Expeller-pressed pomegranate seed (Punica granatum L.) as a protein source for the production of antioxidant peptides

Maryam Rahimipanah
Gorgan University of Agricultural Sciences & Natural Resources
Alireza SadeghiMahoonak (sadeghiaz@gau.ac.ir)
Gorgan University of Agricultural Sciences & Natural Resources
Mohammad Ghorbani
Gorgan University of Agricultural Sciences & Natural Resources
Hoda Shahiri Tabarestani
Gorgan University of Agricultural Sciences & Natural Resources
Mohsen Nabimeybodi
Shahid Sadooghi University of Medical Sciences

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Abstract

Because of health concerns regarding synthetic antioxidants, natural antioxidant compounds are being considered by scientists. Bioactive peptides have been shown to have various physiological functions, such as antioxidative activity. Pomegranate seed protein, the by-product of the pomegranate seed oil industry, can be a good source of bioactive peptides. The optimum conditions for enzymatic hydrolysis of pomegranate seed protein with alcalase were determined using a response surface methodology. The influence of different temperatures (45-55 °C), times (30-180 min), and enzyme to substrate (E/S) ratio (1-3% w/w) on DPPH scavenging power and ferric reducing activity as the responses, were studied. Also, the degree of hydrolysis and the surface hydrophobicity of samples were determined. Moreover, using scanning electron microscopy and electrophoresis technique, microscopic structure and molecular weight of hydrolysate, were studied respectively. Alcalase-derived hydrolysates showed a DPPH scavenging activity (88 ± 0.97 %) and ferric reducing power (0.5 ± 0.83) at optimum conditions of hydrolysis (48.8°C, 97.5 min, E/S ratio 1.3%w/w). The degree of hydrolysis coincided with 36 ±1.2%. An increase in the surface hydrophobicity of the protein during hydrolysis confirmed the unfolding of the pomegranate seed protein structure. The presence of low-molecular-weight peptides was evidenced by the electrophoresis technique. As well as the SEM showed that protein fragments had been reduced to small sizes following enzymatic treatments. According to the results of this study, pomegranate seed protein hydrolysate can be considered a suitable source of antioxidants with an aggregate market value in food formulations

Introduction

Plants are one of the most abundant sources of biopolymers, especially protein. As long as the protein structure is intact, its amino acid sequence is inactive; however, during proteolysis, fermentation, and gastrointestinal digestion, these amino acids are released as oligopeptides ordinally with less than 20 amino acids and below 10 kDa in molecular weight. These peptides are more digestible and can affect biological responses and can exhibit specific bioactive properties such as mineral binding, antimicrobial, immunomodulatory, anti-thrombotic, antihypertensive, and antioxidant properties. In general, hydrolyzed proteins and peptides with specific bioactive sequences decrease the risk of many diseases, such as cancer, diabetes, osteoporosis, and cardiovascular disease, and enhance immunity against infection (Aluko 2015; Chalamaiah et al. 2018; Teshnizi et al. 2020).

The most important free radicals produced in many biological and chemical systems are in different forms of free radicals are reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anions, peroxyl radicals, and nitric oxide radicals. Oxidative stress results from an imbalance between free radicals and antioxidants when ROS in the body increases. A variety of diseases are associated with oxidative stress, including cancer, cardiovascular disease, and accelerated aging. Sometimes external antioxidants are needed outside the body because there are not enough endogenous antioxidants to inhibit free radicals. Because synthetic antioxidants could adversely affect the human body, many researchers have worked to identify and extract antioxidant compounds from natural resources. In this regard, the production of antioxidant peptides as a new generation of natural antioxidants has received much attention(Homayouni-Tabrizi et al. 2017). Many studies have evaluated the antioxidant capacity of protein hydrolysates or

peptides from plant sources, such as tomato seed (Meshginfar et al. 2018), rice bran (Singh et al. 2018), Olive Pomace (Fathi et al. 2022), mung bean (Xie et al. 2019), and pomegranate peel (Hernández-Corroto et al. 2020).

The pomegranate (*Punica granatum L.*) is a member of the family Punicaceae and an important commercial fruit grown in Asia, the Mediterranean, the Middle East, and North Africa (Ozgen et al. 2008; Tehranifar et al. 2010). In the world, Iran is one of the largest producers and exporters of pomegranates, producing nearly 47% of global pomegranate production (Derakhshan et al. 2018). The pomegranate seeds are a by-product of the pomegranate industry and allocate about 9.5–20.5% of the total weight of the fruit (Tehranifar et al. 2010). Pomegranate seeds' protein is rich in essential amino acids, making it an excellent source of protein for human consumption (Kandylis and Kokkinomagoulos 2020).

Producing protein hydrolysates with antioxidant activity adds nutritional value to the by-products of the pomegranate oil industry. According to our knowledge, there isn't any data about the enzymatic hydrolysis of pomegranate seed protein for antioxidant peptides production. This study aimed to determine the feasibility of producing protein hydrolysates from defatted pomegranate seed meals to produce an effective nutritional supplement.

Materials And Methods

Materials

Pomegranate seed meal was provided as a cold press by-product from the Niriorganic company (Yazd, Iran). The chemicals including alcalase 2.4 L. (serine-protease, *Bacillus licheniformis*), DPPH (1,1-Diphenyl-2-picrylhydrazyl), ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)], TCA (trichloroacetic acid), Coomassie brilliant blue (G250), BSA (bovine serum albumin), ANS (8-Anilino-1-naphthalenesulfonic acid ammonium salt), ascorbic acid, and potassium persulfate were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Ferrozine, potassium ferricyanide (III), trichloroacetic acid, and ferrous chloride ferric chloride (FeCl3) were purchased from Merck Co. (Darmstadt, Germany).

pomegranate seed protein isolation

The meal was crushed into powder with an electric mill (Moulinex, France) and sieved with a No. 18 mesh size. The powder was mixed with hexane (1:3 w/v) in a shaker for 3 hours at 25^0C, and 150 rpm to separate the remaining fat. After 48 hours, all solvents were removed at ambient temperature (Singh et al. 2018). Proteins from pomegranate seed meal were isolated using alkaline extraction and acid precipitation. In this method, protein is dissolved in an alkaline solution at a specific pH. The pH is then adjusted with acids until the isoelectric point is reached, after which the proteins are separated (Gerzhova et al. 2016; Olivares-Galván et al. 2020). Extractions of the dry meal in water at 1:10 (w/v), were performed at pH ranging from 1 to 12 under magnetic agitation at room temperature for 60 min to determine the protein solubility at different pH values. HCl (1 N) and NaOH (1 N); were used for the pH adjustment. The supernatant was separated by centrifuging for 20 min at 10000×g and 4^0C (Thermo Fisher Scientific, Germany) for protein quantification according to the method of Bradford (1976) (Bradford 1976) using BSA as standard (Nioi et al. 2012).

Protein extraction at the isoelectric point was followed by neutralizing to pH 7.0, freezing the pellet, and storing it at 18°C until use.

Experimental design for optimization

Different hydrolysis parameters, i.e., temperature (X_1), time (X_2), and enzyme to substrate ratio (X_3), were varied to calculate the combined influence of these independent variables on two response variables, i.e., DPPH scavenging power (Y_1) and ferric reducing ability (Y_2) under appropriate pH. A Face-Centered Central Composite Design was applied to investigate the process and optimize different hydrolysis parameters with response surface methodology (RSM). The symbols and levels are shown in Table 1. Parameters ranges were selected based on the preliminary experimental results. According to Design Expert (Ver. 10), 20 experimental points of the independent variables were selected.

Table 1
Independent variables and their levels used in the RSM

Independent Variable	Symbol	Coded variable levels		
		-α (-1)	0	+α (+ 1)
Temperature (^{0}C)	X_1	45	50	55
Time (min)	X_2	30	105	180
Enzyme to Substrate Ratio (%w/w)	X_3	1	2	3

A second-order polynomial equation expressed the DPPH (Y_1) and ferric reducing power (Y_2) as follows:

$$Y = \beta 0 + \sum_{i=1}^{3} \beta i Xi + \sum_{i=1}^{3} \beta ii Xii + \sum_{i=1}^{2} \sum_{j=2}^{3} \beta ij Xi Xj \quad \text{Eq. 1}$$

Y represents the response variable, β0 is a constant, β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interactive coefficients, respectively, and X_i and X_j are independent variables. Star points were carried out using α = 1. The coefficient of determination (R^2), adjusted R^2, lack of fit, and coefficient of variance of the predicted values were calculated based on the regression equation. Analysis of variance (ANOVA), was used to determine the significance of the model and its terms. Running the design at the central point allowed the estimation of possible pure errors. Surface graphs were generated by varying two of the variables within the experimental range and keeping the remaining variable constant at the central point. For the statistical significance test based on the total error criteria, the confidence level was 95%. The Design Expert analyzed the data using numerical optimization to get the most acceptable result.

pomegranate seed protein concentrate was dissolved in phosphate buffer (20 mM, pH = 8) at a 1:20 (w/v) ratio, blended with the enzyme, and incubated as per experimental design. The agitation level and reaction temperature were controlled at 200 rpm by a shaking incubator. This reaction was terminated by heating at 85°C for 15 min, then cooling and centrifuging at 4°C for 15 min at 10000×g (Hettich, Germany). The supernatant containing the peptides was freeze-dried (Christ, Germany) for analysis (Sierra-Lopera and Zapata-Montoya 2021).

DPPH radical scavenging activity

A method described by Brand-Williams et al. (Brand-Williams et al. 1995) was used to analyze the scavenging activity against DPPH free radicals. 1.5 ml of the ethanolic DPPH radical solution (0.2 mM) was added to 1.5 ml peptide-containing solution (10 mg/ml). After 40 min incubation under dark conditions, followed by centrifugation at 4000×*g* for 10 min, absorbance was measured at 517 nm. The DPPH radical scavenging activity was estimated using the following formula:

DPPH radical scavenging activity (%) = [1- (sample absorbance/blank absorbance)] × 100 Eq. 2

Ferric Reducing Power Assay

The reducing power was measured according to Oyaizu (Oyaizu 1986). A total of 100 µL of samples (10 mg/ml) were mixed with phosphate buffer (500 µL, 0.2 M, pH 6.6) and potassium ferricyanide (500 µL, 1%). After the mixture was incubated at 50°C for 20 min, 500 µL of TCA (10%) was added to this reaction mixture, and then centrifugation at 6000×g for 15 minutes was done. The supernatant (1 ml) was mixed with 1 ml of distilled water and 1 ml of 0.2 ml of 0.1% ferric chloride and absorbance was measured at 700 nm after 10 min incubation. An increase in the absorbance of the reaction mixture indicated the reducing power of samples.

ABTS radical scavenging activity

ABTS radical was generated by mixing ABTS solution (7 mM) with potassium persulfate (2.45 mM final concentration) and keeping it for 14–16 h in the dark to produce stable radicals at ambient temperature. After diluting the stock solution, the ABTS radical solution having an absorbance of 0.70 ± 0.02 at 734 nm was prepared. then aliquots of the samples (30 µL) were added to 3 mL of ABTS diluted solution. Absorbance readings were recorded at 734 nm. The ABTS radical inhibition was calculated as follows (Re et al. 1999):

ABTS radical scavenging activity (%) = [1- (sample absorbance/blank absorbance)] × 100 Eq. 3

Fe2 + chelating assay

The Fe^{2+} chelating activity was determined using the method of Decker and Welch (Decker and Welch 1990). 1 mL of sample solution was premixed with 1.85 mL of distilled water and 0.05 mL of iron (II) chloride solution (2 mM). Afterward, 0.1 mL of Ferrozine solution (5 mM) was added. After standing for 10 minutes, the absorbance of the mixture was measured at 562 nm. The chelating activity was determined as:

Fe^{2+} Chelating activity (%) = [1- (sample absorbance/blank absorbance)] × 100 Eq. 4

Degree of Hydrolysis (DH)

The degree of hydrolysis was estimated as trichloroacetic acid (TCA) solubility index and expressed as nitrogen soluble in 10% TCA (Hoyle and Merritt 1994). 10 mL of hydrolysate was mixed with 10 mL of TCA, strongly mixed, and then centrifuged for 10 min at 10000·g. The degree of hydrolysis was calculated

regarding the amount of protein in supernatant containing TCA to the protein present in the suspension, and they were reported in percentages. The soluble proteins were determined by the Bradford method.

Molecular weight distribution

The molecular size distributions of pomegranate seed proteins and their hydrolysates were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Aliquots of the sample solutions (5 mg mL^{-1}) were mixed with 30 µL of buffer (50 mmol/L Tris pH 6.8, 4% SDS, 0.01% bromophenol blue, 20% glycerol, and 10% β-mercaptoethanol) and heated for 5 min at 90°C. Samples were homogenized and allowed to cool down at room temperature. 10 µL of the samples were loaded onto 15% separating gel. After a constant current (100 mA for 2–2.5 h), gels were stained overnight with a solution of Coomassie Brilliant Blue G-250 and were destained (15%, v/v, methanol, and 10%, v/v, acetic acid). Using protein markers (180 − 11.0 kDa), the MW of peptides was determined.

Surface hydrophobicity

Surface hydrophobicity of pomegranate seed proteins and their hydrolysates using the fluorescence probe, 8-anilino-1-naphthalene sulfonate (ANS), was determined according to a method as described in the literature (Kato and Nakai 1980). In brief, 300 µL of peptide (0.05–1 mg/ml), and 6 µL of ANS (8 mM), were added to the well of a 96-well black plate. The plate was shaken slightly for 3 min, and the fluorescence intensity was measured with a Synergy™ HTX Multi-Mode Microplate Reader (BioTek Instruments Inc.), at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The initial slope of the fluorescence intensity versus sample concentration (mg/mL) plots was used as a surface hydrophobicity index.

Scanning electron microscopy (SEM)

The morphology of pomegranate seed protein before and after enzymatic hydrolysis was observed using a scanning electron microscope (FEI ESEM QUANTA 200), at an accelerating voltage of 25.0 kV and objective aperture of 20 µm. All freeze-dried samples were coated with a gold layer (COXEM). The SEM images of the samples were examined at 1000× magnification (Islam et al. 2021).

Results

Effect of pH extraction on protein content

BSA equivalent concentration in the liquid phase at different pH, showed a high alkaline medium, enabling more protein to be dissolved. However, an increase in pH might harm the essential amino acids might reduce digestibility and biological value (Gerzhova et al. 2016). So, pH = 10.0 was used for the protein extraction. Traditionally alkaline extraction is followed by isoelectric precipitation. Our results showed that the pattern of protein solubility of pomegranate seed proteins was U-shaped with an apparent dip in solubility around pH 4–5, especially at pH 4.4. These results are consistent with the reports of most research in which the isoelectric pH range for most plant proteins was considered to be at pH = 4–5 (Gerzhova et al. 2016; Olivares-Galván et al. 2020). According to the results, the pomegranate seed protein concentrate contains high protein content (70.2 ± 0.36%).

Optimization of enzymatic hydrolysis using RSM

The pomegranate seed protein was hydrolyzed using alcalase, to obtain the optimum condition of hydrolysis. Alcalase is an endopeptidase and a low-specificity food-grade protease to releases several short peptides at a cost-effective rate (Olivares-Galván et al. 2020).

The selected experimental combinations were presented in Table 2. For the response variables, second-order models were developed that included constant, linear, quadratic, and interaction terms.

Table 2
Central composite arrangement for independent variables

	Variable Levels			Response	
Run	X_1	X_2	X_3	Y_1	Y_2
1	50	105	2	87.1	0.512
2	45	180	1	83.9	0.52
3	50	180	2	86	0.5
4	50	105	2	85.6	0.5
5	50	105	2	86	0.52
6	45	30	1	82.9	0.49
7	55	30	1	78	0.42
8	55	105	2	77.9	0.44
9	45	105	2	83	0.51
10	50	30	2	86.7	0.516
11	45	180	3	82.1	0.502
12	50	105	2	88.6	0.51
13	45	30	3	80	0.48
14	50	105	2	84.7	0.519
15	55	180	1	75.4	0.39
16	50	105	2	86.6	0.511
17	55	180	3	70	0.4
18	50	105	3	84.8	0.523
19	55	30	3	71	0.41
20	50	105	1	87	0.513

X_1 (temperature, °C), X_2 (time, min), X_3 (enzyme to substrate ratio, %) and their responses Y_1(DPPH scavenging, %) and Y_2 (Ferric reducing power, absorbance at 700 nm)

The results of the analysis of variance (ANOVA) demonstrate that all statistical models are significant ($p < 0.01$). The lack of fit tests, which indicate the fitness of the models obtained, was non-significant. The coefficients of variance were less than 10% which meant that the models were considered reproducible and can be used to optimize hydrolysis conditions (Table 3).

Table 3
ANOVA for the response surface quadratic polynomial model

	Y1			**Y2**		
Source	F Value	p-value	coefficient	F Value	p-value	coefficient
Model	33.52**	< 0.0001		37.10**	< 0.0001	
X1	89.51**	< 0.0001	-3.96	175.16**	< 0.0001	-0.044
X2	0.082 NS	0.7802	-0.12	0.014 NS	0.9070	-0.0004
X3	21.26**	0.0010	-1.93	0.29 NS	0.6017	-0.0018
X1X2	3.20 NS	0.1038	-0.84	9.49*	0.0116	-0.011
X1X3	4.23 NS	0.0667	-0.96	0.88 NS	0.3707	+ 0.0035
X2X3	0.52 NS	0.4873	+ 0.34	0.16 NS	0.6963	+ 0.0015
$X1^2$	70.08**	< 0.0001	-6.68	52.79**	< 0.0001	-0.046
$X2^2$	0.96 NS	0.3504	-0.78	4.34 NS	0.0638	-0.013
$X3^2$	2.38 NS	0.1538	-1.23	0.26 NS	0.6185	-0.00327
Lack of Fit	0.94 NS	0.5284		3.26 NS	0.1104	
R-Squared	0.9679			0.9709		
Adj R-Squared	0.9390			0.9447		
C.V. %	1.61			2.18		

**: $p < 0.01$, *: $p < 0.05$, NS: Non-significant, X1: Temperature, X2: Time, X3: Enzyme to Substrate ratio, Y1: DPPH scavenging power, Y2: ferric reducing power, C.V.: Coefficient of variance

Regression coefficients showed a significant effect ($p < 0.01$) of temperature (X_1) in linear and quadratic terms and E/S ratio (X_3) in linear terms on the DPPH scavenging power values. The coefficient of determination values was 0.967. The predicted R^2 of 0.868 agrees with the adjusted R^2 of 0.939. Also, regression coefficients showed that temperature had a linear and a quadratic highly significant effect on the

ferric reducing ability values, and the interaction terms of temperature and time (X_1X_2) had a significant ($p < 0.05$) effect. The coefficient of determination values was 0.970. The predicted R^2 of 0.805 is reasonable, with the adjusted R^2 of 0.944.

A high R^2 value, a lack-of-fit that was non-significant, and an adjusted R^2 near 1 suggest these models are applicable for explaining a relationship between the independent variables and the responses.

Analysis of response surface graphs

According to the model, three-dimensional response surface graphs were drawn by varying two independent variables and keeping another independent variable at the central point (Fig. 1).

To investigate the effect of temperature as one of the independent variables, in Fig. 1, diagrams (a) and (b), which respectively show the interaction of temperature-time, and temperature-ratio of the enzyme to the substrate on DPPH scavenging activity, and Also, diagrams (e) and (d), which respectively show the interaction of temperature-time, and temperature-ratio of the enzyme to the substrate on ferric reducing power were studied. Studies have shown that enzymatic reactions are affected by temperature. The optimal temperature for enzyme activity can differ for different substrates, enzyme stability, substrate availability, and by-product formation may affect enzymatic reaction rate (Singh et al. 2018). In current study, Up to 48.8°C, the DPPH scavenging and ferric reducing power increased but then decreased rapidly. In general, the hydrolysis rate goes up with temperature because of exposed peptide bonds. At a lower temperature, the reduced antioxidant activity could be due to incomplete hydrolysis, and at a higher temperature, it could be due to thermal denaturation and the loss of enzyme activity (Singh et al. 2018; Wang and Shahidi 2018). Similar behavior was observed for the proteolysis of bovine plasma protein (Seo et al. 2015), turkey meat (Wang and Shahidi 2018), and Mantle of Cuttlefish (Hamzeh et al. 2019).

A non-linear effect of time on DPPH scavenging power and ferric reducing power was observed in the range of 30–180 min by studying the corresponding interaction diagrams (Fig. 1 (a, c, d, f)). An increase in DPPH scavenging activity and ferric reducing power is achieved by increasing reaction time up to certain levels and then slightly decreasing. This may be because longer treatment results in the hydrolysis of the antioxidant peptides into the production of hydrophilic smaller peptides, which have a lower ability to quench a hydrophobic DPPH molecule and deactivate the enzyme during a long period (Zhuang et al. 2013; Singh et al. 2018; Hamzeh et al. 2019). Similar dependence has been observed for hydrolytic reactions of Mantle of Cuttlefish (Hamzeh et al. 2019), rice bran (Singh et al. 2018), and turkey meat (Wang and Shahidi 2018). The optimal enzymatic hydrolysis period was demonstrated to be 97.5 min by this study.

As shown in Figs. 1 (b, c, e, f), the non-linear effect on DPPH scavenging activity and ferric reducing power, was revealed by the ratio of E/S. Increasing the E/S ratio (up to 4) led to hydrolysates with higher antioxidant activity, but increasing the E/S ratio further did not result in a significant increase in antioxidant activity ($P > 0.05$).

Increased enzyme concentration increases the availability of enzyme active sites, leading to significant degradation of the proteins and cleavage of peptide bonds (Kurozawa et al. 2008). As E/S ratios increase to a certain extent, excessive hydrolysis decreases the chances of enzyme-substrate interactions, eventually

leading to a stationary phase without evidence of hydrolysis. The profile could result from substrate oversaturation resulting in inhibition of the enzyme or auto-digestion (Singh et al. 2018). Enzymatic hydrolysis of flying squid muscle protein (Fang et al. 2012), corn gluten meal (Zhuang et al. 2013), and porcine liver (Maluf et al. 2020) exhibited similar patterns. This study showed that the optimal enzyme/substrate ratio for enzymatic hydrolysis was 1.3% (v/w).

Validation Test

Based on the combination of all the optimal regions, the maximum antioxidant activity of hydrolysates was achieved at a temperature of 48.8°C with a reaction time of 97.50 min and an E/S ratio of 1.3% (w/w). Polynomial models predicted optimal values of DPPH scavenging power and ferric reducing power at the suggested conditions. Samples were analyzed under optimal conditions to obtain experimental values. The results are shown in Table 4. The response predicted by the model showed close agreement with the experimental data. Thus, optimum conditions of hydrolysis were validated by RSM.

Table 4
Experimental and corresponding predicted values for DPPH scavenging power and Ferric reducing power using optimum values of independent variables (n = 3).

Enzyme	Temperature	Time	E/S ratio	DPPH scavenging power		Ferric reducing power	
	^0C	min	%w/w	Predicted	Experimental	Predicted	Experimental
Alcalase	48.8	97.5	1.3	87.87 [a]	88 ± 0.97[a]	0.523 [b]	0.50 ± 0.83[b]

Those with different letters are significantly different, with $p < 0.05$. Comparisons were made between the observed and predicted values for each correspondent response.

Degree of Hydrolysis

The degree of hydrolysis (DH) is an essential factor in tracing or controlling protein hydrolysis reactions, as it is related to chain lengths and peptide cleavage rates. Furthermore, DH is proportional to peptide size or structure, affecting amino acid exposures, biological activities, and the taste of peptides (Akbarbaglu et al. 2019; Cotabarren et al. 2019; Fathi et al. 2022). The DH is determined by the patterns of cleavage and enzyme specificities involved in the hydrolysis. Hydrolysis occurs when a hydrolyzing enzyme is accessible to the scissile peptide bonds. Similarly, the enzyme's affinity for binding substrates, the shape, and structure of its active sites, and how the peptide bond is oriented also play a part (Mirzapour et al. 2016). According to the results, DH of pomegranate seed protein with alcalase is 36 ± 1.2%. Similar results have been reported in the hydrolysis of Iranian wild almond (Mirzapour et al. 2016) and flaxseed protein (Akbarbaglu et al. 2019).

EC_{50}

Antioxidant activity of the pomegranate seed protein and its hydrolysates (produced in optimized conditions) was evaluated by the ABTS, DPPH radicals scavenging, and Fe^{2+} chelating methods. the dose-response curve was used to estimate the EC_{50} value of samples. As an indicator of antioxidant capacity, EC_{50} is a concentration of samples that can scavenge 50% of total radicals. Thus, a lower EC_{50} value indicates better

free radical scavenging abilities. As shown in Table 5 for all methods, hydrolysates showed a significantly lower EC_{50} than non-digested proteins. Other studies also have demonstrated enhanced antioxidant activity by the enzyme hydrolysis of food proteins for a variety of reasons, including the size of peptides or general hydrophobicity (Cotabarren et al. 2019).

Table 5
Comparison of EC50 using different antioxidant test

Test	Non-hydrolyzed	Alcalase hydrolysate	Ascorbic Acid
DPPH radical scavenging activity (mg/ml)	1.2 ± 0.07 [a]	0.18 ± 0.015 [b]	0.0094 ± 0.004 [c]
ABTS radical scavenging activity (mg/ml)	1.3 ± 0.15 [a]	0.4 ± 0.08 [b]	0.027 ± 0.001 [c]
Fe chelating (mg/ml)	1.03 ± 0.3 [a]	0.22 ± 0.07 [b]	0.12 ± 0.06 [b]
There is a significant difference between those with different letters ($p < 0.05$)			

SDS-PAGE patterns of hydrolysates

The molecular weight patterns were characterized by SDS-PAGE. The electrophoretic profile of denatured pomegranate seed protein resulted in bands spanning mainly at 15–75 kDa. The majority showed a molecular weight higher than 20 kDa, and two strong bands of intensity between 20 to 25 kDa and 35 to 48kDa were identified (Fig. 3). Smaller peptide bands and lower band intensity, along with losing some of the peptide fractions, confirm the efficacy of alcalase at cleaving. The electrophoretic patterns of the hydrolysates show peptides at a molecular weight below 11 kDa. The method couldn't separate peptides smaller than 11 kDa.

Other researchers showed that alcalase could hydrolyze the proteins and produce small peptides. The enzyme hydrolysis of peptides releases bioactive peptides with a variety of physiological properties, and these low molecular weight peptides are capable of passing through the intestine and demonstrating biological properties (Mirzapour et al. 2016; Singh et al. 2018; Zang et al. 2019; Teshnizi et al. 2020).

Surface hydrophobicity

The Surface hydrophobicity of pomegranate seed protein and its hydrolysate were compared. ANS was used as a fluorescent probe to measure the fluorescence intensity. As shown in Fig. 4, the enzymatic hydrolysis greatly ($P < 0.05$) improved the surface hydrophobicity.

A similar trend was found in the enzymatic hydrolysis of tomato seed protein [9]. pomegranate seed protein has a low hydrophobic value since it is composed of intact and folded proteins that have the majority of hydrophobic residues in the core site rather than on the surface for stability. Enzymatic hydrolysis may affect the surface hydrophobicity, so the increase in surface hydrophobicity may be explained by the exposure of buried hydrophobic groups. As a result, when polypeptide chains are broken down, nonpolar amino acids are exposed, causing an increase in hydrophobicity. Hydrophilic acidic and basic amino acids, when located close together, can neutralize each other electrostatically, which also results in greater hydrophobicity. In

hydrolyzed proteins, this factor might explain the high surface hydrophobicity. However, longer hydrolysis times can reduce the hydrophobicity by removing nonpolar amino acids. Furthermore, it is thought that the high hydrophobicity of peptides enhances their ability to scavenge free radicals (Meshginfar et al. 2018; Zang et al. 2019). Our results showed that produced hydrolysates have a higher hydrophobicity and higher antioxidant power than intact protein.

Scanning electron microscopy

Figure 5 shows SEM images of hydrolyzed and unhydrolyzed samples. unhydrolyzed protein exhibited complex structures comprised of random sheets of different sizes and shapes. According to SEM images, the protein degraded into small fragments and looser structure with many folds after enzyme hydrolysis, resulting in smaller particles compared with untreated samples with the same SEM parameters. The results are similar to other studies that reported protein had degraded into small fragments, and particle size had been reduced after enzyme treatment (Islam et al. 2021; Fathi et al. 2022).

Conclusion

Pomegranate seed protein concentrate was hydrolyzed enzymatically using alcalase. The effect of independent variables on antioxidant activity was explained. The best conditions for Pomegranate seed protein hydrolysis included a temperature of 48.8°C, hydrolysis period of 97.5 min, and E/S ratio of 1.3 (w/v) which were experimentally validated. The DH was 36 ± 1.2%. Lower EC50 of hydrolysates showed that hydrolysis increases the antioxidant activity significantly. Low molecular weight peptides (< 11.0 kDa) became more intense after cleavage of high molecular weight proteins. The degradation of protein into small fragments was confirmed by SEM. Our results indicated that hydrolysis using alcalase could produce bioactive peptides with antioxidant activities and higher hydrophobicity. Considering the consumer's tendency toward functional foods and present concerns about the 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. In addition, there will be a reduction in waste generated by the pomegranate processing industry. Further studies will need for the isolation and identification of peptides and the evaluation of their possible incorporation in food matrices.

Declarations

Compliance with Ethical Standards

Conflict of interest: The authors declare no conflict of interest.

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Figures

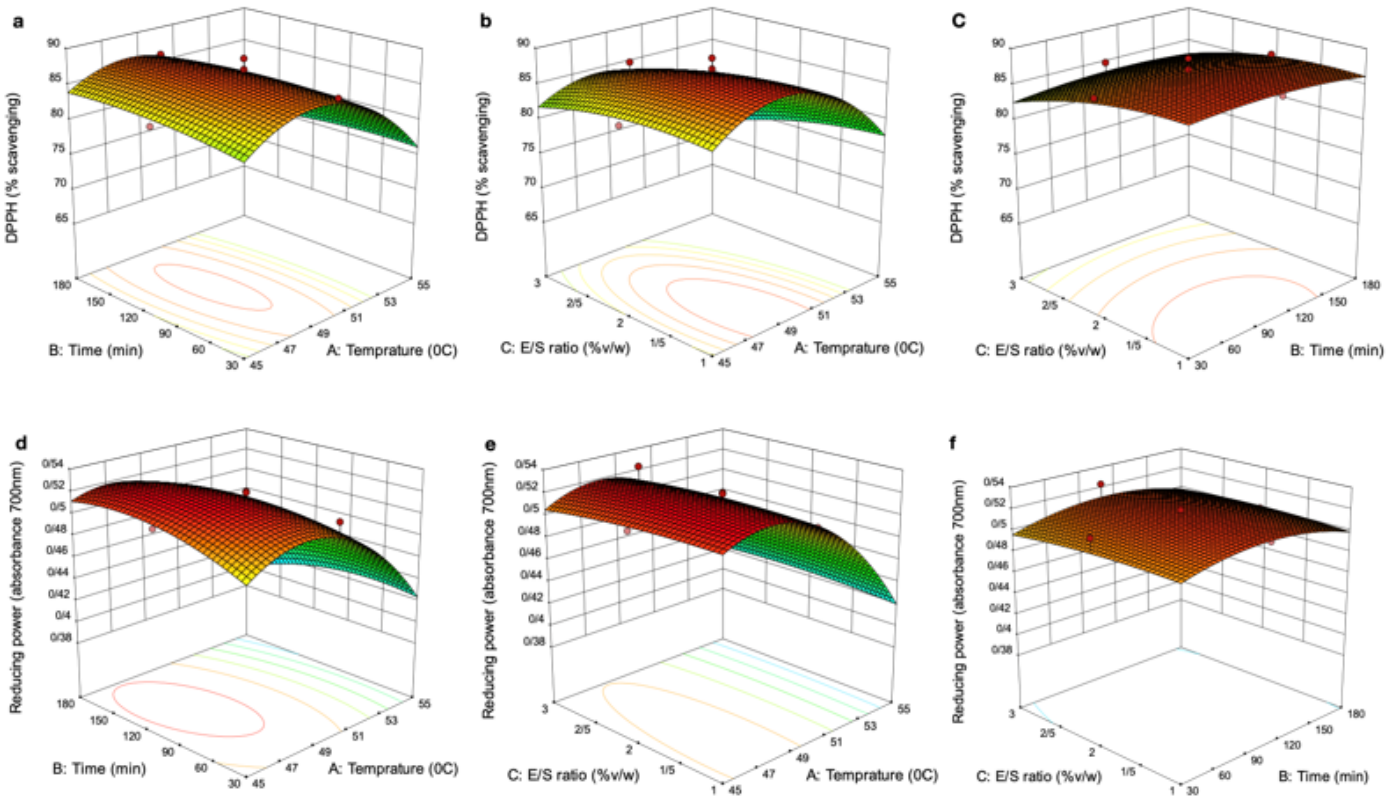

Figure 1

Response surface plots of independent variables on DPPH scavenging power (a, b, c) and on Reducing Power (d, e, f) as a function of various hydrolysis conditions: temperature (A), time (B), and E/S (C)

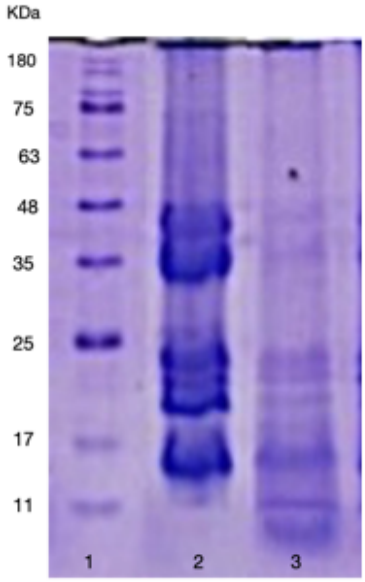

Figure 2

The electrophoretic profiles of pomegranate seed protein and its hydrolysate on SDS-PAGE; 1: Marker, 2: Denatured protein extract, 3: Alcalase hydrolysate

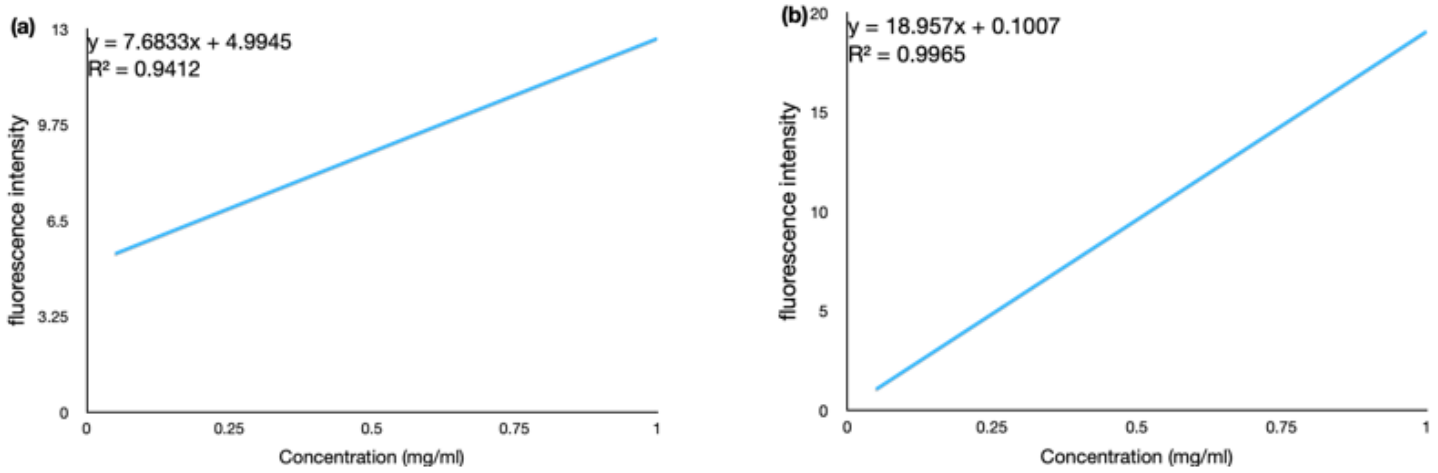

Figure 3

Surface hydrophobic index determined as relative fluorescence intensity versus different concentrations. (a) Unhydrolyzed

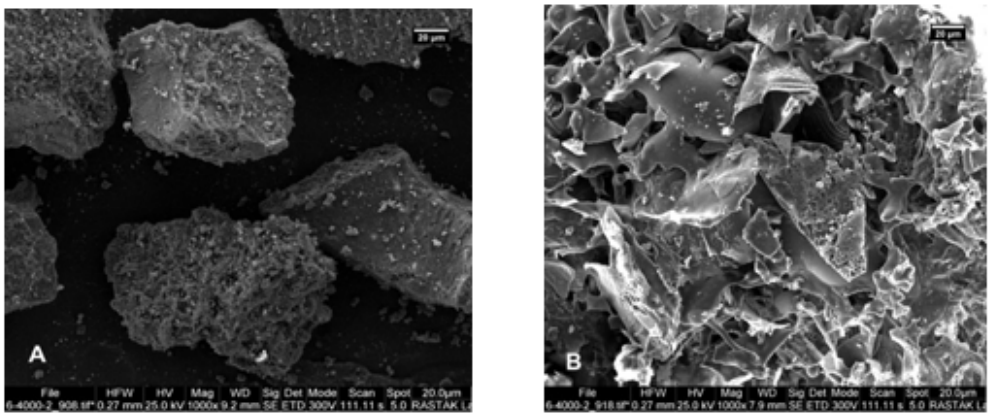

Figure 4

Scanning electron microscope of pomegranate seed protein (A) and its hydrolysates obtained after treatment with alcalase (B) Images with a 20 µm scale bar provided were taken at a magnification of 1000.