Study of antioxidant activity of sheep visceral protein hydrolysate: Optimization using response surface methodology

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Original Article

Abstract

BACKGROUND: The main objective of this experiment was optimal use of none edible protein source to increase nutritional value of production with high biological function, including antioxidant activity.

METHODS: Sheep visceral (stomach and intestine) was used as substrate. Response surface methodology (RSM) was used to optimize hydrolysis conditions for preparing protein hydrolysate from the sheep visceral, using alcalase 2.4 l enzyme. The investigated factors were temperature (43-52 °C), time (90-180 min), and enzyme/substrate ratio [60-90 Anson-unit (AU)/kg protein] to achieve maximum antioxidant activity. Experiments were designed according to the central composite design.

RESULTS: Each of the studied variables had a significant effect on responses ($P < 0.05$). Optimal conditions to achieve antioxidant activity were, temperature (48.27 °C), time (158.78), min and enzyme/substrate ratio (83.35) Anson-unit/kg protein. Under these conditions, antioxidant activity was 68.21%, R^2 for model was 0.983. The values indicated the high accuracy of the model to predict the reaction conditions considering different variables. The chemical analysis of protein hydrolysate showed high protein content (83.78%) and low fat content (0.34%).

CONCLUSION: Our results showed that protein hydrolysate of sheep visceral, can be used as a natural antioxidant with high nutritional value.

Keywords: Antioxidant Peptides, Protein Hydrolysate, Enzyme Hydrolysis, Optimization

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Introduction

Sheep slaughter and edible and non-edible wastes with high content of protein, is considered as the waste of slaughter industry, include internal organs, specially digestive organs (including the stomach and the intestines), if this material is not transfer to refined section, will be suitable source to grow microorganisms. Furthermore, non-use of this material creates financial problems for industrial unit, the visceral amount obtained from sheep slaughter, is around 7-8% of total slaughter weight. In fact if the average weight of one sheep slaughter is around 32 kg. About 2.08 kg of waste will be converted.[1] Biological hydrolysis is the most proper method to produce products with high added value, like bioactive peptides.[2] Bioactive peptides have low molecular mass, which after entering the body are easily digested and absorbed. In fact, the protein absorption in the form of peptide sequences is well-absorbed into amino acids at cellular levels.[3] Bioactive peptides play a more important role in the inner biological conditions. The important functions of these compounds are anti-oxidation, anti-microbial, anti-cancer, and immune system enhancement activities. Antioxidant properties of this compound in vitro system are as well as in vivo system. The results of studies conducted on cardio-vascular diseases and a number of cancers, suggest the existence of an inverse relationship between antioxidant nutrients and progression of these diseases,[3] the necessity of using the natural antioxidants is one of the reason to produce this

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product. The precise mechanism of these antioxidant peptides is not clear, according to different conducted researches; these compounds effectively prevent lipid oxidation, and shows special effort in controlling and scavenging free radicals and metal ions.[4] The antioxidant activity of these compounds is particularly influenced by compositional of the constituent amino acids. Other important and influential factors of bioactive antioxidant peptides related to the amino acid sequences in the peptides, and the special structure of peptides, the reaction condition, type of protease used, and the degree of hydrolysis.[5,6] Several researches were performed to produce hydrolysate protein with antioxidant properties from animal sources.[7-9] Hydrolysate protein is now being used to synthesize medicinal formulations and food formulations as a specific compound with desired characteristic. This product has still good potential for technological and nutritional researches.[10]

This research aimed to produce protein-rich food with high nutritional value, using enzymatic hydrolysis process and evaluate the effects of manufacturing conditions (temperature, time, and amount of enzyme) on product characteristics (antioxidant activity).

Materials and Methods

Initial sample preparation

Sheep visceral was purchased from the city of Gorgan's local slaughterhouses. These raw materials were first completely washed by the high pressure water and kept in −25 °C until the beginning of the test.

To perform the test on the frozen materials, first they were placed in the refrigerator at 4 °C for defrost process, and they were cut into little pieces, then, they were grinded by a meat grinder and eventually the size of the pieces was decreased as much as possible. Grinded and minced mixture was immediately transferred by special containers to the autoclave and was sterilized for 15 min at 121 °C. After cooling at room temperature, the sterilized mixture homogenized by a mixer as much as possible and then centrifuged at 6000 × *g* for 30 min at 4 °C. After centrifugation, the material was divided into three phases, the upper phase included lipid (fat), the middle phase, water, and at last, the enriched and deposited protein was accumulated in the lowest phase. The protein content with low level of fat was collected for further tests.[11]

The preparation of hydrolysate protein

All hydrolysis reactions were performed in 100 ml Erlenmeyer flasks, containing 20 g of protein samples (proteins without any fat) with Tris-HCl buffer of 1:2 (w/v).[12] The alcalase enzyme (from Bacillus licheniformis with a proteolytic activity of 2.4 (AU/ml) (one Anson unit [AU] is defined as the amount of enzyme that will release 1.0 mEq of tyrosine from urea-denatured hemoglobin/min at 25 °C, pH 7.5) was added to the mixture at pH 8 (pH was adjusted with the buffer and was also suitable for the enzyme activity and helped to the stabilization of pH during the process). All reactions were performed in a shaking incubator (Vision, Scientific Co., Ltd.) with constant agitation (200 rpm). At the end of experiment, the enzyme activity was finished by heating the mixture in a water bath at 85° C for 20 min.[13] The mixture's temperature was decreased by using ice-bath, then and centrifuged at 6700 × g for 20 min at 10 °C, for the purpose of collecting the surface liquid.[13] The supernatant was dried using the freeze dryer. Hydrolysate protein production for each treatment was performed in three replications.

Measuring antioxidant activity

1-2,2-Diphenyl-1picryllhydrazyl free radical scavenging assay

For this purpose 1000 µl from each sample with 1000 µl 1-2,2-diphenyl-1picrylhydrazyl (DPPH) (0.1 Mm) produced in 99.5% ethanol were mixed in test tubes. Test tubes were vigorously stirred for 2 min. The mixture was then placed in the room temperature and kept in dark, and then the amount of radical DPPH absorption in 517 nm was measured.[6] It should be noted that, in the control sample instead of using hydrolyzed protein, 1000 µl of ethanol was used. In this experiment, butylated hydroxy toluene (BHT) at a concentration of 0.02 mg/ml was used for comparison.

Chemical analysis

Moisture content was determined by placing approximately 2 g of sample into a pre-weighted aluminum dish. Samples were then heated in an oven at 105 °C until a constant weight.[14] The total crude protein (N × 6.25) in raw materials and protein hydrolysate (liquid and powder) was determined using the Kjeldahl method.[14] Total lipid in samples was determined by Soxhlet extraction.[14] Ash content was determined by heating a pre-burning sample in an electric furnace at 600 °C until a white ash was formed.[14]

Statistical analysis

In this study, the Minitab for Windows (version 16; Minitab Inc., State College, PA, USA) and response surface methodology (RSM) was used to optimize the production condition of hydrolysate protein. RSM

with central composite design and three variables, six replicates at the central point, without block and alpha = 1.414 was considered for this test. Three variables of temperature (X_1), time (X_2), and enzyme ratio (X_3) are shown in table 1.

Results

Chemical analysis

The results related to the chemical compound of the raw material, defatted row material, and protein hydrolysate are presented in table 2.

DPPH free radical scavenging assay

The antioxidant activity was considered as the response variable and was presented in table 3. The effect of each independent variable was eventually examined on the surface of this response. The model proposed for the response is presented in Equation 1:

$$Y_1 = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_{ii}^{\ 2} + \sum_{i=1}^{2} \sum_{j=1}^{3} \beta_{ij} x_i x_j \quad (1)$$

The results of the effect of each variable on the response and model evaluation are presented in table 4.

3D surface plots and contour plots in order to impact of temperature (X_1), time (X_2), enzyme to substrate ratio (X_3) on response are presented in figure 1.

Table 1. Independent factors, their coded, and actual levels used in the experiment

Factor	Levels				
	+α	+1	0	−1	−α
Temperature (°C, X_1)	53.86	52	47.5	43	41.13
Time (min, X_2)	198.60	180	135.0	90	71.37
Enzyme ratio (AU/kg protein, X_3)	96.21	90	75.0	60	53.79

α: 1.414; X_1: Temperature; X_2: Time; X_3: Enzyme ratio

Table 2. Proximate composition (%) of raw materials and protein hydrolysate base on dried weight

Material	Protein	Fat	Moisture	Ash
Fresh viscera	10.30 ± 2.04	4.14 ± 1.04	84.50 ± 1.35	0.13 ± 0.05
Partially defatted visceral	22.55 ± 1.06	2.38 ± 1.45	72.50 ± 1.52	0.17 ± 0.01
Protein hydrolysate	83.78 ± 1.34	0.34 ± 1.03	8.61 ± 0.85	7.05 ± 0.08

All values are means of triplicate determinations (mean ± SD); SD: Standard deviation

Table 3. Experimental design used in response surface methodology studies by using three independent variables showing observed 1-2,2-diphenyl-1picrylhydrazyl free radical

Run no.	X_1	X_2	X_3	DPPH radical scavenging (%)
1	43.00	90.00	60.00	34.00
2	52.00	90.00	60.00	46.32
3	43.00	180.00	60.00	34.20
4	52.00	180.00	60.00	36.70
5	43.00	90.00	90.00	31.93
6	52.00	90.00	90.00	54.12
7	43.00	180.00	90.00	42.31
8	52.00	180.00	90.00	55.21
9	41.13	135.00	75.00	22.35
10	53.86	135.00	75.00	36.60
11	47.50	71.38	75.00	61.63
12	47.50	198.63	75.00	63.89
13	47.50	135.00	53.79	49.50
14	47.50	135.00	96.21	60.80
15	47.50	135.00	75.00	66.13
16	47.50	135.00	75.00	66.17
17	47.50	135.00	75.00	68.17
18	47.50	135.00	75.00	66.30
19	47.50	135.00	75.00	67.50
20	47.50	135.00	75.00	64.62

X_1: Temperature; X_2: Time; X_3: Enzyme ratio; DPPH: 1-2,2-Diphenyl-1picrylhydrazyl

Table 4. ANOVA table for response as affected by independent variables during optimization experiments

Factors	df	Regression coefficient	P
Model	9	−2077.400	< 0.001
Variables			
X_1	1	84.880	< 0.001
X_2	1	0.470	< 0.010
X_3	1	−1.145	< 0.010
Interaction			
$X_1 \cdot X_2$	1	−0.010	< 0.001
$X_1 \cdot X_3$	1	0.040	< 0.001
$X_2 \cdot X_3$	1	0.001	< 0.001
Quadratic effect			
X_1^2	1	−0.890	< 0.001
X_2^2	1	−0.001	< 0.001
X_3^2	1	−0.102	< 0.001
Lack of fitness	5		0.436
R^2-Pred		0.983	
R^2-Adj		0.992	

Df: Degree of freedom; X_1: Temperature; X_2: Time; X_3: Enzyme ratio; X^2: X squared; R^2: Two factors of the regression equations; R^2-Pred: Predicted r-square; R^2-Adj: Adjusted r-square

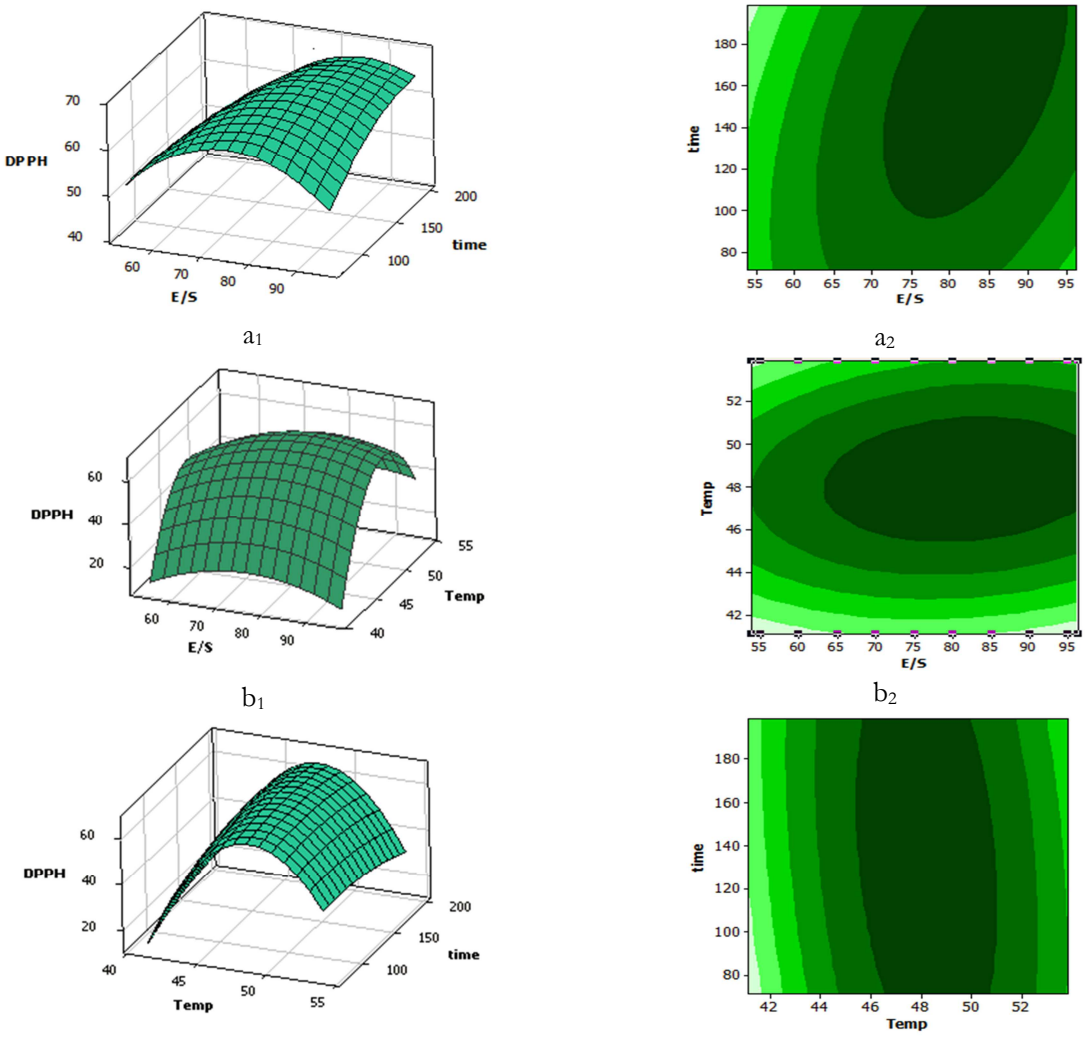

Figure 1. Response surfaces and contour plots for the effect of variables on 1-2,2-diphenyl-1picrylhydrazyl (DPPH) as a function of different hydrolyzing conditions: time and enzyme activity (a), temperature and enzyme activity (b), time and temperature (c)

Discussion

Chemical analysis

These results showed that the highest amounts of protein in hydrolysate protein, defatted raw material, and the fresh material are respectively 83.78 ± 1.34%, 22.55 ± 1.06%, and 10.30 ± 2.04% (based on the dry mass). The results of other researchers also suggest the high amount of protein in hydrolysate protein.[10,15] The amount of fat in the raw material is 4.14 ± 1.04% (based on the dried mass). This amount was drastically decreased after the defatted and separation from the protein ($P < 0.05$). It should be noted that the amount of fat in the hydrolysate protein was also greatly decreased (0.34 ± 1.03%, $P < 0.05$). This could due to release of fat and its sediments along with non-soluble proteins during the high-speed centrifuge.[7,15] Furthermore, some of the fat was seen as a separate layer after centrifuging process on supernatant. The hydrolysate protein is identified as a low fat product. The results of other researchers suggest that the amount of fat in hydrolysate protein is often less than 1%.[10,15]

DPPH free radical scavenging assay

The ANOVA table showed that every variable factor had significant effect on response ($P < 0.05$). The relation between antioxidant activity and hydrolysis parameters was quadratic. The amount of R^2 for the provided model was achieved with high values (0.992), which suggest the good ability of this model to predict the reaction conditions. The results also show that model's lack of fitness with experimental data was not significant (0.436) ($P > 0.05$), which suggest the suitability of the model for the test data.

Surface response graph showed the effect of two variables, whereas the third variable was placed at middle level. The ≤ highest amount of antioxidant activity achieved with applying a temperature: 45 °C and relatively high amount of enzyme (greater than 65 °C), which in these situations the effect of time on the activity rate of free radical is less significant compared to others factors. In fact by creating suitable conditions of temperature and the enzyme content, it is possible to achieve suitable antioxidant activity.

According to the charts-related to the effect of enzyme-time (a) and enzyme-temperature (b), the suitable range of this variable for creating acceptable antioxidant activity has been set between 65 and 95 AU/kg proteins, which tend to achieve the highest amount of antioxidant activity. The chart related to enzyme- time effect also show the amount of enzyme used in the test should be increased, when time of process is increased. The temperature had the most important effect on antioxidant activity rate. Chabeaud et al. in a study on the optimization of antioxidant activity of the hydrolysate fish protein of Saithe, reported that the highest rate of antioxidant activity (66.4%), at 60 °C temperature, pH 8, enzyme to substrate ratio of 8.53 % (AU/kg protein), is achieved after 10.8 min of hydrolysis. These researchers also referred to the effect of peptide properties in antioxidant activity.[16] In another study conducted by Taheri et al. on improving antioxidant activity of hydrolysate sardine protein, the optimal conditions included temperature higher than 45 °C, time between 80 and 120 min, and a moderate range of enzyme. The researchers also reported the improvement of hydrolysis process results in the release of antioxidant peptides from protein chain, but continuing the process of hydrolysis could decrease this activity instead.[12]

Optimizing antioxidant activity and evaluating the validity of model

The optimized conditions for hydrolysate protein antioxidant activity were predicted. These conditions include the temperature of 48.27 °C, time 158.78 min, and enzyme to substrate ratio of 83.35 AU/kg protein, which 67.98% represents free radical scavenging activity. In order to evaluate the statistical model validity, an extra test was carried out under the mentioned conditions and the free radical scavenging activity was estimated as 68.21%. This result shows that the predicted free radical scavenging amount by presented model, is compatible with the amount achieved in the experiment. These conditions represent that the model could appropriately show the effect of three variables such as temperature, time, and enzyme/substrate ratio DPPH free radical scavenging activity.

Conclusion

In the current study, in addition to producing hydrolysate protein products, the focus shifted to examine the effect of different condition on the current feature and its optimization. The results of protein hydrolysis of the antioxidant activity optimization showed that the ideal amount achieved at 48.27 °C, during 158.78 min, and enzyme to substrate ratio of 83.35 AU/kg protein. The highest amount for the antioxidant activity of this product was estimated 68.21%. Temperature had more effect on the antioxidant activity of product in comparison with the other two variables ($P < 0.05$).

The antioxidant behavior of hydrolysate protein was well under the influence of the chemical structure and properties of the peptides. The result of current study shows that the protein hydrolysate of sheep visceral could be considered as a natural antioxidant, instead of synthetic antioxidant.

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Conflict of Interests

Authors have no conflict of interests.

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