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Investigating the Effect of Protease Enzyme Type and Hydrolysis Time on the Antioxidant Properties of Flaxseed Meal (*Linum usitatissimum*) Protein Hydrolysates

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Abstract

In this research, the effect of protease enzyme type (pepsin and pancreatin) and hydrolysis time (40-200 minutes) on the degree of hydrolysis and antioxidant properties (DPPH radical scavenging activity, Fe chelating activity, Fe reducing power and total antioxidant capacity) of flaxseed meal protein hydrolysates was investigated. The results showed that increasing the hydrolysis time increased the degree of hydrolysis, and the samples obtained from pancreatin had a higher degree of hydrolysis than pepsin. The highest activity of Fe^{2+} chelating (53.71 ± 0.45%) and Fe^{3+} reduction (1.32 ± 0.02, absorbance at 700 nm) was achieved by pancreatin after 200 minutes of hydrolysis. Pancreatin samples were more capable of inhibiting DPPH free radicals than pepsin, and their activity increased with increasing time up to 160 minutes. The highest total antioxidant capacity (1.36 ± 0.08 absorbance at 695 nm) among the samples was obtained after 160 minutes of hydrolysis with pancreatin. The antioxidant capacity of flax seed protein hydrolysates in inhibiting DPPH radical, Fe chelating activity, and total antioxidant capacity was lower than the antioxidant capacity of vitamin C at a concentration of 50 (mg/ml), but it had more Fe reducing power than vitamin C. Therefore, it can be concluded that compared to pepsin, pancreatin had a greater ability to produce flaxseed protein hydrolysates with significant antioxidant properties. According to the results, flaxseed protein hydrolysates from pancreatin enzyme and a hydrolysis time of 160 minutes have the ability to be used in food formulations to produce functional products.

Keywords: Antioxidant, Enzymatic hydrolysi, Flax seed, Pancreatin, Pepsin

Introduction

Oxidation of lipids and the production of free radicals play an undeniable role in dangerous diseases such as atherosclerosis, cancer, and cardiovascular diseases. Free radicals are produced in aerobic organs during respiration. They are extremely unstable, quickly react with biological molecules, and lead to irreparable damage to cells (Fiaschi & Chiarugi, 2012). Also, this phenomenon is one of the most important problems of the food industry, because, in addition to the adverse and irreparable effects on the health of consumers, it causes the production of dangerous

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compounds and unpleasant odors and tastes, which causes heavy and irreparable economic losses to the manufacturer (Kaveh *et al*., 2019b). In the food industry, synthetic antioxidants such as BHA[1], BHT[2], PG[3] and TBHQ[4] are used, which have favorable antioxidant potential and reasonable price, but in recent decades, their adverse effects on human health have caused concern among scientists. As a result, in some countries, their application in food formulations has been limited or banned (Sadeghi Mahoonak & Kaveh, 2022).

On the other side, in recent years, public awareness regarding the direct effect of diet on human health has increased, which has increased the demand for food products without synthetic preservatives (Rezazadeh-bari *et al*., 2019). Therefore, identifying natural compounds with antioxidant potential has become a research priority for researchers. Among the natural antioxidants, bioactive peptides are suitable options, which usually have 2-20 amino acid residues and a molecular weight of less than 6000 da. These peptides can be produced by three methods: microbial fermentation, enzymatic hydrolysis, and chemical synthesis (Ulug *et al*., 2021). Recently, protein hydrolysates have been produced using various plant sources such as wheat germ (Karami *et al*., 2019) ،orange seed (Mazloomi *et al*., 2020), soybean (Islam *et al*., 2022), grape seed (Ding *et al*., 2018) and fenugreek (Kaveh *et al*., 2022). One of the suitable vegetable options for production protein hydrolysates is flaxseed, which is a rich source of protein that contains 35-45% oil. Flaxseed contains about 20% of unsaturated fatty acids such as oleic acid and more than 70% of alpha-linoleic acid (omega-3) fatty acids (Martinchik *et al*., 2012).

Among the suitable methods for production protein hydrolysates, enzymatic hydrolysis with protease enzymes is a suitable method that has been proven in various studies such as Farzanfar *et al*., (Farzanfar *et al*., 2024), Alvand *et al*., (Alvand *et al*., 2022), Xia *et al*., (Xia *et al*., 2023) and Islam et al., (Islam *et al*., 2023). Enzymatic hydrolysis is a process usually carried out under controlled conditions (pH, temperature, time, and enzyme concentration) and is the most common method for the production of bioactive peptides (Mora & Toldrá, 2023). In this regard, it has been reported that enzymatic hydrolysis is one of the most effective methods of producing bioactive peptides and protein hydrolysates because it has many advantages, such as high yield, no adverse effect on the nutritional value of proteins, and low side reactions (Habinshuti *et al*., 2023). Therefore, the aim of this research was to investigate the effect of protease enzyme type (pancreatin and pepsin) and hydrolysis time (40-210 minutes) on antioxidant properties (DPPH radical scavenging activity, Fe reducing power, Fe chelating activity, and total antioxidant capacity) and the degree of hydrolysis of hydrolyzed flaxseed protein and comparing it with the antioxidant activity of vitamin C as a synthetic antioxidant and unhydrolyzed flaxseed protein.

Materials and Methods

Pancreatin, pepsin, ammonium molybdate, iron dichloride, ferric chloride, trichloroacetic acid, ferrozine, ascorbic acid, and DPPH from Sigma, ethanol, sodium triphosphate, sulfuric acid, soda, hydrochloric acid, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were purchased from Merck, and flax seeds were purchased from a local store in the center of Tehran. The devices used in this research are listed in Table 1.

1- butylated hydroxyanisole
2- butylatedhydroxytoluene
3- propyl gallate
4- Tertiary butylhydroquinone

Table 1- The used devices

Device	Producer Company	Country
Water bath	Memmert, WNE45	Germany
Centrifuge	HERMLE, 36 HK	Germany
Shaker incubator	Wiggens, WS-600R	South Korea
Hot plate	VELP	Italy
Spectrophotometer	LABNICS, NUVS100	England
pH meter	inoLab, 7110	Germany
Scale	Sartorius	Germany
Freeze dryer	Chrtist	Germany

Fat Removal

To extract protein, flaxseed was first milled with an electric miller. The resulting powder was mixed with hexane at a ratio of 1:4 (w/v) and continuously stirred for 3 hours at room temperature. In the next step, hexane was separated using a Buchner funnel. The defatted powder was dried at 35°C and finally passed through a 40 mesh sieve (Kaveh *et al*., 2022).

Protein Extraction

In order to extract protein from the defatted powder, the resulting powder was mixed with distilled water at a ratio of 1:10 and the pH was adjusted to pH=10 using 1 N NaOH and stirred continuously for 2 hours at room temperature. The resulting solution was then centrifuged at 5000×g for 30 minutes. Then, the pH of the supernatant was adjusted to pH= 4 (the isoelectric pH of flaxseed protein) using 1 N HCl. In the next step, to separate the proteins, the resulting solution was centrifuged at 5000 ×g for 20 minutes, and the pellet was washed twice with distilled water and centrifuged again at 5000 ×g for 5 minutes. Then, the resulting protein isolate was dried with a freeze dryer and kept away from light at 4°C until further analysis (Kaveh *et al*., 2023).

Production of Flaxseed Protein Hydrolysate

Flax seed protein isolate obtained from the previous step was mixed at 5% concentration with 0.2 M phosphate buffer solution pH=7.4 for pancreatin activity, and for pepsin activity, it was mixed with distilled water, and the pH was adjusted to pH=2 using 1 N HCl. The resulting suspensions were continuously stirred for 30 minutes at room temperature in order to ensure complete hydration. Then, the samples were placed in a shaker incubator (40°C for pancreatin and 37°C for pepsin). The amount of enzyme was added at an enzyme-to-protein ratio of 1% (w/w). The reaction time was 40-200 minutes. After each time interval, the samples were transferred into a water bath of 90°C to inactivate the protease enzymes, and after 10 minutes, placed in an ice container to reach the ambient temperature. Then the samples were centrifuged for 20 minutes at 8000 × g, and the resulting supernatant was dried using a freeze dryer and kept in dark containers away from light at -20°C (Fadimu *et al*., 2021).

Degree of Hydrolysis

To evaluate the degree of hydrolysis of the resulting protein hydrolysates, the suspension of hydrolyzed protein and trichloroacetic acid (0.44 M) were mixed in a volume ratio of 1:1 and incubated for 15 minutes at 4°C. Then, the resulting mixture was centrifuged at 10000 rpm for 10 minutes. The amount of protein in the supernatant containing trichloroacetic acid 0.22 M was determined by the Bradford method. Bovine Serum albumin (BSA) was used as a standard (Sarabandi *et al*., 2019). The degree of hydrolysis was determined using Equation 1:

$$DH\ (\%) = \frac{\text{Protein (TCA+Supernatant)}}{\text{Protein (Flax seed hydrolysate suspension)}} \times 100 \quad (1)$$

Evaluation of Antioxidant Properties of Hydrolyzed Protein

DPPH Radical Scavenging Activity

To evaluate the DPPH radical scavenging activity of hydrolyzed flaxseed protein, the samples were dissolved in distilled water at a concentration of 40 mg/ml and vitamin C at a concentration of 50 mg/ml. Then, they were mixed with DPPH ethanol solution at a concentration of 0.15 mM in a ratio of 1:1 and vortexed for 20 seconds for complete mixing. In the next step, the resulting mixture was kept in the dark for 30 minutes and finally centrifuged at 4000 rpm for 15 minutes. The absorbance of the produced supernatant was read at 517 nm. DPPH radical scavenging activity of the samples was calculated using equation 2. A blank is the absorption of the control (DPPH ethanolic solution without hydrolyzed protein), and A sample is the absorption of the sample (Zhang *et al.*, 2015) .

$$I\ (\%) = [\frac{Ablank - Asample}{Ablank}] \times 100 \qquad (2)$$

Total Antioxidant Capacity

To evaluate the antioxidant capacity of protein hydrolysates, 0.1 ml of each hydrolyzed protein was dissolved in distilled water at a concentration of 40 mg/ml or vitamin C at a concentration of 50 mg/ml and mixed with 1 ml of the reagent (sulfuric acid 0.6 M, sodium phosphate 28 mM and ammonium molybdate 4 mM,100 ml of the reagent contained 3.25 ml of sulfuric acid, 0.49 g of ammonium molybdate and 1.064 g of sodium phosphate), then the resulted mixture was incubated in a water bath at 90°C. Finally, after cooling the samples and reaching the ambient temperature, the absorbance of the samples was read at 695 nm. Higher absorbance indicates stronger total antioxidant capacity (Kaveh *et al.*, 2022).

Fe chelating Activity

To evaluate the Fe chelating activity of hydrolyzed protein samples, 1 ml of hydrolyzed protein dissolved in distilled water at a concentration of (40 mg/ml) or vitamin C at a concentration of 50 mg/ml, then 0.05 ml of $FeCl_2$ (2 mM) and 1.85 ml of double distilled water were added. In the next step, 0.1 ml of ferrozine solution (5 mM) was added and the mixture was vigorously vortexed. Finally, it was stored for 10 minutes at ambient temperature and its absorbance was read at 562 nm. The chelating activity of the samples was calculated using equation (3). A blank (the absorbance of the control sample without antioxidant compound and containing distilled water) and A sample (the absorbance of the hydrolyzed sample) (Jamdar *et al.*, 2010).

$$\text{Chelating effect}\ (\%) = [(A_{blank} - A_{sample}/A_{blank})] \times 100 \qquad (3)$$

The Fe Reducing Power

In order to evaluate the Fe reducing power of hydrolyzed proteins, 0.5 ml of hydrolyzed protein solution in distilled water at a concentration of 40 mg/ml or vitamin C at a concentration of 50 mg/ml was mixed with 0.5 ml of 0.2 M phosphate buffer (pH=6.6) and 0.5 ml potassium ferricyanide (1% w/v) and kept in water bath at 50 °C for 20 minutes. Then, 0.5 ml of 10% trichloroacetic acid solution was added to the mixture and centrifuged at 2500 rpm for 10 minutes. Finally, 1 ml of the resulting supernatant was mixed with 1 ml of distilled water and 0.2 ml of ferric chloride (0.1% w/v) and kept at room temperature for 10 minutes. The absorbance of the samples was read at 700 nm. An increase in the absorbance of the mixture indicates an increase in the reducing power (Kaveh *et al.*, 2023).

Statistical Analysis

The statistical analysis of data was carried out using the SPSS 16.0 software (SPSS Inc., Chicago, IL). A means comparison was ascertained by Duncan's test at the 5% significance level using analysis of variance (ANOVA). All experiments were performed in triplicate, and the results were reported as means ± standard deviation.

Results and Discussion

Degree of Hydrolysis

Degree of hydrolysis is a measure of protein hydrolysis by protease enzyme. Fig. 1 shows the effect of hydrolysis time and the type of protease enzyme on the degree of hydrolysis of flaxseed protein. The results of statistical

analysis showed that both factors of hydrolysis time and type of enzyme had a significant effect on the degree of hydrolysis ($p<0.05$), so that after 200 minutes of the hydrolysis process with pancreatin, the degree of hydrolysis was 25.41 ± 0.2% and with pepsin enzyme, it was 24.15 ± 0.18%. The degree of hydrolysis increased significantly with the activity of pepsin and pancreatin enzymes in the first 160 minutes. However, increasing the time of hydrolysis afterward did not have a significant effect on the degree of hydrolysis and the rate of degree of hydrolysis decreased. This trend comes from excessive decomposition of the substrate and the inhibitory effect of the final product, which negatively affects the protease enzyme activity. On the other hand, the decrease in the rate of enzymatic hydrolysis might be as a result of the decrease in the number of peptide bonds available for hydrolysis, as well as the deactivation of the protease enzyme (Kaveh *et al.*, 2024). Our findings are similar to reports of Sherafat *et al.* (Sherafat *et al.*, 2018) and Yasmi *et al.* (Yasemi *et al.*, 2013) in the hydrolysis of after-cooking waste of skipjack fish and carp fish, respectively.

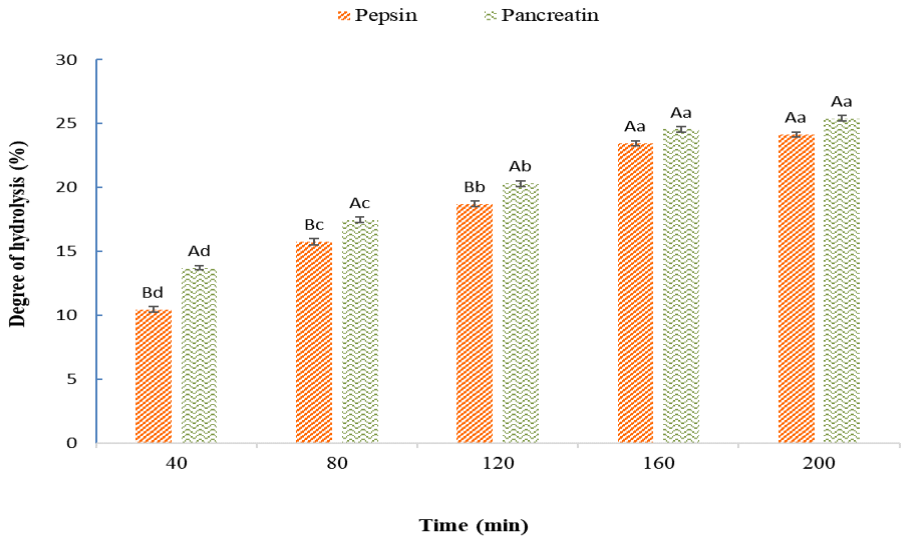

Fig. 1. The effect of hydrolysis time and protease type on the degree of hydrolysis of flax seed protein hydrolysate
(Lowercase letters indicate a significant difference between hydrolyzed samples with the same enzyme at different hydrolysis times, and uppercase letters indicate a significant difference between hydrolyzed samples with different enzymes at the same hydrolysis time, $p < 0.05$)

DPPH Radical Scavenging Activity

DPPH is a fat-soluble free radical that has the highest absorbance at 517 nm, and by receiving hydrogen from compounds with antioxidant activity, it becomes a stable compound, and its absorbance rate decreases (Hashemi *et al.*, 2022). The results of the hydrolysis time investigation showed (Fig. 2) that the lowest level of DPPH radical scavenging activity was related to non-hydrolyzed protein, and increasing the hydrolysis time caused a significant increase in the DPPH radical scavenging activity of hydrolyzed proteins ($p<0.05$). The DPPH radical scavenging activity of protein hydrolysates from pancreatin and pepsin were in the range of 37.54±0.46-51.24±0.37% and 33.12±0.33-45.30±0.26, respectively. DPPH radical scavenging activity for vitamin C at the concentration of 50 (mg/ml) was 70.35 ± 0.94%. Therefore, it can be concluded that increasing the time and degree of hydrolysis causes the release of proton-donating peptides that are capable of reacting with the DPPH free

radical and transforming it into stable compounds, and finally, the radical chain reactions are terminated. On the other hand, in the samples obtained from pancreatin activity, increasing the hydrolysis time more than 160 minutes did not make a significant difference in the inhibitory activity of the resulting hydrolyzed protein, and by pepsin enzyme activity, increasing the hydrolysis time more than 120 minutes caused a decrease in the ability of produced protein hydrolysate in DPPH radical scavenging activity. On the other hand, the negative effect of the excessive increase in enzymatic hydrolysis time can cause the greater effect of the protease enzyme, which causes the breaking and decomposition of a number of antioxidant peptides produced in the initial stages of hydrolysis, as a result of this process, the ability of protein hydrolysates in inhibition of DPPH free radical is reduced (Mazloomi-Kiyapey *et al*., 2019). On the other side, there was a significant difference between the DPPH radical scavenging activity of hydrolyzed proteins obtained from pancreatin and pepsin enzymes. Samples obtained from pancreatin activity had more DPPH radical scavenging activity, so after 200 minutes of hydrolysis by pancreatin enzyme, the DPPH radical scavenging activity was 51.24 ± 0.37%, while the DPPH radical scavenging activity of the hydrolyzed protein resulting from pepsin activity was 42.71 ± 0.42%, this result can be due to the difference in the composition, the amino acid sequence and molecular weight and as a result, the difference in the degree of hydrolysis of the peptides resulting from the activity of these two enzymes (Chalamaiah *et al*., 2015). In this regard, Batista *et al*. (Batista *et al*., 2010) reported on hydrolyzing black crab fish[1] waste protein, stated that DPPH radical scavenging activity increases with increasing degree of hydrolysis. They attributed this finding to the increase of hydrogen-donating peptides with an increase in the degree of hydrolysis, which has a great ability to react with free radicals. Also, Kaveh *et al*. (Kaveh *et al*., 2019a) reported on hydrolysis of fenugreek seed protein, that increasing the enzyme hydrolysis time up to 160 minutes increased the activity of the hydrolyzed protein in inhibiting DPPH free radical by 48%, but increasing the hydrolysis time did not have a significant effect on the inhibitory ability of the resulting hydrolyzed proteins. In accordance with these findings, Mazloomi *et al*. (Mazloomi-Kiyapey *et al*., 2019) and Kaveh *et al*. (Kaveh *et al*., 2022) reported that there is a direct relationship between the DPPH radical scavenging activity and the degree of hydrolysis of both orange seed and fenugreek seed proteins.

Total Antioxidant Capacity

The total antioxidant capacity evaluation test (phosphomolybdenum evaluation) is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity; in fact, the total antioxidant capacity of a compound with antioxidant potential. This method based on the reduction of Mo^{6+} to Mo^{5+}, and as a result of this reaction, a green phosphomolybdenum complex is formed in an acidic environment.

1- black scabbardfish

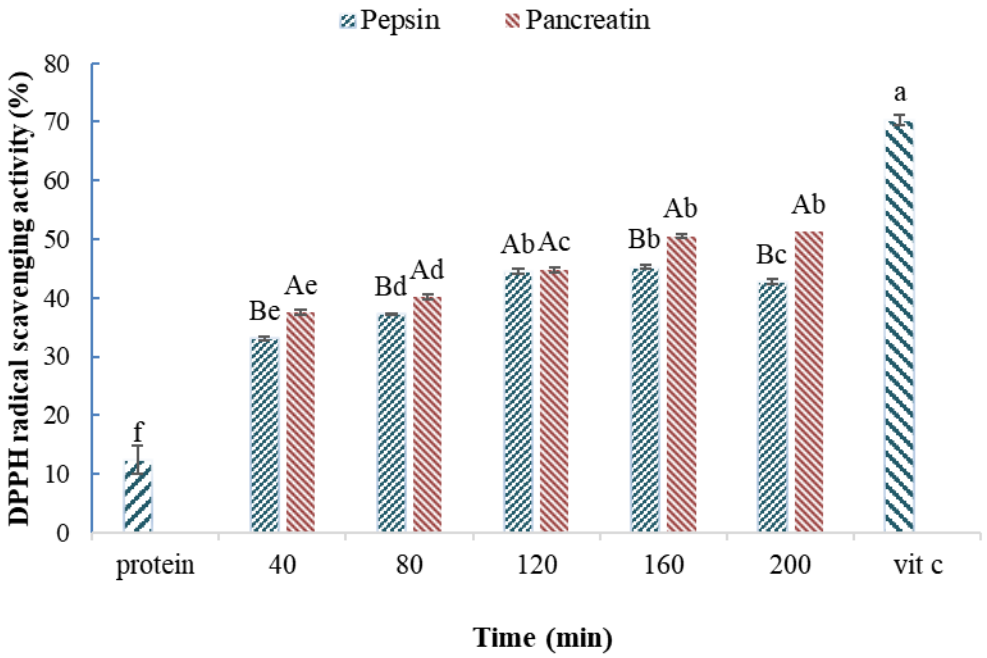

Fig. 2. The effect of hydrolysis time and protease type on DPPH radical scavenging activity of flax seed protein hydrolysate compared to unhydrolyzed protein (40 mg/ml) and Vitamin C (50 mg/ml)
(Lowercase letters indicate a significant difference between hydrolyzed samples with the same enzyme at different hydrolysis times, and uppercase letters indicate a significant difference between hydrolyzed samples with different enzymes at the same hydrolysis time, $p < 0.05$)

As can be seen in Fig. 3, in this research, enzymatic hydrolysis significantly increased the antioxidant capacity of the flaxseed protein, and increasing the hydrolysis time with pepsin and pancreatin enzymes significantly increased the antioxidant activity of samples. For example, after 40 minutes of hydrolysis, the antioxidant capacity of samples obtained from pepsin and pancreatin was 0.710 ± 0.06 and 0.860 ± 0.05 respectively (absorbance at 695 nm), which by progressing the hydrolysis process up to 200 minutes for pepsin enzyme and 160 minutes for pancreatin enzyme, the antioxidant capacity increased significantly and reached to 1.1 ± 0.08 and 1.36 ± 0.08 (absorbance at 695 nm), respectively ($p<0.05$). The total antioxidant activity of all hydrolysates was lower than the total antioxidant capacity of vitamin C (1.65 ± 0.06, absorbance at 695 nm). In this regard, it can be stated that with the increase in the hydrolysis time of the pepsin and pancreatin, the release of peptides with electron-donating properties has increased. These peptides have been able to transform free radicals into stable compounds with less reactivity, which ultimately increased the antioxidant activity of all samples with increasing time (Arabshahi-Delouee & Urooj, 2007). In accordance to these results, Umayaparvathi *et al.* (Umayaparvathi *et al.*, 2014) revealed that the antioxidant capacity of oyster protein hydrolysates was concentration dependent and the highest total antioxidant activity of the hydrolysates was obtained at a concentration of 1 mg/ml, but it was significantly lower than the control sample (vitamin C). Also, Bougatef *et al.* (Bougatef *et al.*, 2009) reported that increasing in the concentration of fish protein hydrolysate had a positive effect on the increasing the total antioxidant capacity of the samples and the hydrolysates obtained from trypsin had higher total antioxidant capacity than samples obtained from pepsin.

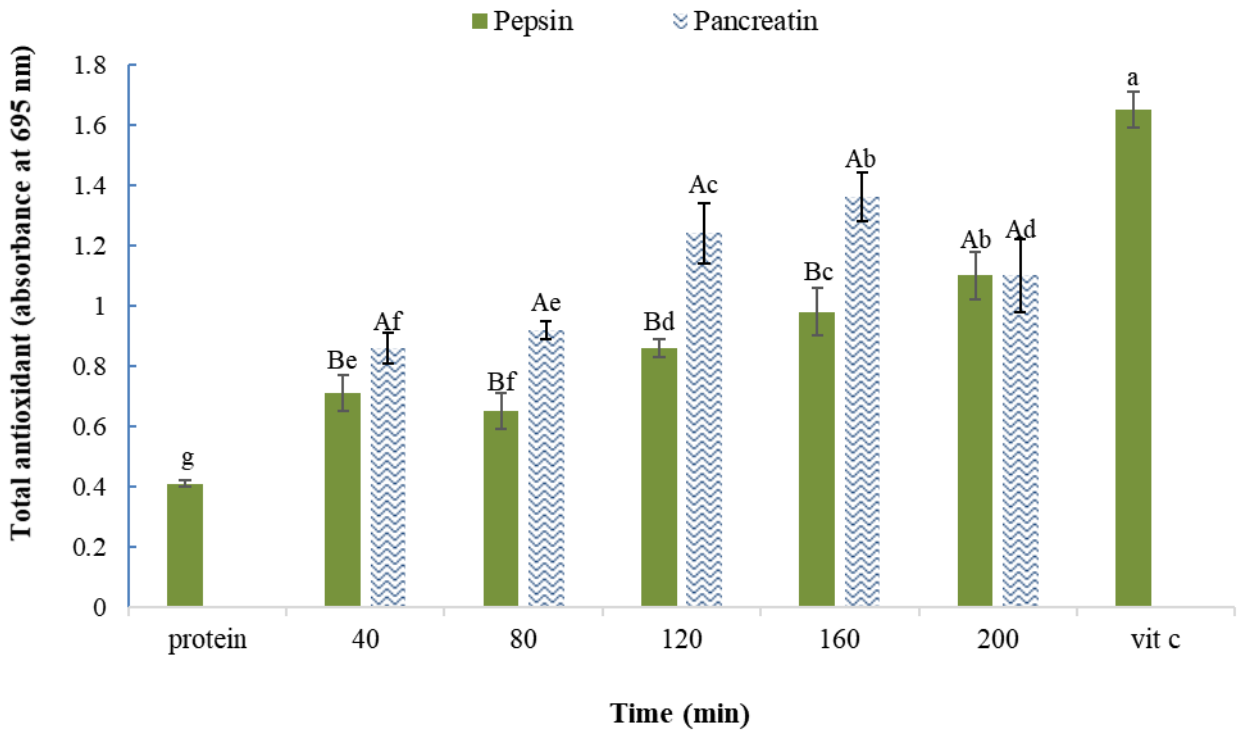

Fig. 3. The effect of hydrolysis time and protease type on total antioxidant capacity of flax seed protein hydrolysate compared to unhydrolyzed protein (40 mg/ml) and Vitamin C (50 mg/ml)
(Lower case letters indicate a significant difference between hydrolyzed samples with the same enzyme at different hydrolysis times and uppercase letters indicate a significant difference between hydrolyzed samples with different enzymes at the same hydrolysis time, $p < 0.05$)

Fe Chelating Activity

In the occurrence of lipid oxidation, metal ions such as Fe^{2+} play an essential role as a catalyst that causes the production and release of dangerous hydroxyl radicals from hydrogen superoxide. These free radical compounds react with the nearby biomolecules at a high speed and lead to the damage of body cells and tissues. Therefore, inhibiting and chelating metal ions plays an essential role in preventing oxidation (Olennikov *et al*., 2014). According to Fig. 4, the chelating activity of hydrolyzed proteins resulting from the activity of pepsin and pancreatin enzymes was significantly higher than the original (non-hydrolyzed) flax seed protein ($p<0.05$); This result indicates the appropriate performance of the enzymes used in this research in the production of peptides with the ability to chelate Fe^{2+} ions. The amount of chelation in hydrolyzed proteins resulting from the activity of pepsin and pancreatin was between 32.21 ± 0.28 ± 46.51 ± 0.28 and 30.76 ± 0.44 - 53.71 ± 0.45, respectively. It should be noted that the level of Fe^{2+} chelating activity of the produced hydrolysates was lower than vitamin C chelating activity at all hydrolysis times. In general, compared to pepsin, the pancreatin enzyme led to the production of a hydrolyzed protein with more Fe^{2+} chelating ability in all hydrolysis times except 40 and 120 minutes. Various studies have shown that the chelating ability of hydrolyzed proteins depends on the type of enzyme, the time and degree of hydrolysis, and the amino acid sequences of the original protein (Zarei *et al*., 2016). Similar to these results, also Sarabandi *et al*. (Sarabandi *et al*., 2018) and Jamdar *et al.* (Jamdar *et al*., 2010) reported an increase in Fe chelating activity of casein and peanut hydrolyzed proteins with increasing time and degree of hydrolysis.

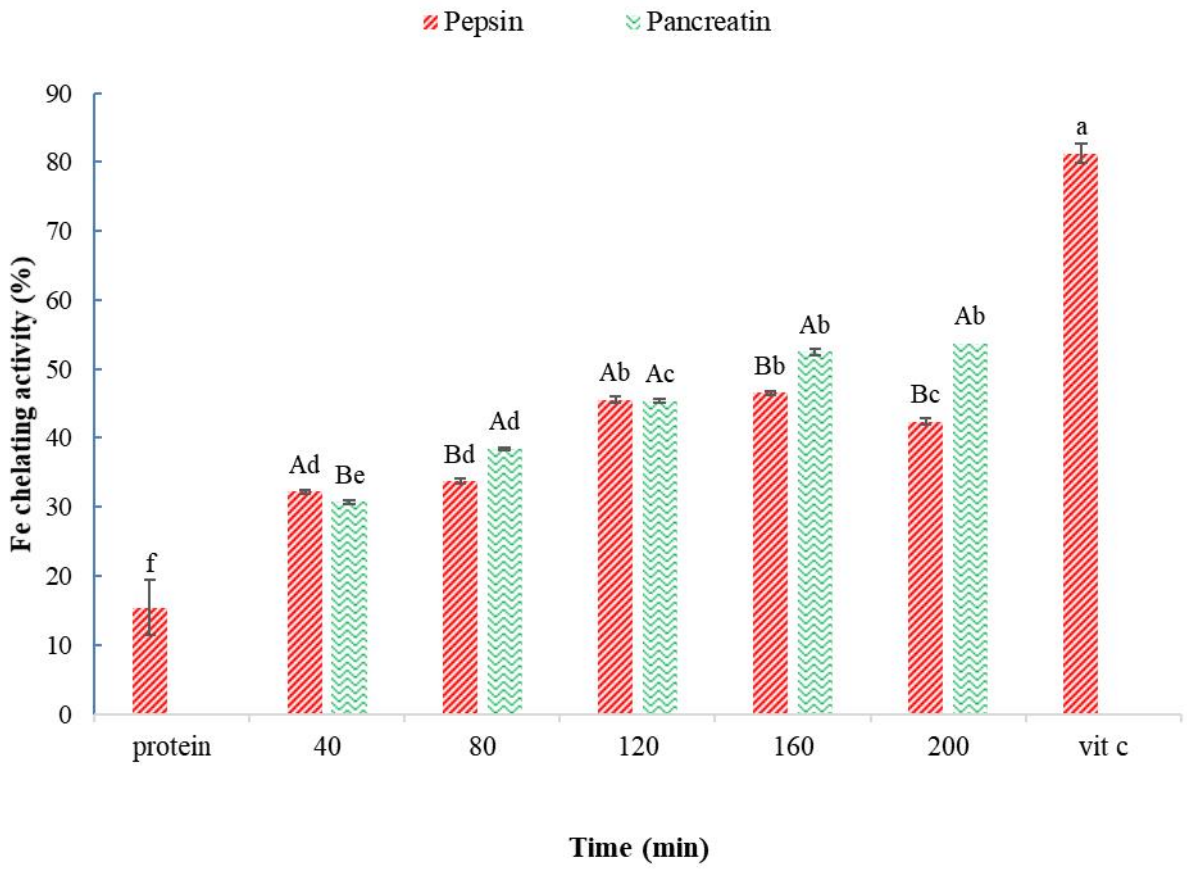

Fig. 4. The effect of hydrolysis time and protease type on Fe chelating activity of flax seed protein hydrolysate compared to unhydrolyzed protein (40 mg/ml) and Vitamin C (50 mg/ml)
(Lower case letters indicate a significant difference between hydrolyzed samples with the same enzyme at different hydrolysis times and uppercase letters indicate a significant difference between hydrolyzed samples with different enzymes at the same hydrolysis time, $p < 0.05$)

Fe Reducing Power

The reducing power test evaluates the electron-donating ability of antioxidant compounds. In other words, this test evaluates a compound's antioxidant ability to donate electrons and convert Fe^{3+} ions to Fe^{2+}. Various studies have shown that there is a direct relationship between the reducing power of bioactive compounds and their antioxidant activity (Vavrusova *et al.*, 2015). Fig. 5 shows the reducing power of hydrolyzed protein resulting from the activity of pepsin and pancreatin compared to unhydrolyzed flaxseed protein and vitamin C. The non-hydrolyzed protein had the lowest reducing power of 0.318 ± 0.02 (absorbance at 700 nm), and all the samples obtained from the activity of pancreatin and pepsin at all hydrolysis times had the reducing power. They had more activity than vitamin C as a positive control sample ($p<0.05$). These results show the positive effect of hydrolysis with pepsin and pancreatin enzymes on increasing the Fe reducing power of unhydrolyzed flax seed protein. In general, it can be stated that increasing the time and degree of hydrolysis caused a significant increase in the reducing power, this finding can have various reasons, including, increasing the amount of hydrolysis with protease enzyme can lead to the release of free amino acids, which

play their role as an additional source of electrons and protons (Zhu *et al*., 2008). On the other side, increasing the degree of hydrolysis increases the availability of electron-donating amino acids such as lysine, histidine, methionine, and tryptophan. As a result, the Fe-reducing power of hydrolyzed protein increases significantly (Jamdar *et al*., 2010). On the other side, the results showed that the Fe reducing power of the hydrolyzed proteins obtained from pancreatin was higher than that of pepsin, which indicates the better performance of pancreatin compared to pepsin in increasing the Fe reducing power. In this regard, Ambigaipalan *et al*. (Ambigaipalan *et al*., 2015) reported that hydrolyzing date kernel protein with alcalase, flavorzyme and thermolysin protease enzymes, and the peptides obtained from alcalase enzyme had lower Fe reducing power. You *et al*. (You *et al*., 2009) and Mazloomi *et al*. (Mazloomi-Kiyapey *et al*., 2019) reported an increase in the reducing power of the hydrolyzed proteins obtained from tian fish and orange seed after the enzymatic hydrolysis process.

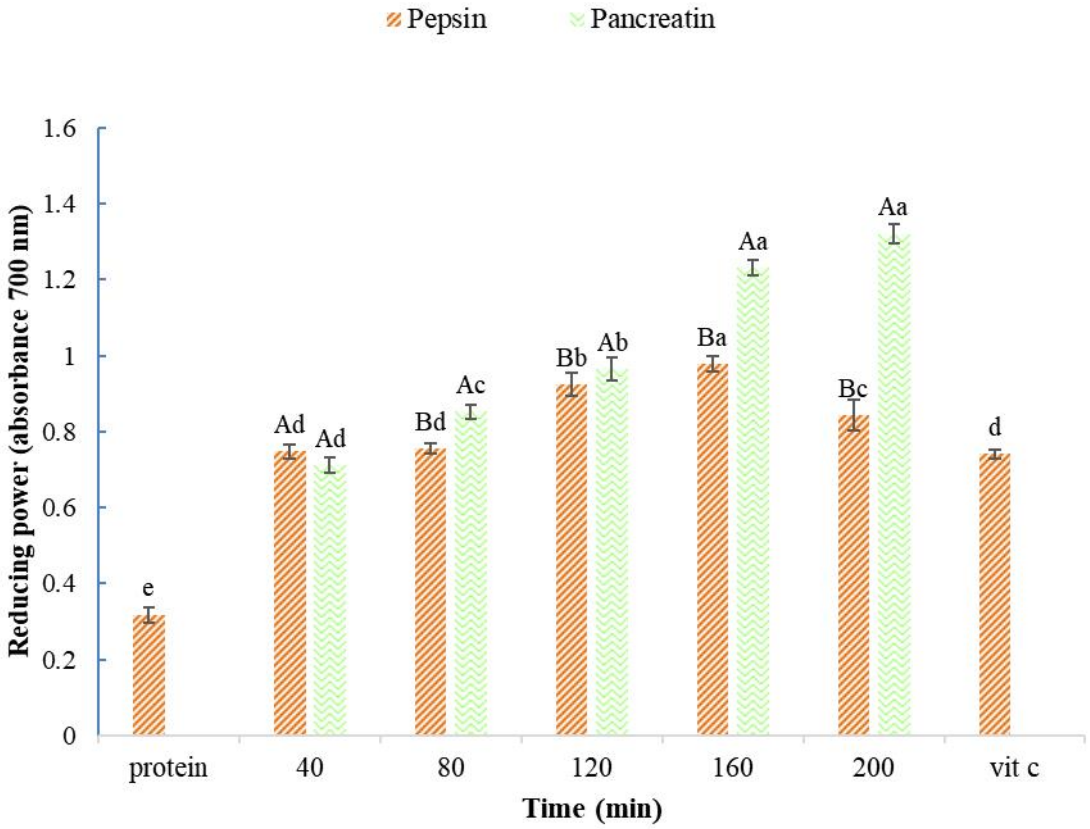

Fig. 5. The effect of hydrolysis time and protease type on Fe reducing power of flax seed protein hydrolysate compared to unhydrolyzed protein (40 mg/ml) and Vitamin C (50 mg/ml)
(Lower case letters indicate a significant difference between hydrolyzed samples with the same enzyme at different hydrolysis times and uppercase letters indicate a significant difference between hydrolyzed samples with different enzymes at the same hydrolysis time, $p < 0.05$)

Conclusion

The application of compounds with antioxidant properties to prevent oxidation is common in the food industry, but the concerns about the adverse effects of synthetic antioxidants have increased the attention of researchers to identify and extract natural compounds with antioxidant properties.

Hydrolyzed proteins are among the natural compounds that have significant antioxidant properties. In this research, the effect of hydrolysis time and type of protease enzyme on antioxidant activity and degree of hydrolysis of flaxseed protein was investigated. The results showed that the antioxidant properties of hydrolyzed flaxseed proteins with pepsin and pancreatin enzymes depend on the degree of hydrolysis of the samples, and with the increase of the degree of hydrolysis, the antioxidant activity of the samples (DPPH radical scavenging activity, Fe chelating activity, Fe reducing power and total antioxidant capacity) increased significantly. After hydrolysis for 200 minutes, the highest DPPH radical scavenging activity (51.24 ± 0.37%), Fe reducing power (1.32 ± 0.02, absorbance at 700 nm) and Fe chelating activity (53.71 ± 0.45%) was related to the samples obtained from the activity of pancreatin enzyme and had no significant difference with the antioxidant activity of the hydrolyzed protein obtained from pancreatin after 160 minutes of hydrolysis; Also, the highest amount of total antioxidant capacity (1.36 ± 0.08, absorbance at 695 nm) was related to the hydrolysates obtained from pancreatin up to 160 minutes of hydrolysis. Therefore, it can be concluded that enzymatic hydrolysis of flaxseed meal protein with pancreatin enzyme after 160 minutes of hydrolysis is a suitable solution for producing peptides with desirable antioxidant properties. Due to the appropriate antioxidant capacity, the resulting hydrolyzed proteins have the potential to be used in food formulations to produce functional products and can compete with synthetic antioxidants, so by conducting in vivo tests, the produced flax seed hydrolyzed protein can be used in the food industry as a substitute for synthetic antioxidants, effectively.

Author Contributions

S.H. Hosseini Ghaboos: Conceptualization, Supervision, Validation, Writing–review and editing; **M. Hashemi:** Formal analysis, Writing–original draft, Methodology, Software; **A. Seraj:** Validation, Visualization, Investigation.

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بررسی تأثیر نوع آنزیم پروتئازی و زمان هیدرولیز بر ویژگی‌های آنتی‌اکسیدانی پروتئین هیدرولیز شده کنجاله بذر کتان (*Linum usitatissimum*)

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چکیده

در این پژوهش بررسی تأثیر نوع آنزیم پروتئازی (پپسین و پانکراتین) و زمان هیدرولیز (۲۰۰-۴۰ دقیقه) بر درجه هیدرولیز و ویژگی‌های آنتی‌اکسیدانی (فعالیت مهار رادیکال DPPH، قدرت شلاته‌کنندگی آهن، قدرت احیاءکنندگی یون آهن و فعالیت آنتی‌اکسیدانی کل) پروتئین هیدرولیز شده کنجاله بذر کتان انجام شد. نتایج نشان داد که افزایش زمان هیدرولیز باعث افزایش درجه‌ی هیدرولیز شد و نمونه‌های حاصل از پانکراتین نسبت به پپسین دارای درجه‌ی هیدرولیز بالاتری بودند. بیشترین میزان فعالیت شلاته‌کنندگی Fe^{2+} (۰/۴۵ ± ۵۳/۷۱ درصد) و احیاءکنندگی Fe^{3+} (۰/۰۲ ± ۱/۳۲، جذب در ۷۰۰ نانومتر) توسط پانکراتین و پس از ۲۰۰ دقیقه هیدرولیز حاصل شد. نمونه‌های حاصل از پانکراتین نسبت به پپسین توانایی بیشتری در مهار رادیکال آزاد DPPH داشتند و فعالیت آن‌ها با افزایش زمان تا ۱۶۰ دقیقه افزایش یافت. بیشترین ظرفیت آنتی‌اکسیدانی کل (۰/۰۸ ± ۱/۳۶ جذب در ۶۹۵ نانومتر) در بین نمونه‌ها پس از ۱۶۰ دقیقه هیدرولیز با پانکراتین به‌دست آمد. قابلیت آنتی‌اکسیدانی پروتئین هیدرولیز شده بذر کتان در مهار رادیکال DPPH، شلاته کنندگی یون آهن و آنتی‌اکسیدانی کل از قابلیت آنتی‌اکسیدانی ویتامین ث در غلظت (mg/ml) ۵۰، کمتر بود اما از خاصیت احیاءکنندگی یون آهن بیشتری نسبت به ویتامین ث برخوردار بود. بنابراین می‌توان نتیجه گرفت که پانکراتین در مقایسه با پپسین توانایی بیشتری در تولید پروتئین هیدرولیز شده بذر کتان با خاصیت آنتی‌اکسیدانی قابل توجه داشت. با توجه به نتایج پروتئین‌های هیدرولیز شده بذر کتان با استفاده از آنزیم پانکراتین و زمان هیدرولیز ۱۶۰ دقیقه، قابلیت استفاده در فرمولاسیون مواد غذایی برای تولید محصولات فراسودمند را دارند.

واژه‌های کلیدی: آنتی‌اکسیدان، بذر کتان، پپسین، پانکراتین، هیدرولیز آنزیمی

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