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Optimized Enzymatic Hydrolysis of Olive Pomace Proteins Using Response Surface Methodology

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Abstract

Background and Objective: Olive pomace is a by-product of olive oil extraction used in livestock and poultry feeds. Nevertheless, it can be a valuable source for the production of bioactive peptides, which include positive effects on the natural functions and health of the body. The objective of this study was to optimize enzymatic hydrolysis conditions of olive pomace protein for the production of hydrolysates with the highest degrees of hydrolysis and investigation of bioactive characteristics of the treatments under optimal conditions.

Material and Methods: After extraction of proteins from olive pomace, response surface method based on the Box-Behnken design was used to optimize the enzymatic hydrolysis of olive pomace protein by trypsin. Degrees of the hydrolysis were considered as response. Antioxidant activity in the hydrolysates was assessed using 2,-2-diphenyl-1-picryl-hydroxyl radical scavenging and iron (II) chelating activity. Antiproliferative activity was assessed on human breast cancer cells (MCF-7) using 3-(4,-5-dimethylthiazol-2-yl)-2,-5-diphenyl tetrazolium bromide assay. Microscopic structure and amino acid content were studied using scanning electron microscopy and reverse phase-high performance liquid chromatography, respectively.

Results and Conclusion: The optimal hydrolysis conditions were as follows: time of 5 h, pH 8.5 and the temperature of 39 °C. Under these conditions, the maximum degree of hydrolysis was achieved as 70.62%. Furthermore, 2,-2-dipheny1-1-picryl-hydroxyl radical scavenging activity and iron (II) chelating activity of the olive pomace protein hydrolysate under optimal conditions respectively were 42.26% ± 2.46 and 30.91% ± 0.26 at a concentration of 3.5 mg ml^{-1}. Although, antioxidant activity of the protein hydrolysate was significantly lower than that of ascorbic acid and ethylene-diaminetetraacetic acid. The highest antiproliferative activity of the olive pomace protein hydrolysate on MCF-7 breast cancer cells was observed at a concentration of 80 mg ml^{-1} and the proportion of cell viability was achieved at 24 and 48 h at 15.06% ±0.40 and 9.71% ±1.32, respectively. Microscopic analysis of the hydrolysate verifies that the enzyme trypsin was able to hydrolyze large protein molecules, converting them into small fragments. The amino acid analysis showed increases in hydrophobic and aromatic amino acids in the hydrolyzed protein. As a conclusion, protein hydrolysates from olive pomace with antioxidant and antiproliferative potentials can be used as functional additive foods in livestock.

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1. Introduction

Olive pomace is a solid by-product of olive oil extraction, which is dark in color and consists of pulp, skin and stone [1]. Olive pomace is lignocellulosic; 90% of its dry weight consists of cellulose, hemicellulose and lignin with a partial quantity of protein. Olive pomace also known as olive cake is produced in high quantities with low price and, can represent a cheap source of dietary fibers and proteins for use in agriculture and aquaculture. According to the Food and Agriculture Organization of the United Nations Static Division (FAOSTAT), Spain with 34%, Greece with 12%, and Italy with11% are the highest olive crop producers worldwide. Iran, with 0.44%, is the 20th olive producer worldwide; however, the country share in the olive residues is more than that of others [2]. Technically, olive pomace or processed olive pomace can be used as a natural source of fuels, fertilizers and feeds in livestock. For bio-augmentation, the olive pomace is fermented to produce probiotic biomasses and used for the extraction of its bioactive peptides [3].

Proteins are usually subjected to hydrolysis processes to achieve peptides. Various methods have been described to produce protein hydrolysates, including enzymatic, fermentation, acid and alkali methods. In enzymatic hydrolysis several factors such as, temperature, pH, enzyme /substrate concentration and time affect activity of the enzyme; thus, these factors make the hydrolysis processes more controllable [4]. Enzymatic methods are more common to produce protein hydrolysates due to their milder processing conditions, and peptides produced to preserve amino acid (AA) configuration and integrity with high specificity. Their bioactive characteristics are majorly linked to enzymatic specificity, solubility, hydrolysis degree, AA composition, molecular mass and hydrophobicity.

Protein hydrolysates (also known as bioactive peptides) are specific protein fragments that include 2-20 AAs residues encrypted in the parent proteins [5]. Bioactive peptides become active when released during enzymatic hydrolysis, microbial fermentation, and chemical hydrolysis. Based on the compositions and sequences of AAs, bioactive peptides include various beneficial health effects such as antioxidant, antihypertensive, hypocholesterolemic, antimicrobial, mineral binding and immunomodulatory effects [6]. Nowadays, bioactive peptides are used widely, including their uses in foods (as emulsifiers), biotechnology, drug production, fermentation (as sources of nitrogen in culture media) and cosmetics (as stimulators of collagen and elastin syntheses) [7]. Assessment of antioxidant characteristics in extracts, hydrolysates, and fractions includes use of various *in vitro* assays and various instruments. Further studies assessing abilities of the proteins and peptides to decrease oxidative stress on cells cultures and animal models are important to verify previous results. In cases, further characterization of the extracts is passed by the identification of peptides [8]. Hence, investigation of novel sources of natural active biological fragments is currently of major interests to the researchers. Recent studies have demonstrated that protein hydrolysates in plant residues such as olive, pumpkin, walnut, corn germ and palm kernal residues include antioxidant and antiproliferative activities [9-13]. Since there are a little information on protein hydrolysates from olive pomace, it is necessary to carry out further investigations. Therefore, the major purpose of the present study was to optimize the hydrolysis conditions of olive pomace protein extracts using response surface methodology to achieve the maximum degrees of hydrolysis. Furthermore, antioxidant and antiproliferative activities of the hydrolysates with the highest hydrolysis rates at various concentrations were assessed.

2. Materials and Methods

2.1 Materials and chemicals

Olive pomace was collected from Gilan Province, Iran September 2019. Trypsin (pancreas protease, 2000 U g^{-1}), sodium dodecyl sulfate (SDS), 2-mercaptoethanol, o-phthaldialdehyde (OPA), tris hydrochloride (Tris-HCl) and, ferrous sulfate ($FeSO_4$) were purchased from Merck, Germany. Moreover, 2-2diphenyl-1-picryl-hydroxyl (DPPH), ferrozine, dithiothreitol (DTT), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, USA. The HPLC grade acetonitrile (ACN), acetone, hydrochloric acid (HCL), methanol(MeOH), penicillin, streptomycin, Dulbecco's modified Eagles medium (DMEM), fetal bovine serum (FBS) and ethylenediami-netetraacetic acid (EDTA) were purchased from bioidea, Iran.

2.2 Protein extraction

Protein extraction of the olive pomace was carried out based on the method by Esteve et al. [9] with some modifications. Briefly, 2g of the olive pomace were mixed with 25 ml of the extraction buffer, including 125 mM of tris–HCl (pH 7.5), 1% (m v^{-1}) of SDS and 0.1% (m v^{-1}) of DTT. Sample was vortexed for 1 min and centrifuged at 4000 g for 20 min at 4 ° C. To precipitate the protein, 20 ml of cold acetone were added to the supernatant and stored at -20 °C for 1 h, then centrifuged at 4 °C for 10 min. Precipitate was dried at room temperature and stored in -20 °C until use.

2.3 Experimental Design and Optimization

In the present study, Design Expert Software (Trial Version 10.0.7.0) was used for the experimental design and prediction of optimal hydrolysis conditions of olive pomace

protein by enzyme trypsin, data analysis and model building. Box-Behnken design (BBD) with three variables were used to show the response patterns and establish models. The three variables used in this study included hydrolysis time (X_1), pH (X_2), and hydrolysis temperature (X_3), with three levels (-1, 0, +1) of each variable. The degree of hydrolysis (DH %) value was selected as the experimental response Y. The experimental design consisted of 17 experimental points and five replicates of the central points (Table 1).

Table 1. Box–Behnken design for the optimization enzymatic hydrolysis conditions

	Independent variables			Responses	
Run	Time (h)	pH	Temperature (˚C)	Degree of hydrolysis%	
	X_1	X_2	X_3	Actual	Predicted
1	2	8	37	30.21	30.48
2	3.5	8	39	61.83	60.22
3	5	8.5	39	70.62	69.64
4	2	8.5	39	55.02	55.90
5	3.5	8	39	61.55	60.22
6	2	7.5	39	45.82	46.79
7	3.5	7.5	41	54.05	55.21
8	3.5	7.5	37	42.84	41.58
9	3.5	8.5	41	63.11	64.37
10	5	7.5	39	52.81	51.95
11	3.5	8	39	59.08	60.62
12	5	8	37	41.71	43.83
13	3.5	8.5	37	60.38	60.38
14	3.5	8	39	57.01	60.62
15	5	8	41	49.54	49.35
16	3.5	8	39	61.62	60.62
17	2	8	41	45.88	43.76

The second-order polynomial regression equation was as follows:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum\sum_{i<j} \beta_{ij} X_i X_j \quad \text{Eq.1}$$

Where Y was the response (degree of hydrolysis %), K was the number of the independent variables, β_0, β_i, β_{ii} and β_{ij} were respectively the coefficients of the intercept, linear, quadratic and interactive terms, and X_i and X_j were the levels of the independent variables.

2.4 Enzymatic hydrolysis of the olive pomace protein

Olive pomace protein extracts [5% (w v^{-1})] were dissolved in phosphate buffer (100 mM). Then, trypsin at 1% (w v^{-1}) was added to the solution and hydrolysis was carried out at 200 rpm for 2_5 h using shaker incubator. Hydrolysis temperatures were 37- 41 °C. At the end of the process, hydrolysates were heated at 85 °C for 15 min to inactivate the enzyme. These were, centrifuged at 4000 g for 30 min and the supernatant was freeze-dried [14].

2.5 Degree of hydrolysis of olive pomace protein hydrolysate

Degrees of hydrolysis were estimated using the o-phthaldialdehyde (OPA) based on the method by Nielsen et al. [15]. The OPA reagent (20 ml) was prepared as follows: 10 ml of 100 mM sodium tetraborate, 1 ml of 20% (m w^{-1}) SDS and 400 µl of 40 mg ml^{-1} OPA dissolved in MeOH, 40 µl of 2-mercaptoethanol and 8.56 ml of water. Then, 10 µl of the hydrolyzed sample were added to 1 ml of OPA reagent and set for 2 min. Absorbance was read at 340 nm. The L-serine was used as standard and water as blank. The %DH was calculated as follows:

$$\%DH = (H/H\,tot) \times 100 \quad \text{Eq.2}$$

$$H = \frac{(\text{serine}-\text{NH2} - \beta)}{\alpha}\ \text{megv/g protein} \quad \text{Eq.3}$$

$\alpha = 1$, $\beta = 0.4$ and $H_{tot} = 8$ were previously set by Nielsen et al. [15].

$$\text{Serine} - \text{NH2} = \frac{\text{OD sample}-\text{OD blank}}{\text{OD standard}-\text{OD blank}} \times 0.9516\left(\frac{n\text{meqv}}{\text{L}}\right) \times 0.1\,\frac{100}{(\text{x}\times\text{p})} \quad \text{Eq.4}$$

Where, serine-NH2 was in meqv serine-NH2/g protein, P was the proportion of the protein present in the sample, X was the quantity of the sample (g), 0.1 L was the volume of the sample and 0.9516meqv l^{-1} was the equivalent weight of serine. Total protein was assessed using Kjeldahl method based on A.O.A.C 976.05[16]. Total protein was reported as 5.1% using the following equation:

$$\text{Nitroge \%} = (\text{volume of acid (ml)} \times \text{acid normality} \times 14)/(\text{weight of sample (g)} \times 1000) \times 100 \quad \text{Eq.5}$$

$$\text{protein \%} = \text{Nitroge \%} \times \text{conversion factor} \quad \text{Eq.6}$$

Conversion factor of 6.25 was used to assess the protein content.

2.6 Assessment of the antioxidant activity

2.6.1 2,-2-diphenyl-1picryl-hydroxyl radical scavenging activity

Briefly, (DPPH) radical scavenging of olive pomace protein hydrolysate and olive pomace protein was assessed based on the method by Xia et al. [17], with some modifications. First, 0.5 mM solution of DPPH in methanol was prepared and 100 µl of this solution was added to 100 µl of hydrolysates at various concentrations (0.5, 1.5, 2.5 and 3.5 mg ml^{-1}). These were then added to 1 ml of methanol and mixed well. The mixture was stored in dark for 60 min at room temperature. Then, absorbance was measured at 517 nm. Ascorbic acid was used as positive control. The DPPH scavenging activity was calculated as follow:

$$\text{DPPH}\ (\%) = (\text{Ac} - \text{As})/\text{Ac} \times 100 \quad \text{Eq.7}$$

Where, A_c was the absorbance of the control reaction (without sample), A_s was the absorbance in the presence of the sample.

2.6.2 Iron (II) chelating activity

The Fe^{2+} chelation activity was assessed using ferrous iron– ferrozine complex based on a method by Hatami et al.[18] with slight modifications. Briefly, 25 µl of $FeSO_4$ solution (1 mM) were added to a mixture, including 1 mL of H_2O and 50 µl of the hydrolysates at various concentrations (0.5, 1.5, 2.5 and 3.5 mg ml^{-1}). Then, 50µl of ferrozine solution (5 mM) were added to the mixture and agitated for 30 s. After agitation, mixture was incubated at room temperature for 10 min. Absorbance of the solution was measured at 562 nm. Furthermore, EDTA was used as positive control. Chelating activity was measured as follow:

$$\text{chelationg activity}\ (\%) = [1 - (\text{As}/\text{Ac})] \times 100 \quad \text{Eq.8}$$

Where, A_c was the absorbance of the control reaction (without sample) and A_s was the absorbance in the presence of the sample.

2.7 Cell culture

The MCF-7 human breast cancer cell line provided by the Pasteur Institute of Iran, Tehran, Iran was used to investigate antiproliferative activity of the olive pomace protein hydrolysates. Cells were cultivated in culture media containing DMEM, 10% of FBS, and 1% of penicillin-streptomycin and incubated at 37 °C using humidified atmosphere containing 5% of CO_2 [19].

The MTT assay

After cell culture, 100 µl of cell suspension containing 8,000-10000 cells were transferred to each well from 96 wells plates and incubated at 37 °C under 5% CO_2 atmosphere. After 24 h the culture media removed and olive pomace protein hydrolysates at various concentrations (5, 10, 20, 40 and 80 mg ml^{-1}) were added to the wells. After 24 and 48 h, 20 µl of 5 mg ml^{-1} MTT were added to each well and incubated at 37 °C for 3 h. MTT solution was removed and 100 µl of DMSO were added to each well and agitated for 15 min to dissolve the precipitates of formazon crystals. Absorbance of the sample was measured at 570 nm [19]. Concentration of the protein hydrolysates that killed 50% of the cells (IC50) was calculated using Microsoft–Excel 2010. Proportion of the cell inhibition was calculated as follows:

$$\text{Cell proliferation inhibition}\ (\%) = [1 - (\text{As}/\text{Ac})] \times 100 \quad \text{Eq.9}$$

Where, A_c was the absorbance of the control reaction (without sample) and A_s was the absorbance in the presence of the sample.

2.8 Scanning electron microscopy

To study microscopic structures of the olive pomace proteins and hydrolysates under optimal conditions, scanning electron microscopy (SEM) (Tescan Vega 3, Czech Republic) was used. Freeze-dried samples were coated with gold using sputter coater and loaded on the system; then, images were captured at the selected magnifications (500 and 150 kx) at 20 kV [20].

2.9 Amino acid composition

The AA contents of the olive pomace proteins before and after hydrolysis under optimal conditions were assessed using phenylthiocarbamy (PTC) AAs and reverse phase-high performance liquid chromatography RP-HPLC (Waters 600, Milford ,MA ,USA) System Controller, Injector U6K, Dual λ Absorbance Detector, Waters 746 Data Module and PICO.TAG Column. Column temperature during operation was maintained at 38 °C using oven heater [21]. Sample was hydrolyzed using 6 N HCl at 110 °C for 24 h in evacuated sealed tubes. The AAs (Asp, Glu, Ser, Gly, His, Thr, Ala, Arg, Pro, Tyr, Val, Met, Cys, Ilu, Leu, PHe and Lys) were used as standards.

2.10 Statistical analysis

Design-Expert Software v.10.0 was used to optimize enzymatic hydrolysis conditions. Comparison between the treatment groups were carried out using one-way analysis of variance (ANOVA), SPSS Software v.23.0 and Tukey HSD with a statistically significance level of $p<0.05$. Results were regenerated three times and expressed as means ±SD (standard deviation).

3. Results and Discussion

3.1 Optimization of hydrolysis condition

Results of 17 treatments designed by Box-Behnken design method are present in Table 1. Factors such as temperature, pH and time included significant effects on degree of hydrolysis ($p<0.05$). In hydrolysis by trypsin, the optimal conditions were as follows: pH, =8.5; temperature, 39 °C and time 5 h. Under the optimal conditions, the highest hydrolysis degree = 70.62% was achieved and a little difference

between the actual and predicted quantities of hydrolysis degrees showed appropriateness of the designs.

Types of the enzymes played important roles in degree of hydrolysis. High degrees of hydrolysis revealed presence of high quantities of short-chain peptides. Trypsin enzyme, as a serine endopeptidase, broke down peptide bonds from the carboxyl AAs of lysine and arginine [22]. Results of this study were similar to those of a study by Ng et al. [13]; in which, optimal conditions of enzymatic hydrolysis of palm kernel cake proteins by trypsin were achieved at pH=8.5 and temperature of 40 °C. Xia et al. [23] reported the optimal conditions for the enzymatic hydrolysis of whey proteins by trypsin at 40 °C, pH=8 and enzyme-substrate ratio of 5%.

Results of the analysis of variance are shown in Table 2. Results demonstrated that X_1, X_2 and X_3(p<0.001) and all the quadratic parameters X_1^2, X_2^2 and X_3^2 (p<0.001), (p<0.01) included significantly effects on the degree of hydrolysis. However, all the interaction parameters of X_1X_2, X_1X_3 and X_2X_3 were insignificant (p>0.05). The Model F-value demonstrated that the model was significant, the *p-value* of the model was <0.0001, showing significance of the model. The lack of fit F-value of 1.36 revealed that the lack of fit was not significant (p>0.05).

The model regression equation was as follows:

$$\text{Degree of hydrolysis} = +60.22 + 4.48X_1 + 6.70X_2 + 4.44X_3 + 2.15X_1X_2 - 1.44X_1X_3 - 2.12X_2X_3 - 8.44X_1^2 + 4.29X_2^2 - 9.41X_3^2 \quad \text{Eq.10}$$

The value of R^2= was 0.9761, which indicated that only 2.39% of the total variations were not explained by the model.

3.2 Effects of the hydrolytic parameters on degree of hydrolysis

Figure 1 (a, b and c) shows three-dimensional (3D) response surface plots and contour plot (d, e and f). Figure 1a shows the effects of hydrolysis time and pH on degree of hydrolysis when the temperature is held at an optimum level.

By increasing hydrolysis time from 2 to 3.5 h during the hydrolysis process, degree of hydrolysis increased quickly. When the hydrolysis time increased from 3.5 to 5 h, degree of hydrolysis increased slowly. Based on the results from other studies, degree of hydrolysis increases significantly ($p<0.05$) with increasing time because with increasing time, enzyme activity increases and the substrate and the peptide bonds are exposed to the enzyme for a longer time [12,24]. As the pH increases, degree of hydrolysis changed gradually and increased almost linearly. Yu and Tan [25] reported that pH included important effects on the catalytic activity of the enzyme because changes in pH affected charge distribution and composition of the molecules and changed protein structure of the enzyme. Figure 1b shows effects of hydrolysis time and temperature on degree of hydrolysis when the pH is held at an optimum level. Based on the results, increases in temperature from 37 to 39 °C included increasing effects on degree of hydrolysis. When temperature increased from 39 to 41 °C, degree of hydrolysis decreased. Moreover, the optimum temperature of trypsin was among 37-55 °C. Catalytic activity of the trypsin enzyme increased with increasing the temperature from 37 to 50 °C. At higher temperatures, enzyme was denatured and its activity decreased [23]. With continuous increases in temperature, activity of the enzyme was affected, which decreased due to the aggregation and deformation of parts of the protein [11]. Generally, each enzyme included the highest catalytic activity and stability at its optimum temperature. Higher or lower temperatures decreased activity and stability of the enzyme. Naturally, enzymes are sensitive to acidic and alkaline environments and are most active only in a certain range of pH [26].

Table 2. Analysis of variance for the degree of hydrolysis quadratic models

Source	sum of squares	df	mean squares	F- value	p-value
Model	1479.24	9	164.36	31.82	<0.0001***
X_1-time	160.65	1	160.65	31.10	0.0008**
X_2-pH	359.25	1	359.25	69.54	<0.0001***
X_3-tem	157.89	1	157.89	30.56	0.0009**
X_1X_2	18.53	1	18.53	3.59	0.1001
X_1X_3	8.24	1	8.24	1.59	0.2471
X_2X_3	17.98	1	17.98	3.48	0.1044
X_1^2	300.13	1	300.13	58. 10	<0.0001***
X_2^2	77.57	1	77.57	15.02	0.0061**
X_3^2	373.25	1	373.25	72.25	<0.0001***
Residual	36.16	7	5.17	_	_
Lack of Fit	18.24	3	6.08	1.36	0.3756
Pure Error	17.92.29	4	4.48	_	_
Cor Total	1515.40	16		_	_

* P < 0.05; ** p < 0.01; *** p < 0.001.

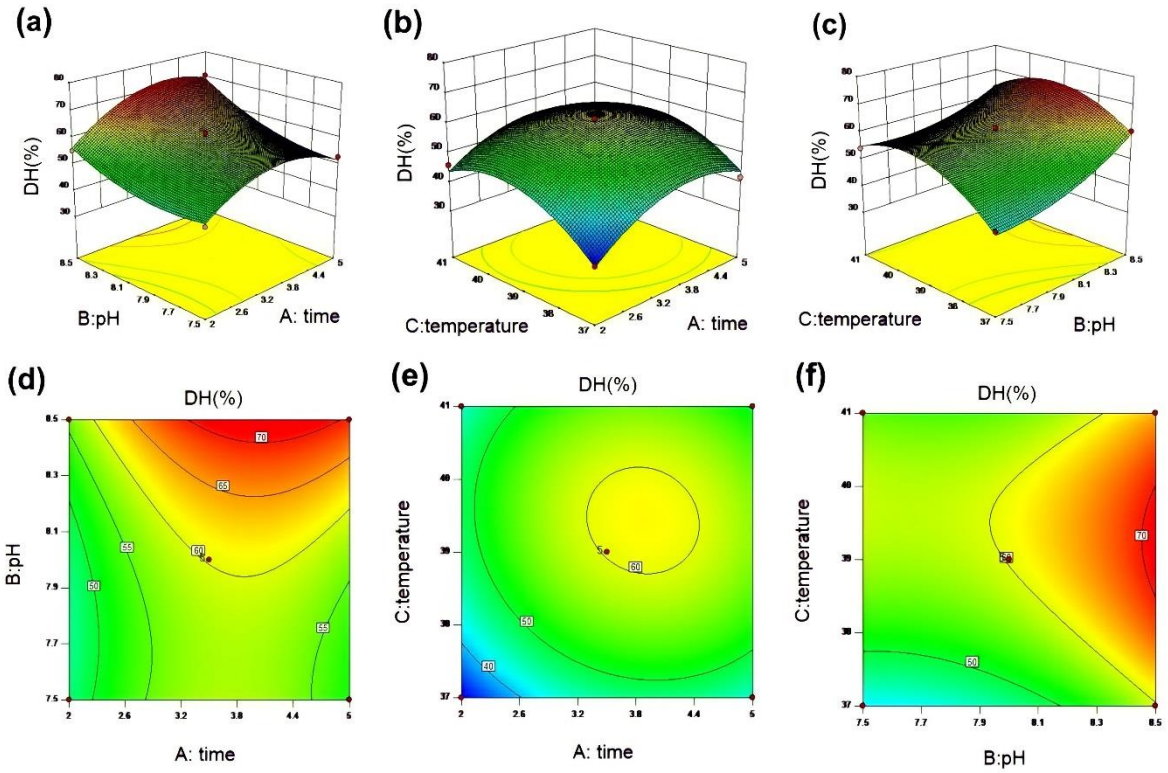

Figure 1. The (3D) response surface plot (a, b and c) and contour plots (d, e and f) for the combined effects of independent variables time, pH and temperature on the degree of hydrolysis of olive pomace protein hydrolysates

By increases in time from 2 to 5 h, degree of hydrolysis increased. Figure 1c shows effects of pH and temperature on degree of hydrolysis when time was held at an optimum level. Based on the results, increases in temperature increased degree of hydrolysis. Moreover, pH included linear relationships with degrees of hydrolysis, and increasing pH lead to increasing the degrees of hydrolysis. By, increasing temperature, enzyme activity increased. Enzyme was inactivated at temperatures above the optimum, and polymer network formation by protein molecules occurred that inhibited function of the enzyme. Eventually, degree of hydrolysis decreased [27].

3.3 The 2,-2-diphenyl-1picryl-hydroxyl radical scavenging activity

Antioxidant activity of the treatment under the optimal conditions at various concentrations (0.5, 1.5, 2.5 and 3.5mg ml^{-1}) using DPPH is shown in Figure.2.a. Results demonstrated scavenging DPPH radical abilities of protein extract and protein hydrolysate at various concentrations. By increasing concentrations the olive pomace protein and hydrolysate to 3.5 mg ml^{-1}, DPPH scavenging activity increased. Protein hydrolysate exhibited the highest DPPH scavenging activity (42.26% ±2 .46 at 3.5 mg ml^{-1}), compared to protein extract (36.03% ± 2.70 at 3.5 mg ml^{-1}).

By increasing concentration of the protein hydrolysate from 0.5 to 3.5 mg ml^{-1}, antioxidant characteristics significantly increased ($p<0.05$). Although, antioxidant activities of the protein and hydrolysate were significantly lower than that of ascorbic acid as positive control. In a study by Ye et al. [28], antioxidant activity of the hydrolyzed protein was higher than that of the protein because of the low molecular weight and composition of AAs in the peptide. In a study by Arise et al. [29], protein hydrolysate from *Citrullus lanatus* seeds after, tryptic digestion at 2.5 mg ml^{-1} exhibited the highest radical scavenging power (56.39% ± 0.45). Naqash and Nazeer.[30] reported that hydrolysates of pink perch muscle showed the highest scavenging activity (48% at 3 mg ml^{-1}). Li et al. [31] reported that scavenging effects increased with increasing concentration of corn and zein peptides. You et al. [24] reported the, dose-dependent DPPH scavenging activity, since the highest activity was reported at the highest hydrolysate concentration of 5 mg ml^{-1}.

3.4 Iron (II) chelating activity

Metal chelating activity of the treatment under optimal conditions at various concentrations (0.5, 1.5, 2.5 and 3.5 mg ml^{-1}) is, shown in Figure.2.b. At the highest concentration (3.5 mg ml^{-1}), metal chelating activity in protein hydrolyte and protein extract was observed at 30.91% ± 0.26 and 17.73% ± 2.30, respectively.

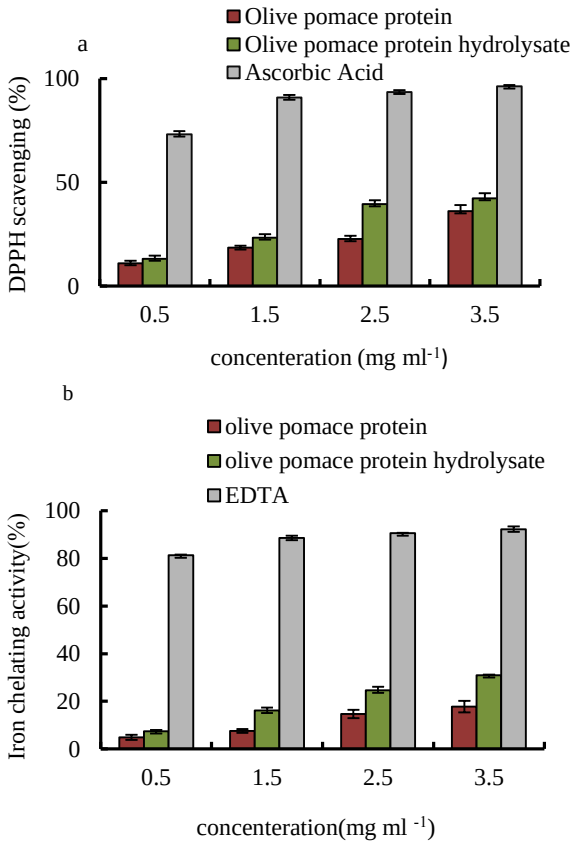

Figure2. Antioxidant activities of the olive pomace protein and olive pomace protein hydrolysate at various concentrations

By increasing the concentration from 0.5 to 3.5 mg ml^{-1} in protein hydrolysate, iron chelating activity increased significantly (p<0.05). Xie et al. [32] reported the highest chelating activity of mung bean meal protein hydrolysates at the highest concentration of 3 mg ml^{-1}. Memarpoor-Yazdi et al.[33] reported that increases in concentration of the hydrolyzed protein of *Zizyphus jujube* fruits from 0 to 1.5 mg ml^{-1} increased, chelating characteristics of the peptide. Venuste et al. [10] showed positive correlations between the peptide concentration and iron chelating activity in pumpkin hydrolysate; hence, the highest concentration of 1 mg ml^{-1} included higher chelating activity. Importantly, antioxidant activities of the two protein and hydrolysate were significantly lower than that of EDTA as positive control.

3.5 Antiproliferative effects of the olive pomace protein hydrolysate on human breast cancer cells

Based on the results from Figure.3, by increasing concentration of the hydrolyzed protein from 5 to 80 mg ml^{-1}, cell viabilities decreased in 24 and 48 h treatments. The lowest cell viabilities achieved at 24 and 48 h at 80 mg mg^{-1} included 15.06% ± 0.40 and 9.71% ± 1.32, respectively. No significant differences (p>0.05) were seen between the concentrations of 20 and 40 mg ml^{-1} in 48 h treatments, but their differences were significant (p<0.05) with a concentration of 80 mg ml^{-1}. Half-maximal inhibitory concentrations (IC50) of 24 and 48 h treatments were achieved at 53. 94 mg ml^{-1} and 28.77 mg ml^{-1}, respectively.

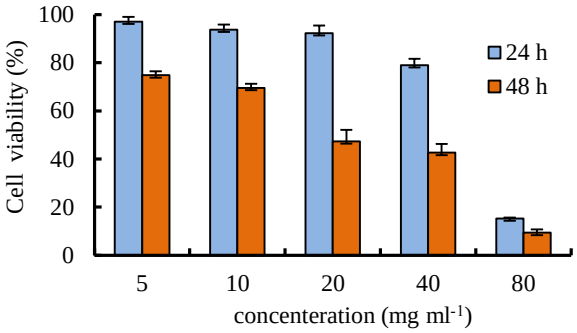

Figure3. Effects of olive pomace protein hydrolysate on MCF-7 cell viability

Shoo-mbuatong et al. [34] reported that antiproliferative activity of peptides was attributed to the electrostatic interactions between the cationic AAs and anionic membranes of the cancer cells. In addition, antiproliferative peptides interfere with cancer cells by causing apoptosis, stimulating necrosis, lysing cell, inhibiting angiogenesis and stimulating host immune system. Lee et al. [35] showed that hydrophobicity of the AAs in hydrolyzed proteins affected cell viability. At concentrations above 40 mg ml^{-1} hydrolyzed protein, cell viability of RAW 264.7 cells decreased due to increased hydrophobicity. In a study by Gupta et al. [19], mung bean hydrolyzed protein by trypsin and alcalase included antiproliferative activity on MCF-7 and MDA-MB 231 cell lines. Another study by Rayaprolu et al. [36] showed antiproliferative activity of soybean meal protein hydrolyzed by alcalase enzyme on MCF-7, kasumi-3, CCRF-CEM and PC-3 cell lines.

3.6 Scanning electron microscopy

In this study, (SEM) was used to investigate changes in structure and surface morphology of the olive pomace proteins before and after enzymatic hydrolysis under optimal conditions Figure.4A-D. Olive pomace proteins without hydrolysis enzymes (Figure.4B, D) represented larger pieces of particles and smooth surface in proteins. Structure of the olive pomace protein after enzymatic hydrolysis (Figure.4A,C) showed that structure of the olive pomace protein was broken, the small particle was clearly visible, the particle surface was rough and the shape was degraded compared to the protein before hydrolysis. These findings indicated that trypsin was able to hydrolyze large protein molecules, converting them into small fragment peptides and AAs. In this study, results from SEM images were similar to those by Bao et al. [20] They reported that the average size decreased in protein hydrolysates from egg yolks with increases in degree of hydrolysis.

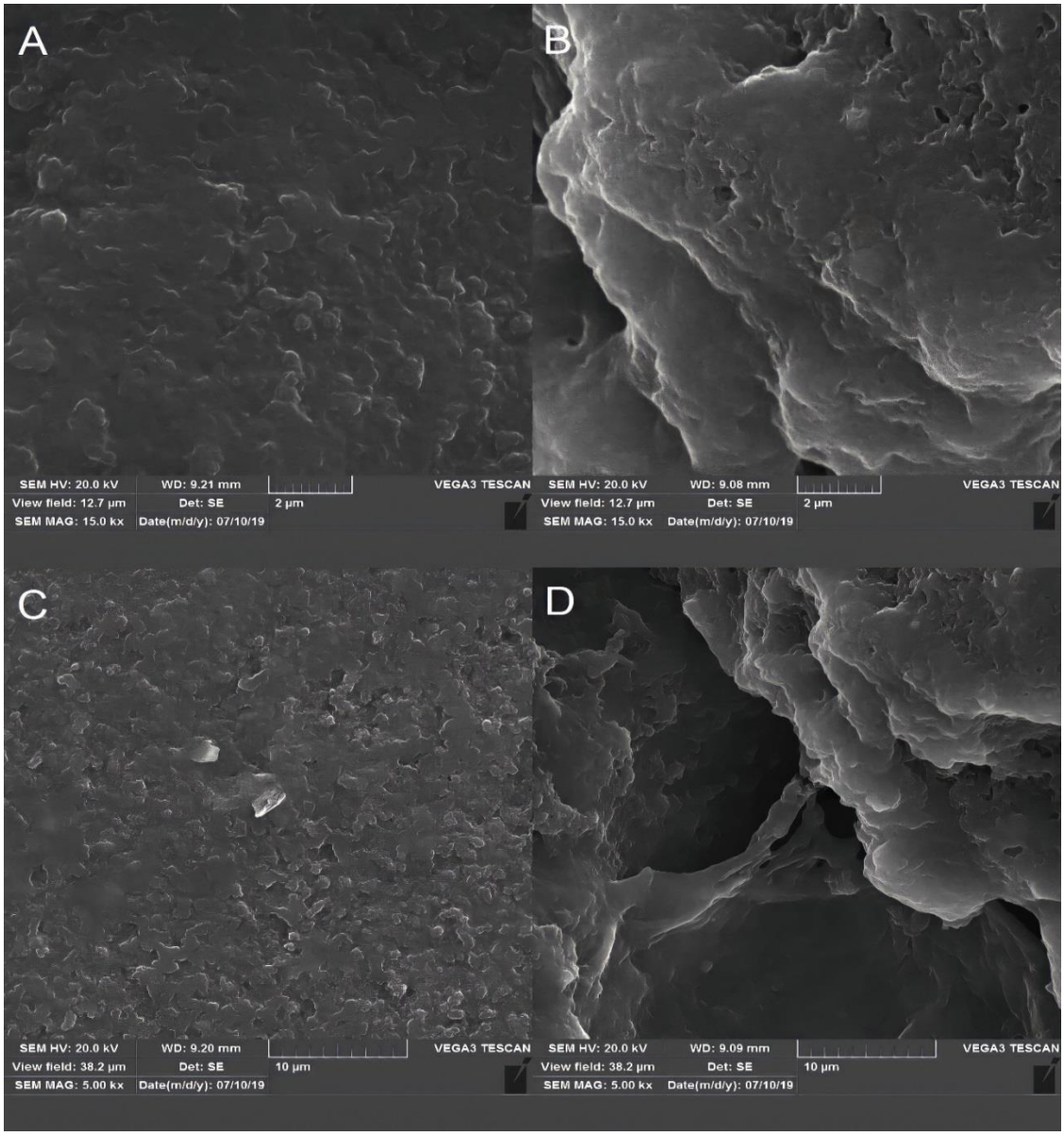

Figure4. Scanning Electron Microscopy of the extracted protein (B and D) protein hydrolysate (A and C) at magnification of 15.0 and 5.00 kx

Ahmed et al. [37] reported that, the particle size of lentil protein hydrolysates decreased (p<0.05) and decreases in protein size occurred due to the function of the enzyme, which converted proteins into smaller peptides. Sun et al. [11] studied walnut proteins using enzymatic hydrolysis and reported that the spherical structure of proteins was degraded after hydrolysis and decreased to smaller particles.

3.7 Amino acid component

The AA compositions of olive pomace protein and olive pomace protein hydrolysate at optimal hydrolysis conditions were analyzed (Table.3). Olive pomace protein and hydrolysate included essential AAs (His, Thr, Val, Ile, Leu, Met, Phe and Lys) as 1.20 and 1.28 mg g^{-1}, respectively.The AAs such as Tyr, Val, Phe and Glu included high contents of AAs in olive pomace protein and hydrolysate. The highest quantity of AAs in olive pomace protein hydrolysate was Tyr (0.83 mg g^{-1}). These results revealed the quantity of hydrophobic AAs increased after enzymatic hydrolysis. This verified increased antioxidant activity of the hydrolyzed protein under optimal conditions. Xia et al. [17] reported that AAs such as Tyr, Thr, and Lys, included significant antioxidant characteristics, which were achieved as 0.98 mg g $^{-1}$ in this study. A study by Firmansyah and Abduh [38] showed that protein hydrolysates included high quantities of hydrophobic AAs, which could verify high scavenge free radicals. Karimi et al. and Nourmohammadi et al. [12,14]

Reported the presence of hydrophobic AAs in proteins hydrolyzed by trypsin increased their solubility in hydrophobic phases, which facilitated transfer of protons to free radicals and interactions between the peptides.

Table3. Amino acid compositions (mg g^{-1}) of olive pomace protein and olive pomace protein hydrolysate

Amino acid	Olive pomace protein(mg g^{-1})	Olive pomace protein hydrolysis(mg g^{-1})
Asp	0. 10	0.12
Glu	0. 22	0.24
Ser	0.08	0.05
Gly	0.13	0.15
His	0. 10	0.16
Arg	0.15	0.13
Thr	0.05	0.04
Ala	0.09	0.12
Pro	0. 15	0.13
Tyr	0. 72	0.83
Val	0.33	0.38
Met	0. 20	0.21
lle	0.08	0.05
leu	0.06	0.05
Phe	0.21	0. 28
Cys	<0.10	<0.1
Lys	0.17	0.11
Total hydrophobic amino acids	1.25	1.37

Moreover, Fe^{2+} chelating activity was due to the presence of basic and acidic AAs (glutamic acid, glutamine, aspartic acid, asparagine, arginine and lysine). Zou et al. [39] reported that increased antioxidant activity in bioactive peptides was due to the presence of hydrophobic and aromatic compounds as well as AAs such as His and Cys that causes antioxidant activity. Zamora et al. [40] reported that the AA composition of olive fruit mesocarp proteins and olive oil included high quantities of AAs such as Asp, Glu and Gly. These results were similar to the results of this study.

4. Conclusion

Results of this study showed that the optimal enzymatic hydrolysis conditions for the olive pomace protein were temperature of 39 °C, pH 8.5, and time of 5 h. The DPPH radical scavenging activity and iron (II) chelating activity of the hydrolysates on optimal conditions were reported as (42.26% ± 2.46) and (30.91% ± 0.26) at 3.5 mg ml^{-1}, respectively. Cell viability achieved within 24 and 48 h at 80 mg ml^{-1} included (15.06% ±0.40) and (9.71% ± 1.32), respectively. These results showed that the quantity of antioxidants from protein hydrolysis at various concentrations was significantly lower than that of positive controls. Results of the microscopic analysis of the hydrolysates verified that trypsin was able to hydrolyze large protein molecules, converting them into small peptides. In conclusion, successful uses of trypsin as an effective hydrolytic enzyme demonstrated characteristics of the antioxidant ingredients with high quantities of hydrophobic AAs (1.37 mg ml^{-1}). Furthermore, antiproliferative activity of the olive pomace was significant, which could prevent or decrease the risks of chronic diseases. Therefore, olive pomace protein hydrolysates as cheap and valuable sources with potential applications can be used as feeds in livestock. Further studies are necessary to produce, purify and identify bioactive peptides from olive pomace, as well as studies on animals and clinical trials to verify these results

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6. Conflict of Interest

The authors report no conflicts of interest.

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بهینه سازی هیدرولیز آنزیمی پروتئین تفاله زیتون با استفاده از روش سطح پاسخ

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چکیده

سابقه و هدف: تفاله زیتون محصول جانبی استخراج روغن زیتون است و در خوراک دام و طیور استفاده می شود. اگرچه، می تواند منبع ارزشمندی برای تولید پپتیدهای زیست فعال باشد که تأثیر مثبتی بر عملکرد و سلامت بدن دارند. هدف از این مطالعه بهینه سازی شرایط هیدرولیز آنزیمی پروتئین تفاله زیتون برای تولید هیدرولیزات با بالاترین درجه هیدرولیز و بررسی خواص زیست فعال تیمار در شرایط بهینه است.

مواد و روش ها: در این تحقیق، پس از استخراج پروتئین از تفاله زیتون، روش سطح پاسخ بر اساس طرح باکس بنکن به منظور بهینه سازی هیدرولیز آنزیمی پروتئین تفاله زیتون توسط تریپسین استفاده شد. درجه هیدرولیز به عنوان پاسخ در نظر گرفته شد. فعالیت آنتی اکسیدانی در هیدرولیزات با مهار رادیکال ۲و۲- دی فنیل -۱- پیکریل هیدرازیل و فعالیت شلاته کنندگی آهن (II) انجام شد. فعالیت ضد تکثیری بر روی سلول های سرطانی سینه انسان (MCF-7) با استفاده از ۳- (۴ ، ۵- دی متیل تیازول-۲-ایل)-۲، ۵- دی فنیل تترازولیوم بروماید بررسی شد. ساختار میکروسکوپی و محتوای اسید آمینه با استفاده از میکروسکوپ الکترونی روبشی و کروماتوگرافی مایع فاز معکوس با کارایی بالا، به ترتیب بررسی شد.

یافته‌ها و نتیجه‌گیری: شرایط بهینه هیدرولیز به شرح زیر بود: زمان ۵ ساعت، pH= ۸/۵و دمای ۳۹ درجه سانتی گراد. در این شرایط، حداکثر درجه هیدرولیز ۷۰/۶۲٪ به دست آمد. فعالیت مهار کنندگی رادیکال ۲و۲- دی فنیل -۱- پیکریل هیدرازیل و فعالیت شلاته کنندگی یون آهن (II) پروتئین هیدرولیز شده تفاله زیتون تحت شرایط بهینه ۴۲/۲۶±۲/۴۶ و ۳۰/۹۱±۰/۲۶ به ترتیب در غلظت ۳/۵ میلی گرم بر میلی لیتر به دست آمد. اگرچه، فعالیت آنتی اکسیدانی پروتیئن هیدرولیز شده به طور قابل توجهی کمتر از آسکوربیک اسید و اتیلن دی آمین تتراستیک اسید بود. بیشترین فعالیت ضد تکثیری پروتئین هیدرولیز شده تفاله زیتون در غلظت ۸۰ میلی گرم بر میلی لیتر بدست آمد، که در این غلظت درصد زنده مانی سلول در تیمار ۲۴ و ۴۸ ساعت به ترتیب، ۱۵/۰۶±۰/۴۰ و ۹/۷۱ ±۱/۳۲ بدست آمد. تجزیه و تحلیل میکروسکوپی هیدرولیزات تأیید می کند که آنزیم تریپسین توانسته مولکول های بزرگ پروتئین را هیدرولیز کرده و آنها را به قطعات کوچک تبدیل کند. آنالیز اسید آمینه نشان دهنده افزایش اسید آمینه آبگریز وآروماتیک در پروتئین هیدرولیز شده است. در نتیجه، پروتئین هیدرولیز شده از تفاله زیتون با پتانسیل آنتی اکسیدانی و ضد تکثیری می تواند به عنوان یک افزودنی کاربردی در تغذیه دام استفاده شود.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.