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Enzymatic Hydrolysis of Defatted *Antheraea pernyi* (Lepidoptera: Saturniidae) Pupa Protein by Combined Neutral Protease Yield Peptides With Antioxidant Activity

Shuhui Ma,[1] Xuejun Li,[1] Yongxin Sun,[1] Rui Mi,[1] Yajie Li,[1] Zhixin Wen,[1] Nan Meng,[1] Li Yi,[2] Xingfan Du,[1,3,●] and Shuying Li[1]

[1]Liaoning Ocean and Fisheries Science Research Institute, Liaoning Academy of Agricultural Sciences, Dalian, China, [2]Shanghai Jianqiao University, Shanghai, China, and [3]Corresponding author, e-mail: dxfan68@163.com

Abstract

In this study, peptides were prepared from defatted *Antheraea pernyi* (Lepidoptera: Saturniidae) pupa protein via hydrolysis with combined neutral proteases. Single-factor tests and response surface methodology (RSM) were used to determine the optimal hydrolysis condition suitable for industrial application. Optimal hydrolysis of the defatted pupa protein was found to occur at an enzyme concentration of 4.85 g/liter, a substrate concentration of 41 g/liter, a hydrolysis temperature of 55°C, and a hydrolysis time of 10 h and 40 min. Under these conditions, the predicted and actual rates of hydrolysis were 45.82% and 45.75%, respectively. Peptides with a molecular weight of less than 2,000 Da accounted for 90.5% of the total peptides generated. Some of the peptides were antioxidant peptides as revealed by sequencing and functional analysis. The antioxidant activity of the mixed peptides was subsequently confirmed by an antioxidant activity assay. The results showed that peptides with high antioxidant activity could be obtained from the hydrolysis of *A. pernyi* pupa protein.

Key words: response surface methodology, defatted *Antheraea pernyi* pupa protein, combined neutral protease, peptides, antioxidant activity

Antheraea pernyi pupa comprises up to 50% protein in mass, although it also contains high levels of fat, amino acids, and other nutrients. The protein in *A. pernyi* pupa is made up of 18 of the 20 common amino acids, and 8 of which are essential amino acids, and they account for more than 40% of the total amino acids. The mass ratio of essential amino acids to nonessential amino acids is 0.73, which is very close to the reference protein model proposed by WHO/FAO (Zhou and Han 2006, Yang et al. 2009). *Antheraea pernyi* pupa is a source of high-quality protein with great potential for exploitation and utilization. At present, *A. pernyi* pupa is mainly used for food; however, the proteins they contain are not easily absorbed by the body due to their complex molecular structure and large molecular mass (Ji et al. 2008). As an alternative, peptides can be obtained by enzymatic hydrolysis of proteins and their absorption, by the gastrointestinal tract is significantly higher than that of single amino acids. Peptides play the role of a messenger in physiological and biochemical reactions that govern human life activities, improving physical fitness, promoting health, and combating diseases (Smacchi and Gobbetti 2000, Daliri et al. 2017, Sánchez and Vázquez 2017). Peptides can have strong biological activities. For example, some peptides are directly involved in the immune function where they can stimulate the proliferation of lymphocytes, enhance the phagocytic function of macrophages, and participate in antigen presentation by antigen-presenting cells (Park and Nam 2015). A large number of bioactive peptides have been isolated from plants, animals, and microorganisms in the 1970s. These bioactive peptides were found to have a variety of biological functions. From the late 1980s to the early 1990s, a combination of chemical and genetic methods and peptide library construction has enabled peptide-based drugs to undergo a revolutionary development. On the basis of a large number of basic and applied research studies on bioactive peptides, a variety of bioactive peptides have been introduced to the market, producing huge economic benefits. The development of bioactive peptides with healthcare effects has become an active field of research in the biochemical industry in the twenty-first century (Sørensen et al. 2008, González-García et al. 2014, Malaguti et al. 2014, Zenezini-Chiozzi et al. 2016). Many of these biologically active peptides have special physiological functions, such as promoting immunity, antibacterial, antiviral, antitumor, antioxidation, lowering blood fat, and pressure (Yang et al. 2003, 2004; Yust et al. 2004; Pihlanto et al. 2010; Rim et al. 2012). They

play various biological roles, and one of the most crucial of which is antioxidant activity. Numerous studies have shown an inverse relationship between the intake of antioxidants and the incidence of diseases, whereby a higher intake of antioxidants would result in a lower incidence of diseases (Sarmadi and Ismail 2010).

Oxidation causes the body to produce free radicals and other substances that damage normal tissue cells, which in turn can cause human fatigue, aging, or chronic diseases. The antioxidant peptides produced from various raw materials are valued for their small molecular weights, easy absorption, and strong activity. They can effectively remove excess reactive oxygen species from the body, protect the structures and functions of the cells and cell organelles (e.g., mitochondria), and prevent the occurrence of lipid peroxidation. Antioxidant peptides have been isolated by hydrolysis of plant, animal, and microbial proteins. The first antioxidant peptide obtained from a protein hydrolysate was isolated from chickpea. The extraction of antioxidant peptides from silkworm pupa has also been reported. In some studies, the enzymolysis of silkworm pupa was carried out using single-enzyme hydrolysis, which often results in poor hydrolysis (Min et al. 2009, Xiao et al. 2011). Other studies have used a multi-enzyme combination, with most studies using alkaline or acid proteases, which require subsequent neutralization and desalination steps, complicating the process (Lin and Jiang 2006, Zhu 2011). The use of complex neutral protease not only could reduce the steps in an enzymatic hydrolysis reaction but could also simplify the process, save time, as well as improving the efficiency of hydrolysis. In this study, single factor tests and RSM analysis were used to establish a high-efficiency and practical protease hydrolysis process for the hydrolysis of defatted pupa protein. Silkworm pupa protein-specific protease, *Aspergillus oryzae* flavourzyme, and *Bacillus subtilis* neutral protease were selected to perform the hydrolysis and the enzyme concentration and combination, substrate concentration, as well as hydrolysis temperature and time required for optimal hydrolysis of the protein were determined. The antioxidant activity of the isolated peptide mixture was also examined. The finding of this study could provide a theoretical basis for the rational utilization of protein in *Antheraea pernyi* pupa and the development of healthcare products.

Materials and Methods

Materials and Main Reagents

Flavourzyme from *Aspergillus oryzae* (specific activity ≥20,000 U/g, CAS: 9001-92-7) and Neutral protease from *Bacillus subtilis* (specific activity of 60,000 U/g, CAS: 9068-59-1) were obtained from Solarbio Technology Co., Ltd. (China). Silkworm pupa protein-specific protease (SPPSP), which is a commercial enzyme preparation, was obtained from Dong Heng Hua Road Biotech Co., Ltd. (China). All other chemicals used were of analytical grade.

Preparation of Defatted *A. pernyi* Pupa Protein Powder

A. pernyi pupa (5 kg) without their silk cocoon was purchased from a market in Dandong, Liaoning Province, China. The mean pupal weight was 9.52 ± 2.35 g. The pupa was washed, boiled for 20 min, dried at 60 °C, and then crushed into a powder using a high-speed pulverizer. The powder was then subjected to supercritical CO_2 extraction and then passed through a 60-mesh sieve, and the sieved powder was used as the starting material in the subsequent hydrolysis.

Optimization Conditions for Enzymatic Hydrolysis

The hydrolysis of defatted pupa protein was investigated using three different proteases: SPPSP, flavourzyme, and the neutral protease, either alone or in combination. The extent of the hydrolysis was used as the evaluation index, while the substrate concentration (20–100 g/liter), hydrolysis temperature (40–60°C), hydrolysis time (3–18 h), protease concentration (0.3–6 g/liter) were selected parameters in the single factor test (Fig. 1). Three replicates were carried out for each hydrolysis. According to the results of the single factor test, the design principle of Box–Behnken was adopted (Sun et al. 2009). Substrate concentration (40, 60, and 80 g/liter), enzyme concentration (1, 3, and 5 g/liter), and hydrolysis time (8, 10, and 12 h) were taken as independent variables, and the degree of hydrolysis was taken as the response value. The process independent variables and their codes and levels used in RSM are shown in Table 1. The Box–Behnken design was used to study the impact of these parameters on hydrolysis. The Box–Behnken design contained 17 groups of experiments (Table 2).

Peptides Preparation Method

The pupa protein powder (180 g) was added to 3,000 ml distilled water. The mixture was then mixed in a sandwich glass reactor. After reaching 55°C, the proteases were added to the protein mixture to start the hydrolysis. The hydrolysis was allowed to proceed for different times and then terminated by heating in a boiling water bath for 10 min. After cooling, the sample was centrifuged at $10,000 \times g$ for 5 min, and a small amount of the supernatant was assayed by the ninhydrin method to determine the degree of hydrolysis, the supernatant was freeze dried, yielding a peptide powder.

Determination of Degree of Hydrolysis

The degree of protein hydrolysis (DH) refers to the number of peptide bonds broken during hydrolysis relative to the total number of peptide bonds in the protein, and it is usually expressed as a percentage. DH (%) = ($- NH_2$/total nitrogen content of the sample) $\times$ 100%. The content of $-NH_2$ produced after hydrolysis was determined by the ninhydrin method (Zhao and Feng, 1995), while the content of total nitrogen was determined by the Kjeldahl method (regarding GB/T 23530-2009).

DPPH Radical Scavenging Assay

DPPH radical scavenging activity was assayed according to the method described by Yamaguchi et al. (1998). The peptide powder was dissolved in deionized water and then diluted to the desired concentrations. The test sample consisted of 2 ml of peptide solution and 2 ml of 0.2 mmol/liter DPPH (dissolved in ethanol). For control, the sample contained Vitamine C instead of the peptide. All samples were incubated at 37°C for 30 min and their absorbances were then measured at 517 nm. DPPH radical scavenging activity was calculated using the following equation:

$$\% \text{ Inhibition} = [1 - (A_1 - A_2)/A_3] \times 100$$

where A_1 and A_2 are the absorbances of the test sample and blank sample, respectively, and A_3 is the absorbance of the DPPH solution.

Hydroxyl Radical Scavenging Activity Assay

Hydroxyl radical scavenging activity was determined according to a previously described method (Wu and Jia 2009). An aliquot (1 ml)
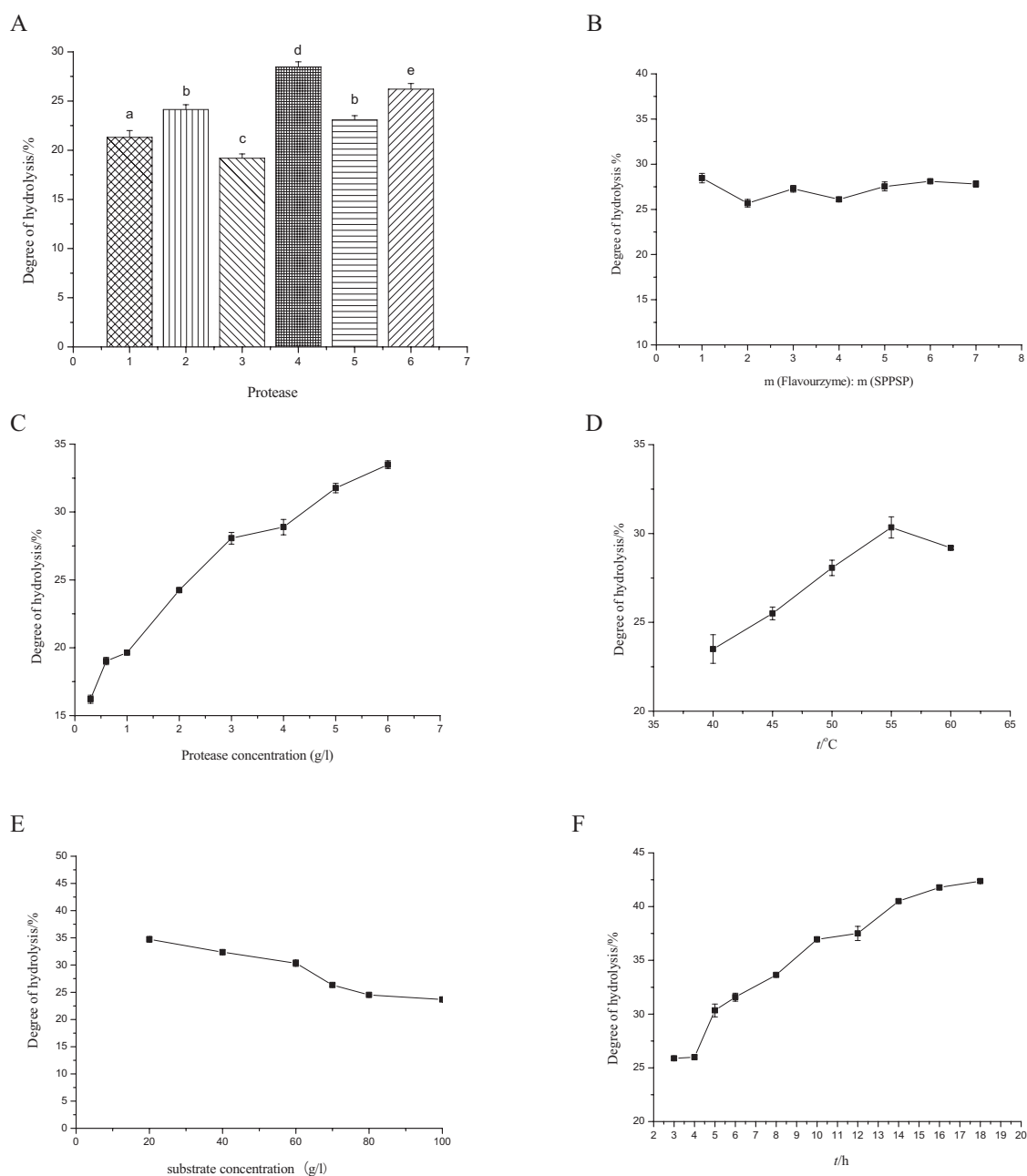
Fig. 1. Effects of different parameters on the hydrolysis of defatted *A. pernyi* pupa protein. The defatted pupa protein was hydrolyzed under conditions where one parameter was varied while the others were fixed. (A) Different combinations of enzymes in the presence of 60 g/liter substrate at 55°C for 5 h; numbers along the *X*-axis are: (1) SPPSP; (2) flavourzyme; (3) neutral protease; (4) flavourzyme + SPPSP; (5) neutral protease + SPPSP; and (6) neutral protease + flavourzyme. Total concentration of enzyme = 3 g/liter. (B) Different ratios of flavourzyme to SPPSP in the presence of 60 g/liter substrate at 55°C for 5 h. (C) Different concentrations of total enzyme (1:1 ratio of flavourzyme to SPPSP) in the presence of 60 g/liter substrate at 55°C for 5 h. (D) Different temperatures in the presence of 3 g/liter enzyme (1:1 ratio of flavourzyme to SPPSP) in the presence of 60 g/liter substrate for 5 h. (E) Different substrate concentrations in the presence of 3 g/liter enzyme (1:1 ratio of flavourzyme to SPPSP) at 55°C for 5 h. (F) Different time in the presence of 3 g/liter enzyme (1:1 ratio of flavourzyme to SPPSP) and 60 g/liter substrate at 55°C

of the diluted peptide solution was added to a centrifuge tube containing 1 ml of 6 mmol/liter ferrous sulfate and 1 ml of 6 mmol/liter H_2O_2 followed by 10 min of mixing. Next, 1 ml of 6 mmol/liter salicylic acids was added to the sample followed by 30 min of incubation at room temperature. Two controls were also prepared; one without peptide and the other without salicylic acid, and both samples were incubated for 30 min at room temperature. After that, the absorbance of each sample was measured at 510 nm, and the hydroxyl radical scavenging activity was calculated according to the following formula:

$$\% \text{ Inhibition} = \left[1 - (A_i - A_j)/A_0\right] \times 100$$

where A_i is the absorbance of the test sample, A_0 is the absorbance of the sample without peptide and A_j is the absorbance of the sample without salicylic acid.

Reducing Power Assay

The reducing power of the mixed peptides was measured according to the method of Oyaizu (1988). First, 2 ml of the peptide solution

Table 1. Independent variables and their levels in Box–Behnken design for the conditions of the enzymatic hydrolysis of defatted *A. pernyi* pupa protein

Level	Factor		
	Protease concentration, g/liter (*A*)	Substrate concentration, g/liter (*B*)	Time, h (*C*)
–1	1	40	8
0	3	60	10
1	5	80	12

Table 2. Box–Behnken experimental design with the independent variables that constitute the conditions of the hydrolysis

Test no.	Protease concentration, g/liter (*A*)	Substrate concentration, g/liter (*B*)	Time, h (*C*)	DH/%, *Y*
1	0	1	1	39.18
2	0	0	0	39.6
3	0	1	–1	34.59
4	0	0	0	40.47
5	–1	0	–1	26.25
6	–1	0	1	28.67
7	0	–1	1	41.31
8	0	0	0	41.15
9	0	0	0	39.52
10	1	0	1	44.66
11	1	–1	0	45.6
12	1	1	0	40.68
13	0	–1	–1	39.11
14	–1	–1	0	32.14
15	1	0	–1	41.31
16	0	0	0	39.78
17	–1	1	0	25.97

was added to 2 ml of 1% potassium ferricyanide in a test tube and the sample was incubated at 50°C for 20 min. Next, 2 ml of 10% trichloroacetic acid (w/v) was added to the sample and the sample was then centrifuged at 3,000 × *g* for 15 min. After that, 2 ml of the supernatant was mixed with 2 ml of distilled water and 0.4 ml of 0.1% ferric chloride in a test tube and kept at room temperature for 10 min. Finally, the absorbance of the sample was measured at 700 nm. An increase in the absorbance would be indicative of a stronger reducing power.

Analysis of the Molecular Weight Distribution of the Peptides

The molecular weight distribution of the peptides was estimated by gel filtration chromatography using an 18-angle laser light scattering gel permeation chromatography system (DAWN HELEOS SYSTEM, Wyatt, USA). Ultrapure water containing 0.2 mol/liter sodium nitrate was used as the mobile phase and the flow rate was set at 0.5 ml/min. This experiment was performed by Sci-Tech Company (China).

Shotgun Mass Spectrometry

The peptide solution was first prepared by dissolving 5 mg peptide powder in 500 µl ultra-pure water. The solution was then filtered through a 0.22 µm filter and the filtrate was transferred to an ultra-filtration cup containing a 10 kDa cutoff membrane followed by the addition of 200 µl of 25 mmol/liter ammonium bicarbonate buffer. The cup was inserted into a collection tube and the whole unit was centrifuged at 14,000 × g and room temperature for 30 min. The filtrate was retained, and another 200 µl of the same buffer was added to the cup followed by additional centrifugation at 14,000 × g and room temperature for 30 min. This step was repeated twice and the three filtrates were pooled and the peptide concentration was estimated by absorbance at 280 nm using an extinction coefficient of 1.1 $M^{-1}cm^{-1}$, calculated based on the frequency of tryptophan and tyrosine in vertebrate proteins. The sample was subsequently subjected to mass spectrometry analysis using an Easy-nLC nanoflow HPLC system connected to Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA). In total, 1 µg peptide was loaded onto a Thermo Scientific EASY column (two columns) using an autosampler set at a flow rate of 200 ml/min. The sequential separation of peptides on a Thermo Scientific EASY trap column (100 µm × 2 cm, 5 µm, 100 Å, C18) and on an analytical column (75 µm × 25 cm, 5 µm, 100 Å, C18) was accomplished using a segmented 1 h gradient consisting of 5 to 28% Solvent B (0.1% formic acid in 100% ACN) in 40 min, followed by 28–90% Solvent B in 2 min and then held at 90% Solvent B for 18 min. The column was re-equilibrated in Solvent A (0.1% formic acid) before each run. The mass spectrometer was operated in a positive ion mode, and the MS spectra were acquired over a range of 350–2,000 *m/z*. The resolving power of the Orbitrap Elite at 100 *m/z* was set to 60,000 for the MS

scan and 15,000 for the MS/MS scan. The top sixteen most intense signals in the acquired MS spectra were selected for further MS/MS analysis. The isolation window was 2 *m/z*, and ions were fragmented through higher energy collisional dissociation with a normalized collision energy of 35 eV. The maximum ion injection time was set to 10 ms for the survey scan and 100 ms for the MS/MS scan. The automatic gain control target values were set to 10^6 and 5×10^4 for the full scan and MS/MS scan modes, respectively. The dynamic exclusion duration was 30 s.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA). Duncan's multiple range test was used to compare the mean differences. All statistical analyses were performed using the SPSS program, version 21.0. Statistical significance was considered at the $P < 0.05$ level.

Results

Hydrolysis Condition

The hydrolysis of defatted pupa protein was evaluated by using three different proteases, SPPSP, flavourzyme, and neutral protease. The hydrolysis was initially carried out at 55°C for 5 h using 60 g/liter protein and 3 g/liter proteases. The result of the hydrolysis is shown in Fig. 1A. Among the three proteases tested, flavourzyme was the most effective, yielding significantly more hydrolysis than the other two proteases, and hydrolyzing about 24.13% of the total protein. Furthermore, the degree of hydrolysis was increased to 28.46% by combining flavourzyme with SPPSP. Therefore, a combination of flavourzyme and SPPSP was used for subsequent experiments. The highest degree of hydrolysis was obtained when a 1:1 mass ratio of flavourzyme to SPPSP was used (Fig. 1B).

The effect of enzyme concentration on the hydrolysis of defatted pupa protein was investigated by varying the concentration of enzyme while fixing the substrate concentration (60 g/liter), temperature (55°C), and hydrolysis time (5 h). The extent of hydrolysis of the protein increased gradually with increasing concentrations of the combined protease, exhibiting a linear relationship when the enzyme

concentration was between 0.5 and 3.0 g/liter (Fig. 1C). When the enzyme concentration reached 3 g/liter, the hydrolysis increased slowly with time.

The effect of temperature on the degree of hydrolysis is shown in Fig. 1D. The hydrolysis was found to increase with increasing temperatures, reaching a maximum at 55°C, but started to decrease with further temperature increases.

The influence of substrate concentration on the hydrolysis was determined by carrying out the hydrolysis using different concentrations of protein (Fig. 1E). The hydrolysis was found to decrease when the substrate concentration was increased beyond 20 g/liter.

The influence of incubation time on the degree of hydrolysis was investigated by varying the time of hydrolysis while maintaining the other parameters at optimum settings. Hydrolysis tended to increase when the incubation time was increased, with a rapid increase occurring during the first 10 h, followed by a more gradual increase thereafter (Fig. 1F).

Establishment of Response Surface Regression Models

To further optimize the condition for the hydrolysis of defatted pupa protein, the Box-Behnken design principle was employed with the response surface regression model (Table 1). The full experimental plan for their coded forms obtained from Box–Behnken experiments is shown in Table 2. Using the Design Expert 8.0.6 statistical software, the data in Table 2 were fitted to quadratic multiple regressions, and the response surface model was established to obtain the quadratic multiple regression equation

$$Y = 40.10 + 7.40A - 2.22B + 1.57C$$
$$+ 0.31AB + 0.23AC + 0.60BC$$
$$- 3.67A^2 - 0.34B^2 - 1.22C^2,$$

where Y is the degree of hydrolysis, A is the concentration of combined protease, B is the concentration of substrate, and C is the hydrolysis time.

Regression Variance Analysis of Response Surface Regression Models

According to the ANOVA in Table 3, the quadratic polynomial model was considered highly significant ($P < 0.0001$), and the lack

of fit value (0.1975) implied that it was insignificant, consistent with the well-fitted regression equation. The coefficient of determination r^2 was 0.9903, indicating good agreement between the experimental and predicted values. The adjustment coefficient r^2_{adj} was 0.9778, and the variation coefficient was 2.37%, indicating that the model could explain the changes in 97.78% of the response values, with high accuracy and reliability. The regression model also showed a satisfactory degree of the fitting. The effects of A, B, C, and A^2 on the degree of hydrolysis were extremely significant ($P < 0.01$). The effect of C^2 on the extent of hydrolysis was significant ($P < 0.05$). Interaction among various factors of the regression model was revealed by the effect diagrams (Fig. 2), which were subsequently generated from the Design Expert 8.0.6 software. The interaction between hydrolysis time and substrate concentration exerted the greatest influence on the hydrolysis, shown as a steep surface in the diagram (Fig. 2A). The effect on the hydrolysis exerted by the interaction between substrate concentration and protease concentration was moderate, shown as a less steep surface in the diagram (Fig. 2B). In contrast, the interaction between hydrolysis time and combined protease had only a minimal effect on the hydrolysis, shown as a fairly flat surface in the effect diagram (Fig. 2C). The F value is generally used to evaluate the influence of various factors on the test indicators. The larger the F value, the more significant the impact. According to the F value, the influence of various factors on the degree of hydrolysis was in the order: enzyme concentration > substrate concentration > time.

Validation and Determination of the Optimal Conditions for the Hydrolysis

To validate the result predicted by the model, the hydrolysis of defatted pupa protein was carried out using the enzyme (4.85 g/liter) and substrate (41 g/liter) concentrations, temperature (55°C), and reaction time (10 h 40 min) predicted by the model. The actual rates of hydrolysis obtained from three parallels experiments were 45.66%, 45.87%, and 45.73%, yielding an average hydrolysis rate of 45.75 ± 0.11%. This value was consistent with the predicted value, demonstrating the accuracy and effectiveness of the response surface analysis.

Peptide Molecular Weight Distribution

The hydrolysis of defatted pupa protein primarily yielded peptides with low molecular weights. Mass spectrometry analysis of these

Table 3. Analysis of variance of the response surface regression models

| Source of variance	Sum of square	df	Mean square	F value	P value
Model	566.01	9	62.89	79.19	<0.0001
A	438.38	1	438.38	551.99	<0.0001
B	39.34	1	39.34	49.53	0.0002
C	19.72	1	19.72	24.83	0.0016
AB	0.39	1	0.39	0.49	0.5057
AC	0.22	1	0.22	0.27	0.6179
BC	1.43	1	1.43	1.80	0.2218
A^2	56.58	1	56.58	71.24	<0.0001
B^2	0.49	1	0.49	0.62	0.4584
C^2	6.22	1	6.22	7.84	0.0265
Residual	5.56	7	0.79		
Lack of fit	3.63	3	1.21	2.51	0.1975
Pure error	1.93	4	0.48		
Total	571.57	16			
r^2	0.9903				
r^2_{adj}	0.9778				
Coefficient of differentiation	2.37%				

A

B

C
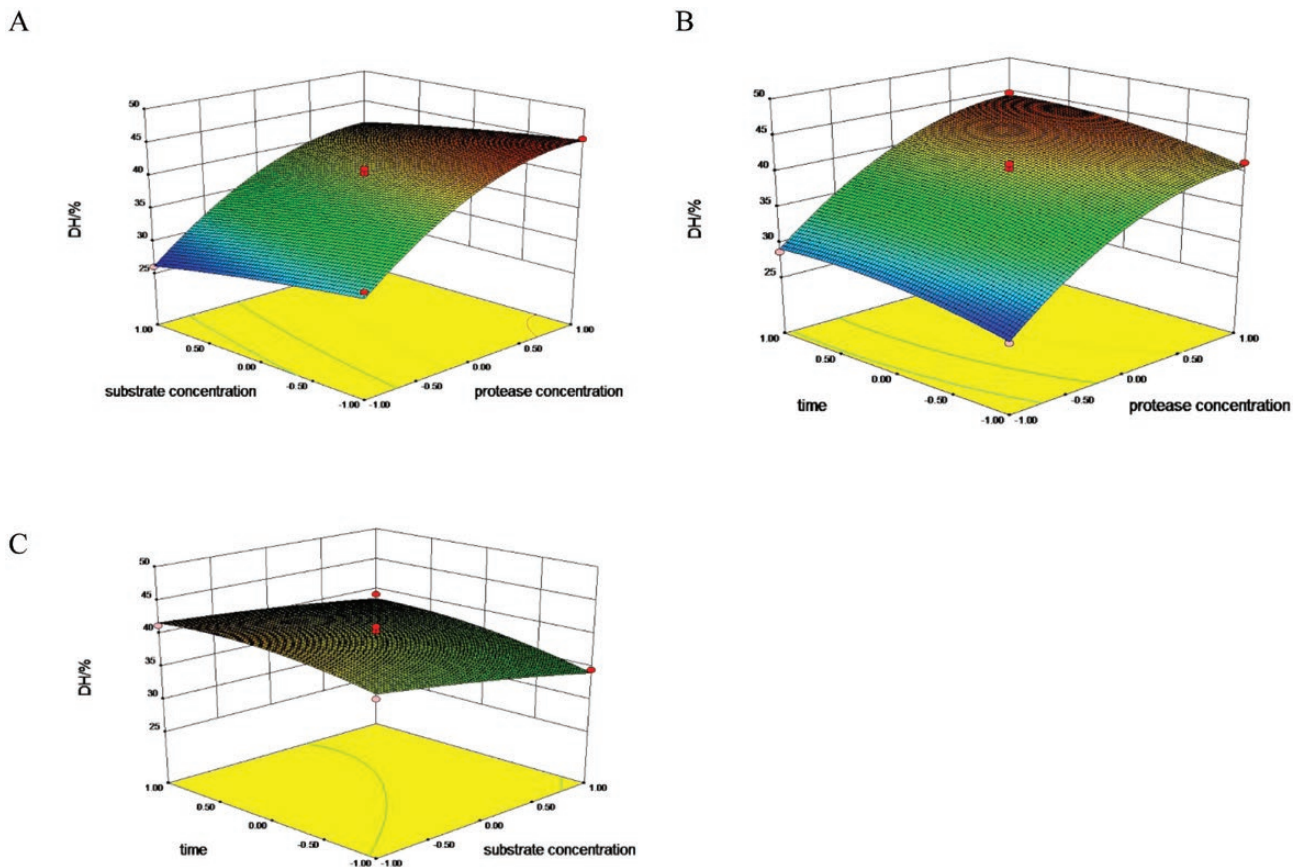
Fig. 2. Effect of different parameters on the hydrolysis of defatted *A. pernyi* pupae protein. Response surface (3-D) showing the degree of hydrolysis of the protein as a function of different parameters: (A) substrate concentration and combined protease concentration; (B) hydrolysis time and combined protease concentration; (C) hydrolysis time and substrate concentration. DH% is the degree of hydrolysis as measured from the percentage of peptide formation.

peptides revealed major peaks at 200–1,000 Da (52.3%) and 1,000–1,500 Da (33.97%) (Fig. 3). Peptides with molecular weights less than 2,000 accounted for 90.5%.

Antioxidant Activity Assay

The antioxidant activity of the mixed peptides was found to increase with increasing peptide concentrations, yielding IC_{50} values of 0.22 and 6.55 mg/ml for the scavenging of DPPH radical and •OH radical, respectively (Fig. 4A, B). The highest rate of DPPH (>90%) and •OH (>80%) scavenging activities was obtained with peptide concentrations of 0.5 and 10 mg/ml, respectively. A peptide concentration of 1.70 mg/ml was found to generate a reducing power equivalent to that produced by 0.05 mg/ml vitamin C (Fig. 4C).

Peptides Sequence and Functional Analysis

The identities of the peptides were determined by Shotgun mass spectrometry. One of the peptides was found to be an inhibitor of angiotensin-converting enzyme (ACE), while the rest of the peptides appeared to be antioxidant peptides (Table 4).

Discussion

Analysis of Hydrolysis Condition

The efficiency of protein hydrolysis usually depends on factors such as the type of hydrolase, enzyme concentration, substrate concentration, temperature, reaction time, and other factors. With the
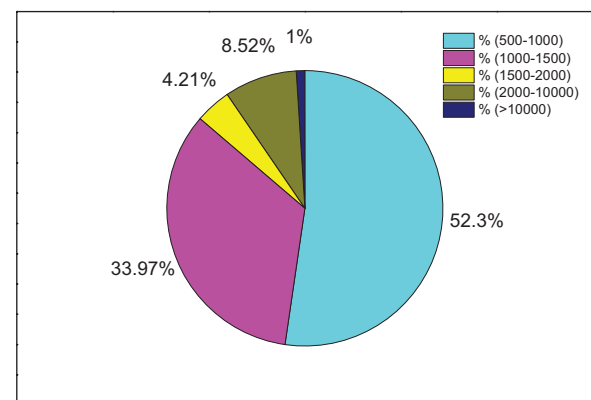

Fig. 3. Molecular weight distribution of peptides generated from the enzymatic hydrolysis of defatted *A. pernyi* pupa protein.

hydrolysis of defatted pupa protein, we first explored these factors one at a time.

The type of enzyme used is an important factor that can affect the efficiency of enzymatic hydrolysis. The use of neutral protease not only can reduce the steps of the enzymatic hydrolysis reaction but can also simplify the process, save time, as well as improving the efficiency of hydrolysis. Huang et al. (2020) used six types of proteases (neutral protease, alkaline protease, protamex, flavor
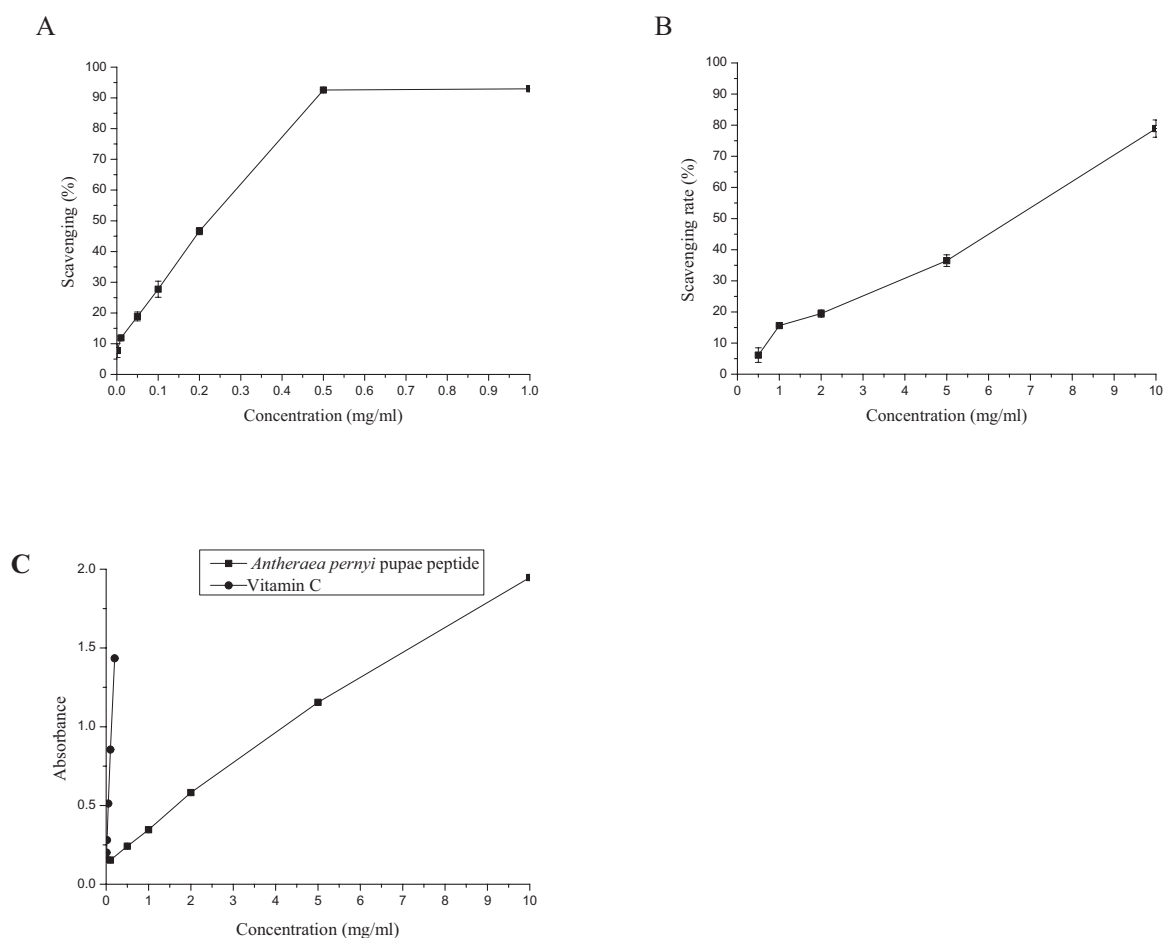
Fig. 4. Antioxidant activity of the peptides generated from the enzymatic hydrolysis of defatted *A. pernyi* pupa protein. Antioxidant activity was determined by measuring the DPPH radical scavenging activity (A), •OH scavenging activity (B), and reducing power (C) of the mixed peptides.

Table 4. Shotgun Mass spectrometry identification of the peptides generated from the hydrolysis of defatted *A. pernyi* pupa protein

Name	Sequence	Chem.mass	EC$_{50}$	Activity
ACE inhibitor	LPLP	438.5540	720.00	
Synthetic peptide	NHH	406.3880	—	Antioxidative
Synthetic peptide	YEY	473.4560	—	Antioxidative
Antioxidative peptide	YLL	407.4900	—	
Antioxidative peptide from as 1-CN(154–156)	YQL	422.4610	—	
f(30–32) of bovine kappa casein	YVL	393.4630	—	Antioxidative
Dipeptidyl peptidase IV Inhibitor	YW	367.3830	10.50	
Synthetic peptide	YYA	415.4190	—	Antioxidative

Note: "—" means no search result.

protease, trypsin, and papain) to hydrolyze silkworm pupa protein and found that neutral protease is the most effective protease for generating peptides from the hydrolysis of *A. pernyi* pupa protein. In our case, SPPSP, *Aspergillus oryzae* flavourzyme, and *Bacillus subtilis* neutral protease were selected to perform the hydrolysis. We found that a 1:1 ratio of flavourzyme to SPPSP increased the hydrolysis by 48.3% compared with the hydrolysis carried out with a single protease, consistent with the result reported in a previous study (Fang et al. 2012), where a combination of enzymes including trypsinase, flavourzyme, and neutral protease was found to increase

the percentage of functional oligopeptides by 45.52% over that obtained with a single enzyme. A similar increase (32.65%) was also reported by Qin et al. (2014) for the hydrolysis of silkworm pupa protein that was carried out with combined enzymes, including flavourzyme and combined protease, suggesting that synergy between the two enzymes could enhance the hydrolysis of silkworm pupa protein.

To the best of our knowledge, the concentration of the enzymatic hydrolysis is related to the stability of the protease. A higher concentration of enzyme would mean more cleavage sites can be acted

upon by the enzyme, and this would translate into more peptides being produced and consequently, a higher degree of hydrolysis. When the enzyme concentration is increased to a certain value, the number of sites on the substrate available for cleavage by the enzyme would become limited, and the rate of hydrolysis would increase slowly (Guerard et al. 2001). Based on the result of hydrolysis (Fig. 1C), the concentration of protease was fixed at 3 g/liter.

Temperature can also exert a significant effect on the hydrolysis of protein. An increase in temperature can impart more kinetic energy to the enzyme and substrate, thereby enhancing their chances of a collision, which is important for an enzymatic reaction to occur. The increase in temperature might also help to open up the protein substrate, making it more vulnerable to attack by the proteases. However, the temperature should not be increased beyond the maximum temperature that the enzyme can tolerate before it starts to lose its conformation, and hence, its activity (Tello et al. 1994). The drop in hydrolysis at higher temperatures could well be due to denaturation in the protease (Wu et al. 2017). In our case, the hydrolysis rate was highest at 55°C, and therefore, the temperature was fixed at 55°C.

The concentration of substrate can also have a marked effect on the rate of hydrolysis. At a low substrate concentration, complete hydrolysis of the protein might occur because all the substrates can form a complex with the enzyme and be converted to products. However, once the substrate concentration exceeded the saturation concentration, the activity of the enzyme decreased as more products accumulated, and some of which could be inhibitory to the enzymes (Guerard et al. 2001). Although the result of substrate concentration was rather unexpected, it was similar to the findings reported by Zhu and Zhang (2012) whereby an increase in substrate concentration led to a gradual decrease in hydrolysis, probably caused by product inhibition of the enzymes.

As for the effect of reaction time, the hydrolysis rate exhibited a time-dependent manner, progressing rapidly during the first 4–9 h and then decreased, similar to the result reported for the hydrolysis of peanut protein by Alcalase, a commercial preparation of subtilisin obtained from *Bacillus licheniformis* (Jamdar et al. 2010). Although protein hydrolysis progressed slowly after 10 h (DH 36.95%), the reaction continued until 18 h to achieve a DH of 42.38%. The slower rate of hydrolysis after 10 h could be due to a decrease in the concentration of hydrolyzable peptide bonds (Guerard et al. 2001). As no further benefit was obtained with prolonged hydrolysis time, a hydrolysis time of 10 h was therefore chosen for subsequent experiments.

Peptide Molecular Weight Distribution
Several studies have already shown that the antioxidant activity of the hydrolysates derived from the hydrolysis of plant proteins depends on the molecular weight distribution of the products (Pena-Ramos et al. 2004, Moure et al. 2006, Wang et al. 2007). Smaller peptides have a higher level of radical scavenging activity than larger peptides (Moosman and Benl 2002). According to a study published by Roberts et al. (1999), peptides with small molecular weight can easily cross the cell membrane and thus exert better scavenging activity. These findings were in agreement with observations reported for silk sericin peptides, in which peptides in the range of 200–3000 Da tend to exhibit stronger antioxidant activity (Fan et al. 2010, Hou et al. 2014).

Antioxidant Activity Assay
The antioxidant activity of the peptide mixture was evaluated by DPPH radical scavenging, •OH inhibition and reducing power

assays, and then compared with that of vitamin C, which was used as a control because it is considered to be one of the most powerful and least toxic natural antioxidants (Weber et al. 1996, Burits and Bucar 2000). The DPPH radical scavenging activity has been widely used to evaluate the free radical scavenging ability of various natural products and has been accepted as a model compound for free radicals (Jao and Ko 2002, Khan et al. 2006, Arabshahi-Delouee and Urooj 2007). According to Lu et al. (2013), the IC_{50} value of the collective antioxidant peptides from silkworm pupa protein for the inhibition of DPPH radicals was 4.24 mg/ml. In our work, the peptides exhibited the most potent inhibition against DPPH radicals (IC_{50} of 0.22 mg/ml), reflecting a better response in DPPH scavenging activity.

Hydroxyl radical •OH is the most reactive species among the different forms of ROS as it can react with almost all the biomolecules, such as amino acids, proteins, and DNA, and inflicts severe damage to the cells (Dreher and Junod 1996, You et al. 2009). The IC_{50} value for the peptides for the inhibition of •OH radicals was 6.55 mg/ml, similar to the IC_{50} value for the silkworm pupa peptides (4.51 mg/ml) reported by Lu et al. (2013).

The reducing ability is an important indicator of the contribution of H• by antioxidants, which can convert free radicals into more stable and less reactive molecules. The antioxidant activity of a peptide is closely related to its reducing ability (Duh et al. 1999). The scavenging of free radicals by an antioxidant usually involves the contribution of H• by the antioxidant, which converts the free radical into a harmless molecule. Thus the reducing power of an antioxidant is closely linked to its antioxidant power. The results indicated that the peptide exhibit good reducing power, similar to the finding reported by Lu et al. (2013), in which an absorbance of 0.434 was obtained when the concentration of the silkworm pupa enzymolysates was 2.0 mg/ml.

Peptides Sequence and Functional Analysis
The antioxidative activity of a peptide is highly influenced by the molecular structure, molecular mass, and amino acid sequence of the peptide (Fan et al. 2010, Wan et al. 2016). Based on the BIOPEP-UWM database analysis (Minkiewicz et al. 2019), the peptides composed of several amino acids with a molecular weight of several hundred daltons shown in Table 4 should be the source of antioxidant activity detected in the assay.

Conclusions
The purpose of this study was to systematically explore the preparation process of the peptides from defatted *A. pernyi* pupa protein and optimize the conditions to improve the degree of hydrolysis. Single-factor tests and RSM were used to determine the optimal hydrolysis condition suitable for industrial application. The model could be used to analyze and predict enzymatic conditions of the hydrolysis of defatted pupa and the degree of hydrolysis. The optimal conditions for the hydrolysis consisted of 41 g/liter the defatted pupa protein, 4.85 g/liter enzymes (a 1:1 ratio of Flavourzyme and SPPSP), a hydrolysis temperature of 55°C and a hydrolysis time of 10 h and 40 min. Collectively, the peptides derived from the enzymatic digestion of defatted pupa protein appeared to exhibit a strong reducing ability and free radical scavenging ability, suggesting that they could be a source of good antioxidant for the food or healthcare product industry, thereby, providing a certain technical reference for the efficient use of *A. pernyi* pupa.

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Author Contributions

Conceptualization: Shuhui Ma, Xingfan Du, Rui Mi. Data curation: Shuhui Ma, Xuejun Li, Yongxin Sun. Formal analysis: Shuhui Ma, Zhixin Wen, Nan Meng. Methodology: Shuhui Ma, Yongxin Sun. Investigation: Shuhui Ma, Yajie Li. Writing - original draft: Shuhui Ma. Writing - review & editing: Shuhui Ma, Li Yi, Shuying Li.

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