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Optimization of Hydrolysis Condition of Pumpkin Seeds with Alcalase Enzyme to Achieve Maximum Antioxidant and Nitric Oxide Inhibition Activity

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Abstract

In this research, optimization of pumpkin (*Cucurbita pepo*) hydrolysis condition was investigated in order to achieve maximum 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and nitric oxide inhibition activity using Design Expert software and response surface method. For this purpose, hydrolysis conditions including concentration of alcalase enzyme were selected within 0.7-3.30%, temperature 32-58 °C and hydrolysis time 30-290 min as independent variable. The results showed that optimum hydrolysis conditions to achieve the maximum DPPH radical scavenging and nitric oxide inhibition activity were temperature of 44 °C, hydrolysis time of 260 min, and enzyme to substrate concentration of 3% which under this condition the DPPH radical scavenging and nitric oxide inhibition activity of hydrolyzate was 72.03 and 89.34%, respectively that was largely similar to the results proposed by the software (75.33 and 84.71%). According to the results, pumpkin seed protein hydrolyzate showed high antioxidant and nitric oxide inhibitory properties and can be used as a suitable ingredient in food formulations.

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Alcalase
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Introduction

Proteins are an important source of nutrition for humans. These compounds provide nitrogen, amino acids and energy for the normal function of the body systems. Hydrolysis acts to break down proteins into free amino acids and peptides of a different size. The most common way of producing peptides is to hydrolyze proteins with the help of enzymes (Korhnen & Pihlanto, 2006). Nowadays, bioactive peptides derived from the hydrolysis of various dietary proteins are known to have various biological roles such as antihypertensive (Li *et al*., 2007), relaxing (Ohinata *et al*., 2007), anticholesterol (Cho *et al*., 2008), antioxidant (Kou *et al*., 2013) and anti-

cancer (Meisel & FitzGerald, 2003), which has led to the consideration of proteins, especially plant types, in the food and drug sector for the production and purification of bioactive peptides. One of the properties of these peptides is inhibition of nitric oxide, which acts as an intermediate compound in the production of nitrosamines. Nitrate and nitrite salts, which are used as an additive to stabilize color and inhibit the growth of some microorganisms in meat products, are very strong oxidants. As a result of the reaction of nitrite with the secondary and tertiary amines, the nitrosamine carcinogen compound is formed; if there is an antioxidant compound in processed meat, it reacts much more rapidly than amines with nitrite and prevents from the formation of nitrosamine (Hauser *et al*., 1980; Coss *et al*., 2004).

Pumpkin seeds (*Cucurbita pepo*) are known as a rich source of protein and contain significant amounts of minerals such as zinc, potassium, calcium, magnesium, iron, copper, phosphorus and β-carotene. The protein isolated from pumpkin is rich in antioxidants and is effective in reducing the malignant effects of malnutrition (Mohamed *et al*., 2009). Nourmohammadi *et al*. (2016) hydrolyzed the protein of pumpkin seed meal using pepsin enzyme using three variables of temperature, time and enzyme concentration. The results showed that produced peptides had more antioxidant power in hydrolysis conditions including 1% concentration of enzyme, temperature of 30 °C and hydrolysis time of 2 h, compared to other treatments obtained from the response surface method and central composite designs. Piri Ghashlaghi *et al*. (2015), Mehregan Niko *et al*. (2013) and Etemadi *et al*. (2014) respectively produced hydrolyzed proteins from whey protein concentrate, crucian carp and soybean meal by alcalase enzyme and the effect of temperature, time, and the ratio of enzyme to substrate on antioxidant activity was investigated.

The results of various experiments have shown that the hydrolyzed proteins derived from the protein hydrolysis of fish (Khantaphant & Benjakul, 2008), the hydrolyzed protein of tracheal meat (Klompong *et al*., 2009), soybean protein (Chen *et al*., 1995), milk proteins such as α- lactalbumin and β-lactoglobulin (Hernandez-Ledesma *et al*., 2005), egg white protein (Davalos *et al*., 2004) and gluten protein (del Castillo *et al*., 2007) showed high antioxidant activity. Lee *et al*. (2012) hydrolyzed the proteins derived from oysters (*Ruditapes philippinarum*) by 8 protease enzymes and ultimately obtained peptides with the highest nitric oxide inhibition property by the alcalase enzyme.

The pumpkin seeds are commonly used in the production of oil. Meal obtained from pumpkin seed oil contains high percentage of protein, which is currently not processed and is a cheap source of protein for the consumption in animal feed. Considering the high potential of this material in the production of bioactive peptides, this study aimed to optimize the enzymatic hydrolysis of pumpkin meal by alcalase enzyme in order to produce protein hydrolyzate with the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and nitric oxide inhibition activity.

Material and methods

Materials

Pumpkin seed meal was purchased from Abkar Gorgan Company. The alcalase enzymes and DPPH radical obtained from Sigma and NaOH, hydrochloridric acid, sulfonilamide, phosphoric acid, sodium nitroprusside and ethanol from Merck, and naphthyl ethylenediamine dihydrochloride were obtained from Ridley Company, Germany. All materials

used were of laboratory grade.

Preparation of pumpkin seed meal

After removing the foreign material, the meal was turned into flour by milling machine (Perten, 3100, Germany) and passed through a sieve with 30 meshes. The resulting flour was de-defatted using hexane with the ratio of 1:3 for 16 h, and after that it was kept at room temperature for 48 h in order to remove the solvent residues completely, and finally kept in the refrigerator (Kaur & Singh, 2007).

Pumpkin meal flour

The defatted meals were ground at this stage by a grinding machine (Perten, 3100, Germany) so that the flour particles could pass through the sieve with 70 meshes. Samples were stored in sealed polyethylene bags at temperature of 4 °C in the refrigerator (Glew *et al.*, 2006).

Preparation of pumpkin protein concentrate

The defatted pumpkin seed meal was dispersed in water with the ratio of 1 to 10 and then the pH of the solution was adjusted to 10 by 1 N NaOH and mixed at room temperature for 1 h. The resulting mixture was centrifuged at 5000g using a refrigerated centrifuge (Combi 514R, South Korea) for 20 min at the temperature of 4 °C. In order to precipitate pumpkin seeds proteins, the pH resulting supernatant was adjusted to pH=5 by 1 N HCL, and centrifuged under similar condition. Then the precipitate obtained was washed with distilled water and dried using a freezing dryer (FD4 model, Operon Company, South Korea) and then kept at freezer at temperature -18 °C for further experiments (Zivanovic *et al.*, 2011).

Hydrolysis of pumpkin seed protein concentrate

Protein concentrate with the proportion of 5% (w/v) was dispersed in a tris-hydrochloridric acid buffer with pH=8 (Optimal pH of alcalase enzyme) and the enzyme was added at concentration of 1-3%. Then, the hydrolysis was carried out in the temperature range of 35-55 °C and time of 60-260 min in a shaking incubator (VS-8480 model, South Korea) with speed of 200 rpm. Finally the enzymatic reaction was stopped at 85 °C for 10 min, and the resulting mixture was centrifuged at the 5000g for 30 min to remove the insoluble compounds (Villanueva *et al.*, 1999). The resulting supernatant was kept at the temperature of -18 °C and freeze dried.

Determination of chemical composition of pumpkin meal

Moisture, ash and protein were determined using the AACC methods of 15-44, 01-08 and 12-46, respectively (AACC, 1999) and the fat content was measured by soxhlet method (Parvane, 2006).

Measurement of DPPH radical scavenging activity

1000 μL of hydrolyzed protein was mixed with 1000 μL of DPPH (0.1 mM) prepared in 96% ethanol and kept at room temperature in the dark for 60 min and finally the absorbance of solution was measured at a 517 nm using spectrophotometer (Bougatef *et al.*, 2009). In the control sample, 1000 μL of distilled water was used instead of the hydrolyzed protein sample. The DPPH radical inhibition activity was calculated as Eq. (1):

(1)

DPPH radical scavenging activity = [(absorption of control-sample absorption)/absorption of control]×100

Measurement of nitric oxide inhibition activity

60 μL of hydrolyzed protein was mixed with 60 μL of sodium nitroprusside in phosphate buffer (0.025 *M*) and placed

in the plates and kept in the incubator for 150 min at room temperature (ascorbic acid was used as a control sample). Then, the equal amount (120 μL) of Grease reagent (including sulfanilamide, naphthyl ethylene dihydrochloride and phosphoric acid) was added to plates and the absorbance was measured at 546 nm (Tsai *et al.*, 2007) and the inhibition activity was calculated as Eq. (2):

(2)

Nitric oxide inhibitory percent = [(absorption of control-sample absorption)/absorption of control] ×100

Optimization of enzymatic hydrolysis conditions

In order to optimize the process from the viewpoint of maximizing the antioxidant and nitric oxide inhibition properties, Design Expert software version 11 and the response surface method with central composite designs were used for 3 independent variables of time (X_1), temperature (X_2) and the ratio of enzyme to substrate (X_3) at 5 levels (-α, -1, 0, +1 and +α). Approximate amount of each independent variable was obtained according to the experimental tests results. The examined responses were DPPH radical scavenging and nitric oxide inhibition activity. Total 20 treatments were randomly selected by the software with six replications at the central point. The different levels of the independent variables and the relevant treatments are presented in Tables (1) and (2), respectively.

Table 1. Levels of independent variables used to optimize DPPH radical scavenging activity and nitric oxide inhibitory activity of pumpkin seeds protein

Independent variables	The limits of change				
	-α	-1	0	+1	+α
Hydrolysis time (min)	30	60	160	260	290
Hydrolysis temperature (°C)	32	35	45	55	58
The ratio of enzyme to substrate (%)	0.70	1	2	3	3.30

The value of α is equal to 1.3.

Table 2. Random treatments and DPPH radical scavenging and nitric oxide inhibitory activity of the hydrolyzed pumpkin seed protein

Treatments	Time (min)	Hydrolysis temperature (°C)	The ratio of Enzyme to Substrate (%)	DPPH radical scavenging activity (%)	Nitric oxide inhibitory activity (%)
1	60	35	3	67.09	80.83
2	160	45	2	79.01	72.71
3	160	45	2	79.73	77.29
4	160	32	2	74.06	86.65
5	60	55	1	78.64	58.89
6	160	58	2	77.21	80.06
7	260	35	3	67.48	85.96
8	160	45	0.70	77.02	83.59
9	260	55	3	76.49	84.89
10	160	45	3.30	81.11	85.73
11	60	55	2	80	85.27
12	290	45	2	72.42	87.27
13	160	45	2	80	77.98
14	260	55	1	70.32	87.04
15	60	35	1	48.46	85.43
16	260	35	1	55.89	88.19
17	160	45	2	79	74.32
18	160	45	2	69.88	74.46
19	30	45	2	75.63	88.57
20	160	45	2	81.45	77.13

The regression models 1 and 2 were proposed to predict the first and second responses (DPPH radical scavenging and nitric oxide inhibition activity) by the following Eq. (3 and 4):

$$R_1=b_0-b_1x_1+b_2x_2+b_3x_3-b_{12}x_1x_2-b_{13}x_1x_3-b_{23}x_2x_3-b_{11}x_1^2-b_{22}x_2^2-b_{33}x_3^2 \quad (3)$$

$$R_2=b_0+b_1x_1-b_2x_2-b_3x_3-b_{12}x_1x_2+b_{13}x_1x_3+b_{23}x_2x_3+b_{11}x_1^2+b_{22}x_2^2+b_{33}x_3^2 \quad (4)$$

In above Eq. (3 and 4), R_1 and R_2 are the responses or dependent variables (DPPH radical scavenging and nitric oxide inhibition activity, respectively), b_0 is a constant, and b_1, b_2 and b_3 are linear effects, b_{11}, b_{22} and b_{33} are quadratic effects and b_{12}, b_{13} and b_{23} are interaction effects.

Analysis of variance, regression coefficients, plotting and optimization were performed by Design Expert software and the significance of the tests were analyzed by Duncan multiple range test and SPSS V16 software at a significant probability level of (P<0.05).

Result and discussion

According to the results of Table (3), the pumpkin seed protein concentrate contain high protein content. The extraction process with the help of alkali led to the separation of non-protein parts from the meal and the accumulation of protein in the remainder section (Khantaphant *et al.*, 2011a). The amount of fat in the concentrate was lower than the fat in the meal. The reason of reducing the fat content of the concentrate compared to the meal was the defatting process done on the pumpkin seed meal before preparation of the concentrate and the process of extracting the protein in alkaline solution and precipitation at the isoelectric point (Khantaphant *et al.*, 2011b). The amount of ash in the meal and protein concentrate was 7.63 and 0.815, respectively, which is equivalent to the values reported by Nourmohammadi *et al.* (2016) and Mazloomi *et al.* (2017). The moisture content of the meal and protein concentrate was 7.03 and 12.56, respectively. The moisture content of protein concentrate was higher than that of the above mentioned sources, and the reason of this matter can be its long-term preservation that causes some moisture absorption.

Table 3. Chemical composition of meal and protein concentrate prepared from pumpkin seed

A matter	Protein	Fat	Moisture	Ash
Defatted Meal	51.8±1.19	9.65±0.15	7.03±0.09	7.63±0.02
Protein Concentrate	76±1	1.29±0.46	12.56±0.01	0.815±0.015

* Results are the average of three replicates.

Linear Eq. (5) and (6), taking into account the regression coefficients for the DPPH radical scavenging activity and nitric oxide inhibition property, respectively, which were suggested by the following factors according to the significance of coefficients (Table 4).

(5)

DPPH radical inhibition activity=+79.33-(0.72×time)+(6.21×temperature)+(3.78×the ratio of enzyme to substrate)-(2.46×time×temperature) -(0.28×time×the ratio of enzyme to substrate)-(2.84×time×the ratio of enzyme to substrate)-(4.70×time×time)-(3.75×temperature×temperature)-(1.72×the ratio of enzyme to substrate×the ratio of enzyme to substrate)

(6)

Nitric oxide inhibition activity= +76.92 + (0.61×time) - (0.52×temperature) - (0.60×the ratio of enzyme to substrate) - (0.89×time×temperature) + (0.10×time×the ratio of enzyme to substrate) + (0.51×temperature×the ratio of enzyme to substrate)+(4.79×time×time)+(2.09×temperature×temperature) + (2.86×the ratio of enzyme to substrate×the ratio of enzyme to substrate)

The value of R_2 was calculated 0.7811 for the first equation (Eq. 5) and 0.7365 for the second equation (Eq. 6), which indicates the relatively suitable distribution of the data. The lack of fitness, which is a criterion for the suitability of the presented model, was 0.1625 and 0.0521 in the first and second equations (Eq. 5 and 6), respectively. The high level of lack of fitness compared to the significant level of probability (95%) or, in other words, the non-significant of this factor indicates the appropriateness of the proposed model and fitness of the model based on the considered responses.

Table 4. Analyze of variance related to DPPH radical scavenging and nitric oxide inhibition test

Factor	Degree of freedom	Sum of squares	Average of squares	F-Value	P-Value
DPPH radical scavenging activity					
Model	9	1101.52	122.39	3.97	0.0214
Time	1	5.88	5.88	0.19	0.6717
Temperature	1	438.30	438.30	14.20	0.0037
Enzyme concentration	1	162.98	162.98	5.28	0.0444
Time×Temperature	1	48.27	48.27	1.56	0.2396
Time×Enzyme	1	0.62	0.62	0.020	0.8900
Temperature×Enzyme	1	64.35	64.35	2.08	0.1793
Time×Time	1	148.23	148.23	4.80	0.0532
Temperature×Temperature	1	94.25	94.25	3.05	0.1111
Enzyme×Enzyme	1	19.82	19.82	0.64	0.4415
Residue	10	308.66	30.87		
Non fitness	5	222.000	44.40	2.56	0.1625
Net error	5	86.66	17.33		
Total	19	1410.19			
Nitric oxide inhibition					
Model	9	367.82	40.87	3.11	0.0460
Time	1	4.27	4.27	0.32	0.5815
Temperature	1	3.05	3.05	0.23	0.6408
Enzyme concentration	1	4.08	4.08	0.31	0.5896
Time×Temperature	1	6.34	6.34	0.48	0.5035
Time×Enzyme	1	0.088	0.088	6.704E-003	0.9364
Temperature×Enzyme	1	2.06	2.06	0.16	0.7006
Time×Time	1	154.05	154.05	11.71	0.0065
Temperature×Temperature	1	29.34	29.34	2.23	0.1662
Enzyme×Enzyme	1	55.00	55.00	4.18	0.0681
Residue	10	131.57	13.16		
Non fitness	5	109.43	21.89	4.94	0.0521
Net error	5	22.14	4.43		
Total	19	499.39			

The effect of independent variables on DPPH radical scavenging activity

In this section, the effect of temperature, hydrolysis time and enzyme to substrate ratio on the DPPH free radicals scavenging activity has investigated. Therefore, using the 3D-graphs obtained by Design Expert software, the effect of mentioned parameters on the antioxidant property was investigated and interpreted and the results were compared and evaluated with the results of previous studies. It should be noted that in each Figure (1, 2 and 3) the effects of the two parameters are investigated when the third parameter was at the optimal point.

The effect of enzyme concentration and hydrolysis temperature on DPPH radical scavenging activity is shown in Figure (1). As it can be seen, the increase in enzyme concentration and

also increase in hydrolysis temperature led to increase in DPPH radical scavenging activity, but the increase in enzyme concentration was less effective than temperature increase in DPPH radical scavenging activity. Therefore, it can be said that the increase of these two parameters has a direct effect on the increase of the amount of free radical inhibition; thus the highest free radical inhibitory power was achieved at temperature of 55 °C and enzyme concentration of 3%.

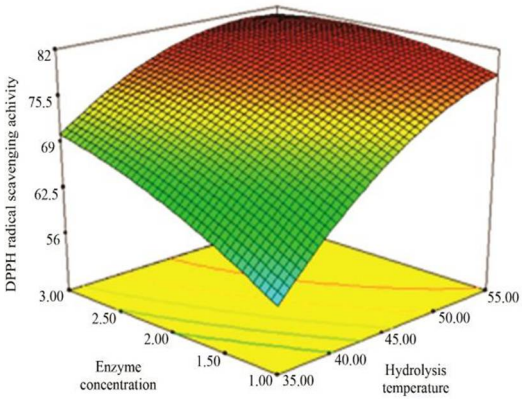

Figure 1. The effect of enzyme concentration and hydrolysis temperature on DPPH radical scavenging activity at optimum hydrolysis time

The DPPH radical scavenging activity is used to check the hydrogenation capability of hydrolyzed proteins. Free radical removal is a mechanism by which antioxidant compounds are capable to prevent oxidative reactions. DPPH radical is one of the few stable radicals at room temperature. When this compound is exposed to a hydrogen donator compound like an antioxidant, it accepts a hydrogen, converts to a stable compound and after radical inhibition, a notable color change from purple to yellow and absorption reduction at 517 nm can be observed (Taheri *et al.*, 2011). The type of initial material, the specificity of the enzyme, the hydrolysis conditions, and the size, amount and structure of amino acids and peptides produced are the factors affecting the antioxidant activity (Mehregan Niko *et al.*, 2013). On the one hand, the antioxidant power of hydrolyzed materials depends highly on the structural arrangement and specific amino acid sequences of the peptide chain. Some researchers believe that histidine, hydrophobic amino acids, and peptides with sequences of proline-histidine-histidine have anti-oxidant potency (Phelan *et al.*, 2009).

The effect of enzyme concentration and hydrolysis time on the DPPH radical scavenging activity is shown in Figure (2). According to Figure (2), the amount of DPPH free radical scavenging activity increased with increasing enzyme concentration, while with increasing hydrolysis time up to 160 min, the scavenging activity reached its highest level and then, by increasing the time, the amount of free radical scavenging activity decreased. Reduction of DPPH radical scavenging activity by hydrolyzed protein with increasing the hydrolysis time can be due to the progression of the hydrolysis and the greater effect of the enzyme on the protein substance, which causes breaking the chain of some antioxidant peptides formed in the early stages of hydrolysis (Taheri *et al.*, 2011). After optimization of production of hydrolyzed protein from tuna fish waste using the response surface method, Ovissipour *et al.* (2009) reported that with increasing hydrolysis time, hydrolysis intensity reduced and after that remained constant. Guerard *et al.* (2002) stated that by increasing the concentration of enzyme, the production of antioxidant peptides increases. They also stated that by increasing hydrolysis time, the degree of hydrolysis increases, and after that the intensity and speed of hydrolysis reducedue to the reduction of the available peptide bands to the enzyme, reduction in the enzyme activity and the formation of inhibitors compounds.

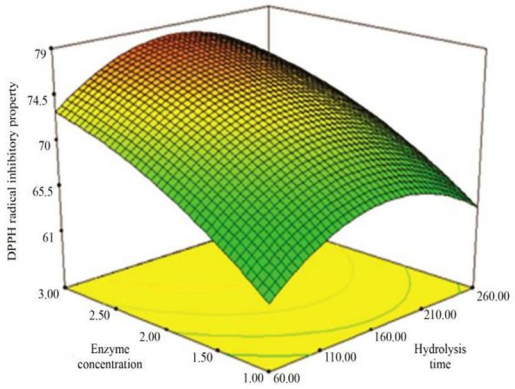

Figure 2. The effect of enzyme concentration and hydrolysis time on DPPH radical scavenging activity at optimum hydrolysis temperature

Figure (3) shows the effect of temperature and hydrolysis time on DPPH radical scavenging activity. As it can be seen in the Figure (3), by increasing the temperature the antioxidant power increases, but the increase in the time up to 160 min increases and then reduces DPPH radical scavenging activity. In a study by Sun *et al*. (2011) on the antioxidant properties of peptides derived from hydrolysis of pig hemoglobin by flavourzyme, papain, alcalase, pepsin and trypsin, the highest DPPH radical scavenging activity (67%) was associated with hydrolyzed produced by pepsin after 60 min of hydrolysis. Nourmohammadi *et al*. (2016) obtained the highest antioxidant activity in peptides derived from hydrolysis of pumpkin seed meal by pepsin after 2 h, hydrolysis.

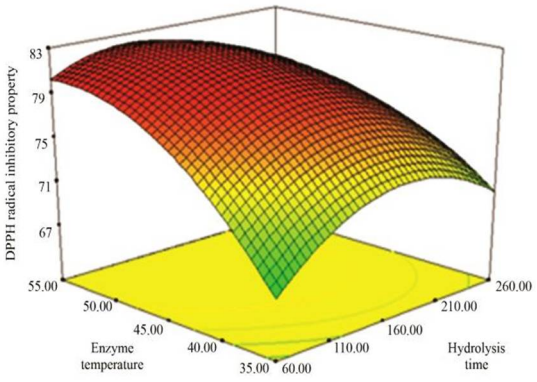

Figure 3. The effect of temperature and hydrolysis time on DPPH radical scavenging activity at optimum concentration of enzyme

According to the results, it can be said that the hydrolysis temperature and the concentration of the alcalase enzyme have a direct effect on the DPPH radical scavenging activity of hydrolysed product, thereby by increasing these two parameters the antioxidant activity can be increased. However, the increase of hydrolysis time up to a certain limit (160 min) can cause an increase in antioxidant activity of pumpkin seed protein; but with the excessive increase in hydrolysis time, the intensity of hydrolysis increases and antioxidant peptides can be degraded, therefore the antioxidant activity decreases (Taheri *et al*., 2011).

The effect of independent variables on nitric oxide inhibitory activity

In this section, as in the previous section, the effect of temperature, hydrolysis time and alcalase enzyme concentration on the nitric oxide inhibition capability was investigated. Therefore, in the same way as previously mentioned, using the three-dimensional graphs obtained by Design Expert software, the effect of hydrolysis parameters on the nitric oxide inhibitory power were investigated and interpreted. It should be noted that in each Figure (4, 5 and 6) the effects of the two parameters are investigated when the third parameter is at its optimal point. The effect of enzyme concentration and hydrolysis temperature on the nitric oxide inhibition property is presented in Figure (4). According to the Figure (4), a decrease in nitric oxide inhibition activity has been observed at lower temperatures (35 to 45 °C) and increase in the enzyme concentration up to 2%, and then, with an increase in temperature and enzyme concentration, the amount of inhibition activity increased with a moderate slope. Thus, the lowest nitric oxide inhibition activity was observed at temperature of 45 °C and the enzyme concentration of 2%. Thus, according to the graph, the

highest nitric oxide inhibition activity (87%) is observed at temperature close to 35 °C and the enzyme concentration of 1%. The nitric oxide inhibitory property depends on the antioxidant property of that compound. The antioxidant property of peptides is related to the selectivity of the protease used, the degree of hydrolysis, the nature of released peptides (such as molecular weight, amino acid sequence and peptide structure), and other characteristics, such as the ability to bond to free radicals, the activity of metal ions, chelation and electron donating power, the structural arrangement and sequence of amino acids and the presence of specific amino acids in their peptide chains. The nitric oxide inhibition property also depends on these factors. The highest nitric oxide inhibition activity observed at the lowest temperature and concentration of pepsin enzyme (35 °C and 1%), but the increase in enzyme concentration (at a certain limit 2%) caused higher enzymatic activity and consequently further breaking of antioxidant and nitric oxide inhibitor peptides occur; so the nitric oxide inhibition activity decreases.

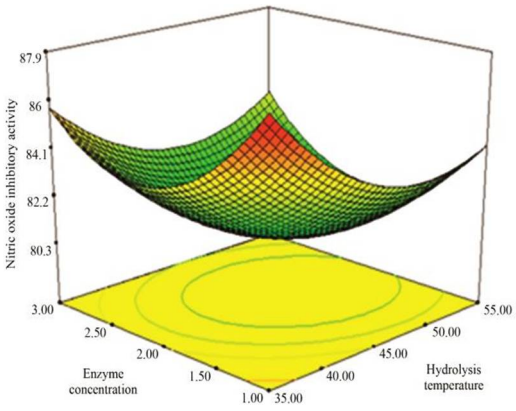

Figure 4. The effect of enzyme concentration and hydrolysis temperature on the nitric oxide inhibition activity at optimum hydrolysis time

The effect of enzyme concentration and hydrolysis time on the nitric oxide inhibition property (Figure 5) shows that in the initial hydrolysis times, with an increase in enzyme concentration up to 2%, the nitric oxide inhibition activity shows a descending trend and by increasing hydrolysis time (after 160 min) the nitric oxide inhibition activity increases. The graph shows that the lowest nitric oxide inhibitory activity (81%) achieve at the hydrolysis time of 160 min and enzyme concentration of 2%, and the highest nitric oxide inhibitory activity observed at the hydrolysis time of 260 min and enzyme concentration of 1%. Lee *et al.* (2012) hydrolyzed the protein derived from oyster by 8 protease enzymes and ultimately peptides with the highest nitric oxide inhibition property obtained by the alcalase enzyme. The hydrolyzed product was then purified using high performance liquid chromatography (HPLC) and finally it was recognized that nitric oxide inhibitory peptide show a sequence of glutamine-cysteine-glutamine-glutamine -alanine-valine-glutamine-serine -alanine -valine at their N terminal section. Therefore, considering the mentioned findings in this section and the research carried out by Lee *et al.* (2012), it can be concluded that by increasing the hydrolysis time, enzyme activity and degree of hydrolysis increases and eventually the peptides with specific sequences obtain that results an increase in the nitric oxide inhibition activity of hydrolyzed products.

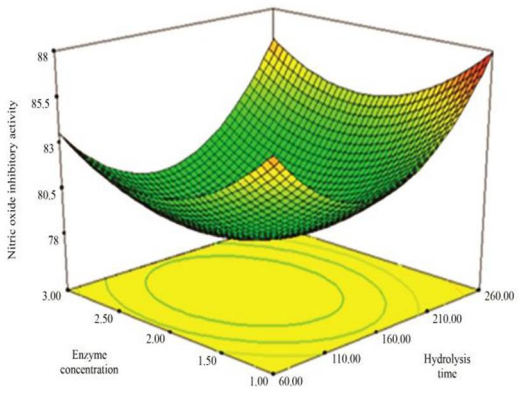

Figure 5. The effect of enzyme concentration and hydrolysis time on the nitric oxide inhibition activity at optimum temperature

Figure (6) shows the effect of hydrolysis time and temperature on the

nitric oxide inhibition activity. In the initial times (before 160 min) and by increasing the temperature up to 45 °C, the nitric oxide inhibition activity shows a descending trend, and by increasing the hydrolysis time to 160 min and temperature to 45 °C, the nitric oxide inhibition show an ascending trend. Therefore, the maximum nitric oxide inhibition activity can be achieved at hydrolysis time of 260 min and temperature of 35 °C. Low nitric oxide inhibition activity of hydrolyzed samples in the early stages of hydrolysis (before time of 160 min) may be due to low hydrolysis time that is insufficient to affect the substrate and to produce peptides with nitric oxide inhibitory activity. With the increase in the hydrolysis time and decrease in the size of produced peptides, the ability to inhibit free radicals by peptides enhances (Nourmohammadi *et al.* 2016).

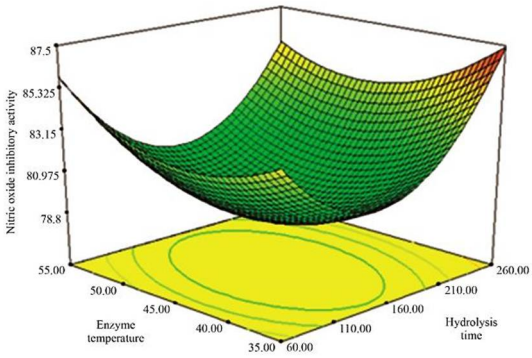

Figure 6. The effect of temperature and hydrolysis time on the nitric oxide inhibition activity at optimum enzyme concentration

Phelan *et al.* (2014) through enzymatic hydrolysis of milk proteins achieved a peptide with amino acid sequence of alanine-valine-proline-tyrosine-proline-glutamine-arginine and tyrosine-tyrosine-alanine-lysine-proline -alanine-alanine-valine-arginine with proper nitric oxide inhibition property.

Also, Kim *et al.* (2013) hydrolyzed the protein of shellfish with 8 different protease enzymes and investigated the nitric oxide inhibition activity of final products. Then, by purification using chromatography system they reported that the peptide which showed high nitric oxide inhibitory property contained 10 amino acids with sequence of glycine-valine-serine-leucine-leucine-glutamine-glutamine-phenylalanine-phenylalanine-leucine. According to the findings for achieving proper nitric oxide radical inhibitory activity of final product, the optimum hydrolysis condition include hydrolysis time of 260 min, temperature 35 °C and enzyme concentration of 1%.

Optimization and validation of the model

Optimal conditions were obtained by Design Expert software. Hydrolysis conditions for preparation of hydrolyzed protein with the highest DPPH radical scavenging activity and nitric oxide inhibition activity were obtained in accordance with temperature of 44 °C, hydrolysis time of 260 min and enzyme to substrate concentration of 3%, which corresponded to the nitric oxide inhibition activity and DPPH free radical scavenging activity of 4.71 and 75.33%, respectively. In order to confirm the suitability of conditions proposed by the software, additional experiments were carried out in the predicted situations obtained by the model. The DPPH free radical scavenging activity and nitric oxide inhibition activity of hydrolysed product were obtained 72.03 and 89.34%, respectively. The obtained results showed almost similar values with the predicted values proposed by the model, which indicated using predictive models it is possible to predict optimal conditions for the production of hydrolyzed protein with high antioxidant and nitric oxide inhibition activity from pumpkin seeds protein. Nourmohammadi *et al.* (2016) stated that the measured antioxidant activity of peptides produced using enzymatic hydrolysis of pumpkin seeds by pepsin at optimal conditions (82.07%) was largely similar to that

proposed by software (80.31%).

Conclusion

Millions of tons of valuable by-products are produced every year during processing of food products. One of these valuable by-products is pumpkin seed meal, which has so far been used as supplement in animal feed. In order to increase the value of this by product which is economically important and due to its high protein content with the appropriate combination of amino acid, this study attempted to use the meal as a suitable protein source for preparation of protein hydrolyzate with high bioactive potential. In this study, optimization of production of hydrolyzed protein with high antioxidant and nitric oxide inhibition activity using alcalase enzyme was performed by response surface method. According to the results, preparation of pumpkin seeds protein hydrolyzate with high antioxidant and nitric oxide inhibition activity is influenced by reaction conditions including temperature, time and the enzyme concentration. Results showed that optimum conditions to achieve maximum DPPH radical scavenging activity and nitric oxide inhibition activity is temperature of 44 °C, hydrolysis time 260 min, and enzyme to substrate concentration of 3%. Under these conditions the antioxidant and nitric oxide inhibition property of protein hydrolyzate were 72.03 and 89.34%, respectively which were largely similar to the results proposed by the software (75.33 and 84.71%). Hence, the pumpkin seed protein hydrolyzate with high antioxidant and nitric oxide inhibitory activity can be used as an alternative to synthetic preservatives in different food formulations including meat products. Considering the consumer's tendency toward functional foods and present concerns about application of synthetic additives, the results of this study lead to the production of a functional ingredient that can be used in various types of food formulation. This product is particularly interesting regarding its nitric oxide inhibitory property which eliminates concerns about the nitrate and nitrite residues and prevention of nitrosamines formation in meat products such as sausage. Also due to its protein structural nature its application increases the nutritional value and creates a product with health promoting advantages.

References

AACC. (1999). *Approved method of the American association of cereal chemists*. St. Paul: America Association of Cereal Chemists.

Bougatef, A., Hajji, M., Balti, R., Lassoued, I., Triki-Ellouz, Y., & Nasri, M. (2009). Antioxidant and free radical-scavenging activities of smooth hound (*mustelus mustelus*) muscle protein hydrolysates obtained by gastrointestinal proteases. *Food Chemistry, 114*(4), 1198-1205. doi: https://doi.org/10.1016/j.foodchem.2008.10.075

Chen, H.M., Muramoto, K., & Yamauchi, F. (1995). Structural analysis of antioxidative peptides from soybean β-Conglycinin. *Journal of Agricultural and Food Chemistry, 43*(3), 574-578. doi: https://doi.org/10.1021/jf00051a004

Cho, S.-J., Juillerat, M.A., & Lee, C.H. (2008). Identification of LDL-receptor transcription stimulating peptides from soybean hydrolysate in human hepatocytes. *Journal of Agricultural and Food Chemistry, 56*(12), 4372-4376. doi: https://doi.org/10.1021/jf800676a

Coss, A., Cantor, K.P., Reif, J.S., Lynch, C.F., & Ward, M.H. (2004). Pancreatic cancer and drinking water and dietary sources of nitrate and nitrite. *American Journal of Epidemiology, 159*(7), 693-701. doi: https://doi.org/10.1093/aje/kwh081

Davalos, A., Miguel, M., Bartolome, B., & Lopez-Fandino, R. (2004). Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection, 67*(9), 1939-1944. doi: https://doi.org/10.4315/0362-028X-67.9.1939

Etemadi, M., Sadeghi Mahoonak, A.R., Ghorbani, M., & Maghsodlou, Y. (2014). Optimization of hydrolysis conditions of soybean protein isolate to achieve maximum antioxidant activity using response surface method, (Unpublished master's thesis). Gorgan University of Agricultural Sciences and Natural Resources. (in Persian)

Glew, R.H., Glew, R.S., Chuang, L.-T., Huang, Y.-S., Millson, M., Constans, D., & Vanderjagt, D.J. (2006). Amino acid, mineral and fatty acid content of pumpkin seeds (*cucurbita spp*) and cyperus esculentus nuts in the republic of niger. *Plant Foods for Human Nutrition, 61*(2), 49-54. doi: https://doi.org/10.1007/s11130-006-0010-z

Guerard, F., Guimas, L., & Binet, A. (2002). Production of tuna waste hydrolysates by a commercial neutral protease preparation. *Journal of Molecular Catalysis B: Enzymatic, 19-20*, 489-498. doi: https://doi.org/10.1016/S1381-1177(02)00203-5

Hauser, E., Yam, I., & Yerman, P. (1980). Nitrosamine in leben esmitteln. *Swiss Food, 2*, 13-22.

Hernandez-Ledesma, B., Davalos, A., Bartolome, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β- actoglobulin. Identification of active peptides by HPLC–MS/MS. *Journal of Agricultural and Food Chemistry, 53*(3), 588-593. doi: https://doi.org/10.1021/jf048626m

Kaur, M., & Singh, N. (2007). Characterization of protein isolates from different Indian chickpea (*cicer arietinum L.*) cultivars. *Food Chemistry, 102*(1), 366-374. doi: https://doi.org/10.1016/j.foodchem.2006.05.029

Khantaphant, S., & Benjakul, S. (2008). Comparative study on the proteases from fish pyloric caeca and the use for production of gelatin hydrolysate with antioxidative activity. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 151*(4, 410-419. doi: https://doi.org/10.1016/j.cbpb.2008.08.011

Khantaphant, S., Benjakul, S., & Ghomi, M.R. (2011a). The effects of pretreatments on antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper (*lutjanus vitta*). *LWT-Food Science and Technology, 44*(4), 1139-1148. doi: https://doi.org/10.1016/j.lwt.2010.10.009

Khantaphant, S., Benjakul, S., & Kishimura, H. (2011b). Antioxidative and ACE inhibitory activities of proteinhydrolysates from the muscle of brownstripe red snapper prepared sing pyloric caeca and commercial proteases. *Process Biochemistry, 46*(1), 318-327. doi: https://doi.org/10.1016/j.procbio.2010.09.005

Kim, E.K, Kim, Y.S, Hwang, J.W, Kang, S.H, Choi, D.K, Lee, K.H, Lee, J.S, Moon, S.H, Jeon, B.T, & Park, P.J. (2013). Purification of a novel nitric oxide inhibitory peptide derived from enzymatic hydrolysates of *mytilus coruscus*. *Fish & Shellfish Immunology, 34*(6), 1416-1420. doi: https://doi.org/10.1016/j.fsi.2013.02.023

Klompong, V., Benjakul, S., Yachai, M., Visessanguan, W., Shahidi, F., & Hayes, K.D. (2009). Amino acid composition and antioxidative peptides from protein hydrolysates of yellow stripe trevally (*selaroides leptolepis*). *Journal of Food Science, 74*(2), 126-133. doi: https://doi.org/10.1111/j.1750-3841.2009.01047.x

Korhnen, H., & Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International Dairy Journal, 16*(9), 945-960. doi: https://doi.org/10.1016/j.idairyj.2005.10.012

Kou, X., Gao, J., Xue, Z., Zhang, Z., Wang, H., & Wang, X. (2013). Purification and identification of antioxidant peptides from chickpea (*cicer arietinum L.*) albumin hydrolysates. *LWT-Food Science and Technology, 50*(2), 591-598. doi: https://doi.org/10.1016/j.lwt.2012.08.002

Lee, S.J., Kim, E.K., Kim, Y.S., Hwang, J.W., Lee, K.H., Choi, D.K., Kang, H., Moon, S.H., Jeon, B.T., & Park, P.J. (2012). Purification and characterization of a nitric oxide inhibitory peptide from *ruditapes philippinarum*. *Food and Chemical Toxicology, 50*(5), 1660-1666. doi: https://doi.org/10.1016/j.fct.2012.02.021

Li, G.H., Qu, M.R., Wan, J.Z., & You, J.M. (2007). Antihypertensive effect of rice protein hydrolysate with in vitro angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats. *Asia Pacific Journal of Clinical Nutrition, 16*(S1), 275-280.

del Castillo, M.D., Ferrigno, A., Acampa, I., Borrelli, R.C., Olano, A., Martinez-Rodriguez, A., & Fogliano, V. (2007). In vitro release of angiotensin-converting enzyme inhibitors, peroxyl-radical scavengers and antibacterial compounds by enzymatic hydrolysis of glycated gluten. *Journal of Cereal Science, 45*(3), 327-334. doi: https://doi.org/10.1016/j.jcs.2006.09.005

Mazloomi, S.N., & Sadeghi Mahoonak, A.R. (2017). Characterization of nutritional properties of protein isolate of pumpkin (*cucurbita pepo* con. pepo var *styriaca*) seeds. *Iranian Journal of Food Science and Technology, 70*(14), 13-25. (in Persian)

Mehregan Nikoo, A.R., Sadeghi Mahoonak, A.R., Ghorbani, M., Taheri, A., & Alami, M. (2013). Optimization of different factors affecting antioxidant activity of crucian carp (*carassius carassius*) protein hydrolysate by response surface methodology. *Electronic Journal of Food Processing and Peservation, 5*(1), 95-110. (in Persian)

Meisel, H., & FitzGerald, RJ. (2003). Biofunctional peptides from milk proteins, mineral binding and cytomodulatory effects. *Current Pharmaceutical Design, 9*(16), 1289-1296. doi: https://doi.org/10.2174/1381612033454847

Mohamed, R.A., Ramadan, R.S., & Ahmed, L.A. (2009). Effect of substituting pumpkin seed protein isolate for casein on serum liver enzymes, lipid profile and antioxidant enzymes in CCl_4-intoxicated rats. *Advances in Biological Research, 3*(1-2), 9-15.

Nourmohammadi, E., Sadeghi Mahoonak, A.R., Sadeghi, M., Aalami, M., & Ghorbani, M. (2016). Identification of the optimum conditions to anti-oxidative peptides production through the enzymatic hydrolysis of pumpkin oil cake protein by pepsin. *Iranian Journal of Food Science and Technology, 61*(13), 123-130. (in Persian)

Ohinata, K., Agui, S., & Yoshikawa, M. (2007). Soymorphins, novel μ opioid peptides derived from soy β-conglycinin β-subunit, have anxiolytic activities. *BioScientific, Biotechnology and Biochemistry, 71*(10):2618-2621. doi: https://doi.org/10.1271/bbb.70516

Ovissipour, M., Abedian, A., Motamedzadegan, A., Rasco, B., Safari, R., & Shahiri, H. (2009). The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from Persian sturgeon (*acipenser persicus*) viscera. *Food Chemistry, 115*(1), 238-242. doi: https://doi.org/10.1016/j.foodchem.2008.12.013

Parvaneh, V. (2006). *Quality control and chemical analysis of food*. (pp.332):Tehran University Publication, (in Persian)

Phelan, M., Khaldi, N., Shields, D.C., & Kerins, D.m. (2014). Angiotensin converting enzyme and nitric oxide inhibitory activities of novel milk derived peptides. *International Dairy Journal, 35*(1), 38-42. Doi: https://doi.org/10.1016/j.idairyj.2013.10.008

Phelan, M., Aherne, A., FitzGerald, R.J, & O'Brien, N.M. (2009). Casein-derived bioactive peptides:biological effects, uses, safety aspects and regulatory status. *International Dairy Journal, 19*(11): 643-654. doi: https://doi.org/10.1016/j.idairyj.2009.06.001

Piri Ghashlaghi, Sh., Sadeghi Mahoonak, A.R., Ghorbani, M, & Aalami, M. (2015). Production of bioactive peptides with high antioxidant activity from whey protein concentrate. *Journal of Research and Innovation in Food Science and Technology, 3*(4), 271-282. doi: http://dx.doi.org/10.22101/jrifst.2015.11.22.437 (in Persian)

Sun, Q., Shen, H., & Luo, Y. (2011). Antioxidant activity of hydrolysates and peptide fractions derived from porcine hemoglobin. *Journal of Food Science and Technology, 48*(1):53-60. doi: https://doi.org/10.1007/s13197-010-0115-0

Taheri, A., Abedian Kenari, A., Motamedzadegan, A., & Habibi-Rezaei, M. (2011). Poultry by-products and enzymatic hydrolysis: optimization by response surface methodology using alcalase® 2.4L. *International Journal of Food Engineering, 7*(5), 1-17. doi: https://doi.org/10.2202/1556-3758.1969

Tsai, P.J., Tsai, T.H., Yu, C.H., & Ho, S.C. (2007). Comparison of no-scavenging and no-suppressing activity of different herbal teas with those of green tea. *Food Chemistry, 103*(1), 181-187. doi: https://doi.org/10.1016/j.foodchem.2006.08.013

Villanueva, A., Vioque, J., Sanchez-Vioque, R., Clemente, A., Pedroche, J., Bautista, J., & Millan, F. (1999). Peptide characteristics of sunflower protein hydrolysates. *Journal of the American Oil Chemists' Society, 76*(12), 1455-1460. doi: https://doi.org/10.1007/s11746-999-0184-2

Zivanovic, I., Vastag, Z., Popovic, S., Popovic, L., & Pericin, D. (2011). Hydrolysis of hull-less pumpkin oil cake protein isolate by pepsin. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering, 5*(3), 94-98. doi: https://doi.org/10.5281/zenodo.1057175

بهینه‌یابی شرایط هیدرولیز پروتئین دانهٔ کدو با آنزیم آلکالاز به‌منظور دستیابی به حداکثر فعالیت ضداکسایشی و مهارکنندگی اکسید نیتریک

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چکیده

در این پژوهش، بهینه‌سازی شرایط هیدرولیز پروتئین دانهٔ کدو (*Cucurbita pepo*) به‌منظور دستیابی به حداکثر خصوصیات مهارکنندگی رادیکال ۲،۲- دی فنیل -۱-پیکریل هیدرازیل (DPPH) و مهارکنندگی اکسید نیتریک با استفاده از نرم‌افزار Design Expert و روش سطح پاسخ موردبررسی قرار گرفت. به‌این‌منظور غلظت آنزیم آلکالاز ۰/۷-۳/۳۰ درصد، دمای ۳۲-۵۸ درجهٔ سانتی‌گراد و زمان ۳۰-۲۹۰ دقیقه به‌عنوان سطوح متغیرهای مستقل انتخاب شدند. نتایج نشان داد که شرایط بهینه برای دستیابی به حداکثر خاصیت مهارکنندگی رادیکال DPPH و مهارکنندگی اکسید نیتریک، دمای ۴۴ درجهٔ سانتی‌گراد، زمان ۲۶۰ دقیقه و غلظت آنزیم به سوبسترا ۳ درصد و با قابلیت ضداکسایشی و مهارکنندگی اکسید نیتریک برابر با ۷۲/۰۳ و ۸۹/۳۴ درصد بود که تا حدود زیادی مشابه با نتایج پیشنهادشده توسط نرم‌افزار (۷۵/۳۳ و۸۴/۷۱ درصد) بود. طبق نتایج به‌دست‌آمده پروتئین هیدرولیزشدهٔ دانهٔ کدو از قابلیت ضداکسایشی و مهارکنندگی اکسید نیتریک مناسبی برخوردار می‌باشد.

واژه‌های کلیدی: آلکالاز، آنتی‌اکسیدانی، دانهٔ کدو، مهارکنندگی اکسید نیتریک، هیدرولیز آنزیمی