Food Chemistry 201 (2016) 230–236

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Heat-pretreatment and enzymolysis behavior of the lotus seed protein

A-Dan Pan, Hong-Yan Zeng *, Gohi Bi Foua Claude Alain, Bo Feng

Biotechnology Institute, College of Chemical Engineering, Xiangtan University, Xiangtan 411105, Hunan, PR China

ARTICLE INFO

Article history:
Received 6 June 2015
Received in revised form 12 January 2016
Accepted 18 January 2016
Available online 19 January 2016

Chemical compounds studied in this article:
Sodium chloride (PubChem CID: 5234)
Hydrochloric acid (PubChem CID: 313)
Trichloroacetic acid (PubChem CID: 6421)
Disodium hydrogen phosphate (PubChem CID: 24203)
Monosodium phosphate (PubChem CID: 23672064)

Keywords:
Lotus seed protein
Enzymolysis
Response surface methodology
Kinetic models
Heat-pretreatment

ABSTRACT

Lotus seed protein (LSP) was heat-pretreated before enzymolysis in order to seek a greater degree of hydrolysis (*DH*) during enzymatic hydrolysis. The parameters including substrate concentration, temperature, pH, and papain concentration were optimized by response surface methodology in the enzymolysis of the heat-pretreated LSP. The influence of substrate concentration on the non-pretreated LSP enzymolysis was assessed, and the enzymolysis was found to obey the Haldane model with inhibition by LSP substrate. The initial concentration of non-pretreated LSP was inferred theoretically to be 11.07 g/L in order to avoid substrate inhibition. On the other hand, Chrastil model was fitted and the diffusion resistance constant values were in the range of 0.5–0.6 for the diffusion-controlled encounter of enzyme and substrate, implying that diffusion was a rate-limiting step. The heat-pretreatment at 60 °C for 60 min could increase the *DH* of the LSP, which enhanced the efficiency of the enzymolysis by papain.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Lotus seed, widely used as food in China (by the name of lianzi), is rich in protein, amino acids, unsaturated fatty acids and minerals. The seed contains approximately 19.85% protein on a dry weight basis, and shows a well-balanced amino acid composition compared with FAO/WHO pattern, with nutritive properties similar to soybean (Zeng, Cai, Cai, Wang, & Li, 2013). The interest in the lotus seed protein (LSP) as a functional food has been increased due to the potential health benefits (Bhat & Sridhar, 2008; Wu et al., 2007; Yen, Duh, & Su, 2005; Zeng et al., 2013).

Hydrolysis of proteins by proteases has undergone development in recent years. Enzymolysis can improve the functional properties of food without affecting its nutritive value. And the degree of hydrolysis (*DH*) is an important parameter for the determination of functional properties of protein hydrolysates (Adler-Nissen, 1979). Hence, protein hydrolysates are extensively incorporated as food ingredients in energy drinks, hypoallergenic formulae and enteral diets for children and sick adults due to their functional, nutritional and immunological properties (Moure, Sineiro, Domínguez, & Parajó, 2006; Prieto, Guadix, & Guadix, 2008; Schmidl, Taylor, & Nordlee, 1994). For the preparation of protein hydrolysates, it is essential to apply pretreatments to increase the accessibility of the enzyme to cleavage sites and consequently to raise the *DH*. Heat-pretreatment is effective for improving enzymatic hydrolysis and considered to be an eco-friendly processing technology since no chemicals are added and decreasing toxic compound formations (Cao, Sun, Liu, Yin, & Wu, 2012; Saha, Yoshida, Cotta, & Sonomoto, 2013). In food industry, heat-pretreatment of proteins before enzymolysis can lead to rearrangements of the inter- and intra-molecular linkages with concomitant changes in protein conformation, making proteins more susceptible to enzymolysis, due to the exposure of previously hidden cleavage sites, thereby enhancing enzymolysis (Adjonu, Doran, Torley, & Agboola, 2013; Boye, Ma, Ismail, Harwalkar, & Kalab, 1997; Chao, He, Jung, & Aluko, 2013; Tang, Chen, & Ma, 2009). In spite of the merits of the proposed approach, the application of heat-pretreatment to the LSP is scarcely described in the literature. In our preliminary work, the *DH* in the enzymolysis of

* Corresponding author.
E-mail address: hongyanzeng99@hotmail.com (H.-Y. Zeng).

http://dx.doi.org/10.1016/j.foodchem.2016.01.069
0308-8146/© 2016 Elsevier Ltd. All rights reserved.

non-pretreated LSP by papain was about 11%, which was too low to improve its functional properties. In order to enhance the *DH*, it is necessary to heat-pretreat the LSP.

The traditional "one-factor-at-a-time approach" is an operation frequently used in optimization to obtain high yields of the desired products. But that method disregards the complex interactions among various physicochemical parameters. The response surface methodology (RSM) is defined as a statistical method using quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate equations. RSM can evaluate the effects of multiple parameters, alone or in combination, on response variables and also predict their behavior under given sets of conditions (Pan, Zeng, Foua, Alain, & Li, 2016).

In the present study, LSP was firstly heat-pretreated, and then the pretreated LSP was enzymolyzed by papain. In order to obtain high *DH*, the effect of the pretreatment temperature on the *DH* was investigated, and the enzymolysis parameters of the pretreated LSP including temperature, initial pH, substrate concentration and papain concentration were also optimized using RSM. Finally, the enzymatic hydrolysis process of non-pretreated LSP was analyzed in terms of kinetic parameters.

2. Materials and methods

2.1. Materials

Papain (EC 3.4.22.2, ⩾99%) was purchased from Sigma–Aldrich Company Ltd in China. All other reagents used in experiments were of analytical grade without further purification. All solutions were made with redistilled and ion-free water. The lotus seeds without seed coats and embryos, cultivated in Hunan Province of China, were purchased from a retail outlet in Xiangtan (China). Prior to experiments, the seeds were milled, and ground to pass through a 100-mesh screen to produce the lotus seed powder. The lotus seed protein (LSP) was extracted as previously described by Zeng et al. (2013). Briefly, the LSP was extracted in the 0.1 mol/L NaCl solution, where 5.0 g lotus seed powder was placed into a 1000 mL flat bottom flask containing 500 mL NaCl solution. The reaction mixture was blended for 1 h at 40 °C under atmospheric pressure and constant magnetic stirring (100 rpm). The slurry was centrifuged using a L535R tabletop refrigerated centrifuge (Xiangyi Centrifuge Instrument Co., Hunan, China) at room temperature (2800 g, 20 min). The supernatant containing the LSP was adjusted to pH 4.2 by adding 0.1 mol/L HCl solution (acid precipitation), and then re-centrifuged (4800 g, 25 min) in order to obtain the crude LSP product (residue). The crude product was washed with deionized water (pH 4.2) three times, and subsequently freeze-dried to give the LSP powder. The LSP powder contained 88.9% protein content, 1.1% fat, 1.8% total sugar content and 2.9% moisture content.

2.2. Differential scanning calorimetric (DSC) analysis

The heat denaturation temperature of the non-pretreated LSP was monitored by differential scanning calorimetry (DSC) in a TA Q100-DSC thermal analyzer (Shimadzu DT-40) (Kumar, Ganesan, Selvaraj, & Rao, 2014). 2.0 mg of the LSP powder was weighed into aluminium liquid pans (Dupont), and 10 μL PBS (0.05 mol/L, pH 7.5) was added. The sealed pans containing the LSP and buffer were equilibrated at 4 °C for more than 12 h. The pans were sealed and heated at a rate of 10 °C/min from 20 to 100 °C in 150 mL/min flow of helium, and subsequently cooled to 20 °C at the same rate. A sealed empty pan was used as a reference. The denaturation temperature, corresponding to the maximum of the transition peak, was determined. Experiments were carried out in triplicate and varied not more than 0.25 °C.

2.3. Heat-pretreatment of the LSP

The assay included two parts of heat-pretreatment and enzymolysis by papain. Firstly, the heat-pretreatment was carried out, where the LSP powder was prepared into a suspension containing 34 g/L LSP concentration. The suspension was pretreated in a rotating vessel (50 rpm) at a temperature range of 50–80 °C for 75 min. Five vessels were used at one time at a specific temperature, where a vessel was removed at each 15 min interval to stop the pretreatment for gaining the pretreated LSP suspension. Secondly, the enzymolysis of the pretreated LSP was performed. All the pretreated LSP suspensions at different temperatures were separately diluted to 15 g/L. Enzymolysis of the pretreated LSP by papain was performed under the conditions of pH 5.5, 45 °C and papain concentration 0.5 g/L for 45 min. All experiments were carried out in triplicate. It was found that the degree of hydrolysis (*DH*) at 60 °C for 60 min was the highest among all the pretreated LSP (Fig. 1B).

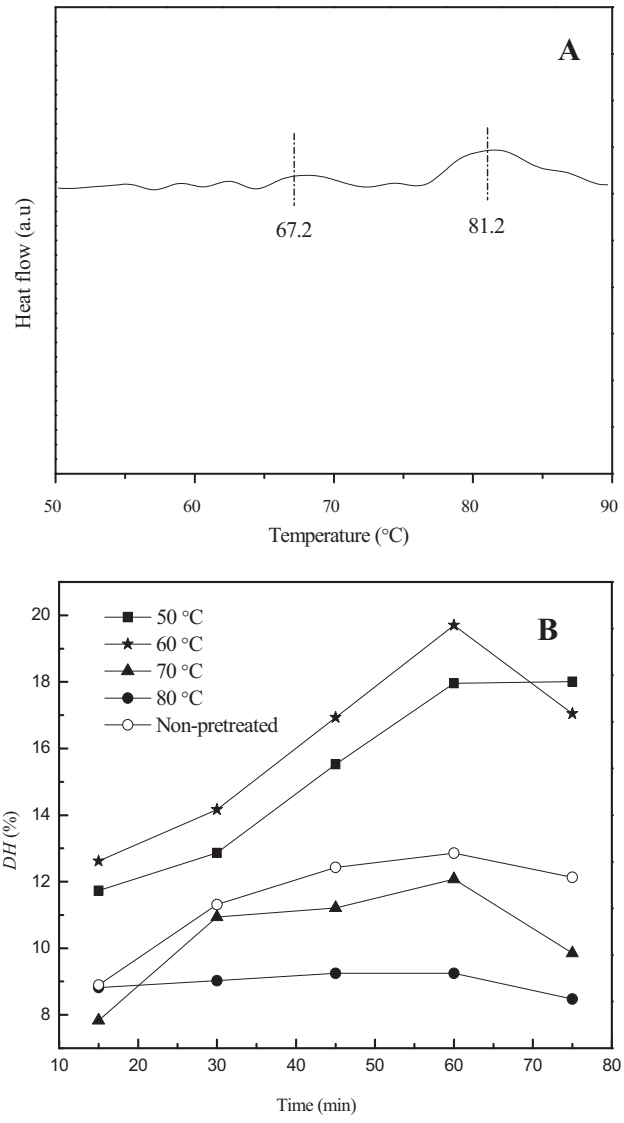


Fig. 1. (A) DSC thermograms of the non-pretreated LSP. (B) Influence of heat-pretreatment temperature on the enzymolysis of the LSP.

2.4. Enzymolysis of the pretreated LSP

Papain solution (1.0 g/L) was obtained by dissolving the enzyme in PBS buffer (0.1 mol/L, pH 7.0), and the protein suspension containing 34 g/L LSP was heat-pretreated at 60 °C for 60 min. According to the experimental design, different concentrations of papain (0.2–0.8 g/L) were added into the pretreated LSP suspension with a specific concentration (2–34 g/L), respectively, where the pH in the reaction system was in the range of 3.5–7.0. The enzymolysis was performed by employing a shake-flask method in a 150 mL flask in a temperature-controlled water bath (40–70 °C). After 45 min, the reaction was stopped at 100 °C for 10 min. The hydrolysate was clarified by centrifuged at 2800 g for 15 min to remove undegraded substrate fragments and residual enzyme. All the experiments were carried out in triplicate under the same condition and average values are reported. The analysis of the hydrolysate was then carried out. The degree of hydrolysis (*DH*) was determined by measuring the nitrogen content in solution using 10% trichloroacetic acid as described by Kim, Park, and Rhee (1990), and was defined as follow:

$$DH\ (\%) = 100 \times N_1/N_2 \tag{1}$$

where N_1 is the soluble nitrogen in the hydrolysate, and N_2 is the total nitrogen content in the protein powder.

In order to describe the enzymolysis kinetics of the non-pretreated LSP, the hydrolysate concentration (*C*, g/L), was calculated as follows (Qu et al., 2012):

$$C = S \times DH \times 0.01 \tag{2}$$

where *S* is the initial protein concentration (g/L).

After approximation of the best conditions by "one-factor-at-a-time" method in our preliminary experiments, RSM was used to test the effect of substrate concentration (*S*), initial pH (*P*), hydrolysis temperature (*T*) and papain concentration (*E*) on the *DH* of the pretreated LSP in the enzymolysis. The optimized response variable was the *DH* (*Y*) and central composite design (CCD) was used to design the experiments in Table S1. After the reaction, the response *Y* was measured. The statistical software package Design Expert software (version 8.0.6) was used for the regression analysis of experimental data and to plot response surface. The second-order polynomial model was applied to predict the response variable (*Y*) as shown below,

$$Y = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii}X_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij}X_iX_j \tag{3}$$

where *Y* is the response value (degree of hydrolysis), β_o, β_i, β_{ii} and β_{ij} are the regression coefficients for interception, linear, quadratic and interaction terms, respectively. X_i and X_j are the independent variables.

2.5. Enzymolysis kinetics

As the knowledge of the enzymolysis kinetics, it is essential to understand the capacity of papain in the LSP enzymolysis. The non-pretreated LSP was directly degraded by papain in the solution (pH 5.5) containing initial substrate concentration range from 2 to 34 g/L with a specific papain concentration at 55 °C for 45 min. At the same time, the pretreated LSP (13 g/L) made at 60 °C for 60 min was investigated as control under the same conditions except 0.5 g/L papain. The hydrolysate concentration (*C*) in the reaction solution was monitored at regular intervals.

3. Results and discussion

3.1. Thermal properties of the LSP

Thermal denaturation of the non-pretreated LSP was studied by DSC, and the thermogram curve is as shown in Fig. 1A. The non-pretreated LSP exhibited two exothermic transitions due to heat denaturation, where the minor exothermic transition temperature was at about 67 °C and the major one at about 81 °C. The appearance of the minor exothermic transition peak might be due to the presence of the impurities such as impure protein and carbohydrates in the non-pretreated LSP powder, and the second main transition peak presumably represented the denaturation of the non-pretreated LSP. Aggregation of the denatured molecules involved formation of new intermolecular bonds could give rise to an exothermic process in DSC (Fitzsimons, Mulvihill, & Morris, 2007; Kumar et al., 2014). There was reason to infer that high temperature (higher than 81 °C) could result in aggregation of the denatured proteins so that it was difficult to hydrolyze the proteins by papain, implying that thermal pretreatment did not lead to protein aggregation denaturation in the LSP when it was below 81 °C. According to the DSC analysis, pretreatment temperatures of the LSP were chosen to be below 81 °C.

3.2. Heat-pretreatment

Fig. 1B shows the change in the *DH* during the enzymolyses of the LSP pretreated under different temperatures (50–80 °C). Comparing with the non-pretreated LSP at a specific enzymolysis time, the *DH* of the pretreated LSP at 50 and 60 °C were significantly higher, while the *DH* of the pretreated LSP at 70 and 80 °C were lower. In all the pretreated LSP samples, the *DH* increased with time in the first 60 min, and then declined, except for the sample at 80 °C that levelled off for the first 50 min. For 60 min, the *DH* was 17.85% at 50 °C and 19.70% at 60 °C. However, the *DH* decreased to 12.08 and 9.25% at 70 and 80 °C, respectively. It could be because that the denaturation at about 67 °C made the non-pretreated LSP to agglomerate leading to the *DH* decreases. Excessively high temperature (>60 °C) began to cause the LSP denaturation (thermal aggregation) leading to decrease of the *DH*. At lower than 60 °C, non-covalent bonds and the disulfide bonds in the LSP molecules were gradually broken to cause the disaggregation and unfolding of proteins with increasing temperature, which facilitated more enzyme-cutting sites of the LSP exposure to papain (Adjonu et al., 2013; Boye et al., 1997; Chao et al., 2013). So, the heat-pretreatment at 60 °C prior to enzymolysis could best enhance the LSP hydrolysis. Hence, the heat-pretreatment at 60 °C for 60 min was used for the following the optimation of enzymolysis and kinetics as a control for the pretreated LSP.

3.3. Enzymolysis of the pretreated LSP

3.3.1. Central composite design (CCD) analysis

A CCD of the RSM is the most commonly used in optimization experiments. The values of the independent variables and the responses obtained in the multivariate study for each experiment are shown in Table 1. The statistical analysis of variance (ANOVA) was performed by the software in order to investigate the fitness and significance of the model and the effects of the individual variables and interactions on the response (Table S2). According to the ANOVA, the model was highly significant with a *p*-value ($p < 0.0001$) to predict the response values. In terms of the significant coefficients, the *S*, *P*, *ST*, *PT*, S^2, P^2 and T^2 were high significant factors ($p < 0.0001$), and *T*, *TE*, and E^2 were significant term ($p < 0.05$), but *E*, *SP*, *SE* and *PE* were insignificant ($p \geqslant 0.05$). The

Table 1
Central composite design matrix (CCD) and its response and predicted value.

Run	Experimental variables				
	S (g/L)	*P*	*T* (°C)	*E* (g/L)	Expt.
1	12	4.5	50	0.4	14.36
2	18	4.5	50	0.4	9.62
3	12	5.5	50	0.4	23.41
4	18	5.5	50	0.4	21.23
5	12	4.5	60	0.4	21.21
6	18	4.5	60	0.4	8.30
7	12	5.5	60	0.4	25.69
8	18	5.5	60	0.4	15.84
9	12	4.5	50	0.6	14.29
10	18	4.5	50	0.6	6.75
11	12	5.5	50	0.6	24.51
12	18	5.5	50	0.6	17.36
13	12	4.5	60	0.6	22.84
14	18	4.5	60	0.6	11.54
15	12	5.5	60	0.6	26.65
16	18	5.5	60	0.6	15.32
17	9	5.0	55	0.5	20.48
18	21	5.0	55	0.5	3.00
19	15	4.0	55	0.5	11.57
20	15	6.0	55	0.5	25.74
21	15	5.0	45	0.5	18.21
22	15	5.0	65	0.5	20.49
23	15	5.0	55	0.3	21.25
24	15	5.0	55	0.7	21.15
25	15	5.0	55	0.5	24.57
26	15	5.0	55	0.5	24.31
27	15	5.0	55	0.5	23.94
28	15	5.0	55	0.5	22.98
29	15	5.0	55	0.5	24.87
30	15	5.0	55	0.5	24.64

elimination of the insignificant terms improved the regression model, and the quadratic model was given as:

$$Y = 24.22 - 4.25S + 3.75P + 0.85T - 1.49ST - 1.37PT + 0.63TE - 3.17S^2 - 1.44P^2 - 1.27T^2 - 0.81E^2 \quad (4)$$

To test the fit of the model, the regression equation and determination coefficient (R^2) were evaluated. In this case, the value of the determination coefficient ($R^2 = 0.9905$) indicated that the model could explain 99.05% of the variability. The predicted R^2 and adjusted R^2 were both close to 1, which indicated the adequacy of the model. Adequate precision value was found to be 38.878 (greater than 4), revealing that the model was sufficient for discrimination. On the other hand, a relatively lower value of the coefficient of variation (CV 4.61%) demonstrated a good precision and reliability of the experiments (Yuan et al., 2008). The non-significant value of lack of fit ($p > 0.05$) also showed that the quadratic model was valid for the present study.

3.3.2. Interactions between the variables

The model suggested that there were high significant interactions between substrate concentration and hydrolysis temperature ($p < 0.0001$), pH and hydrolysis temperature ($p < 0.0001$), and between enzyme concentration and hydrolysis temperature ($p < 0.05$). Therefore, it was of great interest to further characterize the interactions. Fig. 2 represents the 2D contour plots and 3D response surfaces for the optimization of the *DH*, and each figure presents the effect of two variables on the *DH*, while the other two variables were held at zero level. Fig. 2A shows the combined effect of hydrolysis temperature and substrate concentration on the *DH*. The temperature displayed a linear effect on the response from 45.00 to 59.22 °C where the *DH* increased with temperature, and then reached equilibrium when the temperature further increased. Similarly, *DH* also increased to a maximum with the increasing substrate concentration and then levelled-off as substrate concentration further increased (>12.73 g/L). The combined effect of hydrolysis temperature and pH on the *DH* is shown in Fig. 2B. Both temperature and pH exerted the effect on the *DH* when the temperature and pH were under 52.57 °C and 5.76, respectively, and then achieved a balance with increasing temper-

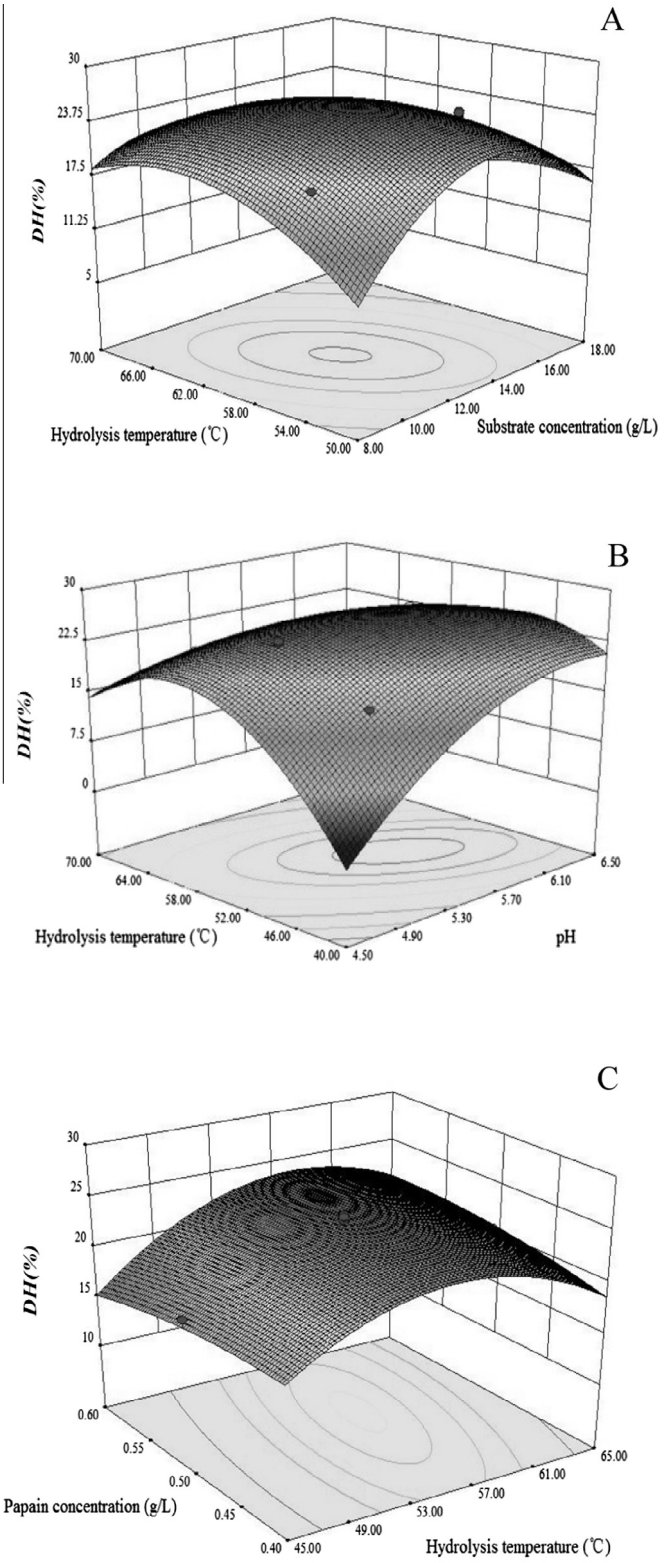


Fig. 2. Response surface of predicted *DH* versus hydrolysis temperature and substrate concentration (A), hydrolysis temperature and pH (B), hydrolysis temperature time and papain concentration (C).

ature and pH. It is clear from Fig. 2C that the combine effect of enzyme concentration and hydrolysis temperature was significant with the contour curve of oval shape. The enzyme concentration had almost no influence on the *DH*, however, the temperature demonstrated a quadratic effect on the response, where the *DH* increased up to about 24.38% at 56.87 °C, followed by a decline with increase in temperature.

3.3.3. Optimization analysis

One of the aims of this study is to find the optimum process parameters to maximize the *DH* using the developed mathematical model equation. The model predicted the maximum *DH* of 27.73% appeared under the conditions of substrate concentration 12.99 g/L, pH 5.50, temperature 56.10 °C and papain concentration 0.51 g/L. The model validation was determined by additional independent experiments under the optimal conditions of 13 g/L pretreated LSP, pH 5.5, 55 °C and 0.5 g/L papain for 45 min. The results indicated that the experimental *DH* (26.84%) was not significantly different from the predicted *DH* (27.43%), which confirmed the efficacy of the predicted model.

3.4. Enzymolysis kinetics for the non-pretreated LSP

3.4.1. Effect of initial papain concentration

At the 13.0 g/L initial non-pretreated LSP, *C* variation in different initial papain concentrations (E_o) with time is shown in Fig. 3A. The results show that *C* increased with an increase in papain concentration and enzymolysis time, where *C* increased rapidly in each concentration for the first 20 min and then slowed progressively. At 0.5 g/L papain, the pretreated LSP gave nearly twice as many hydrolysate concentrations (*C*) as the non-pretreated LSP, implying that heat-pretreatment could increase *C* due to disaggregation and unfolding of the protein (Adjonu et al., 2013; Boye et al., 1997; Chao et al., 2013; Tang et al., 2009).

3.4.2. Effect of initial substrate concentration

The effect of initial non-pretreated LSP substrate concentration on the enzymolysis was investigated, which the *DH* and hydrolysate concentrations (*C*) were obtained in the scopes of 2 to 34 g/L initial LSP (Fig. 3B and C). At low substrate concentrations (about 2∼13 g/L), the *DH* increased with substrate concentration. However, at higher substrate concentrations (> 13 g/L), the *DH* decreased as the concentration increased (Fig. 3B). It might be because the viscosity of the reaction system increased with the concentration, which further slowed the diffusion of the substrate to the active center of the enzyme molecule resulting in reduction of enzymatic activity. The results suggested that the non-pretreated LSP in high concentrations (> 13 g/L) inhibited the papain activity, which possibly existed substrate inhibition (Pan et al., 2016).

3.4.3. Kinetic constants

The enzymolysis kinetics is usually described by the pseudo-first-order and pseudo-second-order models (as shown in Table 2) due to their good representation of the experimental data (Wu, 2007). Based on the existing experimental data in Fig. 3C, the kinetic parameters were calculated by the two models and the results are shown in Table 2. The results show that all the correlation coefficients R^2 in the two models are above 0.99, demonstrating their applicability. Pseudo-second-order model was more suitable to describe the enzymolysis process than pseudo-first-order model based on a lower residual standard deviation *s* (Table 2). In the

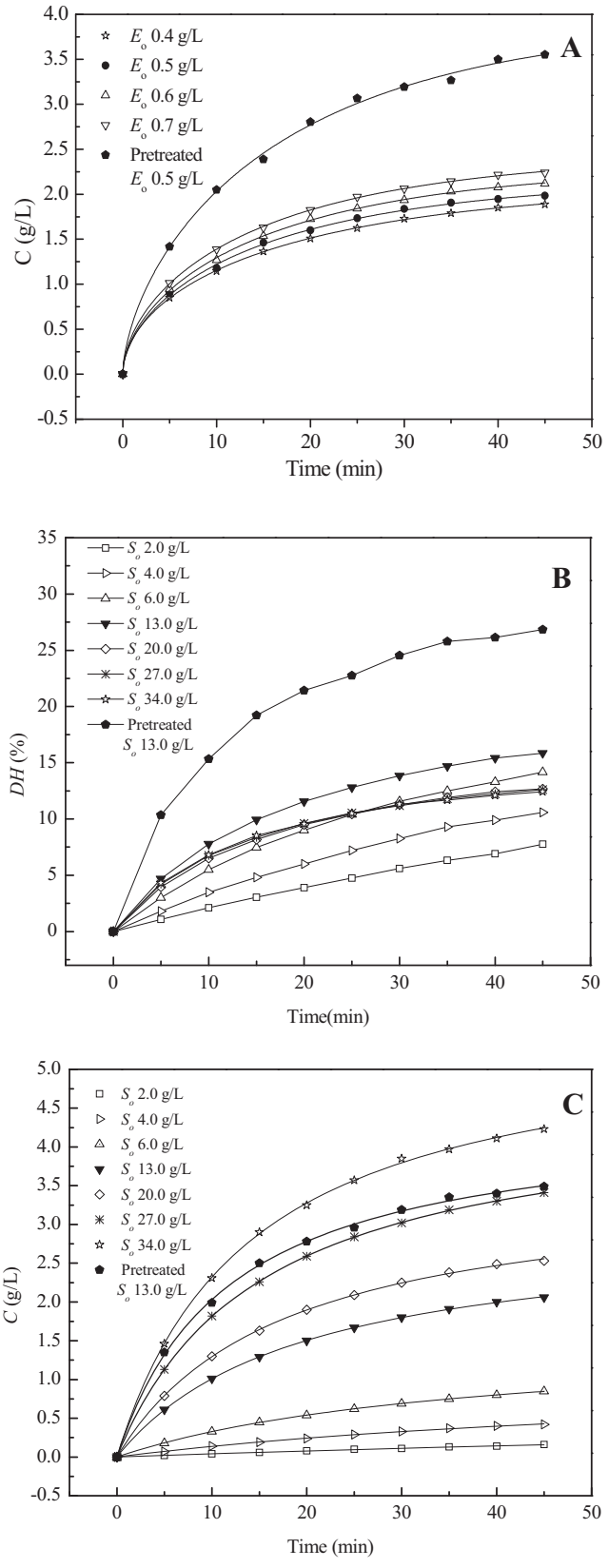


Fig. 3. Enzymolysis of non-pretreated and pretreated LSP. (A) Change of the hydrolyzed LSP concentrations (*C*) at different enzyme concentrations (E_o). (B) Change of the *DH* at different initial substrate concentrations (S_o). (C) Change of the hydrolyzed LSP concentrations (*C*) at different initial substrate concentrations (S_o).

Table 2
Kinetic parameters for the LSP enzymolysis by papain at 55 °C.

Concentration (g/L)	Pseudo-first-order* $C_t = C_e(1 - \exp(-k_1 t))$ *			Pseudo-second-order $t/C_t = 1/(k_2 C_e^2) + t/C_e$*		
	k_1 (1/min)	R^2	s	k_2 (L min/g)	R^2	s
2	0.00132	0.9912	0.014	0.00669	0.9980	0.007
4	0.02332	0.9994	0.010	0.01364	0.9991	0.012
6	0.03742	0.9995	0.018	0.01746	0.9999	0.008
13	0.05999	0.9985	0.072	0.01754	0.9999	0.013
20	0.06456	0.9983	0.095	0.01625	0.9997	0.038
27	0.07085	0.9975	0.155	0.0145	0.9999	0.018
34	0.07496	0.9976	0.187	0.01287	0.9997	0.068
13 (Pretreated)	0.08401	0.9953	0.216	0.01932	0.9992	0.088

* C_t and C_e are hydrolyzed LSP concentration at t and equilibrium, respectively. k_1 and k_2 are the rate constants of first-order and second-order models, respectively.

pseudo-second-order model, the value of k_2 was the same as the value of the specific hydrolysis rate (v) (Pan et al., 2016).

The specific hydrolysis rate v increased with increasing the substrate concentration from 2 to 13 g/L and then decreased, which trend was consistent with the experimental results (Fig. 3B). At 13 g/L initial substrate concentration, the v value of the non-pretreated LSP was maximum, i.e. 0.0187 g/(L min), and the v value (0.01932 g/(L min)) of the pretreated LSP substrate was higher than that of the non-pretreated LSP. The may possibly be due to the dis-aggregation and unfolding of the protein molecules during heat-pretreatment (Adjonu et al., 2013; Boye et al., 1997; Chao et al., 2013; Tang et al., 2009). The result further confirmed that heat-pretreatment could enhance the enzymolysis of the LPS by papain. The decrease in C and v at higher initial substrate concentration (>13 g/L) might be due to the inhibitory effect of the LSP substrate on the papain (Pan et al., 2016) as the initial LSP concentration was further increased. High-substrate inhibition was a form of uncompetitive inhibition that was characterized by a decrease in the enzymolysis rate at high substrate concentrations. The kinetic behavior of high-substrate inhibited enzymatic process followed the Haldane model (Dona, Pages, Gilbert, & Kuchel, 2010). The Haldane equation was selected for assessing the dynamic behavior of the enzymolysis (Ayhan, Ayhan, Pişkin, & Tanyolac, 2002; Dona et al., 2010).

$$v = \frac{V_{\max} S}{K_m + S + \frac{S^2}{K_{ss}}} \tag{5}$$

where v is specific rate of enzymolysis, which value is equal to one of the enzymolysis rate constant k_2. K_{ss} is the high-substrate inhibition constant, $V_{\max}$ is the maximum rate of enzymolysis, K_m is the Michaelis rate hydrolysis constant, and S is substrate concentration.

The v values were used to estimate the inhibited enzymolysis kinetic parameters, and the v of enzymolysis and model predicted are shown in Fig. 4. It could be seen that Haldane model was found to fit the data well. The R^2 value was 0.9255, which indicated the regression curve agreed with the experimental data very well. So, the values of parameters $V_{\max}$ = 0.0681 g/(L min), K_m = 14.6377 g/L and K_{ss} = 8.3520 g/L were obtained using analysis of Origin 9.0. Of the three kinetic constants, K_{ss}, the inhibition constant, is a measure of sensitivity to inhibition by inhibitory substances. It should be noted that the Haldane equation is simplified to the Michaelis–Menten equation when K_{ss} is very large, i.e., there is not substrate inhibition (Li, Yan, Xia, Wang, & Wang, 2014). K_m, half-saturation constant, is defined as the substrate concentration at which v is equal to half $V_{\max}$, which K_m values show the affinity of enzyme to substrate. It can be noted that it is impossible to observe an actual $V_{\max}$ if the substrate is inhibitory. Thus, K_m takes on a hypothetical meaning, and it is impossible to observe an actual $V_{\max}$. According

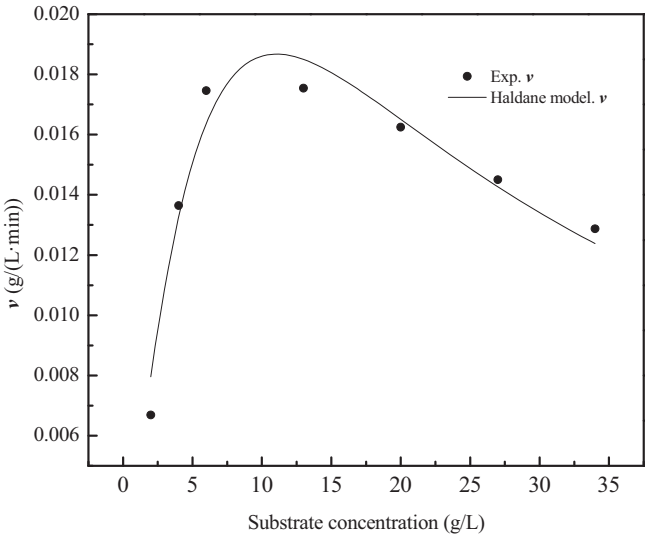


Fig. 4. The dependence of specific hydrolysis rate (v) on initial substrate concentration.

to Eq. (5), the plots of v versus S were taken, in which it can be shown that the Eq. (5) will go through a maximum value ($d_v/d_S = 0$) at a substrate concentration S^*, and the value, v^*, can be calculated as the v value corresponding to the S^* (D'Adamo et al., 1984; Nuhoglu & Yalcin, 2005; Rozich, Gaudy, D'adamo, 1985):

$$S^* = \sqrt{K_m K_{ss}} \tag{6}$$

$$v^* = \frac{V_{\max}}{1 + 2\sqrt{K_m/K_{ss}}} \tag{7}$$

According to these values ($V_{\max}$, K_m and K_{ss}), S^* and v^* were computed as 11.07 g/L and 0.0187 g/(L min), respectively, hence the minimum LSP inhibitive concentration was 11.07 g/L. The calculated results suggested that the process should be operated at a LSP concentration below 11.07 g/L in order to avoid substrate inhibition, which was close to the experimental value of 13 g/L (Fig. 3C).

The calculated results also showed that the v^* value was significantly lower than the $V_{\max}$ value. It was therefore reasonable to assume that the particular kinetic profile of the enzymolysis was at least partially conditioned by diffusional restrictions (Carrillo, Lis, Colom, López-Mesas, & Valldeperas, 2005). Chrastil (1988) proposed an exponential model which accounted for diffusion inhibition as follows,

$$C = C_\infty [1 - \exp(-k' E_o t)]^n \tag{8}$$

where C and C_∞ are the product concentrations at time t and at equilibrium, respectively, k' is a rate constant proportional to the diffusion coefficient, and n is a parameter related to structural diffusion resistance. When enzyme diffusion is not the rate-limiting step, n tends to be 1 and the reaction is of first order. When the reaction rate is strongly limited by the diffusion of the enzyme, n becomes 0.5–0.6 (Carrillo et al., 2005).

The Eq. (8) offered means to calculate diffusion coefficients from the data at different initial enzyme concentrations (Fig. 3A). These coefficients could be used to give information concerning rate-limiting steps and the results are shown in Table S3. The results indicated that the Chrastil diffusion model presented good fit to the experimental data ($R^2 \geqslant 0.998$). For the non-pretreated LSP, the enzyme diffusion was a rate limiting step due to the n values ranging from 0.52 to 0.56. There was no significant difference

in the n values (about 0.55) in various initial papain concentrations (0.4–0.7 g/L), which confirmed that the papain concentration had no significant effect on the enzymolysis of the LSP. At 0.5 g/L initial papain, the diffusion coefficient n (0.61) for the pretreated LSP was higher than that for the non-pretreated LSP (0.56), which indicated less diffusion resistance for the pretreated LSP than the non-pretreated LSP. As a result, it was reasonable to infer that the heat-pretreatment could reduce diffusion resistant during the enzymolysis to make papain more accessible to substrate due to the unfolding by breaking sub bonds of the LSP protein.

4. Conclusions

Heat-pretreatment of the LSP at 60 °C for 60 min before enzymolysis could accelerate the enzymolysis. The enzymolysis parameters of the pretreated LSP were optimized by RSM, and the *DH* was 27.43% under the optimal conditions of substrate concentration 13 g/L, pH 5.5, 55 °C and papain concentration 0.5 g/L. The enzymolysis of the non-pretreated LSP followed the pseudo-second-order and Haldane models. The kinetic parameters of the enzymolysis for non-pretreated LSP based on the two models were gained, i.e., V_{max} = 0.0681 g/(L min), K_m = 14.6377 g/L and K_{ss} = 8.3520 g/L. The non-pretreated LSP concentration to avoid substrate inhibition had been inferred theoretically to be 11.07 g/L, and the heat-pretreated LSP was easier enzymolyzed than the non-pretreated LSP. At the same time, it was found that Chrastil diffusion model could also well describe the enzymolysis behavior, where all the n values were less than or equal to 0.6, implying that diffusion was the rate controlling step.

Acknowledgment

This work was supported by National Natural Science Foundation of China (21105085, 31270988).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.01.069.

Reference

Adjonu, R., Doran, G., Torley, P., & Agboola, S. (2013). Screening of whey protein isolate hydrolysates for their dual functionality: Influence of heat pre-treatment and enzyme specificity. *Food Chemistry, 136*, 1435–1443.

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*, 1256–1262.

Ayhan, F., Ayhan, H., Pişkin, E., & Tanyolac, A. (2002). Optimization of urease immobilization onto non-porous HEMA incorporated poly (EGDMA) microbeads and estimation of kinetic parameters. *Bioresource Technology, 81*, 131–140.

Bhat, R., & Sridhar, K. R. (2008). Nutritional quality evaluation of electron beam-irradiated lotus (*Nelumbo nucifera*) seeds. *Food Chemistry, 107*, 174–184.

Boye, J., Ma, C.-Y., Ismail, A., Harwalkar, V., & Kalab, M. (1997). Molecular and microstructural studies of thermal denaturation and gelation of β-lactoglobulins A and B. *Journal of Agricultural and Food Chemistry, 45*, 1608–1618.

Cao, W., Sun, C., Liu, R., Yin, R., & Wu, X. (2012). Comparison of the effects of five pretreatment methods on enhancing the enzymatic digestibility and ethanol production from sweet sorghum bagasse. *Bioresource Technology, 111*, 215–221.

Carrillo, F., Lis, M., Colom, X., López-Mesas, M., & Valldeperas, J. (2005). Effect of alkali pretreatment on cellulase hydrolysis of wheat straw: Kinetic study. *Process Biochemistry, 40*, 3360–3364.

Chao, D., He, R., Jung, S., & Aluko, R. E. (2013). Effect of pressure or temperature pretreatment of isolated pea protein on properties of the enzymatic hydrolysates. *Food Research International, 54*, 1528–1534.

Chrastil, J. (1988). Enzymic product formation curves with the normal or diffusion limited reaction mechanism and in the presence of substrate receptors. *International Journal of Biochemistry, 20*, 683–693.

D'adamo, P. D., Rozich, A. F., & Gaudy, A. F. (1984). Analysis of growth data with inhibitory carbon sources. *Biotechnology and Bioengineering, 26*, 397–402.

Dona, A. C., Pages, G., Gilbert, R. G., & Kuchel, P. W. (2010). Digestion of starch: *In vivo* and *in vitro* kinetic models used to characterise oligosaccharide or glucose release. *Carbohydrate Polymers, 80*, 599–617.

Fitzsimons, S. M., Mulvihill, D. M., & Morris, E. R. (2007). Denaturation and aggregation processes in thermal gelation of whey proteins resolved by differential scanning calorimetry. *Food Hydrocolloids, 21*, 638–644.

Kim, S. Y., Park, P. S., & Rhee, K. C. (1990). Functional properties of proteolytic enzyme modified soy protein isolate. *Journal of Agricultural and Food Chemistry, 38*, 651–656.

Kumar, K. S., Ganesan, K., Selvaraj, K., & Rao, P. S. (2014). Studies on the functional properties of protein concentrate of Kappaphycus alvarezii (Doty) Doty-An edible seaweed. *Food Chemistry, 153*, 353–360.

Li, N., Yan, Y., Xia, B. Y., Wang, J. Y., & Wang, X. (2014). Novel tungsten carbide nanorods: An intrinsic peroxidase mimetic with high activity and stability in aqueous and organic solvents. *Biosensors and Bioelectronics, 54*, 521–527.

Moure, A., Sineiro, J., Domínguez, H., & Parajó, J. C. (2006). Functionality of oilseed protein products: A review. *Food Research International, 39*, 945–963.

Nuhoglu, A., & Yalcin, B. (2005). Modelling of phenol removal in a batch reactor. *Process Biochemistry, 40*, 1233–1239.

Pan, A. D., Zeng, H. Y., Foua, G. B., Alain, C., & Li, Y. Q. (2016). Enzymolysis of chitosan by papain and its kinetics. *Carbohydrate Polymers, 135*, 199–206.

Prieto, C. A., Guadix, E. M., & Guadix, A. (2008). Influence of temperature on protein hydrolysis in a cyclic batch enzyme membrane reactor. *Biochemical Engineering Journal, 42*, 217–223.

Qu, W., Ma, H., Jia, J., He, R., Luo, L., & Pan, Z. (2012). Enzymolysis kinetics and activities of ACE inhibitory peptides from wheat germ protein prepared with SFP ultrasound-assisted processing. *Ultrasonics Sonochemistry, 19*, 1021–1026.

Rozich, A. F., Gaudy, A. F., & D'adamo, P. C. (1985). Selection of growth rate model for activated sludges treating phenol. *Water Research, 19*, 481–490.

Saha, B. C., Yoshida, T., Cotta, M. A., & Sonomoto, K. (2013). Hydrothermal pretreatment and enzymatic saccharification of corn stover for efficient ethanol production. *Industrial Crops and Products, 44*, 367–372.

Schmidl, M. K., Taylor, S. L., & Nordlee, J. A. (1994). Use of hydrolysate-based products in special medical diets. *Food Technology, 48*, 77–85.

Tang, C.-H., Chen, L., & Ma, C.-Y. (2009). Thermal aggregation, amino acid composition and *in vitro* digestibility of vicilin-rich protein isolates from three *Phaseolus* legumes: A comparative study. *Food Chemistry, 113*, 957–963.

Wu, C.-H. (2007). Adsorption of reactive dye onto carbon nanotubes: equilibrium, kinetics and thermodynamics. *Journal of Hazardous Materials, 144*, 93–100.

Wu, J.-Z., Zheng, Y.-B., Chen, T.-Q., Yi, J., Qin, L.-P., Rahman, K., & Lin, W.-X. (2007). Evaluation of the quality of lotus seed of *Nelumbo nucifera* Gaertn from outer space mutation. *Food Chemistry, 105*, 540–547.

Yen, G.-C., Duh, P.-D., & Su, H.-J. (2005). Antioxidant properties of lotus seed and its effect on DNA damage in human lymphocytes. *Food Chemistry, 89*, 379–385.

Yuan, X., Liu, J., Zeng, G., Shi, J., Tong, J., & Huang, G. (2008). Optimization of conversion of waste rapeseed oil with high FFA to biodiesel using response surface methodology. *Renewable Energy, 33*, 1678–1684.

Zeng, H. Y., Cai, L. H., Cai, X. L., Wang, Y. J., & Li, Y. Q. (2013). Amino acid profiles and quality from lotus seed proteins. *Journal of the Science of Food and Agriculture, 93*, 1070–1075.