





## Production of antioxidant peptides through hydrolysis of medicinal pumpkin seed protein using pepsin enzyme and the evaluation of their functional and nutritional properties

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### Original Article

#### Abstract

**BACKGROUND:** A hydrolyzed protein composition is a mixture of peptide and amino acids that have been achieved through hydrolysis by the enzyme from different sources, acid or caustic soda. These peptides show important health improving properties including anti-oxidation, antimicrobial, anti-cancer, anti-diabetic, anti-hypertensive activity.

**METHODS:** The aim of the present study was to hydrolyze the protein extracted from medicinal pumpkin seed (*Cucurbita Pepo* Con. *Pepo* Var *Styriaca*) seed meal by pepsin enzyme to obtain bioactive peptides with the highest antioxidant capacity. For this, response surface method (RSM) and central composite design were used at different enzyme concentrations (1%-2%), hydrolysis times (2-5 hours), and temperatures (30-40 °C) as independent variables. Then, the functional properties (emulsifying capacity, foaming capacity, water absorption capacity, and oil absorption capacity), heat and pH stability, and amino acid analysis were measured for the optimum treatment.

**RESULTS:** 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of peptides achieved in optimum conditions (82.07%) was highly similar to the results predicted by the software (80.31%) and their functional properties were significantly different from the initial protein ( $P > 0.050$ ). Amino acid profile showed that the antioxidant capacity of the hydrolysates could be due to the total hydrophobic amino acid content that accounts for 39.85% of total amino acids in pumpkin seed meal.

**CONCLUSION:** According to the results, pumpkin seed meal hydrolysates, with outstanding functional properties, can be used in different food formulation to improve their physical and chemical properties and extend their shelf life, and as antihypertensive and antioxidant agents in the prevention of cardiovascular disease.

**Keywords:** Pumpkins, Hydrolysis, Antioxidants, Antihypertensives, Amino Acid Analysis

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#### Introduction

Proteins are vital components of health because they provide the nitrogen, amino acids, and energy needed by the body. However, applications of proteins are limited due to some of their properties such as solubility. Protein hydrolysis is a widespread strategy to improve their chemical, functional, and nutritional properties.<sup>1</sup> During the hydrolysis, proteins are broken into small peptides and amino acids. Because the enzymatic hydrolysis is performed in relatively mild conditions and no

amino acid damage occurs, this kind of hydrolysis is preferred to acidic and alkaline hydrolysis.<sup>2</sup>

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Because of the potential applications of bioactive peptides in functional foods production, these compounds have great importance and application in human nutrition and health. Bioactive peptides from various food proteins can be used as natural substitutes for most expensive chemical medicines that are usually used for chronic diseases.<sup>2</sup> Hydrolysed proteins showed antioxidative activity and angiotensin-converting-enzyme (ACE) inhibiting effect which depended on the type of enzyme and was increased with increase in the hydrolysis time.<sup>3</sup> As synthesized anti-hypertensive drugs have been reported to exhibit several undesirable side effects or other health complications following long-term administration, food-derived ACE inhibitory peptides are considered to be safer in combating hypertension.<sup>4</sup> Therefore, many researchers have searched for natural sources of ACE inhibitors in the past decade. Recent studies on proteins and bioactive peptides with the aim to improve human health and prevent chronic diseases have led to further investigations about these bioactive peptides. Rayaprolu *et al.* purified and characterized bioactive peptides from soybean for cancer cell proliferation inhibition.<sup>5</sup> Lee *et al.* investigated the antioxidant and anticancer effects of functional peptides obtained from ovotransferrin hydrolysates.<sup>6</sup> Ghassem *et al.* identified two novel antioxidant peptides obtained from protein hydrolysates obtained from the Edible-nest Swiftlet (*Aerodramus fuciphagus*).<sup>7</sup>

Results of scientific literature show that bioactive peptides obtained from plant proteins through enzymatic hydrolysis have suitable functional properties such as emulsifying and foaming capacities (e.g., functional properties of soybean),<sup>8</sup> water and oil absorption, and outstanding health improving functions such as high antioxidant activity (peptides from soybean, black wheat, cotton seed, sunflower seed, peanut, and pumpkin),<sup>9</sup> cholesterol reduction (fish hydrolysates),<sup>10</sup> antimicrobial activity (peptides from cereals, legumes, and mushrooms),<sup>11</sup> antihypertensive activity (peptides from wheat, soybean, rice, and azufrado beans),<sup>12</sup> and metal chelating ability (peptides from collagen).<sup>13</sup> Takenaka *et al.* studied the antioxidant activity of soybean protein and its hydrolysates. They found that a diet containing 20% soybean protein and its hydrolysates has a preventive effect on thiobarbituric acid reactive substances (TBARS) in rats, and peptides obtained from soybean hydrolysis can decrease blood

cholesterol more than non-hydrolyzed soybean proteins.<sup>14</sup> Lahart *et al.* investigated reasons for the prevention of oxidative processes in the presence of bioactive peptides and concluded that this preventive effect could be due to the metal chelating activity of peptides or some certain groups in their amino acid side chains that preferably bind with free radicals in fatty acids and prevent the oxidation process.<sup>15</sup> Klompong *et al.* studied the protein hydrolysates of yellowstripe scad (*Selaroides leptolepis*) obtained through hydrolysis using alcalase and flavourzyme, and suggested that these hydrolysates have proper functional properties.<sup>16</sup> During hydrolysis by both of the enzymes, protein solubility increased more than 85%, and interfacial activity (emulsifying, and foaming capacities and stabilities) of the hydrolysates was dependent upon the degree of hydrolysis and type of protease.

Pumpkin is of the Cucurbitaceae, and in this family, medicinal pumpkin seed (*Cucurbita pepo*) has the highest diversity. A large amount of oil products are obtained from plant resources each year. Medicinal pumpkin seed with 23-35% protein and 20-55% oil is a rich source of protein and oil. Pumpkin seed oil contains high amounts of unsaturated fatty acids such as linoleic acid and oleic acid. Thus, pumpkin seed is an appropriate source for various industries.<sup>17</sup> The issue at hand is the amount of remaining wastes that mostly consist of pumpkin meal. Disposing of this amount of wastes is not cost-effective and causes environmental problems, and these wastes have nutritional and medicinal value; thus, they can be used in various processes to decrease waste and produce products with high nutritional values.

In the present study, enzymatic hydrolysis of proteins of pumpkin meal was carried out using protease enzyme pepsin, and therefore, the best treatment was selected using Design-Expert software (version 6.0.2, Stat-Ease Inc., Minneapolis, MI, USA) with response surface method (RSM) as optimum treatment for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. Subsequently, functional properties (emulsifying, foaming, water absorption, and oil absorption capacities), pH and heat stability, and amino acid profile for optimum treatment were investigated and compared to those of pumpkin seed protein isolate.

## Materials and Methods

Medicinal pumpkin seed meal (*Cucurbita pepo* con. *Pepo* var. *Styriaca*), with 48.57% ± 3.51% protein, 10.32% ± 0.58% lipid, 4.24% ± 0.52% moisture,

and  $6.045 \pm 0.17$  ash, was purchased from a soybean factory in Gorgan, Iran, and pepsin enzyme and DPPH were purchased from Merck KGaA, Darmstadt, Germany. All chemicals used were of analytical grade.

#### **Pumpkin protein isolate production:**

Pumpkin protein was produced according to the process reported by Horax et al. Pumpkin meal flour was mixed with distilled water with 1:10 (w/v) ratio at room temperature and reached the highest solubility pH (pH = 10) with 1N NaOH. The sample was stirred for 1 hour at room temperature and centrifuged at  $5000 \times g$  for 20 minutes (Combi514R, South Korea). The supernatant reached isoelectric point (pH = 3.49) with 1N HCL, and was left to settle at room temperature for 30 minutes. The resulting suspension was centrifuged at room temperature at  $5000 \times g$ . The pellet was washed with 20 ml distilled water and dried using a freeze dryer (FD<sub>4</sub>, Operon, South Korea).<sup>18</sup>

**Optimization of the hydrolysis process by the response surface method:** In the present study, in order to evaluate the effect of hydrolysis conditions on protein hydrolysate properties and to optimize this process, response surface design with three variables was used to find the relation between responses and variables. Effects of independent variables, enzyme to substrate ratio ( $X_1$ ), time ( $X_2$ ), and temperature ( $X_3$ ) are presented in table 1.

**Table 1.** Independent variables and their levels used in the central composite design (CCD)

Independent Variables	Symbol	Coded Level		
		-1	0	+1
Enzyme/substrate Ratio (ml/g)	$X_1$	1	1.5	2
Time (minute)	$X_2$	2	3.5	5
Temperature (°C)	$X_3$	30	35	40

**Enzymatic hydrolysis:** According to the amounts calculated using the Design-Expert software, enzyme pepsin (from porcine stomach mucosa, 6907 U/g) was added in concentrations of 1, 1.5, and 2%, and hydrolysis time as independent factor was 2, 3.5, and 5 hours in a 200 rpm shaker incubator (8480-VS, South Korea). Hydrolysis temperatures were 30, 35, and 40 °C. After deactivating the enzyme at 85 °C for 15 minutes, the suspension was centrifuged at 4 °C for 30 minutes at  $4000 \times g$ , and supernatant was collected as the protein hydrolysate.<sup>19</sup> The collected supernatants were lyophilized.<sup>20</sup>

**Degree of hydrolysis:** The degree of hydrolysis

(DH) was estimated according to the method presented by Kaewka et al.; 10 ml protein hydrolysate was mixed with 10 ml trichloro acetic acid (TCA) 10% and centrifuged. Nitrogen content (N) of the supernatant and total nitrogen was measured through the Kjeldahl method and DH was measured using the following equation (1).<sup>21</sup>

$$\text{Degree of Hydrolysis} = \frac{\text{N in TCA}}{\text{N in whole hydrolysed sample}} \quad (1)$$

**DPPH radical scavenging activity:** Hydrolysate at a concentration of 1000  $\mu\text{l}$  was added to DPPH 0.1 M at a concentration of 1000  $\mu\text{l}$  in ethanol (96%) and left to settle for 60 minutes at room temperature in a dark place. Absorbance was measured at 517 nm.<sup>22</sup> Ethanol was used as control. DPPH scavenging activity was calculated using equation (2).

$$\text{DPPH Scavenging Activity (\%)} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{Absorbance of Control}} \times 100 \quad (2)$$

**Emulsifying capacity:** Emulsifying capacity was investigated based on the method reported by Pearce and Kinsella.<sup>23</sup>; 10 ml corn oil was mixed with 30 ml protein hydrolysate 0.1%/protein solution through centrifugation at 14489 g for 1 minute. Then, 1 ml of emulsion was removed from the bottom and mixed with sodium dodecyl sulfate (SDS) 0.1% with the ratio of 1:100. The absorbance of the solution was measured at 500 nm immediately after emulsion formation at room temperature ( $A_0$ ). Emulsifying activity ( $\text{m}^2/\text{g}$ ) indicated the surface area that was stabilized by 1 g protein hydrolysate and was calculated using the following equation (3):

$$\text{Emulsifying activity} = \frac{2 \times 2.303 \times A_0}{F \times (\text{g proteinweight})} \times 100 \quad (3)$$

Where F is the oil volume of the emulsion (0.25).

**Foaming capacity:** Foaming capacity was measured according to the method presented by Adebowale and Lawal<sup>24</sup> with slight modifications; 1 g protein/protein hydrolysate was mixed with 50 ml distilled water in a mixer (SBG-5725, Japan) with high speed for 5 minutes. Then, the solution was poured into a 250 ml scaled cylinder and foam volume was read after 30 seconds. Foaming activity was calculated using the following equation (4):<sup>24</sup>

$$\text{FC (\%)} = \frac{(A_0 - B) \times 100}{B} \quad (4)$$

Where FC is foaming capacity (%),  $A_0$  is sample volume after stirring, and B is sample volume before stirring.

**Water absorption capacity:** Water absorption capacity was measured according to the method reported by Kaur and Singh<sup>25</sup> with slight modifications; test tubes were weighted, and 10 ml distilled water was added to 1 g protein/ protein hydrolysate and they were mixed. After 30 minutes in room temperature, tubes were centrifuged for 30 minutes at 6000 ×g. After removing the supernatant, the tubes remained in 45 °C for 25 minutes in a 45-degree angle until surface water was removed. Then, the tubes were weighted again. Water absorption percentage is reported as g of absorbed water/ g of sample.<sup>25</sup>

**Oil absorption capacity:** Oil absorption capacity was measured according to the method presented by Kaur and Singh.<sup>25</sup> Test tubes were weighted and 0.5 g of protein/ protein hydrolysate was added to 6 ml sunflower oil and stirred with a metal spatula for 1 minute. After 30 minutes in room temperature, the tubes were centrifuged for 30 minutes at 6000 ×g. After removing the supernatant, the tubes were kept upside-down for 25 minutes to remove the excess oil, and then, weighted. Oil absorption percentage is reported as g of absorbed oil/ g of sample.<sup>25</sup>

**pH and heat stability:** To investigate pH stability, the protein hydrolysate sample was incubated at room temperature and exposed to pH 1-11 by 1M NaOH or HCl for 1 hour. Then, the pH of samples was set at 7 and DPPH radical scavenging activity was measured.

For heat stability investigation, 5 ml of each protein hydrolysate sample at a pH of 7 was poured into a test tube and heated at 100 °C in a water bath for 0, 30, 60, 90, 120, and 180 minutes, and cooled in cold water immediately after removal from the water bath. The control sample was kept at 25 °C. Then, DPPH radical scavenging activity was measured.<sup>26</sup>

**Amino acid profile analysis:** Amino acid profile analysis was carried out using reverse phase high performance liquid chromatography (RP-HPLC). pumpkin seed protein hydrolysate hydrolysate was used for this analysis; 30 mg of the hydrolysate sample was hydrolyzed with 6 N HCl at 110 °C for 24 hours. RP C-18 columns (15 × 0.46 cm) with 5 μ internal particle size were used for this analysis.

The treatments were optimized using RSM in the form of a central compound design in Design-Expert software. In the present study, in order to evaluate hydrolysis conditions on protein hydrolysates properties and to optimize this process, response surface design with three discrete variables [enzyme to substrate ratio ( $X_1$ ), time ( $X_2$ ),

and temperature ( $X_3$ )] was used to find the relation between response (DPPH scavenging activity), that it is as continuous variables, and variables.

The data collected from experiments on the hydrolysate in optimized conditions was analyzed through a completely random design in SPSS software (version 21, IBM Corporation, Armonk, NY, USA). Each experiment was repeated three times. Duncan's test was used to compare the means of all evaluated parameters and the difference between different treatments were evaluated using t-test at probability level of 5% ( $\geq 5\%$ ). Microsoft Excel spreadsheet software (Microsoft Corporation, Redmond, WA, USA) was used to draw charts.

## Results

**DPPH radical scavenging activity:** According to the experiment results, the hydrolysate sample with 1% enzyme concentration at 30 °C for 2 hours showed the highest DPPH scavenging activity (82.07%), with the R-squared and adjusted R-squared of 0.9092 and 0.9522 ( $P = 0.091$ ), respectively, for optimization treatment. Therefore, it was suggested as the optimum treatment for free radical scavenging activity (degree of hydrolysis: 28%) by Design-Expert software. Table 2 shows the DPPH scavenging activity of hydrolysates obtained from pumpkin protein isolate.

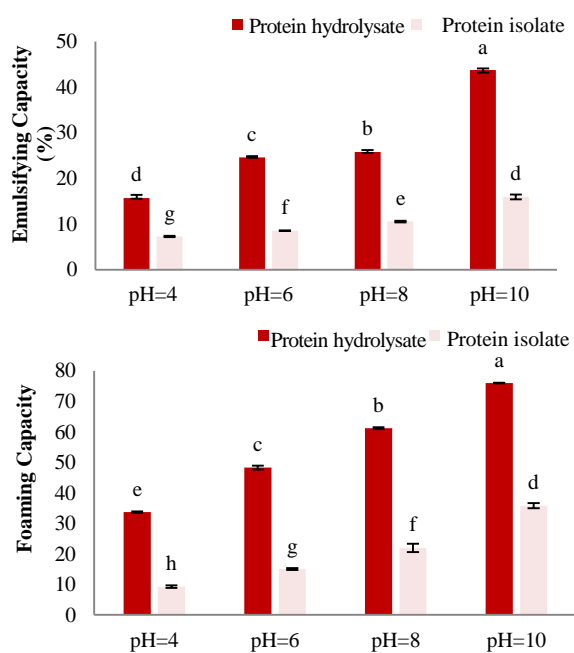
**Emulsifying capacity of pumpkin seed protein isolate and hydrolysate:** Emulsifying capacity was significantly ( $P > 0.050$ ) influenced by the degree of hydrolysis. As can be seen in figure 1, hydrolysis has a positive effect on emulsifying capacity. Changes in pH had a significant effect ( $P > 0.050$ ) on emulsifying capacity, and the lowest and highest emulsifying capacities were observed at a pH of 4 and 10, respectively. As the pH range of most processed foods is 4-10, this range was used for evaluation of the functional properties of protein isolate and concentrate.

**Foaming capacity of pumpkin seed protein isolate and hydrolyste:** Changes in foaming capacities of pumpkin seed protein isolate and hydrolysates in different pH are shown in figure 1. Both hydrolysis process and pH variation showed significant effects ( $P > 0.050$ ) on foaming capacity. As can be seen in figure 1, protein hydrolysates, within the whole pH range considered in this study, had higher foaming capacity than protein isolate. Changes in pH showed significant ( $P > 0.050$ ) effects on the foaming capacity of pumpkin seed protein isolate and hydrolysates. The lowest and highest foaming capacities for protein isolate and hydrolysate were observed in a pH of 4 and 10, respectively.

**Table 2.** Antioxidant activities of different treatments used for enzymatic hydrolysis of pumpkin seed protein

Treatment	Enzyme concentration (%)	Temperature (°C)	Time (hour)	DPPH radical scavenging activity (%)	Average (%)	Standard deviation	P
1	2.0	30	2.0	68.11	67.41	0.61	0.169
2	1.0	30	2.0	82.07	81.39	0.53	0.091
3	2.0	30	5.0	72.00	71.17	0.62	0.032
4	1.0	30	5.0	70.31	69.56	0.61	0.967
5	1.5	30	3.5	50.18	49.27	0.65	0.101
6	1.5	35	3.5	65.90	64.77	0.81	0.040
7	1.5	35	2.0	70.34	69.53	0.61	0.228
8	1.5	35	3.5	65.13	64.53	0.64	0.398
9	1.5	35	3.5	68.00	67.35	0.62	0.409
10	1.0	35	3.5	79.24	78.34	0.64	0.064
11	1.5	35	3.5	68.44	67.54	0.64	0.255
12	1.5	35	3.5	62.39	61.77	0.63	0.155
13	1.5	35	3.5	67.84	67.11	0.61	0.321
14	1.5	35	5.0	68.93	68.45	0.72	0.048
15	2.0	35	3.5	79.00	78.46	0.68	0.065
16	1.0	40	5.0	62.67	61.90	0.61	0.155
17	1.5	40	3.5	43.52	42.70	0.61	0.015
18	2.0	40	2.0	68.72	68.33	0.79	0.271
19	1.0	40	2.0	76.83	75.95	0.63	0.197
20	2.0	40	5.0	65.31	65.51	1.47	0.041

DPPH: 2,2-diphenyl-1-picrylhydrazyl

**Figure 1.** Emulsifying and foaming capacities of pumpkin seed protein isolate and hydrolysate

Data correspond to the mean  $\pm$  standard deviation (SD) of the three independent experiments. ANOVA was performed in GraphPad Prism (version 6.0, GraphPad Software Inc., CA, USA). Values with different letters (a, b,...) indicate significant differences with emulsifying and foaming capacities of pumpkin seed protein isolate and hydrolysate ( $P < 0.050$ ).

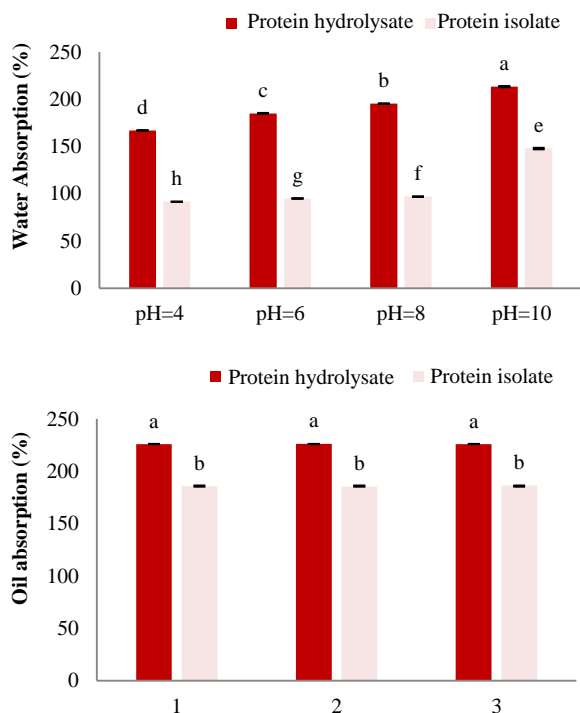
Duncan's test was used to compare the means of all evaluated parameters and the difference between different treatments were evaluated using t-test at probability level of 5 % ( $\geq 5\%$ ).

The lowest foaming capacity at a pH of 4 was related to the lower solubility and more compact structure of protein at pH near the isoelectric point.

**Water absorption capacity of pumpkin seed protein isolate and hydrolysate:** Water absorption capacity is defined as the ability of particles to absorb water molecules in a limited amount of water. Figure 2 shows the water absorption capacity of pumpkin seed protein isolate and hydrolysates. Enzymatic hydrolysis of pumpkin seed protein isolate had a significant ( $P > 0.050$ ) direct effect on its water absorption capacity.

Changes in pH had significant ( $P > 0.050$ ) effect on the water absorption of pumpkin seed protein isolate and hydrolysates. The lowest and highest water absorption capacity of both isolate and hydrolysates were at a pH of 4 and 10, respectively.

**Oil absorption capacity of pumpkin seed protein isolate and hydrolysate:** The oil absorption capacity increased in pumpkin seed protein hydrolysate compared to its isolate (Figure 2) which can be related to an increase in surface hydrophobicity after enzymatic hydrolysis that leads to an increase in physical entrapment of oil droplets.<sup>27</sup> Zhang et al. found similar results in their study on oil absorption capacity of rice bran protein hydrolysates compared to its isolate.<sup>27</sup>



**Figure 2.** Water and oil absorption capacities of pumpkin seed protein isolate and hydrolysate

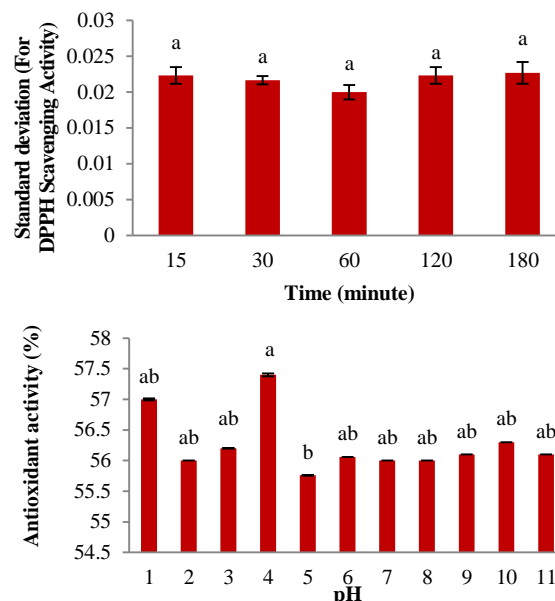
Data correspond to the mean  $\pm$  standard deviation (SD) of the three independent experiments. ANOVA was performed in GraphPad Prism. Values with different letters (a, b,...) indicate significant differences with water and oil absorption capacities of pumpkin seed protein isolate and hydrolysate ( $P > 0.050$ ).

Duncan's test was used to compare the means of all evaluated parameters and the difference between different treatments were evaluated using t-test at probability level of 5 % ( $\geq 5\%$ ).

**Heat stability:** Figure 3 shows changes in the antioxidant activity of hydrolysates after incubation in 100 °C for 0, 15, 30, 60, 90, 120, and 180 minutes. There was no significant difference in the results ( $P > 0.0500$ ). This shows that antioxidant activity in peptides after 180 minutes at 100 °C remains approximately unchanged.

**pH stability:** Changes in antioxidant activity of pumpkin seed protein hydrolysates with pH variation are shown in figure 3. There was no significant ( $P > 0.050$ ) difference in antioxidant activity of samples in different pHs. As antioxidant activity of hydrolysates in most of the pHs had no significant difference with that in the pH of 7, pumpkin seed protein hydrolysates can be used as an antioxidant in food systems in the whole range of pH.

**Amino acid analysis:** Amino acid profile has a key role in the antioxidant activity of peptides.<sup>28</sup> This property has been evaluated to find a relation between antioxidant activity and amino acid content in pumpkin seed protein hydrolysate.



**Figure 3.** Heat (up) and pH (down) stabilities of pumpkin seed protein hydrolysate

Data correspond to the mean  $\pm$  standard deviation (SD) of the three independent experiments. ANOVA was performed in GraphPad Prism. Values with different letters (a, b,...) indicate significant differences with heat (up) and pH (down) stabilities of pumpkin seed protein hydrolysate ( $P > 0.050$ ).

Duncan's test was used to compare the means of all evaluated parameters and the difference between different treatments were evaluated using t-test at probability level of 5 % ( $\geq 5\%$ ). DPPH: 2,2-diphenyl-1-picrylhydrazyl

As can be seen in table 3, glutamic acid, leucine, and aspartic acid are the main amino acids in pumpkin seed protein hydrolysate.

**Table 3.** Amino acid composition of hydrolysed pumpkin seed proteins (mg/100 g)

Amino acid	Protein hydrolysate	FAO
Alanine	3046	
Serine	2889	
Aspartic acid	7049	
Glutamic acid	13643	
Histidine	1772	1900
Tyrosine	3383	6300
Arginine	2575	
Glycine	4029	
Threonine	4417	1400
Methionine	3143	2500
Valine	3344	3500
Leucine	7121	6600
Isoleucine	2722	2800
Lysine	2730	5800
Phenylalanine	4284	
Hydrophobic amino acids	25689	
Total	64449	

FAO: Food and Agriculture Organization

The estimated amount of aspartic and glutamic acid may be more than their real amount, because they may be produced by the deamination of glutamine and asparagine during the acidic treatment of samples.<sup>29</sup> According to table 3, although the amount of histidine is lower than other amino acids, it meets the human need (1.7 g/100 g body weight).

### Discussion

**DPPH radical scavenging activity:** The pepsin enzyme can hydrolyze peptide bounds next to leucine and aromatic amino acids (phenylalanine, tryptophan, and tyrosine), and it has been acknowledged that the terminal phenyl group in the resultant peptide chain has free radical scavenging activity. Furthermore, it has been recognized that proteins' denaturation and loss of natural structure through hydrolysis leads to unfolding and exposure of active amino acids that can react with free radicals. There is a direct relation between free radical scavenging and hydrogen donating of amino acids. The change in peptide chain length during hydrolysis has an important effect on antioxidant activity, and peptides with smaller molecular weight have stronger antioxidant activities.<sup>20</sup>

**Emulsifying capacity of pumpkin seed protein isolate and hydrolysate:** The emulsifying mechanism of protein hydrolysate samples is defined as their adsorption of oil droplets, covering them, and prevention of their association after homogenization.<sup>30</sup> As emulsifying capacity is dependent upon the degree of hydrophobicity and molecular weight of hydrolysates, samples with hydrophobic amino acids and longer chain have higher emulsifying capacity.<sup>30</sup> During hydrolysis, the solubility of hydrolysates increases, and they migrate to the water-oil interface.<sup>27,31</sup>

As protein hydrolysates contain hydrophobic and hydrophilic sequences that are necessary for interfacial properties, these components are considered as surface active agents.<sup>30</sup> During hydrolysis, the balance between hydrophobic and hydrophilic sequences of hydrolysates leads to an increase in the emulsifying capacity of hydrolysates.<sup>31</sup> Emulsifying capacity at the pH of 4 can be related to the lower solubility of protein near the isoelectric point, while the increase in emulsifying capacity of the sample by increasing the pH could be related to negative charges association that not only causes electrostatic repulsion, but also increases the flexibility of protein and facilitates its propagation through the water-oil interface.<sup>31</sup>

**Foaming capacity of pumpkin seed protein isolate and hydrolysate:** Because of their high interfacial activity, proteins are responsible for foaming in flours. Soluble proteins can reduce the surface tension in the fluid-air interface and prevent the association of bubbles. Moreover, protein molecules can unfold, react with each other, and form a multilayer protein film that leads to higher flexibility in the water-air interface; consequently, breaking the bubbles becomes difficult and firm foam is formed.<sup>24</sup>

To show the foaming capacity, proteins have to easily disperse in water, readily migrate to the interface, and easily unfold to form a layer around the air/gas bubbles.<sup>27</sup> Protein flexibility, presence of hydrophobic amino acids, and reduced surface tension are factors that influence foaming properties.<sup>16</sup> The observed increase in foaming capacity of pumpkin seed hydrolysates compared to that of protein isolate might be related to the increase in solubility of hydrolysates due to enzymatic hydrolysis. Klompong et al. studied the enzymatic hydrolysis of yellowstripe scud by alcalase and flavourzyme in different degrees of hydrolysis (5%, 15%, and 25%).<sup>16</sup> They found that foaming capacity is influenced by type of enzyme and degree of hydrolysis, and increasing the degree of hydrolysis decreases the foaming capacity.<sup>16</sup> Increase in foaming capacity due to hydrolysis was also reported by Li et al.<sup>31</sup>

**Water absorption capacity of pumpkin seed protein isolate and hydrolysate:** Water absorption capacity has an important role in viscose systems such as soups and prevents water loss in bakery products like cakes.<sup>27</sup> Water holding capacity is related to the nitrogen solubility index, and higher nitrogen solubility index leads to higher water absorption capacity. As nitrogen solubility increases during hydrolysis,<sup>27</sup> hydrolysates have higher water absorption capacity compared to protein isolate.

Increase in water absorption capacity at a pH of 10 can be related to the increase in repulsion forces due to negative charge in basic pH, and decrease in this capacity can be related to a decrease in solubility around the isoelectric point.<sup>24</sup>

**Oil absorption capacity of pumpkin seed protein isolate and hydrolysate:** Generally, oil absorption is strongly affected by both solubility and surface hydrophobicity of protein<sup>27</sup> and because of the ability of protein to bridge between water and oil, oil absorption capacity has an important role in some food systems such as meat products, especially sausages.<sup>32</sup> Oil absorption is a key factor

in meat substitutes due to the role of this functional property in the enhancement of flavor and generation of a better mouthfeel.<sup>32</sup>

**Heat stability:** The slight change in antioxidant activity of some treatments can be due to the breakdown or aggregation of antioxidant peptides because of the heat. In general, proteins are sensitive to heat, so they aggregate. However, there are reports that smaller hydrolysed proteins are resistant to aggregation in higher temperatures. As this resistance is seen in our samples, these hydrolysates can be used in food processed under heating operations, without significant changes in antioxidant activity. The results of heat stability experiment are similar to those of Nalinanon *et al.* on fish (*Nemipterus hexodon*) hydrolysates.<sup>26</sup>

**pH stability:** Generally, pumpkin seed protein hydrolysates can exhibit 97-99% of their antioxidant activity in the whole pH range. Nalinanon *et al.* evaluated the pH stability of peptides from enzymatic hydrolysis of fish (*Nemipterus hexodon*) muscle by pepsin and reported that radical scavenging activity of peptides was stable at a pH of 1-10, while a slight decrease was observed at a pH of 11. They concluded that antioxidant peptides may be losing some of their activity in higher pHs.<sup>26</sup>

**Amino acid analysis:** Generally, pumpkin seed protein hydrolysates are a suitable source of essential amino acids, threonine, tyrosine, phenylalanine, leucine, cysteine, and methionine, and sufficient source of isoleucine, valine, and lysine that is the first limiting amino acid. In the non-hydrolyzed protein, glutamic acid, leucine, and aspartic acid were also the main amino acids and lysine was the first limiting amino acid. Similar results have been reported by Zhong *et al.*<sup>28</sup> for the amino acid composition of wheat germ protein and hydrolysate, and Khantaphant *et al.*<sup>33</sup> for stripe red snapper brown fish hydrolysate obtained by alcalase and flavourzyme. In the present study, tryptophan content was not estimated due to its sensitivity and decomposition during hydrolysis. According to Bougatef *et al.*, sensitive amino acids such as methionine and tryptophan are rarely observed after protein hydrolysis.<sup>22</sup> Amino acid composition has an important role in antioxidant activity of protein hydrolysates. This factor, to a large extent, depends on the type of protease used for the hydrolysis.<sup>34</sup> Alcalase show high specificity for aromatic (alanine, tryptophan, and tyrosine), acidic (glutamic acid), sulfur containing (methionine), aliphatic (leucine, and isoleucine), hydroxylated (serine), and basic (lysine) amino acids.<sup>35</sup> It has been approved that

aspartic and glutamic acid,<sup>34</sup> in addition to prolamins, arginine, methionine, histidine, leucine, isoleucine, alanine, tyrosine, and valine,<sup>36</sup> have strong antioxidant activities. According to the amino acid content of pumpkin seed's hydrolysates, it is clear that, in addition to the amount of aspartic acid and glutamic acid, there is also a correlation between antioxidant activities and amount of leucine, Phenylalanine, Threonine and Glycine. Furthermore, the hydrophobic amino acid content of hydrolysed pumpkin seed proteins, which positively correlates with its antioxidant activity, was estimated as 25689 mg/100 g that accounts for 39.85% of total amino acid content of pumpkin seed hydrolysates. According to the amino acid scoring pattern for adults provided by the Food and Agriculture Organization (FAO) of the United Nations, although the tyrosine, histidine, and lysine content of hydrolysed pumpkin seed proteins is less than the suggested amount, it can be a balanced source of essential amino acids (threonine, methionine, valine, leucine, isoleucine, and phenylalanine); therefore, it can provide the major amino acids requirement for better health performance, and can be used as dietary supplement in diets contain food protein's deficient in essential amino acids.

## Conclusion

Application of antioxidants is a way of extending the shelf life of high fat foods. Presently, the application of synthetic antioxidants is decreasing due to their chemical nature and risk of being carcinogenic. Bioactive protein hydrolysates obtained from enzymatic hydrolysis are components that can be a good substitution for synthetic antioxidants in foods, and can be used as effective antioxidants and antihypertensive agents in preventing cardiovascular disease. Pumpkin seed protein hydrolysates contain a large amount of hydrophobic amino acids and the high antioxidant activity of these hydrolysates can be related to their hydrophobic sequences. Furthermore, the results showed that enzymatic hydrolysis can improve functional properties such as emulsifying and foaming capacities and water and oil absorption in food formulations like meat and bakery products and some protein food supplements, and thus, improve their physical and chemical properties and extend their shelf life. Pumpkin seed hydrolysates contain essential amino acids (except lysine), so they can be a suitable source of such amino acids according to FAO's amino acid scoring pattern for adults.



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### Conflict of Interests

Authors have no conflict of interests.

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