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Optimization of apple seed protein hydrolysis using alkalase enzyme and investigation of its antioxidant properties

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ABSTRACT

With increasing concerns about the harmful effects of synthetic antioxidants on health, the use of natural antioxidants, especially bioactive peptides, has become increasingly important. In this study, the optimization of the enzymatic hydrolysis process of apple seed protein by alkalase enzyme and ultrasound pretreatment was investigated. Enzymatic hydrolysis of apple seed protein with alkalase enzyme was performed at temperatures of 40 to 55°C, enzyme to substrate ratio of 1 to 2%, and for a period of 30 to 180 minutes. The protein solution was treated with a pulsed ultrasound device (200 W, 60 Hz) for 5 minutes before hydrolysis. Antioxidant activity was evaluated by measuring DPPH radical scavenging, reducing power, and total antioxidant capacity. Samples without ultrasound pretreatment showed the highest antioxidant activity. The optimal conditions for antioxidant activity in this case were, respectively, hydrolysis time of 149.60 minutes, temperature of 51.96°C, and enzyme to substrate ratio of 1.80%. The total antioxidant capacity test (at a wavelength of 695 nm) showed that the absorbance of the ultrasonically pretreated apple seed protein hydrolyzed samples was between 0.521 and 0.817, and that of the non-pretreated samples was between 0.513 and 0.982. In the iron reducing power test (at a wavelength of 700 nm), the absorbance of the ultrasonically pretreated samples was between 0.793 and 0.870, and that of the non-pretreated samples was between 0.623 and 1.095. The DPPH radical scavenging activity for the ultrasound-pretreated samples ranged from 45.17 to 63.89% and for the non-pretreated samples ranged from 45.22 to 66.97%. The results of this study indicate that these peptides can be used as natural antioxidants in the formulation of food products to reduce oxidation and increase their shelf life.

1- Introduction

To prevent food spoilage and protect the body against a wide range of diseases, it is essential to inhibit lipid peroxidation and prevent the formation of free radicals in food products and living cells. Although synthetic antioxidants are used as food additives to prevent quality deterioration, there are concerns about their safety and health-related consequences. Therefore, the development and application of natural antioxidants as a suitable alternative to synthetic types has attracted the attention of many researchers [1]. In recent years, the growing awareness of consumers about the role of nutrition in maintaining and promoting health has led to a significant increase in the desire to use functional foods [2]. Bioactive peptides, which are composed of 3 to 20 amino acids, are formed during the process of protein degradation and the production of short peptides and free amino acids [3]. These peptides are specific parts of the protein structure that, in addition to their nutritional value, can have beneficial physiological effects on health. However, these sequences are inactive in the original protein and only after being released in the form of short peptides, they can express their health-promoting properties [4]. The most common method for producing bioactive peptides is the enzymatic hydrolysis of proteins. One of the common enzymes used in enzymatic hydrolysis is alcalase, which is produced by the bacterium Bacillus licheniformis. This enzyme is a type of alkaline and non-specific protease with high catalytic activity. Alcalase produces a suitable degree of hydrolysis in a shorter time and at alkaline pH compared to other natural and acidic enzymes. Its high proteolytic power and specific function make alcalase a suitable choice for the hydrolysis of plant proteins [5]. In the study of Abbasi et al. (2022), the effectiveness of alcalase and trypsin on the production of antioxidant peptides from quinoa protein was investigated. The findings showed that alcalase produced the highest amount of antioxidant peptides within 30 minutes and trypsin after 4 hours, respectively [6]. Extensive research has been conducted in the field of hydrolyzed protein production. However, enzymatic hydrolysis processes face limitations such as low enzyme efficiency, long reaction time, and reduced substrate-to-product conversion rate [7]. For this reason, today, several technologies such as ultrasound, microwave, freezing, and high-pressure processes are used as protein pretreatment methods to overcome the aforementioned limitations [8]. Ultrasound, which is known as a green, low-cost, and efficient technology, is widely used in the extraction of proteins and antioxidant compounds [9, 10]. Although this process leaves the primary structure of the protein intact, it causes changes in the tertiary and quaternary configurations, a change that can affect the functional and biological properties of the protein [11, 12]. Pacheco et al. (2023) investigated the effect of ultrasound pretreatment on the enzymatic hydrolysis of pumpkin seed protein concentrate. The results showed that the ultrasonically pretreated samples had a higher free radical scavenging capacity than the conventionally hydrolyzed samples [13]. The processes associated with the transformation and preparation of fruits and vegetables generate a significant volume of vegetable waste that can be recycled and reused; however, a large part of these materials are left unused and not considered [14]. Apple, scientifically known as Malus domestica, is one of the most widely consumed and popular fruits in the world, which has attracted attention due to its pleasant taste, high nutritional value, and diverse applications in the food industry. Statistics show that its global production has exceeded 14.86 million tons in 2024. A major part of global apple production is used to prepare products such as juice, and during this process, a pomace remains, which constitutes about 20 to 35% of the original weight of the fruit. This pomace includes parts such as skin, flesh, and a small amount of seeds [15]. Studies have shown that using these wastes in animal feed not only reduces waste, but also helps increase protein content and improve intestinal condition in animals, while also having good degradability [16]. Apple seeds are often not used due to the presence of anti-nutritional compounds such as amygdalin [17]. Amygdalin, a cyanogenic glycoside, is the most toxic compound in apple seeds, which can be converted to cyanide by beta-glucosidase in the intestine under appropriate conditions. However, recent studies have shown that the usual amounts consumed in moderate

amounts by humans or animals do not cause significant toxic effects, and some anti-inflammatory and antiproliferative effects have also been reported [18]. Apple seeds are a rich source of phenolic compounds, including phenolics (such as chlorogenic acid), flavonoids (catechins, quercetin, phloridzin), and terpenoids. These compounds have high antioxidant, anti-inflammatory, and anticancer activities [18]. Studies have shown that these seeds are rich in nutritional and bioactive compounds. The protein content of whole apple seeds (not defatted) is reported to be about 49.5 g per 100 g and its fat content is about 24 g per 100 g. These seeds also contain 2.5 to 4.31% ash, significant amounts of dietary fiber and phenolic compounds. These characteristics make apple seeds a potential source for the production of plant proteins and food and pharmaceutical applications[19]. The aim of this study is to optimize the enzymatic hydrolysis process of apple seed protein with alcalase enzyme to achieve the highest antioxidant activity.

2-Materials and Methods

2.1. Materials

Alkalase enzyme, trichloroacetic acid, potassium ferricyanide, ammonium hepta-molybdate, iron(III) chloride, hexane, sulfuric acid 95-98%, ethanol 96%, hydrochloric acid, dipotassium hydrogen phosphate, DPPH, Coomassie Brilliant Blue (G250), sodium dihydrogen phosphate monohydrate, sodium hydroxide were purchased from reputable companies such as Sigma and Merck. The implementation stages of this research were carried out in the laboratory of the Food Science and Technology Department of Gorgan University of Agricultural Sciences and Natural Resources. Apple seeds were prepared from waste from the pilot dried fruit section of Gorgan University of Agricultural Sciences and Natural Resources.

2.2.Preparation and oil extraction of raw materials

First, the seeds were separated manually and obtained as a uniform powder in an electric grinder (Asan Toos Shargh-A500) [20]. Then, for oil extraction, apple seed powder was mixed with hexane solvent in a ratio of 1:10 (weight-volume) and stirred for 72 hours with a shaker at a speed of 440 rpm. Then, the solvent residue in the meal was separated through a fan oven (Memmert-1720-Germany) at a temperature of 40 ° C for 24 hours. Finally, the resulting powder was passed through a sieve (Damavand Sieve Industries-Iran) with a mesh of 40 and the defatted apple seed powder was stored at refrigerator temperature until protein extraction[21].

2.3.Preparation of apple seed protein concentrate

To extract apple seed protein, defatted apple seed powder was dispersed in distilled water in a ratio of 1:10 (weight-volume). To denature the proteins, the pH of the mixture was adjusted to 11 (the solubility pH of apple seeds) using 1N sodium hydroxide solution, and then the mixture was stirred for one hour at room temperature with the help of a magnetic stirrer (Genoa-UK). In order to separate the soluble components, the samples were centrifuged at 5000 rpm for 20 minutes at 4°C in a refrigerated centrifuge (Hanil-Combi 514R-South Korea). After collecting the supernatant, the solid residue was redissolved in distilled water and the extraction step was repeated. Then, the supernatants obtained were combined and their pH was adjusted to 4 (the isoelectric pH of apple seeds) using 1N hydrochloric acid. Next, centrifugation was repeated under the same conditions, and finally, the resulting precipitate was dried using a freeze dryer (Operon-FDB 5503-South Korea) and stored in closed containers in a dry and cool environment[22].

2.4. Ultrasound pretreatment

First, a solution with a concentration of 5% (w/v) of apple seed protein concentrate in distilled water and a pH of 8 was prepared. To apply ultrasound pretreatment, a pulsed ultrasound device with a constant frequency of 60 Hz was used for 5 minutes and a power of 200 watts. In order to prevent temperature increase during the ultrasound operation, an ice bath was used[9].

2.5. Enzymatic hydrolysis

In order to prepare protein hydrolysate from apple seed protein concentrate, the enzyme alcalase was used. Alcalase enzyme was added to the protein solution at concentrations of 1 to 2% (weight ratio of enzyme to substrate) and the hydrolysis process was carried out at temperatures between 40 and 55°C and at pH 8, using a shaking incubator (Vision-VS 8480-South Korea) and for time intervals between 30 and 180 minutes. To inactivate the enzyme activity, the resulting mixture was heated in a bain-marie (Memmert-WNB 22-Germany) at 90°C for 15 minutes and then placed in a cold water bath for 10 minutes. In order to separate insoluble materials, the samples were centrifuged at 4000 rpm for 15 minutes and then the liquid phase (supernatant) was powdered using a freeze dryer and stored at -18°C until use[23].

2.6. Determination of the degree of hydrolysis

The degree of protein hydrolysis was determined by calculating the ratio of proteins soluble in 10% trichloroacetic acid to the total proteins in the sample. For this purpose, the sample was mixed with 20% trichloroacetic acid in a ratio of 1:1 and after 10 minutes vortex (Labortron-LS 100-Germany) at a speed of 4000 rpm, centrifuged for 20 minutes. The amount of protein in the soluble part (supernatant) was measured by the Bradford method at a wavelength of 595 nm with a spectrophotometer (PG Instrument-T80-England) and the degree of hydrolysis was calculated using equation(1) [24].

(1) DH (%) = { Protein (TCA+ Supernatant)/ Protein (almond hydrolysate suspension) }× 100

TCA: Trichloroacetic acid

2.7. Chemical composition analysis

Various chemical tests were performed on raw grain, defatted grain and the resulting protein concentrate. The percentage of moisture, protein, fat and ash (based on wet weight) were measured according to the AOAC (2003) method. The total carbohydrate content was obtained by subtracting the protein, ash, moisture and fat values from 100[25].

2.8. Investigation of antioxidant properties of hydrolyzed protein

To evaluate the antioxidant properties of hydrolyzed protein, three tests were performed including DPPH free radical scavenging, iron reducing power and total antioxidant capacity.

2.8.1. DPPH free radical scavenging measurement

In this method, first 1000 µl of the final supernatant obtained from hydrolysis was mixed with 1000 µl of DPPH solution with a concentration of 0.15 mM and after stirring for 20 seconds, the mixture was kept in the dark for 30 minutes. Then, the samples were centrifuged at 2500 rpm for 10 minutes and the absorbance of the supernatant was measured at a wavelength of 517 nm. The percentage of DPPH radical scavenging was calculated using equation (2). In the control sample, 1000 µl of distilled water was used instead of the supernatant obtained from hydrolysis[26].

Percentage of DPPH free radical inhibition= ($A_{blank} - A_{sample} / A_{blank}$) ×100

2.8.2. Iron ion reducing activity

First, 1000 µL of the final supernatant obtained from hydrolysis was mixed with 2500 µL of 0.2 M phosphate buffer with pH 6.5 and 500 µL of 1% potassium ferricyanide solution. This mixture was placed in a hothouse at 50°C for 30 minutes. Then, 500 µL of 10% (w/v) trichloroacetic acid solution was added to it. The resulting mixture was centrifuged for 10 minutes at 3000 rpm. In the next step, 1000 µL of the supernatant phase was combined with 1000 µL of distilled water and 200 µL of 0.1% ferric chloride solution. After a few minutes, the absorbance of the solution was measured at a wavelength of 700 nm. In the control sample, 1000 µL of distilled water was used instead of the supernatant obtained from hydrolysis. The higher the absorption rate, the higher the reducing power of the sample[27].

2.8.3. Total antioxidant capacity

First, 100 µL of the final supernatant obtained from hydrolysis was mixed with 1000 µL of reagent (0.6

M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and placed in a hot water bath at 90°C for 90 minutes. After cooling, the absorbance of the samples was measured at a wavelength of 695 nm. In the control sample, 100 µL of distilled water was used instead of the supernatant obtained from hydrolysis. Higher absorbance values indicated higher total antioxidant activity[28].

2.9. Statistical analysis

The experiments were conducted using the response surface methodology (RSM), using Design Expert version 13 software based on the central composite design (CCD), for three independent variables: time (A), temperature of the enzymatic hydrolysis process (B), and enzyme to substrate ratio (C) (Table 2). For this purpose, 20 random treatments were proposed by Design Expert software, considering 6 replications at the central point. The surfaces used were at a distance of 1+, 1-, α+, and α- from the central point (0). The α factor was equal to 1.414. The responses examined were total antioxidant capacity, iron reducing power, and DPPH radical scavenging activity. Different levels of the independent variables are shown in Table 1.

Table 1- Levels of independent variables used to optimize the conditions for hydrolysis of apple seed protein with alcalase enzyme with and without ultrasound application

	Levels variables				
	+α	+1	0	-1	– α
Independent variables code					
Hydrolysis process time (A)	180	149.595	105	60.4047	30
Process hydrolysis temperature (B)	55	51.9595	47.5	43.0405	40
Enzyme to substrate ratio % (C)	2	1.7973	1.5	1.2027	1

Table 2- Treatments designed using the response surface method in two modes with and without ultrasound

Treatment	Enzyme to substrate ratio (%)	Temperature (°C)	Time (minutes)
1	1.50	47.50	30
2	1.20	43.04	60.40
3	1.80	51.96	60.40
4	1.80	43.04	60.40
5	1.20	51.96	60.40
6	1.50	40	149.60
7	1.50	47.50	149.60
8	1.50	47.50	149.60
9	1.50	47.50	149.60
10	1.50	47.50	149.60
11	1.50	47.50	149.60
12	1.50	47.50	149.60
13	2	47.50	149.60
14	2	47.50	149.60
15	1.50	55	149.60
16	1.20	43.04	149.60
17	1.80	51.96	149.60
18	1.80	43.04	149.60
19	1.20	51.96	149.60
20	1.50	47.50	180

3-Results and Discussion

3.1. Approximate Compositions of Non-Fat, Defatted Apple Seed Powder and Protein Concentrate

The results of the analysis of the approximate compositions of non-fat apple seed powder, fat-containing apple seed powder and apple seed protein concentrate used in the present study are presented in Table 3.

Table 3- Approximate compositions of defatted, non-defatted and apple seed protein concentrate powders

Sample	Protein (%)	Moisture (%)	Oil (%)	Ash (%)	Carbohydrate (%)
defatted apple seed powder	28.78 ± 0.01[b]	9.58 ± 0.35[a]	7.20 ± 0.20[b]	2.64 ± 0.00[a]	51.80
Apple seed powder with fat	19.51 ± 0.01 [c]	9.42 ± 0.29[a]	19.88 ± 0.42[a]	2.55 ± 0.01[b]	48.64
Apple seed protein concentrate	72.01 ± 0.26[a]	5.68 ± 0.09[b]	2.20 ± 0.20[c]	0.75 ± 0.01[c]	19.36

Means with the same letter in each column indicate no significant difference at the p>0.05 level. Values are mean ± standard deviation.

The protein content of defatted apple seed powder was lower than that of defatted apple seed protein concentrate (defatted), while the moisture, fat, and ash content of defatted apple seed powder was higher than that of defatted apple seed protein concentrate (defatted). In line with the results obtained in this study, Bazi et al. (2024) reported after examining defatted pumpkin seed meal and protein isolate that the fat, moisture, and ash content of defatted pumpkin seed meal was higher than that of its protein isolate, and the protein content of defatted pumpkin seed meal was lower than that of its isolate. The protein (19.51 ± 0.01), moisture (42.9 ± 0.29), fat (19.88 ± 0.42), and ash (2.55 ± 0.01) values of the non-defatted apple seed powder obtained in this study were lower than the values of these compounds (34, 10.2, 27.7, and 4.1 percent, respectively) in the study of Yu et al. (2007) [29]. The reason for the difference in the chemical compositions of the samples in this study and previous studies can be attributed to the difference in the type of variety.

3.2. Calculating the degree of hydrolysis

The degree of hydrolysis is a common index for measuring the degree of protein hydrolysis. This index is defined as the ratio of broken peptide bonds to the total peptide bonds in the protein and is important in evaluating the biological activities of hydrolyzes and functional properties [30]. Based on the data, the degree of enzymatic hydrolysis of apple seed protein under ultrasonic pretreatment was reported to be 21.13% and 24.39% under conditions without ultrasonic pretreatment. 3-3- Optimization of production conditions of hydrolyzed apple seed protein using response surface methodology

To achieve the highest DPPH free radical scavenging activity, total antioxidant capacity and iron(III) ion reducing property, the response surface methodology was used. After performing the hydrolysis process according to the treatments specified by the Design expert software, each of the treatments was examined in terms of the mentioned tests.

3-3-1- Effect of hydrolysis time and temperature and enzyme to substrate ratio on DPPH radical scavenging activity of hydrolyzed apple seed protein with and without ultrasound pretreatment

Electron-donating and reducing compounds such as antioxidants, when exposed to the purple DPPH radical, convert it into a non-radical solution with a yellow to colorless color, and as a result, the absorption decreases. It is expected that apple seed hydrolysates contain peptides or amino acids with electron-donating ability and can scavenge free radicals [31]. According to the results, the DPPH radical scavenging activity of hydrolyzed apple seed protein without ultrasonic pretreatment was in the range of 45.22 to 66.97% and with ultrasonic pretreatment in the range of 45.17 to 89.63%

(Table 4). According to (Figure 1a), at a temperature of 51.96°C with increasing enzymatic hydrolysis process time, the DPPH radical scavenging activity of hydrolyzed apple seed protein pretreated with ultrasound increases. While according to (Figure 1d) when no ultrasonic pretreatment was used, at a temperature of 43.04°C with increasing hydrolysis process time, the highest DPPH radical scavenging activity of hydrolyzed apple seed protein was observed. (Figure 1b) The response surface plot shows the DPPH radical scavenging activity as a function of hydrolysis time and enzyme to substrate ratio. At the hydrolysis time of 149.60 min, it was found that the DPPH radical scavenging activity of the hydrolyzed proteins pretreated with ultrasound was at its highest value at enzyme to substrate ratios of 1.20 and 1.80%. As shown in the three-dimensional response surface plot (Fig. 1e) of the interaction between hydrolysis time and enzyme to substrate ratio, the DPPH radical scavenging activity of the hydrolyzed proteins without ultrasound pretreatment was maximum at the maximum values of hydrolysis time and enzyme to substrate ratio. The increased radical scavenging activity of hydrolyzed apple seed proteins is probably due to structural changes in the protein, which leads to a greater ability to scavenge radicals. The disruption of the structure of apple seed proteins by enzymatic hydrolysis may open the protein and expose amino acid residues that are electron donors and can react with free radicals [32]. The results of this part of the present study are consistent with the findings of Promjeen et al. (2025), who showed that increasing enzyme concentration and increasing hydrolysis time increased DPPH radical scavenging activity. This increase was primarily attributed to structural changes, molecular weight reduction, and increased exposure to functional groups [33]. Looking at (Figure 1c), it can be seen that the highest DPPH radical scavenging activity of proteins hydrolyzed by ultrasound pretreatment was observed at a hydrolysis temperature of 43.04°C and an enzyme to substrate ratio of 1.20%. Vaštag et al. (2010) stated in a study that temperature strongly affects the antiradical activity of hydrolysates and higher hydrolysis temperatures (close to 50°C) are preferred for producing hydrolysates with antioxidant properties [34]. As shown in (Figure 1f) the simultaneous effect of hydrolysis temperature and enzyme to substrate ratio, the highest DPPH radical scavenging activity of hydrolyzed proteins without ultrasound pretreatment was observed in the temperature range of 51.96°C and enzyme to substrate ratio of 1.80%.

Table 4- DPPH free radical scavenging activity data with and without ultrasound pretreatment

Treatment	DPPH	DPPH(U)
1	60.85	49.10
2	58.14	62.95
3	58/57	50.86
4	51.71	62.32
5	60.88	57.97
6	62.05	50.39
7	57.27	50.96
8	59.24	50.21
9	59.79	50.39

10	61.05	50.58
11	60.07	45.17
12	52.87	45.18
13	60.31	58.95
14	52.06	56.47
15	52.59	52.16
16	64.38	63.89
17	66.97	59.46
18	59.15	62.00
19	45.22	61.01
20	56.72	60.19

U: ultrasound pretreatment

DPPH: DPPH radical scavenging activity(%)

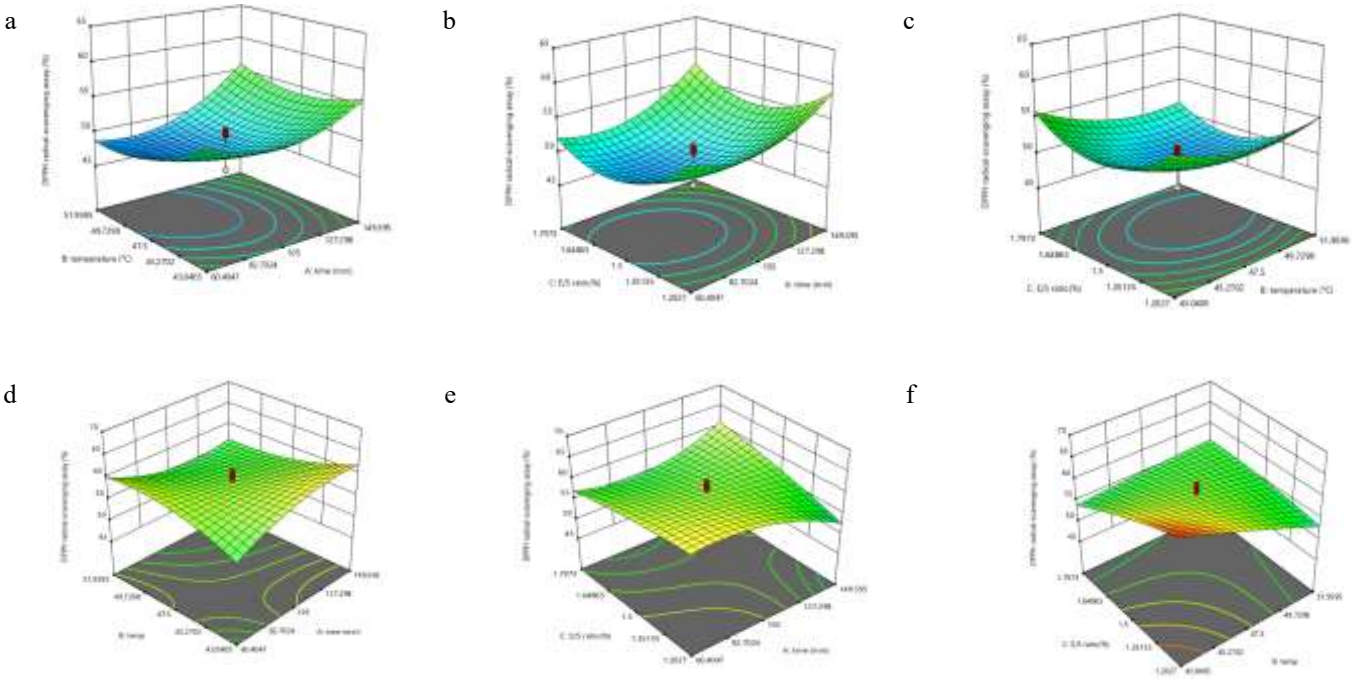


Figure 1- Three-dimensional response surface plot of the effect of hydrolysis time (minutes), hydrolysis temperature (°C), and enzyme to substrate ratio (percent) on changes in DPPH radical scavenging

activity of hydrolyzed apple seed proteins with (a-c) and without ultrasound pretreatment (d-f).

3.3.2. Effect of hydrolysis time and temperature and enzyme to substrate ratio on total antioxidant activity of hydrolyzed apple seed protein with and without ultrasonic pretreatment

This test is based on the reduction of Mo(VI) (molybdenum ion with a positive charge of 6+) to Mo(V) (molybdenum ion with a positive charge of 5+) by the sample and the formation of a green Mo(V)/phosphate complex at acidic pH [27]. An increase in the absorption rate indicates a higher total antioxidant activity [36]. According to the results of the experiments (Table 5), the absorption of hydrolyzed apple seed protein samples under ultrasonic pretreatment in the total antioxidant activity test (at a wavelength of 695 nm) was between 0.521 and 0.817, and in samples without ultrasonic pretreatment, it was between 0.513 and 0.982. The interaction effect of time on hydrolysis temperature on the total antioxidant activity of hydrolyzed apple seed proteins pretreated with ultrasound pretreatment (Figure 2a) and without ultrasound pretreatment (Figure 2d) showed that at a fixed enzyme to substrate ratio (1.50%), the total antioxidant activity of the samples reached a maximum when the hydrolysis time was 149.60 min and the temperature was 51.96 °C. It has been reported that with the passage of time and increasing the hydrolysis temperature, due to the progress of the hydrolysis process, the breaking of bonds between peptides and their conversion into smaller peptides, the length of the peptide chain changes, and this has a significant effect on its antioxidant properties, such that it has been found that peptides produced with lower molecular weight have higher antioxidant capacity [37]. Izanloo et al. (2022) after studying the hydrolysis of edible mushroom protein found that increasing both the temperature and hydrolysis time parameters had an upward effect on the total antioxidant activity [38]. According to the response surface diagram (Figure 2b), it can be seen that the highest total antioxidant activity of hydrolyzed apple seed proteins pretreated with ultrasound was obtained at the end of the hydrolysis process (in the range of 127.30 to 149.60 minutes) and the enzyme to substrate ratio was 1.20 to 1.70%. According to the response surface diagram (Figure 2e), it can be seen that the maximum total antioxidant activity of hydrolyzed apple seed proteins without ultrasound pretreatment was obtained when the enzymatic hydrolysis process time and enzyme to substrate ratio were at their highest (time 149.60 minutes and enzyme to substrate ratio 1.80%). The interaction effect of temperature and enzyme to substrate ratio on total antioxidant activity is shown in (Figure 2c), the highest total antioxidant activity of the samples was obtained at temperatures of 43.04 to 45.27 °C and enzyme to substrate ratio of 1.20 to 1.35%. The three-dimensional graph (Figure 2f) shows the interaction effect of temperature and enzyme to substrate ratio (at a constant time of 105 minutes) on the total antioxidant activity of the hydrolyzed protein. It is clear that at a temperature of 51.96 °C, the total antioxidant activity of the samples increases with increasing enzyme to substrate ratio. In general, it can be concluded that the alcalase enzyme, by hydrolyzing peptide bonds in apple seed protein, was able to release peptides with electron-donating properties, which converted free radicals into stable compounds and ultimately led to an increase in total antioxidant capacity [39].

Table 5- Total antioxidant capacity data with and without ultrasound pretreatment

Treatment	AOT	AOT(U)
1	0.581	0.556
2	0.653	0.806

3	0.528	0.591
4	0.657	0.556
5	0.561	0.620
6	0.513	0.697
7	0.654	0.721
8	0.616	0.693
9	0.609	0.671
10	0.621	0.667
11	0.618	0.675
12	0.682	0.757
13	0.611	0.586
14	0.682	0.581
15	0.687	0.627
16	0.621	0.821
17	0.982	0.817
18	0.706	0.521
19	0.573	0.753
20	0.659	0.756

U: ultrasound pretreatment

AOT: total antioxidant activity

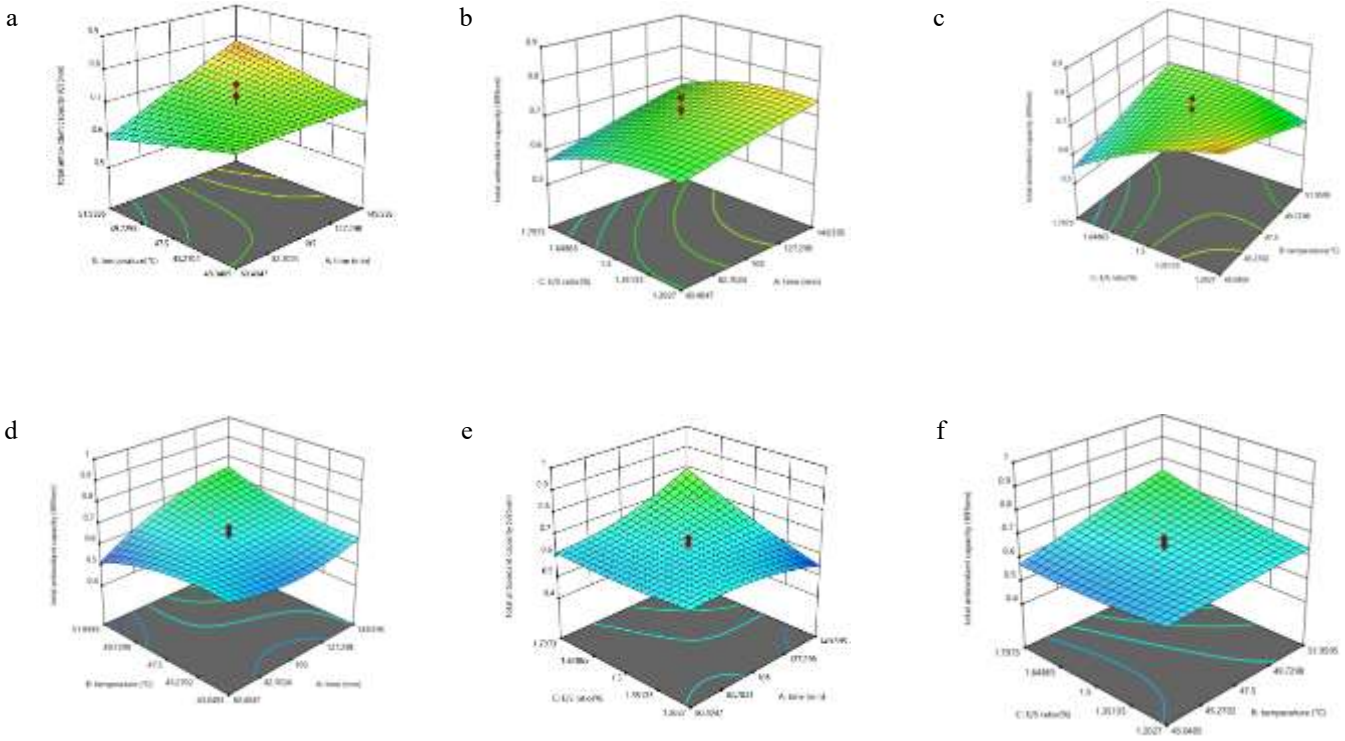


Figure 2- Three-dimensional response surface plot of the effect of hydrolysis time (minutes), hydrolysis temperature (°C), and enzyme to substrate ratio (%) on changes in total antioxidant activity of hydrolyzed apple seed proteins with (a-c) and without ultrasound pretreatment (d-f).

3.3.3. Effect of hydrolysis time, temperature and enzyme to substrate ratio on the iron ion reducing power of hydrolyzed apple seed protein with and without ultrasonic pretreatment

Reducing power measurement is often used to evaluate the electron donating ability of antioxidants. This test is usually related to reductants. Reductants play their antioxidant role by donating hydrogen and disrupting chain reactions, forming free radicals or reacting with some peroxide precursors and preventing their formation [27]. Based on the results, the absorption of ultrasonically pretreated apple seed protein samples in the iron ion reducing power test (at a wavelength of 700 nm) was obtained in the range of 0.793 to 0.87 and without ultrasonic pretreatment in the range of 0.623 to 1.095 (Table 6). With increasing enzymatic hydrolysis time at a fixed enzyme to substrate ratio (1.50%), the iron reducing power of the hydrolyzed samples increased with a gentle slope (Figure 3a). For hydrolyzed apple seed proteins without ultrasonic pretreatment, based on the response surface model at a fixed enzyme to substrate ratio (1.50%), the highest iron reducing power of the samples was obtained at 105 min and 51.96°C (Figure 3d). The hydrolysis reaction probably changed the structure of the apple seed protein and led to the exposure of amino acid residues that are electron donors and capable of reacting with free radicals [40]. Consistent with the results obtained in this study, Zhidong et al. (2013) found that hydrolysis temperature and time improved the reducing power of whey protein within a certain range, but at high temperature and time, the reducing power decreased. According to the results obtained by the iron reduction power of hydrolyzed samples subjected to ultrasound pretreatment (Figure 3b), the highest desired response was observed with increasing the hydrolysis process time from 110 to 149.60 minutes and the enzyme to substrate ratio from 1.55 to 1.80%. Zhidong et al. (2013) found that the reducing power increased with increasing the enzyme to substrate ratio [35]. With increasing the hydrolysis time and decreasing the size of the produced peptides, the ability of the peptides to remove free radicals increased [41]. Samadi Varedesara et al. (2021) after investigating the hydrolysis of grape seed protein with alcalase enzyme stated that with increasing the hydrolysis time, the reducing power of ferric ion increased, which is consistent with the results obtained in the present study. According to (Figure 3c), it was observed that the simultaneous increase in the enzyme to substrate ratio and temperature improves the iron reduction power in the hydrolyzed samples. The highest iron reducing power of the samples subjected to ultrasonic pretreatment was observed at the maximum enzyme to substrate ratio when the hydrolysis temperature was low (43.04 to 45.27 °C). However, when no ultrasonic pretreatment was used (Fig. 3f) at a high enzyme to substrate ratio (1.80%), the iron reducing power of the hydrolyzed apple seed samples increased steeply with increasing hydrolysis temperature. The high reducing power of apple seed protein hydrolysates is probably due to the increased availability of free hydrogen groups resulting from peptide hydrolysis[32].

Table 6- Iron reduction property data with and without ultrasound pretreatment

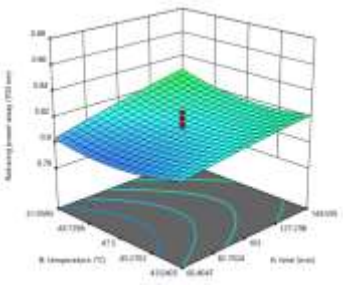
Treatment	Fe	Fe(U)
1	0.691	0.799
2	0.763	0.793
3	0.638	0.803
4	0.767	0.843

5	0.671	0.802
6	0.623	0.811
7	0.764	0.814
8	0.726	0.794
9	0.719	0.824
10	0.731	0.804
11	0.728	0.819
12	0.792	0.809
13	0.721	0.795
14	0.792	0.840
15	0.797	0.835
16	0.731	0.814
17	1.095	0.845
18	0.816	0.870
19	0.683	0.834
20	0.769	0.803

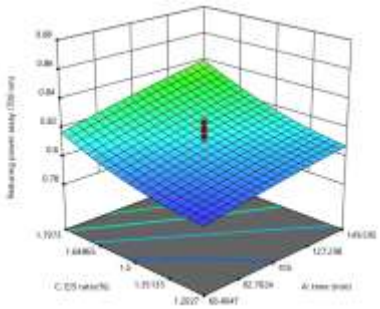
U: ultrasound pretreatment

Fe: Iron reducing power

a



b



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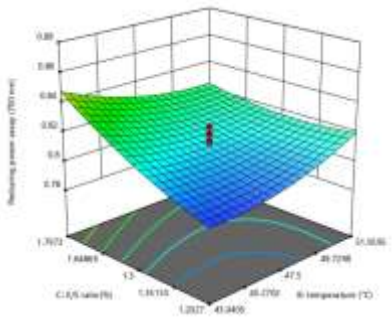

d e f

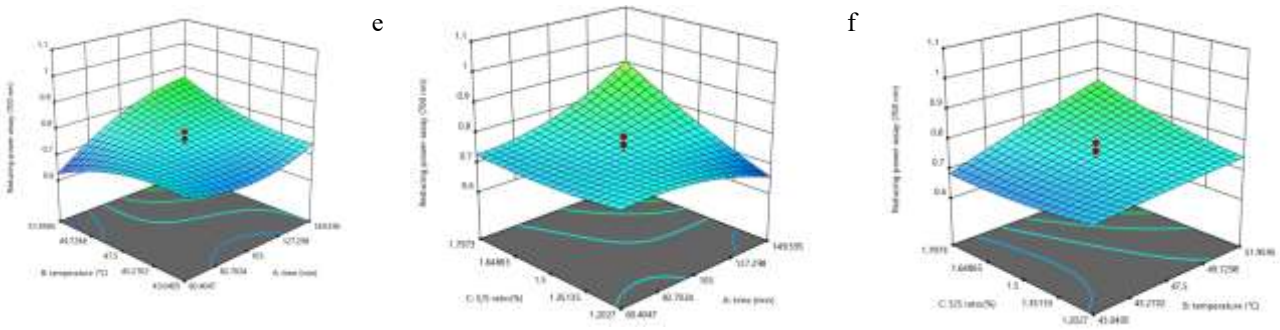

Figure 3- Three-dimensional response surface plot of the effect of hydrolysis time (minutes), hydrolysis temperature (°C), and enzyme to substrate ratio (%) on changes in the iron reducing power of hydrolyzed apple seed proteins with(a-c) and without ultrasound pretreatment (d-f).

3.3.4. Determination of optimal hydrolysis conditions for the production of hydrolyzed apple seed proteins with high antioxidant properties using response surface methodology

The results obtained showed that the optimal conditions for the hydrolysis of apple seed protein with optimal activity, by the alcalase enzyme with and without ultrasound pretreatment, were obtained with hydrolysis process time of 149.60 minutes, hydrolysis process temperature of 51.96 °C, enzyme to substrate ratio of 1.80% (the optimal point was the same for both processes). Under such conditions, the total antioxidant capacity (at a wavelength of 695 nm), iron reducing power (at a wavelength of 700 nm), and DPPH radical scavenging activity with and without ultrasound pretreatment were 0.80 and 0.97, 0.84 and 1.08, 58.90 and 66.67%, respectively (Table 7).

Table 7- Predicted and actual values of dependent variables under optimal enzymatic hydrolysis conditions

Target factors	Total antioxidant activity (absorbance at 695nm)	Iron reduction power (absorbance at 700nm)	DPPH radical scavenging activity (%)
Predicted value by applying ultrasound pretreatment	0.80	0.84	58.90
Predicted value without applying ultrasound pretreatment	0.97	1.08	66.67
Actual value with ultrasonic pretreatment	0.92	0.78	47.27
Actual value without ultrasonic pretreatment	0.77	0.93	59.42

4-Conclusion

In this study, first, experiments were conducted using the response surface methodology (RSM), using Design Expert software based on the central composite design (CCD), for three independent variables: time and temperature of the enzymatic hydrolysis process and the enzyme to substrate ratio. The enzymatic hydrolysis process was carried out at temperatures of 40 to 55 ° C, an enzyme to substrate ratio of 1 to 2%, and a time interval of 30 to 180 minutes. The total antioxidant capacity test (at a wavelength of 695 nm) showed that the absorption of the hydrolyzed apple seed protein samples pretreated with ultrasound was between 0.521 and 0.817, and the samples without pretreatment were between 0.513 and 0.982. In the iron reduction power test (at a wavelength of 700 nm), the absorbance of the ultrasound-pretreated samples was in the range of 0.793 to 0.870 and that of the samples without pretreatment was in the range of 0.623 to 1.095. The DPPH radical scavenging activity was between 45.17 and 63.89 percent for the ultrasound-pretreated samples and between 45.22 and 66.97 percent for the samples without pretreatment. The results showed that the optimal conditions for hydrolysis of apple seed protein with the highest total antioxidant activity, iron reducing power, and DPPH free radical scavenging activity by alcalase enzyme without ultrasound pretreatment were obtained with hydrolysis time of 149.60 min, hydrolysis temperature of 51.96 °C, and enzyme to substrate ratio of 1.80%. Apple seeds, as a rich source of protein and bioactive compounds, have a high potential for producing plant peptides with antioxidant activity. These results indicate that peptides produced from apple seeds can be used as natural antioxidant additives in food products and reduce oxidation and increase their shelf life. The findings also indicate the importance of recovering fruit waste and utilizing plant protein sources for the development of healthy and functional food products.

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بهینه‌یابی هیدرولیز پروتئین دانه سیب با استفاده از آنزیم آلکالاز و بررسی ویژگی آنتی‌اکسیدانی آن

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چکیده

با افزایش نگرانی‌ها درباره اثرات مخرب آنتی‌اکسیدان‌های مصنوعی بر سلامت، استفاده از آنتی‌اکسیدان‌های طبیعی به‌ویژه پپتیدهای زیست‌فعال، اهمیت فزاینده‌ای یافته است. در این پژوهش بهینه‌یابی فرآیند هیدرولیز آنزیمی پروتئین دانه سیب توسط آنزیم آلکالاز و پیش‌تیمار فراصوت بررسی شد. هیدرولیز آنزیمی پروتئین دانه سیب با آنزیم آلکالاز، در دماهای ۴۰ تا ۵۵ درجه سانتی‌گراد، نسبت آنزیم به سوبسترا ۱ تا ۲ درصد و در بازه زمانی ۳۰ تا ۱۸۰ دقیقه انجام شد. محلول پروتئینی پیش از هیدرولیز به‌مدت ۵ دقیقه با دستگاه فراصوت پالسی (۲۰۰ وات، ۶۰ هرتز) تیمار شد. ارزیابی فعالیت آنتی‌اکسیدانی شامل سنجش مهار رادیکال DPPH، قدرت احیاکنندگی و ظرفیت آنتی‌اکسیدانی کل صورت گرفت. نمونه‌های بدون پیش تیمار فراصوت بیشترین فعالیت آنتی‌اکسیدانی را از خود نشان دادند. شرایط بهینه فعالیت آنتی‌اکسیدانی در این حالت، به‌ترتیب شامل زمان هیدرولیز ۱۴۹/۶۰دقیقه، دمای ۵۱/۹۶ درجه سانتی‌گراد و نسبت آنزیم به سوبسترا ۱/۸۰ ٪ بود. آزمون ظرفیت آنتی‌اکسیدانی کل (در طول موج ۶۹۵ نانومتر) نشان داد که جذب نمونه‌های پروتئین هیدرولیز شده دانه سیب پیش‌تیمار شده با فراصوت بین ۰/۵۲۱ تا ۰/۸۱۷ و نمونه‌های بدون پیش‌تیمار بین ۰/۵۱۳ تا ۰/۹۸۲ قرار داشت. در آزمون قدرت احیاء آهن (در طول موج ۷۰۰ نانومتر)، جذب نمونه‌های پیش‌تیمار شده با فراصوت در بازه ۰/۷۹۳ تا ۰/۸۷۰ و نمونه‌های بدون پیش‌تیمار در بازه ۰/۶۲۳تا ۱/۰۹۵ به‌دست آمد. فعالیت مهارکنندگی رادیکال DPPH برای نمونه‌های پیش‌تیمار شده با فراصوت بین ۴۵/۱۷ تا ۶۳/۸۹ درصد و برای نمونه‌های بدون پیش‌تیمار بین ۴۵/۲۲ تا ۶۶/۹۷ درصد قرار داشت. نتایج این پژوهش نشان می‌دهد که این پپتیدها می‌توانند به‌عنوان آنتی‌اکسیدان طبیعی، در فرمولاسیون محصولات غذایی استفاده شده و موجب کاهش اکسیداسیون و افزایش ماندگاری آن‌ها شوند.