5 g/l, e_0 = 0.50 g/l, pH = 2.0, and T = 35°C (Figure omitted). The occurrence of a rapid increase in the hydrolysis rate as a result of this addition ($P < 0.05$) indicated the existence of enzymatic inactivation and, at the same time, confirmed the existence of a sufficient amount of peptide bonds available for hydrolysis (Moreno & Fernandez Cuadrado 1993; He *et al*. 2002).

Determination of the exponential kinetic equation

Effects of s_0, e_0, and T on parameters a and b. According to the time-course hydrolysis curves given in Figures 1, 3, and 4, the values of parameters *a* and *b* (Table 1) corresponding to different experimental conditions were calculated using the non-linear regression analysis (by Matlab software) in accordance with the exponential equation (Eq. 20). It can be observed that while *a* presents a clear dependence upon the initial enzyme concentration e_0, substrate concentration s_0, and temperature *T*, it decreases with the initial substrate concentration, s_0. The value of *b* remains constant when e_0 varies and its values lie within a very small range, with an average value of 1.260, but it decreases when s_0 and *T* increase while the parameter *a* increases with e_0 and temperature. This consideration of the temperature effect on parameters *a* and *b* is supplementary for the kinetic mechanism of enzymatic hydrolysis of proteins (He *et al*. 2002).

Calculation of the reaction kinetic constants. According to *a* and *b* expressions derived from the

Table 1. Values of kinetic parameters *a* and *b* of Eq. 20 (pH = 2.0)

T (°C)	s_0 (g/l)	e_0 (g/l)	a (min^{-1})	b
35	87.5	0.10	19.82	1.257
35	87.5	0.30	49.07	1.269
35	87.5	0.50	85.84	1.264
35	87.5	0.80	113.5	1.248
35	105.0	0.50	72.74	1.245
35	175.0	0.50	50.27	1.226
35	262.5	0.50	32.85	1.204
35	350.0	0.50	18.76	1.178
30	105.0	0.50	42.85	1.261
35	105.0	0.50	84.56	1.248
40	105.0	0.50	109.4	1.232
45	105.0	0.50	207.7	1.218

Table 2. Values of kinetic and thermodynamic constants for peptic hydrolysis of EWP

K_S (g/l)	k_2 (1/min)	$k_d=k_4 K_M$(1/min)	E_a (kJ/mol)	E_d (kJ/mol)
3916.5	17202.86	21962.03	56.89	51.99

substrate inhibition (Eq. 17), two straight lines, a^{-1}~e_0^{-1} and b^{-1}~s_0, were drawn as shown in Figure 6. As a result of the SAS analysis, the coefficients of determination r^2 were found to be 0.9941 and 0.9978, respectively. The good linear relationships between the dependent and independent variables demonstrated the validity of the proposed reaction model of EWP-pepsin system.

Furthermore, based on the linear regression method, the reaction kinetic constants (K_S, k_2, $k_4 \times K_M$) were calculated (Table 2) in accordance with the slope and intercept of these lines.

Since parameter a was related to the reaction rate constant k_2, and $a \cdot b$ related to the enzyme inactivation constant $k_d = k_4 \times K_M$ (Table 2), the changes of a and $a \cdot b$ caused by the temperature will follow the Arrihenius equation.

$$\ln a = \frac{E_a}{RT} + A_a, \quad \ln(ab) = -\frac{E_d}{RT} + A_d$$

Where:

A_a, A_d – frequency factors

R – gas constant 8.314J/mol/K

The values of activation energy E_a and E_d (Table 2) can be calculated through the slope of regression straight lines ($\ln a = -6.8425/T + 26.6602$, $r^2 = 0.9695$; $\ln(ab) = -6.2534/T + 24.9885$, $r^2 = 0.9630$).

As can be seen from Table 2, the values of activation energy E_a and E_d are similar, which means that the two reactions need to overcome similar energy barriers. This confirms the previously described correlation between parameters a and s_0, e_0, T, and between b and s_0, T.

To sum up, the kinetic constants were determined by varying s_0 and e_0, and were subsequently used to establish complete kinetic equations. In addition, activation energy E_a, E_d can be determined by varying the temperature.

Hydrolysis curve fitting and kinetic model application

Theoretical hydrolysis curves corresponding to different values of s_0 with 105.0 g/l, 175.0 g/l, 262.5 g/l, and 350.0 g/l (e_0 = 0.50 g/l, pH = 2.0 and T = 35°C) and different values of e_0 with 0.1 g/l, 0.3 g/l, 0.5 g/l, and 0.8 g/l (s_0 = 87.5 g/l, pH = 2.0, and T = 35°C) were obtained by substituting each kinetic constant into Eq. 20. The average of the relative error (ARE) between the calculated values and the experimental data was less than 5.0% (1.15%, 0.60%, 0.31%, and 2.41% for the above mentioned different substrate concentrations, as well as 0.17%, 3.47%, 2.27%, and 2.71% for the above mentioned different enzyme concentrations), which demonstrated again that the proposed reaction mechanism and kinetic model are reasonable. Meanwhile, the kinetic model can also be used to predict the time-course relationships of EWP-pepsin system at different substrate and enzyme concentration values under eligible pH and temperature conditions.

CONCLUSIONS

The mechanism of peptic hydrolysis of egg white protein consists of a series of consecutive and parallel bioreactions involving the substrate inhibition and enzyme deactivation, depending upon the substrate concentration in the appropriate range of temperature and pH values. The proposed kinetic model clearly appears to correlate with the experimental data, and can be used for fitting the data from the batchreactor experiments with protein hydrolysis. For the preparation of bioactive peptides, the empirical kinetic model can be used to predict the course of peptic hydrolysis of egg white protein at different reaction times, or reveal the relationship between the DH and biological activity.

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Corresponding author:

Dr. Yu-Jie Chi, Northeast Agricultural University, College of Food Science, 59 Mucai Street, Harbin, Heilongjiang, 150030, P. R. China
tel./fax: + 86 451 551 917 93, e-mail: yjchi@126.com