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Production of Bovine Collagen Hydrolysate with Antioxidant Activity; Optimized by Response Surface Methodology

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Abstract: Antioxidants are widely used in pharmaceutical industries. Gelatin is a byproduct of the meat industry and its hydrolysates showed several functionalities, such as antioxidant activity. The purpose of this study was to describe and optimize the enzymatic hydrolysis conditions including time, temperature, pH, and enzyme/substrate ratio (E/S) to produce protein hydrolysate with antioxidant functionality from bovine gelatin by RSM; the scavenging activity was evaluated using the DPPH method. The model observed was fitted with desirable adequacy and sufficiency. We found that the antioxidant activity increased significantly ($p < 0.05$) with the increase in pH value, E/S ratio, and time of enzymatic process; however, the temperature had no significant ($p < 0.05$) effect on the antioxidant activity of the hydrolysate. The optimum hydrolysis conditions were observed at a temperature of 35.3 °C, pH of 8.0, and E/S ratio at 2.5 after 2 h hydrolysis by trypsin enzyme. The results showed that the hydrolysate under these conditions, optimized by RSM, could be more effective on antioxidant activity. Regarding the antioxidant potential, gelatin hydrolysate can be used as an antioxidant supplement in pharmaceutical industries.

Keywords: bovine gelatin; protein hydrolysate; antioxidant activity; response surface methodology

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1. Introduction

Collagen is the predominant structural protein in vertebrate and invertebrate animals and the principal fibrous protein constituent in skin, bones, and cartilages. Gelatin is a soluble protein derived from collagen and obtained by a partial hydrolysis process [1]. Gelatin is widely used for its functional and nutritional properties in the food and pharmaceutical industries. Currently, collagen is mainly extracted from the skin and bones of cows and pigs. It is worthwhile to note that collagen and gelatin are the main proteinaceous byproducts of meat industries. However, the disposal and utilization of these byproducts can reduce the cost of production and generate income for producers [1,2]. Gelatin-derived peptides and hydrolysates showed several functional activities and potential biological benefits, including antioxidant, anticancer, cholesterol lowering, and antihypertensive effects [3]. More than half of the amino acid residues in collagen α-chain contain glycine, proline, and hydroxyproline amino acids. The presence of both proline and glycine amino acids are associated with radical scavenging and antioxidant activity of gelatin hydrolysate as a bioactive peptide [4].

Bioactive peptides, namely biopeptides, have been characterized as specific fragments of native proteins that have positive effects on human body conditions, which eventually promote health and well-being. Several studies showed the biological functionalities of

different biopeptides, including antioxidant, immunomodulatory, antidiabetic, mineral binding, anti-thrombotic, opioid, antimicrobial, and antihypertensive activities [5]. Three main biopeptide production methods include enzymatic hydrolysis, microbial fermentation, and chemical synthesis [6]. Enzymatic digestion is the most reliable, productive, and efficient method to produce biopeptides with the highest antihypertensive and antioxidant activity. Antioxidants and biopeptides with antioxidant activity are used for removing free radicals and for the prevention of oxidation reactions in the food and pharmaceutical industries [7]. Various types of proteases, such as trypsin, chymotrypsin, and papain have been used for the production of antioxidant biopeptides from food proteins [8]. It is worthwhile to note that to be more effective and efficient, all enzymatic reactions are highly recommended to be performed under the most optimized conditions. There are four main parameters, including time, temperature, pH, and enzyme/substrate that cooperatively affect the enzyme efficiency and activity; thereby, making the bioprocess more productive and controllable [7]. Response surface methodology (RSM) is a statistical method, which has been developed and frequently used to optimize the conditions of enzymatic reactions using all of the variables simultaneously [9,10]. Furthermore, RSM has been applied in several studies to design the experiments and optimize the enzymatic reactions for the production of antioxidant biopeptides from food protein sources [11]. However, there is not any data about the application of RSM to optimize the bioprocess of antioxidant biopeptides production from bovine gelatin by enzymatic hydrolysis. The objective of this study was to optimize the enzymatic hydrolysis conditions, including time, temperature, pH, and enzyme/substrate ratio to produce biopeptides with antioxidant functionality from bovine gelatin by RSM.

2. Materials and Methods

2.1. Preparation of Gelatin Hydrolysate

Bovine gelatin in powder form (Sigma chemical Co., St. Louis, MO, USA.) as the substrate and trypsin (buffer, 0.1 M Na_2HPO_4–NaH_2PO_4; temperature 37 °C; pH 8.8) (Sigma chemical Co., USA.) for enzymatic hydrolysis were used in this study. Furthermore, all chemicals used here were of analytical grade. For gelatin 1% *w/v* solution preparation, 1 g of bovine gelatin powder was mixed with 100 mL phosphate buffered saline (pH = 7.4), heated, and stirred at 50 °C for 2 h. To inactivate any enzymatic activity, gelatin solutions were heated at 95 °C for 15 min in a boiling water bath. For pH adjustment, hydrochloric acid 0.1 mol/L (Merck, Darmstadt, Herisau, Germany), sodium hydroxide 0.1 mol/L (Merck, Germany), and a pH meter device (Metrohm 713, Switzerland) were used. Gelatin solutions were prepared and incubated in definite various pH levels, enzyme/substrate ratios (E/S), temperatures, and times determined through the design of experiment-based RSM optimization and modeling approach. The proteolysis index was randomly measured and used to confirm the degradation of the protein sources by using the method described by López-Pedrouso et al. [12].

2.2. Optimization and Modeling of Enzymatic Hydrolysis Process by Response Surface Methodology

Response surface methodology was used to model and optimize the hydrolysis conditions of bovine gelatin hydrolysis by using a trypsin enzyme. The optimization of four variables, including temperature (X_1 = 30, 35, 40, 45, and 50 °C), pH level (X_2 = 5, 6, 7, 8, and 9), E/S (X_3 = 2, 2.5, 3, 3.5, and 4), and time (X_4 = 1, 2, 3, 4, and 5 h) for the level of antioxidant activity (Y), was carried out using Design-Expert software package version 10.0.3.1 (Stat-Ease Inc., Minneapolis, MN, U.S.A.). Notably, the upper and lower limits of the levels of variables in this study have been chosen considering some initial random experiments before designing the main experiment.

2.3. Antioxidant Activity

The antioxidant activity of the hydrolyzed gelatin solutions was measured using a DPPH (1, 1-diphenyl-2-picrylhidrazyl) assay. The DPPH method was carried out as previously described by Kedare and Singh [13]. In the presence of antioxidant compounds, DPPH is able to accept a hydrogen atom or an electron from the antioxidant molecules contributing to a reduction of DPPH radicals and producing a non-color ethanol solution. A total of 60 µL of hydrolyzed gelatin solution (or ethanol as the negative control) was mixed with 60 µL of DPPH solution (60 µM; prepared in ethanol) for 10 s, then was transferred into a quartz capillary tube. After 30 min incubation, the scavenging activity of the hydrolyzed gelatin solution on DPPH radicals was evaluated spectrophotometrically and the absorbance of the samples was measured at 517 nm. The antioxidant activity of the samples was calculated by the following formula:

$$\text{Antioxidant activity (\%)} = (\text{Ac} - \text{As/Ac}) \times 100$$

where, Ac and As represent the absorbance values of the control and sample, respectively. The positive control (gallic acid) was used as the reference drug in this evaluation procedure.

3. Results

A second-degree fractional polynomial model was used to describe all relationships between the independent process factors (time, temperature, pH, and E/S ratio) and only the dependent variable (antioxidant activity) because most of the responses' variability was appropriately explained by the developed model and good fits were achieved regarding the significant coefficients of multiple determination. The experimental design is presented in Table 1. Each variable contained five distinct levels; a total of 30 runs were performed using a central composite rotatable design (CCRD) to scrutinize the impacts of independent variables. The response function (Y) was associated with the coded variables (X1, X2, X3, and X4) by a second order polynomial equation using the least squares method, and the results of experiments were fitted with the following equation:

$$\begin{aligned} Y = \beta 0 + \beta 1X_1 + \beta 2X_2 + \beta 3X_3 + \beta 4X_4 + \beta 11X_1X_1 + \beta 22X_2X_2 + \beta 33X_3X_3 + \beta 44X_4X_4 \\ + \beta 12X_1X_2 + \beta 13X_1X_3 + \beta 14X_1X_4 + \beta 23X_2X_3 + \beta 24X_2X_4 + \beta 34X_3X_4 \end{aligned}$$

where, Y represents the level of antioxidant activity response; β0, is the offset term; β1, β2, β3, and β4 are the linear effect terms; β11, β22, β33, and β44 are the squared effects; β12, β13, β14, β23, β24, and β34 are the interaction effects; and X_1, X_2, X_3, and X_4 are the variables. Analysis of variance (ANOVA) was used to evaluate the significance level ($p < 0.05$) of the polynomial regression model terms for the response value. R^2, adjusted and predicted R^2 values are determination coefficients analyzed by the F-test. The adequacy of the regression model was assessed by the calculation of adequate precision (ADP), coefficient of variance (CV), lack of fit, and the PRESS values. The fitness of the model was improved by omitting the non-significant ($p > 0.05$) terms. The model was verified by comparing the response values from experimental design and the predicted responses from the fitted and optimized model. The results of protein indexes were in accordance with the results from antioxidant activities. However, these results were not included in the model.

Table 1. Experimental design to optimize antioxidant activity of trypsin hydrolysis of bovine gelatin under various hydrolysis conditions.

Run No.	Temperature (°C)	pH	E/S[a]	Time (h)	Antioxidant Activity (%)
1	45	8	2.5	4	15.1
2	45	6	3.5	4	18.5
3	40	9	3	3	31.3
4	40	7	3	3	3.9
5	35	8	2.5	4	22.6
6	30	7	3	3	41.5
7	45	8	3.5	4	18.3
8	35	8	3.5	4	18.6
9	40	7	3	3	3.3
10	40	7	3	1	14.7
11	50	7	3	3	30.4
12	40	7	3	3	3.1
13	45	6	3.5	2	12.4
14	40	7	4	3	8.6
15	35	6	2.5	2	18.7
16	35	8	3.5	2	22.2
17	40	5	3	3	13.3
18	45	6	2.5	4	13.5
19	40	7	3	3	3.6
20	40	7	3	3	3.3
21	35	6	3.5	4	14.9
22	35	8	2.5	2	17.9
23	35	6	2.5	4	13.5
24	35	6	3.5	2	26.7
25	40	7	2	3	9.6
26	40	7	3	3	3.5
27	45	8	3.5	2	28.6
28	45	6	2.5	2	11.3
29	45	8	2.5	2	18.9
30	40	7	3	5	7.6

In this study, the mathematical model represents the antioxidant activity (Y) of bovine gelatin hydrolyzed by trypsin enzyme as a function of independent variables, including temperature (X_1), pH (X_2), E/S ratio (X_3), and time (X_4) within the region under evaluation, and these were presented by the following equation:

$$Y^{0.5} = 11.44 - 0.33X_1 - 0.93X_2 - 0.67X_3 - 0.23X_4 + 0.004X_{12} + 0.69X_{22} + 0.11X32 + 0.036X_{42}$$

All independent variables significantly ($p < 0.05$) affected the antioxidant activity level of the hydrolysate. Not significant ($p = 0.986$) lack of fitness was observed for the response. Regression coefficients including R^2 and predicted and adjusted R^2 values were observed for the model obtained in this study as 0.92, 0.84, and 0.90, respectively, indicating a good regression model fitness. The adequate precision (ADP) value, indicating signal to noise ratio, was 20.71 for the antioxidant activity model; however, the CV value was observed as 10.5%. Furthermore, the PRESS value was calculated as 0.073 for the model. Studentized residuals plot and normal plot of residuals (Figure 1A, B) also indicated the adequacy of fitness for the model. Consequently, fitted interaction and a quadratic equation developed in the present study was able to describe and predict significantly the relationships between the independent variables and the response value with desirable sufficiency and adequacy.

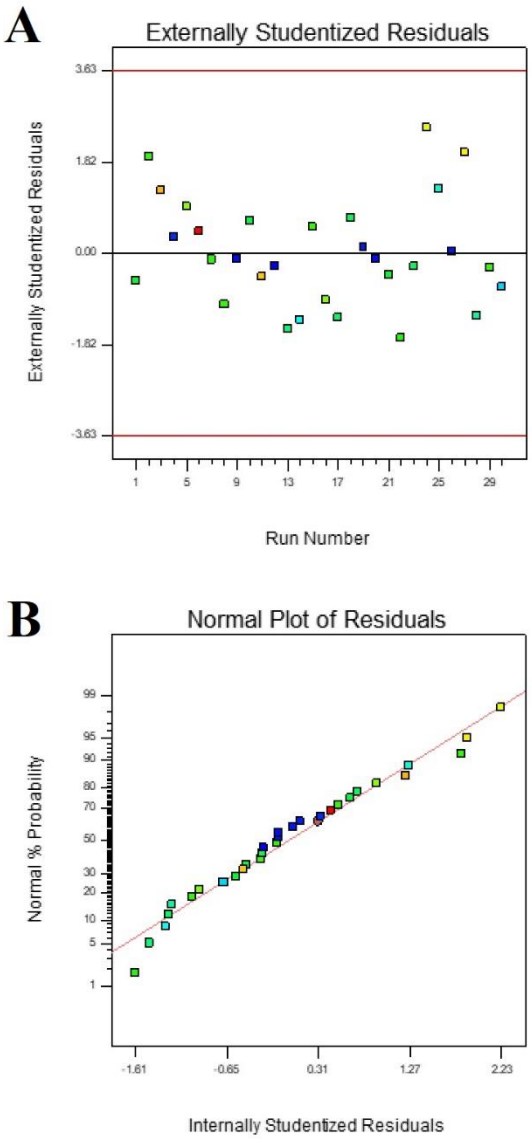

Figure 1. Studentized residuals plot (**A**) and normal plot of residuals (**B**) for the fitted model of antioxidant protein hydrolysate production from bovine gelatin by trypsin using RSM.

The effect and interaction of temperature, pH, E/S ratio, and time of the enzymatic reaction on antioxidant activity of the gelatin hydrolysate are shown in response surface plots (Figure 2A–D) when one variable remains constant. We found that the antioxidant activity of the gelatin hydrolysates significantly increased with the increase in E/S ratio, pH level, and time of the hydrolysis process by trypsin enzyme. However, temperature (ranged between 30 and 50 °C) did not significantly affect the antioxidant activity of gelatin hydrolysate in this study. The results of the optimization indicated that the optimum antioxidant activity with the desirability of 85% was observed in the hydrolysis conditions including temperature 35.3 °C, pH 8.0, and E/S ratio at 2.5 after 2 h hydrolysis of bovine gelatin using a trypsin enzyme. The optimized conditions were tested and the obtained results significantly ($p < 0.05$) confirmed the results released from the optimized conditions by the model.

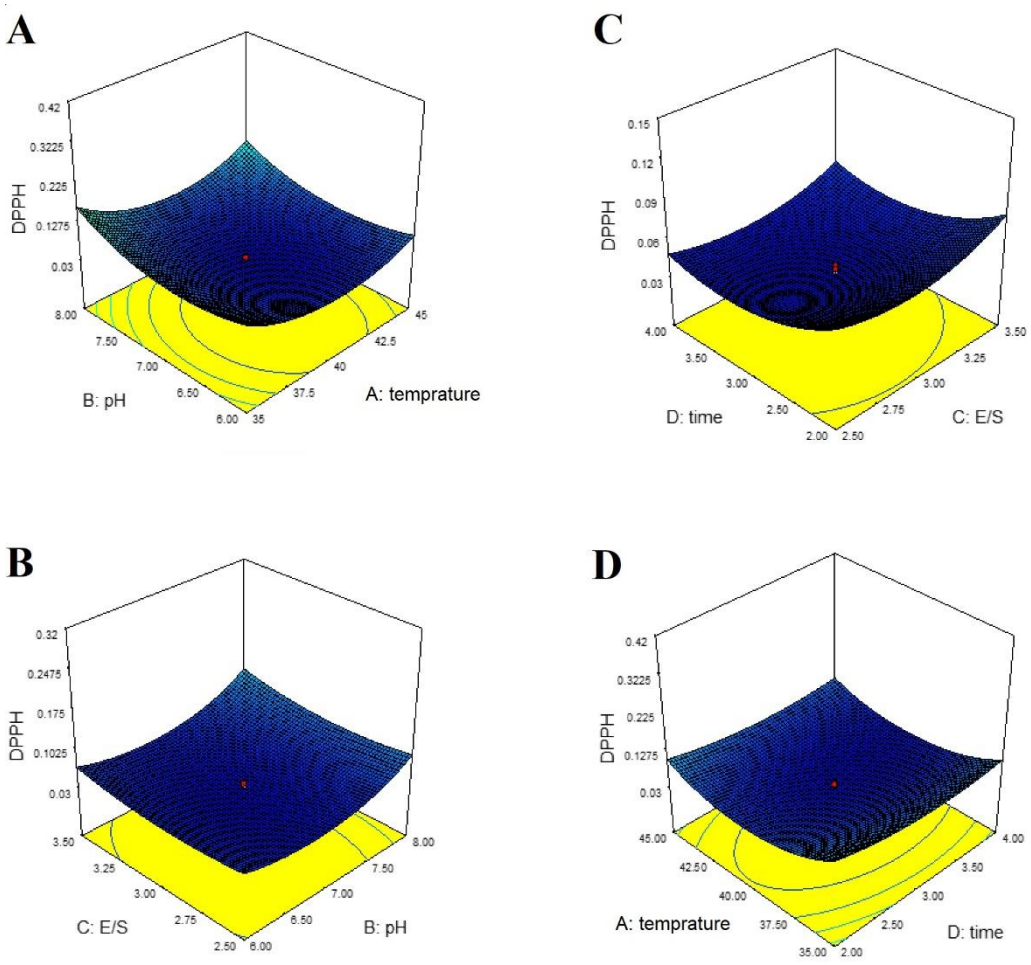

Figure 2. The 3D plots of effect of temperature and pH (**A**); pH and E/S (**B**); time and E/S (**C**); and time and temperature (**D**) on the DPPH value of gelatin protein hydrolyzed by trypsin enzyme.

4. Discussion

We developed a fitted quadratic and interaction equation to model and predict the significant relationships between the independent variables, including temperature, pH value, E/S ratio, and time, and the response of antioxidant activity of the hydrolyzed gelatin by trypsin enzyme; we eventually provided a regression model using the RSM method with desirable adequacy, sufficiency, and accuracy to describe and optimize the enzymatic process efficiently. We showed that the antioxidant activity was significantly increased with the increase in pH value, E/S ratio, and time of the process; however, temperature had no significant impact on the antioxidant activity. An increase in antioxidant activity is commonly attributed to the conditions of the hydrolysis process. Changes in the molecular characteristics of proteins, such as increased molecular charge, decreased molecular weight, and structural exposure of hydrophobic groups, may lead to an augmentation in antioxidant activity of gelatin hydrolysate [14].

The results achieved in this study are comparable with the findings of Naik et al. They found that the optimum conditions for producing whey protein hydrolysate using a trypsin enzyme and optimization with RSM were 7.3 pH value, 0.05 E/S ratio, and 8 h hydrolysis time; the antioxidant activity increased with the increase in pH, E/S, and process time [15]. Our findings are also close to the results obtained by Halim and Sarbon. They optimized the hydrolysis conditions of eel protein to produce biopeptides with antioxidant activity using RSM and showed that an increase in pH and E/S ratio contributed to an increase in antioxidant activity [16]. Several studies evaluated and optimized the enzymatic production of antioxidant protein hydrolysates by using RSM. Intiquilla et al. reported that

the highest scavenging activity was found in tarwi protein hydrolyzed by alcalase with an E/S ratio of 1.87% after 138 min. They also found that releasing more peptides containing aromatic and hydrophobic amino acids could contribute to the higher antioxidant activity observed [10]. According to Qiu et al., the composition of hydrophobic amino acids, such as glycine, is pivotal in antioxidant activity mediated by peptides produced from the hydrolysis of gelatin, which is especially rich in this amino acid. They also found that the hydrolysis of gelatin by using alcalase and trypsin enzymes contributes to the production of hydrolysates with higher scavenging activity. It is also shown that specific hydrolysis activity of trypsin is higher at alkaline pH levels [17]. Moreover, Naqash and Nazeer showed that the peptide containing glycine, alanine, and arginine, which is produced through the hydrolysis of gelatin, exhibited antioxidant activity [18]. Regarding antioxidant potential, gelatin hydrolysate can be used as an antioxidant supplement in pharmaceutical industries. It is worthwhile to note that the main limitation of this study is that we have optimized the variables for only one response and just one functionality (antioxidant activity). However, it is strongly recommended for future studies to design experiments and develop multi-response models for describing the enzymatic production of bioactive peptides released from collagen and gelatin with different functionalities.

5. Conclusions

In this study, we developed a fitted and adequate model using RSM to describe and optimize the conditions of enzymatic hydrolysis of bovine gelatin by trypsin to produce protein hydrolysate with antioxidant activity. We found that the antioxidant activity increased with the increases in pH value, E/S ratio, and process time; however, the temperature had no significant effect on the antioxidant activity. The optimum hydrolysis conditions were a temperature of 35.3 °C, pH of 8.0, and E/S ratio at 2.5 after 2 h hydrolysis by trypsin enzyme. Bovine gelatin, as a byproduct of the meat industry, has the potential to produce antioxidant bioactive peptides.

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Conflicts of Interest: The authors declare no conflict of interest.

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