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Optimization of Hydrolysis Conditions for Obtaining Antithrombotic Peptides from *Tenebrio Molitor* Larvae

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Abstract: *Tenebrio molitor* larvae was hydrolyzed by pepsin and trypsin for preparing high antithrombotic activity hydrolysate. The significant variables were selected according to the Plackett-Burman design and further optimized by the Response Surface Methodology (RSM). Four factors, including substrate concentration, pepsin digestion time, pepsin amount and trypsin amount, respectively, influenced significantly on the hydrolysis effectiveness. The best hydrolysis conditions obtained using RSM experiments are as following: substrate concentration of 19.8 mg/mL, pepsin digestion time of 1.8 h, pepsin amount of 1634 U/mL and trypsin amount of 126 U/mL, respectively. Under the optimal hydrolysis conditions, the antithrombotic activity was 82.8% predicted by the RSM model, which was basically consistent with the 80.6% obtained in the three verification experiments. Moreover, the antithrombotic activity of the hydrolyzate had no linear relationship with the degree of hydrolysis.

Keywords: *Tenebrio molitor* Larvae, Enzymatic Hydrolysis, Antithrombotic Activity, Plackett–Burman Design, Response Surface Methodology

Introduction

Thromboembolic diseases include disseminated intravascular coagulation, deep vein thrombosis, transient ischemic attack and pulmonary embolism, which are serious diseases that threaten human health and even cause death (Wakefield *et al*., 2008). According to statistics, the incidence of venous thromboembolism per 1,000 people in industrialized countries was 1-3 cases per year (Heit *et al*., 2005). Thrombosis is usually caused by abnormal blood clots in the circulating blood, or by blood deposits on the inner wall of the heart or blood vessels (Mackman, 2012). The development of blood clots involves the complex interaction of vascular endothelium, platelets and coagulation factors (Syed and Mehta, 2018). Currently, antithrombotics such as heparin, warfarin are most often used for treatment (Einhäupl *et al*., 1991, Prasad *et al*., 2009). However, it's gradually found that the long-term use of these drugs can cause severe bleeding in the clinical application (Levine *et al*., 2001; Prasad *et al*., 2009). In addition, gastrointestinal system injury (Derry and Loke, 2000; Grossman, 2015; Kkf *et al*., 2018), drug-induced thrombocytopenia (Abraham, 2011) and fetal malformation (Schivazappa *et al*., 1982) are also common adverse reactions. Therefore, it is the objective to find the antithrombotic agent which is safer and effective.

During the past three decades, various peptides have been separated and identified from natural substances and have exhibited many bioactive functions (Lima and Moraes, 2018). Nowadays, vary of peptides or hydrolysates of protein derived from the insects (especially the hematophagous and venomous insects) have been shown to have antithrombotic activity *in vitro*. Peptides derived from insects bind to coagulation factors through the molecular interaction in the intrinsic pathway or extrinsic pathway of blood coagulation (Syed and Mehta, 2018). In addition, there are some researchers found that they can also antagonize platelet membrane glycoprotein integrin and prevent platelet aggregation (Ciprandi *et al*., 2003).

Generally, proteins are cleaved into smaller active peptides or free amino acids using enzymatic hydrolysis. These low sequence peptides often contain original functional activities compared to the original protein. The yellow mealworms (*Tenebrio molitor*), belong to the family of *Tenebrionidae*, are used to be considered pests due to their eating habits (Tang *et al*., 2018). However, these larvae have a protein content of more than 25% (fresh weight) that considered as the extremely rich

protein resource. Rich protein of *T. molitor* may produce various bioactivity peptides after enzymatic hydrolysis. Recently, pepsin and trypsin have been used as commonly used enzymes for protein hydrolysis to obtain antithrombotic peptides. (Qiao *et al*., 2018; Tian *et al*., 2016). Compared with plant proteases, pepsin and trypsin have high hydrolysis rates and short reaction cycles. Moreover, the enzymatic hydrolysates obtained by simulating biological functions are more suitable for functioning in living organisms (Huang *et al*., 2009). In our previous preparations, it was found that enzymatic hydrolysis gave the degree of hydrolysis of 21.5% and an anticoagulant activity of 60.78% (Csample = 30 mg/mL) under gastrointestinal conditions. Therefore, in the present study, we optimized the enzymatic hydrolysis conditions of *T. molitor* larvae with pepsin and trypsin in order to obtain the higher proteolytic and antithrombotic peptides.

Materials and Methods

Materials

T. molito larvae were bought from a local flower and bird market in Hangzhou, China and were brought to the laboratory in a living state. The species of the *T. molitor* was identified by observation and comparison of morphology. Bovine fibrinogen and thrombin were purchased from Sigma. The pepsin (3000 U/mg) and trypsin (250 U/mg) were of biochemical grade and purchased from local chemical company. And other chemicals used in this experiment were of analytical grade without special mention.

The *T. molitor* larvae were starved for 24 h to empty the intestines and then washed several times with distilled water. They were then dried at 50°C until the moisture content was reached 5%. The dry insects were ground into a powder with the stirrer (Tang *et al*., 2018). *T. molitor* powder was degreased by isopropyl alcohol at 50°C for 1 h. The defatted *T. molitor* slurry was dried and stored at -20°C.

Preparation of Hydrolysate

The main operation of the enzymatic hydrolysate for preparing the *T. molitor* larvae protein was as follows. The pH, incubation time and enzyme amounts were set according to experiment design shown in corresponding tables. The defatted *T. molitor* powder was suspended in distilled water and adjusted to designated pH. The requisite amount of pepsin solution was added and started to hydrolysis at oscillation water bath. At the end of pepsin hydrolysis, adjust pH to a certain value with 1 M NaOH and the trypsin was added, incubating with agitation during a period of time. After that, protease activity was stopped by heating at 95°C for 15 min. Finally, the mixture was centrifuged at 12000×g for 15 min and the supernatant was collected.

Determination of the Degree of Hydrolysis (DH)

The DH was tested by the method of Balson and Lawson (1936) with a little modification. The method is based on free amino groups combined with formaldehyde and releasing H^+ from free amino groups. The amount of free amino groups can be calculated by titrating the released H^+ with the alkali. The hydrolysis process is mainly the cleavage of peptide bonds, resulting in the formation of free amino groups. Therefore, the degree of hydrolysis can be obtained by calculating the change in the content of the free amino group before and after the hydrolysis. Simply, 8 mL hydrolysate was diluted with 60 mL distilled water and adjusted the pH 7. After then, 10 mL neutral formaldehyde solution was added and stirred constantly for 5 mins. The solution was then titrated to pH 9.2 with a 0.05 M NaOH standard solution and the consumption volume of NaOH was recorded as V_1 (mL). Repeated the above steps, taking the unhydrolyzed protein solution as a blank experiment and noted that NaOH consumption volume as V_2 (mL). Protein content was determined using the K1100 automatic Kjeldahl analyzer (Hanon, Shandong, China). And the protein conversion factor was 6.25.

The DH value was expressed as the Equation 1:

$$DH\% = 0.05 \times \left(V_1 - V_2\right) \times \left(1000 / 8C_{wo}\right) \times \left(1 / 6.25\right) \times 100 \quad (1)$$

where, C_{wo} is the protein concentration of the sample solution (g/L).

Determination of Antithrombotic Activity

Antithrombotic activity was determined according to the method of Yang *et al*. (2007). Samples, thrombin and fibrinogen were separately dissolved in 0.05 mol/L Tris-HCl buffer (pH 7.4). The absorbance was measured at 405 nm using the IMark microplate reader (Uji-shi, Kyoto, Japan). 140 µL of 1 mg/mL fibrinogen and 40 µL of the sample were added to the 96 microplate well, mixed and the absorbance *Sb* was recorded. Then, 10 µL of thrombin (12 IU/mL) was added to the well at 37°C for 10 min and the value was S. The control group contained 40 µL of Tris-HCl buffer instead of the sample and the absorbances were *Cb* and *C*, respectively. The antithrombotic activity was expressed as the Equation 2:

$$Antithrombotic\ activity\left(\%\right) = \frac{\left(C - C_b\right) - \left(S - S_b\right)}{C - C_b} \times 100 \quad (2)$$

Selection of Significant Variables by Plackett–Burman Design

In the previous experiment, it was confirmed that the hydrolysate with antithrombotic activity could be obtained by hydrolyzing the protein of the mealworm with pepsin and trypsin. A total of nine parameters were tested and identified by the Plackett-Burman Design (PBD) to select significant variables for proteolysis. Each factor had a low level and a high level, where X_{10} and X_{11} were the comparative virtual factors set by the estimation error (Table 1). Proteolysis had a significant effect on factors above 95% confidence level ($P<0.05$). The first-order polynomial model was used for mathematical modeling as follows (Dayana and Bakthavatsalam, 2016):

$$Y = \beta_0 + \sum \beta_i x_i \qquad (3)$$

where, Y is the predicted response (DH of proteolysis), β_0 is the model intercept and β_i is the linear coefficient and x_i is the level of the independent variable.

Optimization by Response Surface Methodology (RSM)

The Execution Factor Box-Behnken Design (BBD) was used to determine the optimal value for each variable to achieve maximum antithrombotic activity after PBD screening. In this study, a four-independent-factor-three-level BBD was employed with 29 runs. The four significant factors were substrate concentration (mg/mL), pepsin digestion time (h), pepsin amount (U/mL) and trypsin amount (U/mL) which were denoted as X_1, X_2, X_3 and X_4 respectively (Table 2). At the same time, other non-critical factors were fixed: Pepsin reaction pH 2.0, pepsin reaction temperature 37°C, trypsin digestion time 5 h, trypsin reaction pH 8, trypsin reaction temperature was 37°C. After enzymatic hydrolysis process, the mixture was centrifuged at 12000×g for 15 min. The supernatant was used to determination the antithrombotic activity and DH.

The antithrombotic activity and DH data were fitted by a multiple regression program (Equation 4):

$$Y = a_0 + \sum_{i=1}^{k} a_i x_i + \sum_{i=1}^{k} a_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} a_{ij} x_i x_j + e_0 \qquad (4)$$

where, Y is predicted value, k is the amount of factors, α_0 is the intercept, α_i, α_{ii} and α_{ij} are coefficients of the model. x_i or x_j are the coded factors.

Statistical Analysis

Experimental data were analyzed by ANOVA. The statistical software package Design-Expert V 8.0.6 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for statistical analysis of PBD and RSM.

Results

Screening of Significant Factors Using PBD

The PBD was used to select the significant variables necessary to enhance DH in this study. Factors above the 95% confidence level are considered to have a significant effect on the outcome and apply to the next optimization phase. The results (Table 3) showed that the effect of substrate concentration (mg/mL), pepsin digestion time (h), pepsin amount (U/mL) and trypsin amount (U/mL) have a positive influence on proteolysis and hence were further determined by an RSM design.

The model equation for DH (Y, %) was as follows:

Table 1: Plackett-burman design of factors (in coded levels) with proteolysis as response

	Coded levels[a]											DH (%)	
Run order	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Observed[b]	Predicted
1	30	2.5	1000	2.5	40	5.00	100	7.5	35	1	-1	4.50	5.19
2	5	2.5	2500	2.0	40	5.00	250	7.5	35	-1	1	23.75	23.06
3	30	1.0	2500	2.5	35	5.00	250	8.0	35	-1	-1	13.03	13.51
4	5	2.5	1000	2.5	40	3.33	250	8.0	40	-1	-1	14.40	14.89
5	5	1.0	2500	2.0	40	5.00	100	8.0	40	1	-1	9.98	10.67
6	5	1.0	1000	2.5	35	5.00	250	7.5	40	1	1	16.23	15.75
7	30	1.0	1000	2.0	40	3.33	250	8.0	35	1	1	5.91	5.43
8	30	2.5	1000	2.0	35	5.00	100	8.0	40	-1	1	6.28	5.59
9	30	2.5	2500	2.0	35	3.33	250	7.5	40	1	-1	12.80	13.49
10	5	2.5	2500	2.5	35	3.33	100	8.0	35	1	1	15.12	14.64
11	30	1.0	2500	2.5	40	3.33	100	7.5	40	-1	1	1.40	0.71
12	5	1.0	1000	2.0	35	3.33	100	7.5	35	-1	-1	6.94	7.42

[a]The meaning of each code representative: X_1:Substrate concentration (mg/mL), X_2:Pepsin digestion time (h), X_3:Pepsin amount (U/mL), X_4:Pepsin reaction pH, X_5:Pepsin reaction temperature (°C), X_6:Trypsin digestion time (h), X_7:Trypsin amount (U/mL), X_8:Trypsin reaction pH, X_9:Trypsin reaction temperature (°C), X_{10}, X_{11}:Virtual factor; [b]The observed values of DH were the mean values of duplicates.

[b]The observed values of DH were the mean values of duplicates.

Table 2: Experimental design and results of BBD

	Coded levels[a]				DH (%)		Antithrombotic activity (%)	
Run order	X_1	X_2	X_3	X_4	Observed[b]	Predicted	Observed[b]	Predicted
1	5	1	1750	175	5.94	5.89	46.23	45.42
2	30	1	1750	175	6.06	6.38	38.67	37.08
3	5	2.5	1750	175	19.70	19.50	40.93	40.19
4	30	2.5	1750	175	0.77	0.95	59.96	58.44
5	17.5	1.75	1000	100	8.90	9.26	62.40	61.35
6	17.5	1.75	2500	100	6.50	6.36	74.33	74.79
7	17.5	1.75	1000	250	7.05	7.32	54.48	51.69
8	17.5	1.75	2500	250	3.67	3.44	41.91	40.63
9	5	1.75	1750	100	19.30	19.27	53.55	52.46
10	30	1.75	1750	100	3.22	2.52	81.90	83.15
11	5	1.75	1750	250	8.38	9.12	56.51	56.28
12	30	1.75	1750	250	7.76	7.82	33.39	35.50
13	17.5	1	1000	175	5.09	5.54	30.81	31.44
14	17.5	2.5	1000	175	8.39	8.03	56.04	58.46
15	17.5	1	2500	175	0.14	0.55	52.99	51.59
16	17.5	2.5	2500	175	6.65	6.24	40.30	40.70
17	5	1.75	1000	175	17.97	17.46	59.69	60.86
18	30	1.75	1000	175	11.75	11.54	48.93	48.54
19	5	1.75	2500	175	17.14	17.18	43.08	44.77
20	30	1.75	2500	175	4.70	5.04	66.88	67.01
21	17.5	1	1750	100	0.56	0.34	52.39	53.53
22	17.5	2.5	1750	100	5.29	6.03	63.31	62.59
23	17.5	1	1750	250	0.42	-0.49	30.58	32.61
24	17.5	2.5	1750	250	1.95	2.00	39.52	39.68
25	17.5	1.75	1750	175	28.09	28.37	78.24	79.23
26	17.5	1.75	1750	175	28.18	28.37	79.66	79.23
27	17.5	1.75	1750	175	28.85	28.37	79.44	79.23
28	17.5	1.75	1750	175	28.37	28.37	78.46	79.23
29	17.5	1.75	1750	175	28.35	28.37	80.35	79.23

[a]The meaning of each code representative: X_1:Substrate concentration (mg/mL), X_2:Pepsin digestion time (h), X_3:Pepsin amount (U/mL), X_4:Trypsin amount (U/mL)
[b]The observed values of DH and antithrombotic activity were the mean values of duplicates.

Table 3: Effect and statistical analysis of factors using PBD

Variables	Coefficient	%Contribution	Standard error	Sum of squares	P-value
Intercept	10.86	-	-	-	-
Model	-	-	-	421.44	0.0443**
X_1	-3.54	35.36	0.42	150.52	0.0139**
X_2	1.95	10.68	0.42	45.47	0.0438**
X_3	1.82	9.32	0.42	39.68	0.0497**
X_4	-0.082	0.019	0.42	0.080	0.8642
X_5	-0.87	2.14	0.42	9.12	0.1745
X_6	1.43	5.79	0.42	24.65	0.0766
X_7	3.49	34.37	0.42	146.30	0.0143**
X_8	-0.075	0.016	0.42	0.067	0.8751
X_9	-0.68	1.3	0.42	5.55	0.2480

R^2-0.9900; Adjusted-R^2-0.9449; Adeq precisior-16.771; 95% significant level. **Significant at P<0.05

$$Y = 18.07328 - 0.28333X_1 + 2.59556X_2 + 2.42444 \times 10^{-3}X_3 - 0.32667X_4 - 0.34867X_5 + 1.71657X_6 + 0.046556X_7 - 0.30000X_8 - 0.27200X_9 \quad (5)$$

The P value of the analysis of variance model was 0.0443 and the adjeq precision was 16.271>4 indicating that the regression region of the model fits well and is satisfactory. R^2 = 0.9900 and Adj R^2 = 0.9449, indicating that the regression model can account for 94.5%.

Optimization by RSM

The experiments conducted in this study consisted of a quadratic model consisting of 29 trials. The corresponding results and predicted values of the RSM experiments for the four variables (substrate concentration, pepsin digestion time, pepsin amount and trypsin amount) were shown in Table 2. Table 4 showed the variance analysis of the response surface quadratic model.

Table 4: ANOVA for antithrombotic activity

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob>F
Model	7282.678000	14	520.191300	158.651700	<0.0001**
X_1-Substrate concentration	73.674760	1	73.674760	22.469860	0.0003*
X_2-Pepsin digestion time	195.233300	1	195.233300	59.543660	<0.0001**
X_3-Pepsin amount	4.253434	1	4.253434	1.297243	0.2738
X_7-Trypsin amount	1440.768000	1	1440.768000	439.415900	<0.0001**
X_1X_2	176.692900	1	176.692900	53.889060	<0.0001**
X_1X_3	298.484500	1	298.484500	91.033950	<0.0001**
X_1X_7	662.287900	1	662.287900	201.989400	<0.0001**
X_2X_3	359.338700	1	359.338700	109.593700	<0.0001**
X_2X_7	0.983260	1	0.983260	0.299882	0.5926
X_3X_7	150.125300	1	150.125300	45.786300	<0.0001**
X_1^2	949.570400	1	949.570400	289.606800	<0.0001**
X_2^2	3096.076000	1	3096.076000	944.263700	<0.0001**
X_3^2	908.216100	1	908.216100	276.994300	<0.0001**
X_7^2	685.372900	1	685.372900	209.030000	<0.0001**
Residual	45.903560	14	3.278826		
Lack of Fit	42.865600	10	4.286560	5.644000	0.0549
Pure Error	3.037959	4	0.759490		
Cor Total	7328.582000	28			

*Significant at P≤0.05
**Significant at P≤0.01

The model F value of 158.65 indicated that the model was significant (P<0.001). A "lack of fitted F value" of 0.055 indicates that the model was well suited. X_1, X_2, X_4, X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_3X_4, X_1^2, X_2^2, X_3^2 and X_4^2 were shown to be significant by ANOVA analysis (P<0.05). The coefficient of R^2 is equal to 0.9937 indicating that the model has good accuracy. The anti-thrombotic activity of $R^2(\text{ad}_j)$ was 0.9875, indicating that the model can account for 98.75% of the antithrombotic activity. This pattern does not account for 1.25% of the total variation.

The following regression equation indicated antithrombotic activity (Y):

$$\begin{aligned} Y(\%) &= 252.28552 + 2.45725X_1 + 159.94138X_2 \\ &+ 0.10684X_3 + 0.939717X_4 + 0.70894X_1X_2 \\ &+ 9.21424\times10^{-4}X_1X_3 - 0.013725X_1X_4 \\ &- 0.016850X_2X_3 - 8.81418\times10^{-3}X_2X_4 \\ &- 1.08912\times10^{-4}X_3X_4 - 0.077435X_1^2 - 38.83994X_2^2 \\ &- 2.10362\times10^{-5}X_3^2 - 1.82741\times10^{-3}X_4^2 \end{aligned} \quad (6)$$

Construct a three-dimensional response surface by plotting the Z-axis response (antithrombotic activity) against any two independent variables while keeping the other variables at zero, in order to determine the optimal value for each variable (for maximum antithrombotic activity) (Fig. 1). The antithrombotic activity increased until substrate concentration, pepsin digestion time, pepsin amount (even if not significant) and trypsin amount reached a maximum point and then decreased following further these factors increase.

The predicted model obtains the optimal hydrolysis conditions as follows: substrate concentration of 19.8 mg/mL, pepsin digestion time of 1.8 h, pepsin amount of 1633.7 U/mL and trypsin amount of 126.5 U/mL. The highest antithrombotic activity by the predicted model was 82.8%. Based on these, we chose substrate concentration of 19.8 mg/mL, pepsin digestion time of 1.8 h, pepsin amount of 1634 U/mL and trypsin amount of 126 U/mL as a condition of hydrolysis. which was consistent with the average of three replicates of 80.6% obtained in the validation experiments. And the DH was 21.5% under this condition. The antithrombotic activity of validated experiments was uniformity to that of predicted value. Therefore, the predict model for antithrombotic activity during hydrolysis might be used for preparing hydrolysate with high antithrombotic activity.

Relationship of DH and Antithrombotic Activity

During enzymatic hydrolysis, the protein peptide bonds broke and released more peptides, that is, the increase in DH during the hydrolysis process. At the same time, the molecular weight of the peptide was also reduced. On the other hand, the antithrombotic activity of the hydrolysate changed during enzymatic hydrolysis. The relationship of DH and antithrombotic activity was shown in Fig. 2. The linear correlation coefficient R^2 between the hydrolysate and the protein DH was 0.2951, indicating that there is no correlation between them.

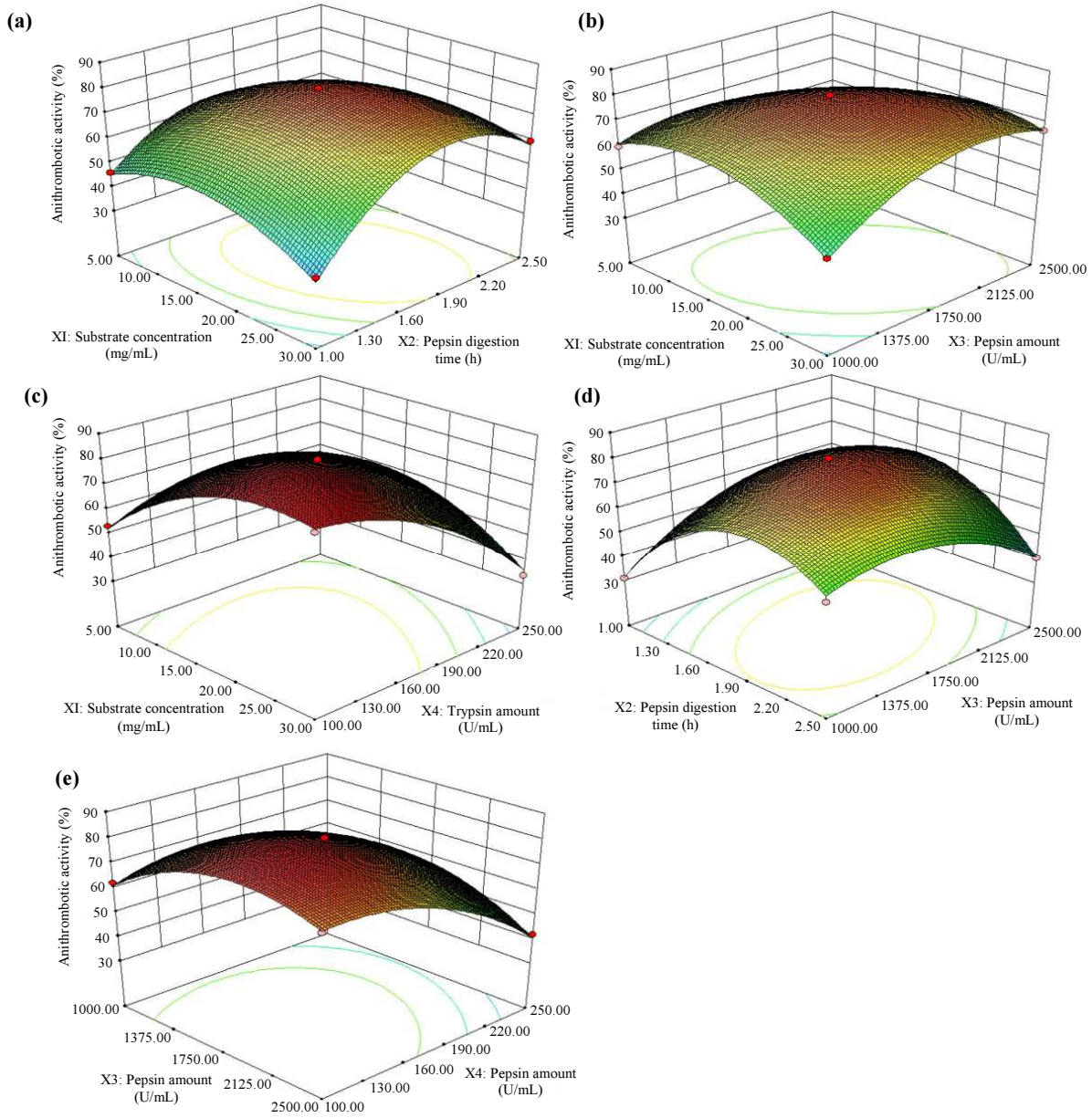

Fig. 1: The 3-D plots for the effects of variables on antithrombotic activity: (a) substrate concentration and pepsin digestion time; (b) substrate concentration and pepsin amount; (c) substrate concentration and trypsin amount; (d) pepsin digestion time and pepsin amount; (e) pepsin amount and trypsin amount.

Discussion

Peptides provide an exciting opportunity as drug candidates because they combine the benefits of small molecules (cost, compositional constraints, membrane permeability) and proteins (natural ingredients, target specificity, high potency) (Syed and Mehta, 2018). However, natural antithrombotic peptides have low yield and poor stability. Therefore, many scholars have been using specific enzymes to cleave proteins to obtain biologically active fragments. Some antithrombotic peptides by proteolytics have been confirmed in the past few years, such as *Mytilus edulis*, peanut and amaranth (Qiao *et al*., 2018; Sabbione *et al*., 2016; Zhang, 2016). The use of statistical models to optimize protein enzymatic conditions has increased in today's bio industry due to its ease of application and adaptability. We hope to get more antithrombotic peptides with low molecular weight and high activity. The amount and size of the protein that is cleaved into a polypeptide is positively correlated with DH (Zhang *et al*., 2016).

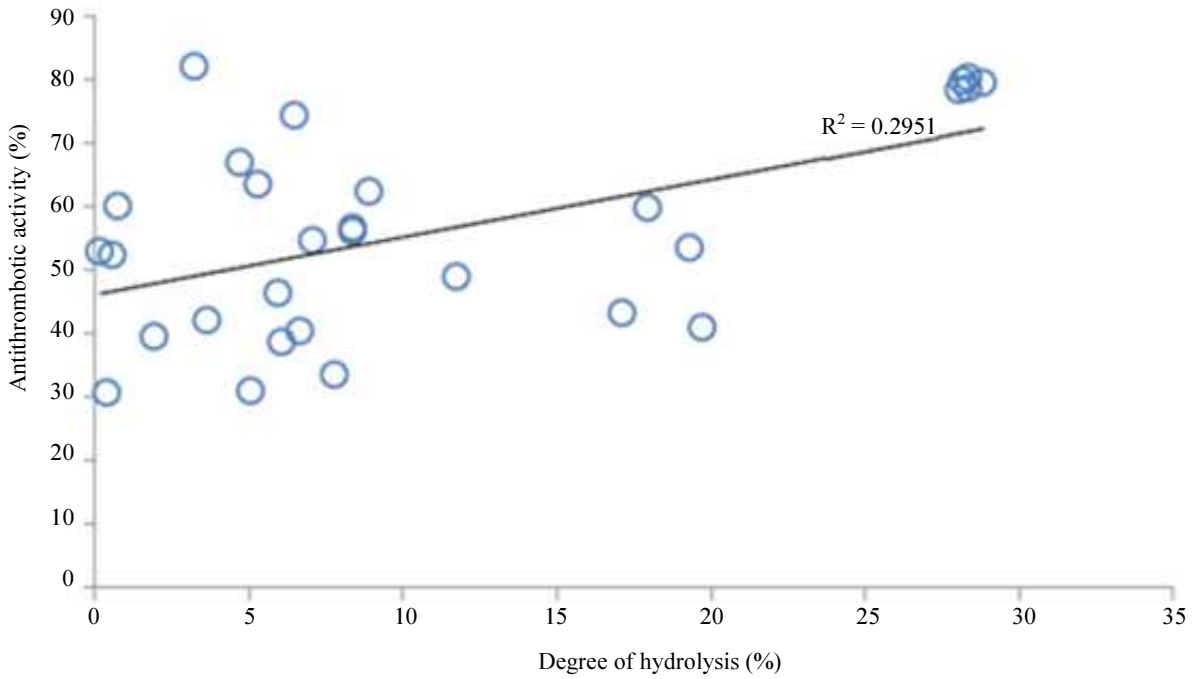

Fig. 2: The relationships of antithrombotic activity and degree of hydrolysis (DH)

Substrate concentration and digestion time are common factors affecting the activity of hydrolyzed peptides (Roslan *et al*., 2015; Zhang *et al*., 2015). The antithrombotic activity for protein hydrolyzates may be related to the amino acid end of the active peptide is arginine (Rogozinskaya and Lyapina, 2017). Arginine has been identified in many natural or synthetic antithrombotic peptides (Jo and Kim, 2008; Qiao *et al*., 2018). Trypsin specifically cleaves the peptide bond amount of arginine carboxy formed, forming a polypeptide having a terminal arginine. Qiao *et al*. (2018) used trypsin to hydrolyze *Mytilus edulis* protein to obtain a hydrolysate with an IC_{50} of 1.49 mg/mL. Conversely, although the amount of pepsin significantly affected DH, there was no significant effect on anticoagulant activity. It may be that pepsin acts on a specific site of the protein due to selectivity, resulting in no significant effect of the resulting crude peptide against thrombogenic activity (Zhang, 2016). Generally, the substrate cannot be fully enzymatically interpreted for antithrombotic activity at low levels. And at high levels, the active hydrolysate is excessively hydrolyzed and the activity is reduced. At higher substrate concentrations, due to the certain phenomena, such as changes in the fluidity of the solution, the enzyme is not sufficiently contacted with the substrate, resulting in reduced hydrolysate activity (Guerard *et al*., 2002). Therefore, we can find the optimal hydrolysis conditions through the RSM. Amaranth protein was hydrolyzed by simulating gastrointestinal digestion showing a dose-response behavior (IC_{50}=0.23±0.02 mg/mL) exhibited inhibition of thrombin activity (Sabbione *et al*., 2016). And the DH of amaranth hydrolysate about 51.1% was much higher than *T. molito* larvae hydrolysate by treated with the same enzyme, probably because of the different number of restriction sites for different proteins.

After the *T. molitor* protein is hydrolyzed, some of the active peptide sequences originally buried in the protein molecule was released upon hydrolysis. In general, a high degree of hydrolysis makes it more likely to obtain more short peptides. The information collected in the literature indicates that peptides with antithrombotic activity are often short (Hyun *et al*., 2006; Sabbione *et al*., 2016), although some of the peptides described have higher molecular weights (Jo and Kim, 2008). Notably, there is no obvious the relationship between antithrombotic activity and degree of hydrolysis. That is, the inhibition rate corresponding to high hydrolysis degree is not necessarily the highest. In fact, when the degree of hydrolysis is low, the proteolysis is incomplete, which may result in the partial hydrolysis of the polypeptide with stronger anticoagulant activity; and excessive hydrolysis may cause the partial anticoagulant activity of the polypeptide to be hydrolyzed to peptide, causing the weakening of anticoagulant activity. Similar results have been reported in previous surveys (Zhang, 2016).

Conclusion

This study aimed to optimize the parameters in various enzymatic processes, including substrate concentration, pepsin digestion time, pepsin amount and trypsin amount, for obtaining high antithrombotic peptides. The nine variables were tested using the PBD and four variables (i.e., substrate concentration, pepsin digestion time, pepsin amount and trypsin amount) exerted significant effects on DH. In order to optimize low cost and high efficiency enzymatic conditions for high antithrombotic peptides, we conducted RSM experiments. The optimal level of each parameter was as follows: substrate concentration of 19.8 mg/mL, pepsin digestion time of 1.8 h, pepsin amount of 1634 U/mL and trypsin amount of 126 U/mL. Under these optimal hydrolysis conditions, the predicted antithrombotic activity was 82.8%, which was consistent with the average of 80.6% of the three replicates obtained in the validation experiment. In order to improve its application as a new antithrombotic drug, future studies should base on antithrombotic peptide purification, stability of antithrombotic peptides and actual absorption in intestinal cells.

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Author's Contributions

Fangyuan Chen: Performed the experiments and wrote the manuscript.

Han Jiang, Yini Gan and Wenwei Chen: Participated in partial experiments.

Guangrong Huang: Developed the idea and analyzed the data.

Ethics

The authors declare that they had no conflict of interest.

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