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Evaluation of Antioxidant and Functional Properties of broccoli sprout Protein Hydrolysates using enzymatic Hydrolysis

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ABSTRACT

Bioactive peptides are among the functional compounds that have recently been recognized in food products. Enzymatic hydrolysis has been used to improve the functional properties of plant proteins. The aim of this study was to enzymatically hydrolyze broccoli sprout protein and investigate the functional and antioxidant properties of the resulting hydrolysates. For this purpose, two enzymes, Alcalase and Flavourzyme, and different enzymatic hydrolysis times (30, 60, and 90 min) were used. The results showed that the protein hydrolysates obtained by Alcalase have smaller chain length and higher amounts of hydrophobic amino acids, degree of enzymatic hydrolysis, protein recovery, hydrolyzed protein content, solubility, emulsifying capacity, emulsion stability, foaming capacity, foam stability and antioxidant activity compared to the hydrolysates prepared by Flavourzyme. Increasing the enzymatic hydrolysis time from 30 to 90 min resulted in increased solubility and decreased emulsifying capacity, emulsion stability, foaming capacity, and foam stability of the produced protein hydrolysates (p<0.05). In general, due to the antioxidant activity of broccoli sprout protein and its significant improvement after enzymatic hydrolysis, especially by Alcalase enzyme at 90 min, the prepared protein hydrolysates are recommended as natural antioxidants to maintain the quality and safety of food products.

1.Introduction

Peptides and protein hydrolysates are classified as functional food components that can be used to increase the shelf life of food products [1]. Bioactive peptides have antioxidant and antimicrobial properties and also show health-promoting effects. The antimicrobial activity of bioactive peptides depends on various factors such as peptide chain length, solubility, structure, etc. [2]. One of the most common methods for producing protein hydrolysates rich in bioactive peptides is enzymatic hydrolysis using various protease enzymes [3]. In enzymatic hydrolysis, the type of enzyme used is of great importance, as it determines the patterns of peptide bond cleavage. Alcalase and flavourzyme enzymes are among the most widely used proteolytic enzymes used to produce protein hydrolysates because they have shown peptides with high antioxidant and antimicrobial properties. [4]. The advantages of protein hydrolysates as natural preservatives compared to synthetic preservatives and antioxidants include health benefits, their role in reducing diseases, lower production cost, high digestibility, safety, easy absorption, low allergenicity, higher stability under different conditions, high nutritional value, preservation of activity, biofunctional properties, etc. [5]. Plant protein isolates and their hydrolyzed proteins are used in meat, soup, dairy and bakery formulations. A significant part of the functional properties of food materials is related to proteins [6]. Any change in the structural arrangement of proteins also causes changes in the functional properties of the protein [7]. Solubility is one of the most important and generally the first functional property to be considered during the development of new protein components because it has a significant effect on other functional properties [8]. Low solubility causes an undesirable appearance and a sandy mouthfeel in the final product [9]. Foaming property is also of particular importance in the food industry because it provides a desirable and unique texture to aerated foods and beverages, including ice cream, bread, cakes and beer. Since consumer perception of food quality is influenced by appearance, the stability of food foams is essential from the point of view of consumer acceptance. Broccoli, scientifically known as *Brassica Oleraceae* L. var. italic, is a plant belonging to the *Brassicacea* family. Broccoli sprouts are obtained by germinating broccoli seeds over a period of 3 to 5 days. Broccoli sprouts contain various bioactive compounds and exhibit antimicrobial, antioxidant, antidiabetic, anticancer, and anti-inflammatory activities [10]. Broccoli seeds contain significant amounts of protein, and research has shown that during germination, the protein content of the seeds increases, such that the protein content of broccoli seeds in the study of Tarasevičienė et al. (2009) was initially 26.10% and increased to 29.87% after 120 h of germination [11]. Since there is no comprehensive information regarding the functional and antioxidant properties of hydrolyzed broccoli sprout protein, this study investigated the effect of using alcalase and flowerzyme enzymes in producing hydrolyzed proteins with functional and antioxidant properties.

2- Materials and methods

2.1. Materials

Broccoli seeds were purchased from a local store. Alcalase enzyme extracted from

Bacillus licheniformis and flavourzyme enzyme extracted from *Aspergillus oryzae* were obtained from Novozyme, Denmark. Hydrochloric acid, sodium hydroxide, ethanol, and potassium dihydrogen phosphate were obtained from Titracam. All materials were of laboratory grade.

2.2. Preparation of broccoli sprout protein hydrolysates

2.2.1. Germination of broccoli seeds

Sterilization of seeds was performed by immersing them in 5 g/L 70% ethanol for 60 s and then immersing them in a solution of sodium hypochlorite (1.5%) for 15 min. The sterilized seeds were soaked in a 10:1 ratio in water at room temperature for 12 h (in the dark and upside down). Seed germination was carried out for 5 days, and the resulting sprouts were washed with deionized water and dried in an oven for 24 hours at 40°C [11].

2.2.2. Extraction of broccoli sprout protein

First, broccoli sprout powder was defatted by hot extraction with Soxhlet and using normal hexane solvent (solvent to powder ratio 4 to 1) for 9 h. Then, the defatted sprout was mixed with distilled water in a ratio of 1 to 10 and the mixture was made alkaline using 1N sodium hydroxide and its pH reached 10. After stirring for one hour at room temperature, centrifugation was performed at 4°C and 9000 rpm for 30 min. After separating the lower solid phase, in order to precipitate broccoli sprout proteins, the pH of the supernatant phase was adjusted to 5 with 1N hydrochloric acid and centrifuged again under the previous conditions. The resulting precipitate, which is the broccoli sprout protein concentrate, was dried in a vacuum oven (at 45°C) and the resulting powder was stored in a freezer until further use [12].

2.2.3. Enzymatic hydrolysis of broccoli sprout protein

50 g of sample was poured into a 250 ml Erlenmeyer flask, then 100 ml of distilled water was added to the Erlenmeyer flask in a ratio of (2:1) and homogenized with a digital mixer for 2 min. Then, it was placed in a water bath at 85°C for 20 min to inactivate internal enzymes [13]. Then, by adding 0.2 N sodium hydroxide, the optimum pH for enzyme activity (alcalase 8.5, flavourzyme 7) was reached. The samples were placed in a moving water bath at 55°C to produce hydrolyzed protein with a constant speed of 200 rpm, then the enzyme (1% of the original sample protein) was added to it and after each sampling (time 30 and 60 min) and at the end of the experiment (time 90 minutes) it was placed in a water bath at 95°C for 15 min to stop the enzymatic reaction. After cooling, the hydrolyzed proteins were centrifuged at a constant speed of 6700 rpm for 20 min and the supernatant was collected and the hydrolyzed protein was stored in a freezer, then it was powdered using a freeze dryer [14].

3.2.4.1. Measurement of the degree of hydrolysis

The degree of hydrolysis of broccoli sprout protein was determined by mixing the hydrolysate (1 mL) with 0.44 M trichloroacetic acid (1 mL), centrifuging at 4°C and 7800 × g for 10 min. The protein content of the supernatant solution was measured using the Bradford method and the degree of protein hydrolysis was obtained through Equation 1 [15]:

Equation 1:

$$\text{Hydrolysis degree (\%)} = \frac{TCA-soluble\ protein\ hydrolysate}{Total\ protein\ of\ the\ initial\ suspension} \times 100$$

$$\text{DPPH (\%)} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

3.2.4.4. Measurement of the amount of hydrolyzed protein in broccoli sprouts

Based on the Koldahl method, the samples were digested and then the amount of total precipitated protein in the aqueous phase was calculated by titration of (6.25×N) [16].

3.2.4.5. Examination of the amino acid profile

The amino acid profile of broccoli sprout protein hydrolysates was examined using a reversed-phase high-performance liquid chromatography (RP-HPLC) system equipped with a fluorescence detector and an RP-C18 column with dimensions of 150 mm × 5 mm × 6.4 mm. Acetate buffer (50 mM) with pH = 3.4 and flow rate of 1.3 mL/min was used as the mobile phase. Initially, the protein hydrolysates were treated with 6 M hydrochloric acid for 24 h at 110°C, and then the treated sample was derivatized with orthophthalaldehyde and injected into the RP-HPLC column. The amino acid contents in the protein hydrolysates were reported as mg/100g protein [17].

2.3. Evaluation of antioxidant activity

2.3.1. Evaluation of antioxidant activity by DPPH method

To evaluate the antioxidant activity of protein hydrolysates by DPPH radical scavenging method, protein hydrolysate (1 mL) was mixed with 0.1 mM DPPH solution (1 mL) and after incubation in the dark for 30 min, its absorbance was recorded at 517 nm. The percentage of DPPH radical scavenging was finally obtained through Equation 2 [17].

Equation 2:

2.3.2. Evaluation of antioxidant activity by FRAP method

To evaluate the antioxidant activity of hydrolysates by the ferrous reducing power method or FRAP, first 0.5 mL of hydrolysate (with a concentration of 40 mg/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer solution (pH = 6.6) and 2.5 mL of potassium ferricyanide solution (1% w/w), and the resulting solution was kept at 50°C for 30 min, and then 2.5 mL of trichloroacetic acid solution (10%) was added to it. After that, the solution was centrifuged at 3000 rpm for 10 minutes, and then 2.5 ml of the supernatant was mixed with 2.5 ml of ferric chloride (0.1%) and 2.5 ml of distilled water and kept at room temperature for 10 minutes, and finally its absorption was recorded by spectrophotometer at a wavelength of 700 nm [18].

2.4. Examination of functional properties

2.4.1. Measurement of solubility in water

To examine the solubility of protein hydrolysates in water, 200 mg of protein hydrolysate was mixed with 20 ml of distilled water at room temperature, and then the pH was adjusted to 1, 3, 5, 7, 9 and 11 using hydrochloric acid or sodium hydroxide (1 N). The mixture was stirred at room temperature for 30 minutes, then centrifuged at 10,000 × g for 10 min. After that, two phases were formed and the amount of protein in the supernatant was determined by the Bradford method (1976) and the amount of total protein in the sample was determined by the Koldel method. The solubility was calculated in percent based on equation 3, then a

solubility graph was plotted against different pH values. The pH that showed the lowest solubility in the graph was considered as the isoelectric point [19].

Equation 3:

$$\text{Solubility (\%)} = \frac{Protein\ content\ of\ supernatant}{Total\ protein\ content\ of\ sample} \times 100$$

2.4.2. Measurement of emulsifying capacity and emulsion stability

To investigate the emulsifying capacity of hydrolysates, 2 ml soybean oil was added to 10 ml of 1% protein hydrolysate solution with pH=7 and the mixture was homogenized for 2 min at 10,000 rpm. Then, 50 µl of the emulsion sample was removed from the bottom of the container at times zero and 10 min after homogenization and 5 ml of sodium dodecyl sulfate solution (0.1%) was added to it. Finally, using a spectrophotometer, the absorbance of the solutions was recorded at 500 nm and the emulsifying capacity and emulsion stability of the samples were obtained by equations 4 and 5, respectively, where: A0 is the absorbance of the emulsion immediately after homogenization, dilution factor 100, Δt: 10 minutes and ΔA: the difference between the absorbances at two times zero and 10 minutes [20].

Equation 4:

$$\text{EAI } (m^2/g) = \frac{(2)(2.303)(A0)(dilution\ factor)}{(0.17)(protein\ concentration)(10000)}$$

Equation 5:

$$\text{ESI (min)} = A_0 \times \frac{\Delta t}{\Delta A}$$

2.4.3. Measurement of foaming capacity and foam stability

To measure foaming capacity, 20 ml of protein solutions were poured into a 50 ml graduated cylinder. Then, it was stirred for one minute with an Ultratorx at a speed of 10,000 rpm. The volume of the final mixture was recorded in ml. Finally, using equations 6 and 7, the foaming capacity and foam stability of protein hydrolysates were obtained, respectively [21].

Equation 6:

$$\text{Foaming capacity (\%)} = \frac{Foam\ volume\ immediately\ after\ mixing}{Sample\ volume\ before\ foam\ formation} \times 100$$

Equation 6:

$$\text{Foam stability (\%)} = \frac{Foam\ volume\ 20\ minutes\ after\ stirring}{Foam\ volume\ immediately\ after\ stirring} \times 100$$

2.5. Statistical analysis

A completely randomized design was used to analyze the data and examine the information obtained from the experiment. All experiments were repeated 3 times and SPSS 22.0 software and ANOVA analysis of variance were used to statistically analyze the data obtained from the experiments. Statistically significant differences between treatments were expressed using Duncan's multiple range test at a probability level of 95%. The final results were reported as mean ± standard deviation.

3. Results and discussion

3.1. Degree of hydrolysis of broccoli sprout protein hydrolysates

The degree of hydrolysis is an important parameter that indicates the degree of protein hydrolysis and can be used to evaluate the biological activities and functional properties of protein hydrolysates [19]. The average values of the degree of hydrolysis of broccoli sprout protein hydrolysates prepared by protease enzymes and different enzymatic hydrolysis times are compared in Figure 1.

The results show that the hydrolysates prepared by the alcalase enzyme had a significantly higher degree of hydrolysis than the hydrolysates prepared by the flavourzyme enzyme ($p<0.05$). In the case of both enzymes, with increasing the enzymatic hydrolysis time from 30 to 90 min, the degree of hydrolysis increased significantly ($p<0.05$). In general, the highest degree of hydrolysis was obtained for the hydrolysate prepared by the alcalase enzyme and the hydrolysis time was 90 min (13.24%), and the lowest degree was obtained for the hydrolysate prepared by the flavourzyme enzyme and the hydrolysis time was 30 min (5.96%). The higher degree of hydrolysis of protein hydrolysates prepared by the alcalase enzyme compared to the flavourzyme has also been reported in previous studies. For example, Xu et al. (2020) also observed, in agreement with the results of the present study, that the degree of hydrolysis of chickpea protein hydrolysates prepared by the alcalase enzyme was significantly higher than that of the flavourzyme enzyme [20]. In the study of Cui et al. (2021), the degree of hydrolysis of milk protein hydrolysates prepared by the alcalase enzyme was higher than that of the flavourzyme and Protamax enzymes. These researchers also showed that with increasing the enzymatic hydrolysis time, the degree of hydrolysis increased, but this increase was not significant until 90 min [22]. Zheng et al. (2019) also reported in their study the effect of increasing the hydrolysis time on increasing the degree of hydrolysis of black bean proteins by different enzymes, which was consistent with the results of the present study. These researchers also observed that during 120 min of enzymatic hydrolysis, the alcalase enzyme produced the highest degree of hydrolysis among the different proteases [23]. In another study, the degree of hydrolysis of sesame protein hydrolysates prepared by the alcalase enzyme was higher than that of the flavourzyme enzyme, and an increase in the degree of protein hydrolysis was observed with increasing enzymatic hydrolysis time [24].

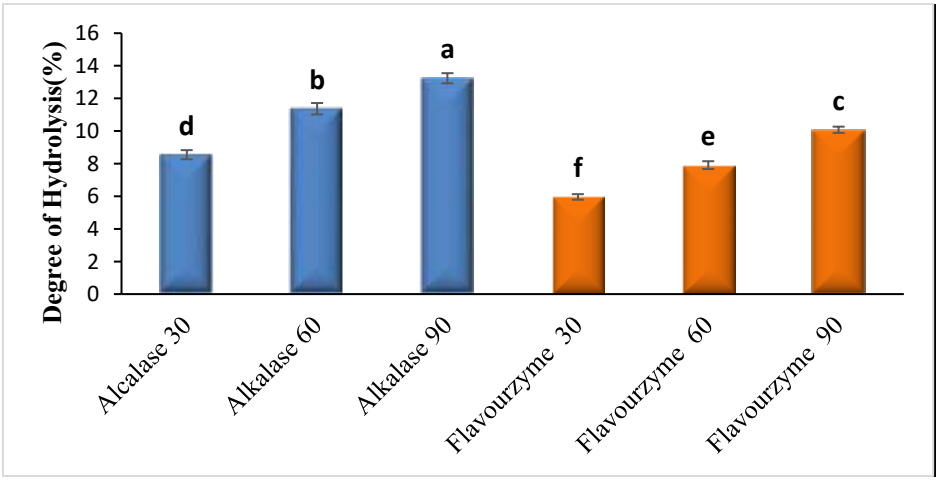

Fig 1. Comparison of degree of hydrolysis of broccoli sprout protein hydrolysates (%)

Values represent mean ± SD. Different letters indicate significant difference among samples at 5% probability level.

3.2. Hydrolyzed protein content of broccoli sprout protein hydrolysates

The average hydrolyzed protein values of broccoli sprout protein hydrolysates

prepared by protease enzymes and different enzymatic hydrolysis times were shown in Figure 2. The results show that the hydrolysates prepared by the alcalase enzyme had significantly higher hydrolyzed protein content than the hydrolysates prepared by the flavourzyme enzyme ($p<0.05$). For both enzymes, the hydrolyzed protein content increased significantly with increasing enzymatic hydrolysis time from 30 to 90 min ($p<0.05$). Overall, the highest amount of hydrolyzed protein was related to the hydrolysates prepared by the alcalase enzyme and the hydrolysis time of 90 minutes (91.44%). In the study of Ghanbarinia et al. (2022), the protein yield of sesame protein hydrolysates prepared by the alcalase enzyme was higher than that of the flavourzyme enzyme, and the protein yield increased significantly with increasing the enzymatic hydrolysis time [24]. Nemati et al. (2019) reported an increase in the protein recovery rate of fish waste protein hydrolysates prepared by the alcalase enzyme due to increasing the enzymatic hydrolysis time [25]. The effect of increasing the enzymatic hydrolysis time on increasing protein recovery has also been observed in the studies of Ovissipour et al. (2012), and Rafatinia and Roomiani (2018) [26,27].

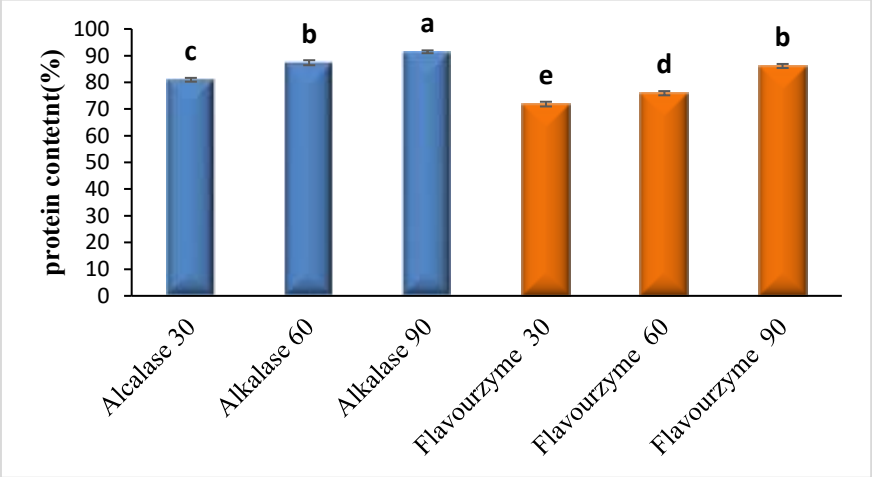

Fig 2. Comparison of protein content of broccoli sprout protein hydrolysates (%)

Values represent mean ± SD. Different letters indicate significant difference among samples at 5% probability level.

3.3. Amino acid profile of broccoli sprout protein hydrolysates

The amino acid composition of protein hydrolysates is important because amino acids have high nutritional value and the amino acid composition also affects the functional properties of the hydrolysates. The physiological activities of protein hydrolysates are largely dependent on their amino acid profile [28]. The amino acid profile of broccoli sprout protein hydrolysates is given in Table 1 and shows that glutamine, followed by leucine, were the dominant amino acids in broccoli sprout protein hydrolysates. The type of enzyme used for enzymatic hydrolysis of broccoli sprout protein and the time of enzymatic hydrolysis influenced the amino acid profile of the hydrolysates produced. The amounts of aromatic and hydrophobic amino acids in the hydrolysates prepared by the alcalase enzyme, including histidine,

isoleucine, leucine, valine, phenylalanine, tyrosine, and proline, were higher than those prepared by the flavourzyme enzyme, and with increasing enzymatic hydrolysis time in both protease enzymes, an increase in the amount of these hydrophobic amino acids was observed ($p<0.05$). The total sum of these amino acids in the hydrolysates prepared by the alcalase and flavourzyme enzymes were obtained in the range of 34.49-39.23 mg/100g and 25.66-31.94 mg/100g, respectively. The amounts of essential amino acids in the hydrolysates prepared by the alcalase enzyme were higher than those prepared by the flavourzyme enzyme, but the amounts of non-essential amino acids were lower. An increase in the content of essential amino acids was observed with increasing enzymatic hydrolysis time. With increasing enzymatic hydrolysis time, the ratio of essential to non-essential amino acids and the ratio of essential to total amino acids gradually increased ($p<0.05$), and these ratios were higher in hydrolysates prepared with the alcalase enzyme than in those prepared with the flavourzyme enzyme. The amino acids, isoleucine, valine, leucine, histidine, phenylalanine, tyrosine, proline, and tryptophan, are hydrophobic and aromatic amino acids, and their levels in broccoli sprout protein hydrolysates prepared by the alcalase enzyme were significantly higher than those prepared by the flavourzyme enzyme, which is a clear reason for the higher antioxidant activity of the hydrolysates produced by the alcalase enzyme. Due to the more hydrophobic nature of these amino acids, they have a good ability to bind to hydrophobic radicals such as DPPH radical and therefore show significant antioxidant activity [30].

Table 1. Amino acid composition of the of broccoli sprout protein hydrolysates (g/100g protein)

Treatments	Flavourzyme 90	Flavourzyme 60	Flavourzyme 30	Alcalase 90	Alcalase 60	Alcalase 30
Asparagine	10.41±0/04 ^{b}	10.43 ±0.06 ^{b}	10.59 ± 0.07 ^{a}	4.93± 0.09 ^{d}	5.01 ± 0.02 ^{d}	5.67 ± 0.06 ^{c}
Serine	4.43 ±0.07 ^{d}	4.65 ±0.12 ^{abc}	4.53 ± 0.06 ^{bcd}	4.50 ± 0.03 ^{cd}	6.69 ± 0.11 ^{ab}	4.73 ± 0.05 ^{a}
Gluramine	23.61±0.03 ^{d}	22.72 ±0.07 ^{e}	21.20 ± 007 ^{f}	24.72± 0.08 ^{b}	24.31 ± 0.05 ^{c}	24.92 ± 0.10 ^{a}
Glycine	2.86 ±0.04 ^{c}	2.92 ±0.04 ^{c}	2.64 ± 0.10 ^{d}	3.15 ± 0.01 ^{b}	3.68 ± 0.05 ^{a}	2.54 ± 0.11 ^{d}
Histidine^{ab}	2.67 ±0.01 ^{c}	2.46 ±0.05 ^{d}	2.11 ± 0.08 ^{e}	3.40 ± 0.11 ^{a}	3.38 ± 0.13 ^{a}	3.09 ± 0.07 ^{b}
Arginine^{a}	6.98 ±0.04 ^{b}	3.99 ±0.04 ^{b}	5.83 ± 0.07 ^{e}	6.71 ± 0.02 ^{c}	6.32 ± 0.04 ^{d}	7.95 ± 0.13 ^{a}
Threonine^{a}	3.12 ±0.09 ^{d}	3.02 ±0.03 ^{de}	3.77 ± 0.05 ^{a}	3.28 ± 0.02 ^{c}	3.42 ± 0.01 ^{b}	2.96 ± 0.05 ^{e}
Proline^{ab}	2.47 ±0.09 ^{a}	1.86 ±0.09 ^{b}	1.70 ± 0.05 ^{c}	2.64 ± 0.09 ^{a}	2.02 ± 0.07 ^{b}	1.57 ± 0.09 ^{c}
Alanine	6.58 ±0.03 ^{c}	7.37 ±0.07 ^{b}	6.55 ± 0.03 ^{c}	6.31 ± 0.05 ^{d}	7.06 ± 0.09 ^{b}	8.50 ±0.08 ^{a}
Systeine	1.43 ±0.02 ^{c}	1.46 ±0.07 ^{c}	1.67 ± 0.09 ^{b}	1.93 ± 0.09 ^{a}	1.71 ± 0.03 ^{b}	2.04 ± 0.14 ^{a}
Tyrosine^{ab}	3.26 ±0.04 ^{c}	3.02 ±0.06 ^{d}	2.16 ± 0.08 ^{e}	3.87 ± 0.10 ^{b}	4.09 ± 0.08 ^{a}	4.03 ± 0.03 ^{a}
Valine^{ab}	4.03 ±0.05 ^{b}	4.02 ±0.02 ^{b}	3.85 ± 0.02 ^{c}	2.24 ± 0.04 ^{a}	4.30 ± 0.04 ^{a}	3.96 ± 0.09 ^{bc}
Methionine^{a}	2.42 ±0.08 ^{b}	2.42 ±0.10 ^{b}	2.26 ± 0.04 ^{c}	1.03 ± 0.07 ^{e}	1.72 ± 0.11 ^{d}	3.28 ± 0,02 ^{a}
Lysine^{a}	4.70 ±0.11 ^{a}	3.49 ±0.06 ^{b}	3.36 ± 0.08 ^{b}	2.76 ± 0.06 ^{c}	1.97 ± 0.08 ^{d}	1.65 ± 0.05 ^{e}
Isoleucine^{ab}	4.35 ±0.04 ^{c}	3.99 ±0.07 ^{e}	3.14 ± 0.05 ^{d}	5.47 ± 0.07 ^{a}	5.39 ± 0.12 ^{a}	4.83 ± 0.04 ^{b}
Leucine^{ab}	10.60±0.09 ^{d}	10.18 ±0.02 ^{e}	9.18 ± 0.06 ^{f}	12.73 ± 0.11 ^{a}	12.00± 0.08 ^{b}	10.92 ± 0.07 ^{c}
Phenilalanine^{ab}	4.56 ±0.08 ^{d}	4.36 ±0.06 ^{e}	3.52 ± 0.12 ^{f}	7.06 ± 0.01 ^{a}	6.79 ± 0.03 ^{b}	6.09 ± 0.01 ^{c}
Total	98.48± 0.28 ^{a}	95.35± 0.19 ^{c}	88.06 ± 0.33 ^{d}	98.55 ± 0.41 ^{a}	97.86± 0.21 ^{b}	98.73 ± 0.25 ^{a}
Hydrophobice	31.94± 0.23 ^{d}	29.89± ۰.۱۷ ^{e}	25.66 ± 0.24 ^{f}	39.23 ± 0.19 ^{a}	37.97± 0.35 ^{b}	34.49 ± 0.20 ^{c}
E AA^{a}	46.69± 0.15 ^{c}	43.98± 0.21 ^{d}	3918 ± 0.18 ^{e}	50.55 ± 0.10 ^{a}	49.38± 0.16 ^{b}	49.16 ± 0.13 ^{b}
NEAA^{c}	51.79± 0.21 ^{a}	51.40± 0.14 ^{b}	48.88 ± 0.15 ^{d}	48.00 ± 0.17 ^{f}	48.48 ± 0.11 ^{e}	49.57 ± 0.025 ^{c}

EAA/NEAA	0.90 ± 0.04 [c]	0.86 ± 0.03 [c]	0.80 ± 0.02 [d]	1.05 ±0.03 [a]	1.02 ± 0.05 [ab]	0.99 ± 0.02 [b]
EAA/Total (%)	47.41± 0.11 [d]	46.09± 0.18 [e]	44.49 ± 0.05 [f]	51.29 ± 0.09 [a]	50.45± 0.14 [b]	49.79 ± 0.07 [c]

Values represent mean ± SD. Different letters indicate significant difference among samples at 5% probability level.

[a] Essential Amino Acids (EAA)

[b] hydrophobic amino acids

[c] Non Essential Amino Acid (NEAA)

3.4. Antioxidant activity of broccoli sprout protein hydrolysates

3.4.1. DPPH radical scavenging activity

DPPH radical scavenging activity is a widely used method to investigate the ability of compounds to act as hydrogen donors or free radical scavengers and indicates the antioxidant potential of compounds. The presence of a proton donor as an antioxidant leads to radical scavenging [29]. The average percentage values of DPPH radical scavenging of broccoli sprout protein hydrolysates prepared by protease enzymes and different enzymatic hydrolysis times are compared in Figure 3. The results show that the initial crude protein had the lowest DPPH radical scavenging activity (10.28%) and the enzymatic hydrolysis process significantly increased the DPPH radical scavenging activity of broccoli sprout protein ($p<0.05$). Hydrolysates prepared by alcalase enzyme had significantly higher DPPH radical scavenging percentage than hydrolysates prepared by flavourzyme enzyme ($p<0.05$). In the case of both enzymes, with increasing enzymatic hydrolysis time from 30 to 90 min, the percentage of DPPH radical scavenging increased significantly ($p<0.05$). In general, the highest DPPH radical scavenging activity was related to the hydrolysate prepared by alcalase enzyme and hydrolysis time of 90 min (73.98%) and the lowest was obtained in the hydrolysate prepared by flavourzyme enzyme and hydrolysis time of 30 min (42.20%). The higher antioxidant activity of hydrolysates prepared by alcalase enzyme in DPPH radical scavenging method is probably related to its more hydrophobic nature. Because research has shown that hydrolysates produced by alcalase are hydrophobic and have the ability to bind to hydrophobic DPPH radicals [30]. In the study of Zheng et al. (2019), black bean protein hydrolysates prepared by alcalase enzyme showed the highest DPPH radical scavenging activity at a hydrolysis time of 120 min. These researchers stated that the extraction conditions and the type of enzyme used have a significant effect on the antioxidant activity of protein hydrolysates [23]. Xu et al. (2020) reported a direct relationship between the degree of hydrolysis and DPPH radical scavenging activity of chickpea protein hydrolysates [20]. Rahimipanah et al. (2023) showed that increasing the enzymatic hydrolysis time to 136 minutes increased the DPPH radical scavenging activity of pomegranate seed protein hydrolysates, and further increasing the hydrolysis time decreased the radical scavenging activity [31].

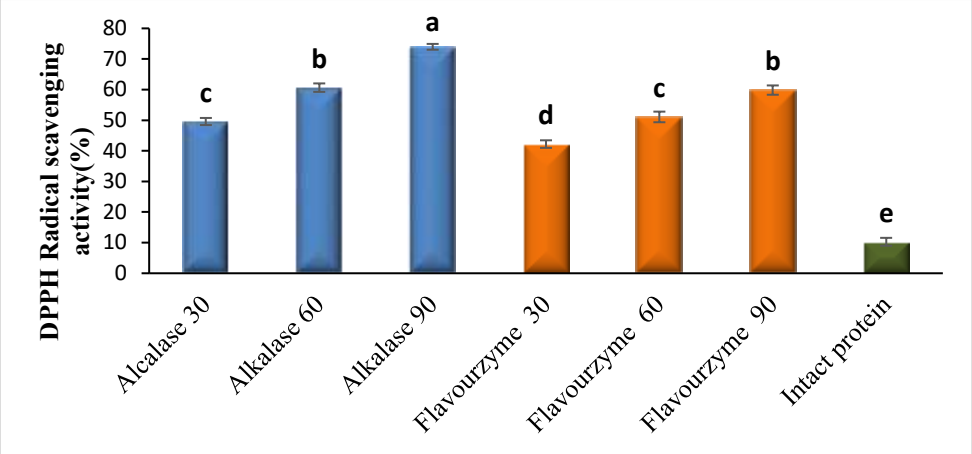

Fig 3. DPPH radical scavenging activity broccoli sprout protein hydrolysates (%)

Values represent mean ± SD. Different letters indicate significant difference among samples at 5% probability level.

3.4.2. Iron reducing capacity (FRAP)

The results of the statistical analysis of the data showed that the treatments studied had a statistically significant effect on the FRAP rate of broccoli sprout protein hydrolysates ($p<0.05$). The average FRAP values of broccoli sprout protein hydrolysates prepared by protease enzymes and different enzymatic hydrolysis times are compared in Figure 4. The results show that the initial crude protein had the lowest FRAP rate (0.064) and the enzymatic hydrolysis process increased the iron reducing capacity of broccoli sprout protein ($p<0.05$). In the case of both enzymes, with increasing the enzymatic hydrolysis time from 30 to 90 min, the FRAP rate increased significantly ($p<0.05$). In general, the highest FRAP value was obtained for the hydrolysate prepared by the alcalase enzyme and the hydrolysis time was 90 min (0.394) and the lowest value was obtained for the hydrolysate prepared by the flavourzyme enzyme and the hydrolysis time was 30 min (0.165). The degree of hydrolysis, the decrease in molecular weight, the status of hydrophobic groups and the number of ionizable groups are some of the factors that affect the antioxidant activity of protein hydrolysates [32]. The results of the antioxidant activity of broccoli sprout protein hydrolysates in the DPPH radical scavenging and FRAP methods showed that the enzymatic hydrolysis process significantly improved the antioxidant activity of broccoli sprout protein.

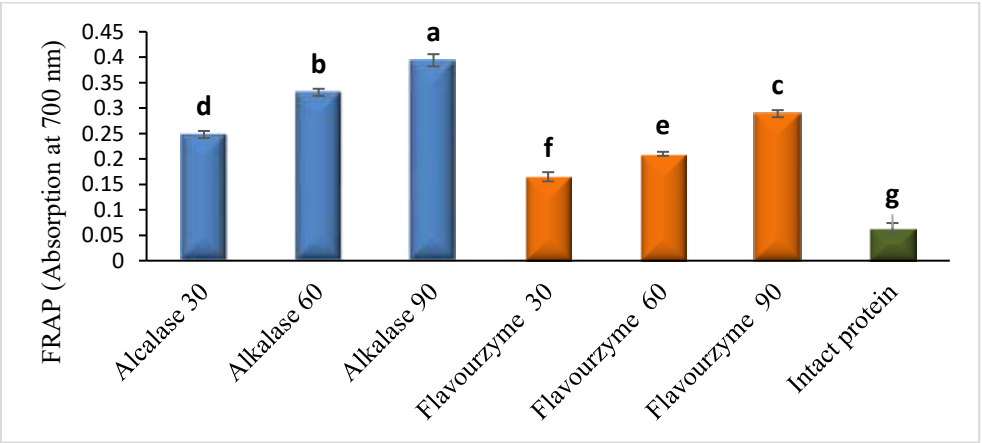

Fig 4. DPPH radical scavenging activity broccoli sprout protein hydrolysates (%)

❋Values represent mean ± SD. Different letters indicate significant difference among samples at 5% probability level.

3.5. Functional properties of broccoli sprout protein hydrolysates

3.5.1. Solubility

Protein solubility is an important and critical functional property for food applications. This property also affects other functional properties of proteins such as foaming and emulsifying properties [33]. The average solubility values of broccoli sprout protein hydrolysates prepared by protease enzymes and different times of enzymatic hydrolysis at different pHs are compared in Figure 5. The results show that the initial crude protein had the lowest solubility at all pHs studied and the enzymatic hydrolysis process increased the solubility percentage of broccoli sprout protein ($p<0.05$). Hydrolysates prepared by the alcalase enzyme had significantly higher solubility than hydrolysates prepared by the flavourzyme enzyme at different pHs ($p<0.05$). For both enzymes, with increasing enzymatic hydrolysis time from 30 to 90 min, the solubility percentage of the hydrolysates increased significantly ($p<0.05$). With increasing pH values, the solubility of the original protein and protein hydrolysates initially decreased and then increased ($p<0.05$). The lowest solubility was at pH = 5, which represents the isoelectric point of broccoli sprout protein. These observations are related to the higher degree of hydrolysis of the hydrolysates prepared by the alcalase enzyme and the production of shorter-chain peptides with higher solubility. With increasing enzymatic hydrolysis time, an increase in the solubility of protein hydrolysates was also observed due to the increase in the degree of hydrolysis. So that these researchers observed the effect of increasing the enzymatic hydrolysis time on increasing the degree of hydrolysis and solubility of milk protein hydrolysates and found that the alcalase enzyme had a higher degree of hydrolysis than the flavourzyme and Protamax enzymes and the hydrolysates prepared with it also showed higher solubility. These results were also consistent with the findings of Najafian (2022), and these researchers also reported a positive relationship between the degree of hydrolysis and solubility of protein hydrolysates [34]. The higher solubility of chickpea protein hydrolysates prepared by the alcalase enzyme compared to the flavourzyme enzyme was also observed in the study of Shuai et al. (2022), and these researchers also showed that with increasing the degree of enzymatic hydrolysis, the percentage of solubility of protein hydrolysates increased, which was consistent with the results of the study [21].

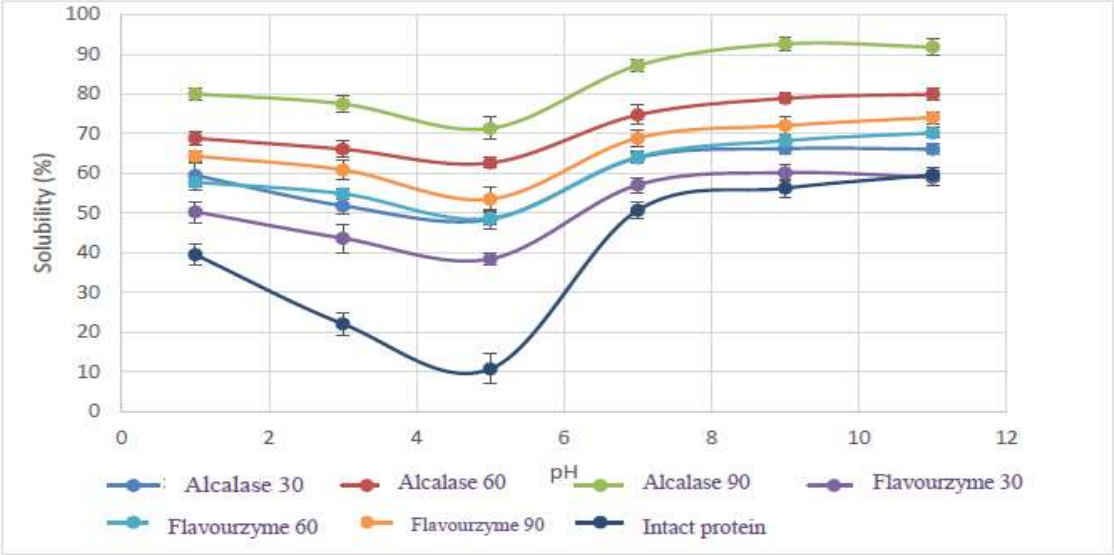

Fig 5. Comparison of solubility of broccoli sprout protein hydrolysates (%)

3.5.2. Emulsifying capacity and emulsion stability

The results of statistical analysis of the data showed that the treatments studied had a statistically significant effect on the emulsifying capacity and emulsion stability of broccoli sprout protein hydrolysates ($p<0.05$). The average values of emulsifying capacity and emulsion stability of broccoli sprout protein hydrolysates prepared by protease enzymes and different enzymatic hydrolysis times are compared with each other in Figures 6 and 7, respectively. The results show that the initial crude protein had the lowest emulsifying capacity (97.19 m^2/g) and emulsion stability (38.24%), and the enzymatic hydrolysis process increased these functional properties of broccoli sprout protein ($p<0.05$). Hydrolysates prepared by alcalase enzyme had significantly higher emulsifying capacity and emulsion stability than hydrolysates prepared by flavourzyme enzyme ($p<0.05$). For both enzymes, as the enzymatic hydrolysis time increased from 30 to 90 min, the emulsifying capacity and emulsion stability of protein hydrolysates gradually decreased ($p<0.05$). This decrease is probably related to the production of shorter chain length peptides that have less ability to reduce surface tension [35]. In general, among protein hydrolysates, the highest emulsifying capacity (194.23 m^2/g) and emulsion stability (50.81%) were obtained for the hydrolysate prepared by the alcalase enzyme and the hydrolysis time was 30 min, and the lowest values of these properties were obtained for the hydrolysate prepared by the flavourzyme enzymeand the hydrolysis time was 90 min (146.37 m^2/g and 43.10%, respectively). Researchers have found that protein hydrolysates prepared by the enzyme Catalase have small droplets and high hydrophobicity, which causes more interaction of these droplets at the interfaces of oil-protein emulsions and creates higher stability with less merging of droplets with each other [30]. Zheng et al. (2019) reported the emulsion stability index of black bean protein hydrolysates prepared by alcalase enzyme at different hydrolysis times in the range of 138.37–821.40 min

[23]. The results of the present study showed that enzymatic hydrolysis of broccoli sprout proteins significantly increased the emulsifying capacity of these proteins ($p<0.05$), which can be attributed to the presence of small peptides and hydrophobic residues that can be rapidly released and immediately adsorbed to the surfaces of newly formed oil droplets during homogenization [36]. Also, this improvement in emulsifying capacity after enzymatic hydrolysis of sprout protein at low hydrolysis degree can be attributed to the improvement of solubility and flexibility of peptide chains [23]. The effect of increasing solubility on improving the emulsifying capacity of protein hydrolysates was also shown in the study of Ai et al. (2019) [37].

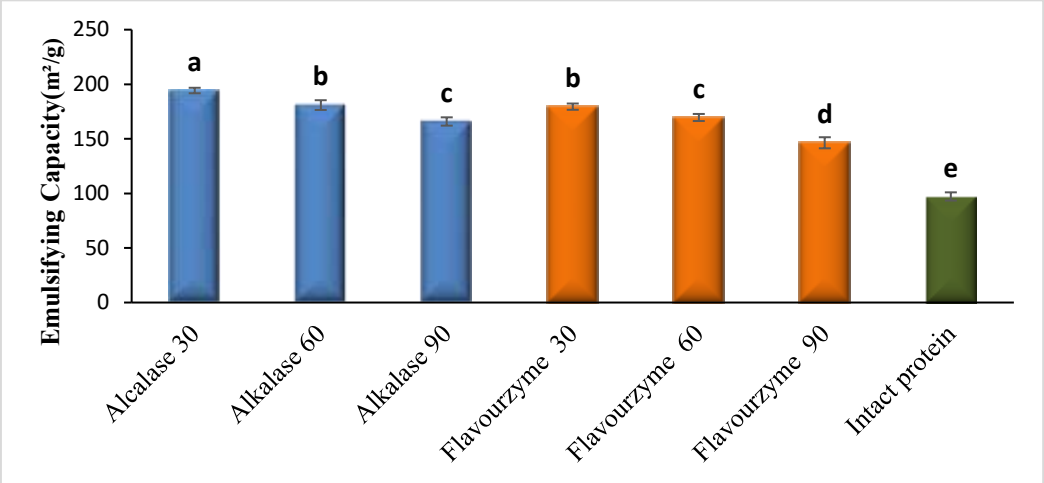

Fig 6. Comparison of emulsifying capacity of broccoli sprout protein hydrolysates (m^2/g)

Values represent mean ± SD. Different letters indicate significant difference among samples at 5% probability level.

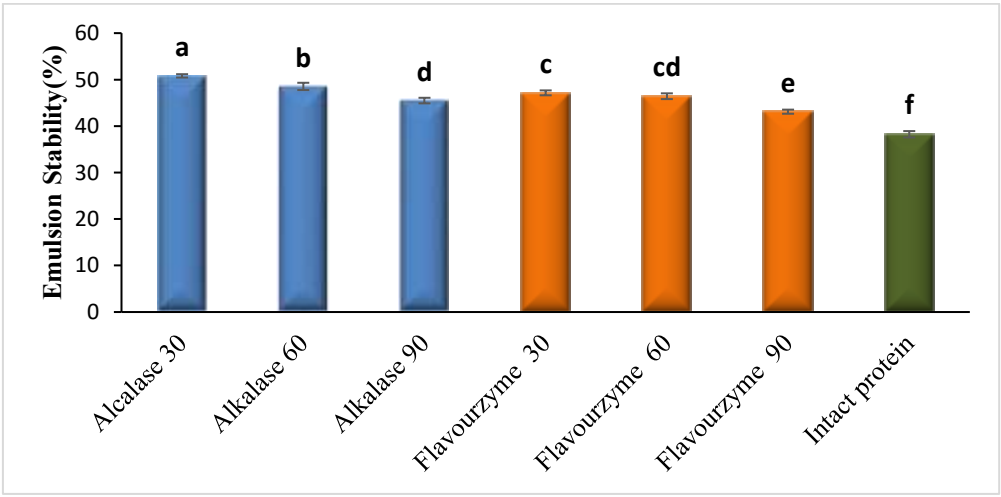

Fig 7. Comparison of emulsion stability of broccoli sprout protein hydrolysates (%)

Values represent mean ± SD. Different letters indicate significant difference among samples at 5% probability level.

3.5.3. Foaming capacity and foam stability

The results of statistical analysis of the data showed that the treatments studied had a statistically significant effect on the foaming

capacity and foam stability of broccoli sprout protein hydrolysates ($p<0.05$). The average values of foaming capacity and foam stability of broccoli sprout protein hydrolysates prepared by protease enzymes and different enzymatic hydrolysis times are compared with each other in Figures 8 and 9, respectively. The results show that the initial crude protein had the lowest foaming capacity (44.82%) and foam stability (73.59%) and the enzymatic hydrolysis process increased these functional properties of broccoli sprout protein ($p<0.05$). Hydrolysates prepared by the alcalase enzyme had significantly higher foaming capacity and foam stability than hydrolysates prepared by the flavourzyme enzyme ($p<0.05$). For both enzymes, with increasing enzymatic hydrolysis time from 30 to 90 min, the foaming capacity and foam stability of protein hydrolysates gradually decreased ($p<0.05$). In general, among protein hydrolysates, the highest foaming capacity (65.24% and 64.15%, respectively) and foam stability (86.97% and 86.10%, respectively) were related to hydrolysates prepared by the alcalase enzyme and hydrolysis times of 30 and 60 min (13.24%), and there was no statistically significant difference between these two treatments. The lowest levels of these properties were obtained in the hydrolysates prepared by the flavourzyme enzyme and hydrolysis time of 90 min (47.30% and 75.14%, respectively). In line with the results of the present study, Shuai et al. (2022) also found that enzymatic hydrolysis of chickpea protein increased the foaming capacity and foam stability of chickpea protein hydrolysates. With increasing the degree of hydrolysis of chickpea protein, the foaming capacity and foam stability initially increased and then decreased. These researchers stated that by increasing the degree of hydrolysis to the desired level, increasing solubility can increase the foaming capacity of the hydrolysate, but a higher increase in the degree of hydrolysis can reduce the foaming capacity by destroying the globular structure of the protein. The balance between hydrophilic and hydrophobic proteins also creates the desired foaming capacity, which excessive hydrolysis reduces this balance [21]. However, Vogelsang-O'Dwyer et al. (2023) reported no significant effect of the enzymatic hydrolysis process by different protease enzymes (alcalase, novozyme, and flavourzyme) on the foaming capacity and foam stability of lentil protein hydrolysates, and only a slight increase in the emulsifying capacity of hydrolysates produced by alcalase and novozyme enzymes and a decrease in foam stability in the hydrolysates were observed compared to the original crude protein [38].

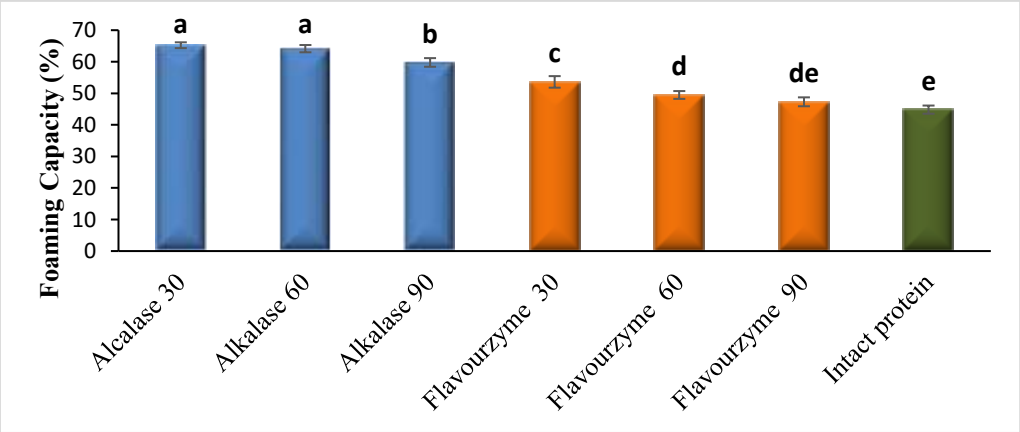

Fig 8. Comparison of foaming capacity of broccoli sprout protein hydrolysates (%)

Values represent mean ± SD. Different letters indicate significant difference among samples at 5% probability level.

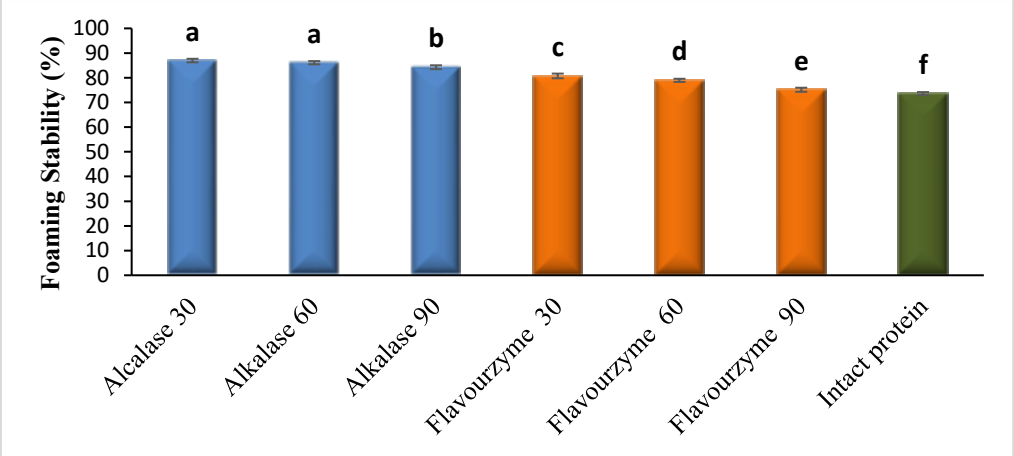

Fig 9. Comparison of foaming stability of broccoli sprout protein hydrolysates (%)

Values represent mean ± SD. Different letters indicate significant difference among samples at 5% probability level.

4- Conclusion

In this study, broccoli sprout protein hydrolysates were prepared using two protease enzymes, alcalase and flavourzyme, and three enzymatic hydrolysis times (30, 60, and 90 min), and the antioxidant and functional properties of the protein hydrolysates were investigated. The results showed that the degree of enzymatic hydrolysis, hydrolyzed protein content, DPPH radical scavenging activity, and FRAP of the hydrolysates prepared by alcalase were significantly higher than those prepared by flavourzyme. The initial crude protein had the lowest solubility, emulsifying capacity, emulsion stability, foaming capacity, and foam stability, and the enzymatic hydrolysis process increased these functional properties of broccoli sprout protein. The hydrolysates prepared by alcalase had significantly higher solubility, emulsifying capacity, emulsion stability, foaming capacity, and foam stability than the hydrolysates prepared by flavourzyme. For both enzymes, with increasing enzymatic hydrolysis time from 30 to 90 min, solubility increased, but emulsifying capacity, emulsion stability, foaming capacity, and foam stability of protein hydrolysates gradually decreased. The hydrophobic amino acid content of hydrolysates prepared by alcalase enzyme was higher than that of flavourzyme enzyme. Hydrolysates prepared by alcalase enzyme and enzymatic hydrolysis time of 90 min were selected as the best treatment.

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مقاله علمی_پژوهشی

ارزیابی خواص آنتی اکسیدانی و عملکردی پروتئین جوانه کلم بروکلی هیدرولیز شده به روش هیدرولیز آنزیمی

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چکیده

پپتیدهای زیست‌فعال جزء ترکیبات عملکردی محسوب می‌شوند که اخیراً در مواد غذایی شناخته شده‌اند. روش هیدرولیز آنزیمی به منظور بهبود ویژگی‌های عملکردی پروتئین‌های گیاهی مورد استفاده قرار گرفته است. هدف از این تحقیق هیدرولیز آنزیمی پروتئین جوانه بروکلی و بررسی خصوصیات عملکردی و آنتی اکسیدانی هیدرولیزات‌های حاصله بود. برای این منظور از دو آنزیم‌های آلکالاز و فلاورزایم و زمان‌های مختلف هیدرولیز آنزیمی (۳۰، ٦۰ و ۹۰ دقیقه) استفاده گردید. نتایج نشان داد که هیدرولیزهای پروتئینی حاصله توسط آنزیم آلکالاز دارای طول زنجیره کوچکتر و مقادیر بالاتر اسیدهای آمینه هیدروفوب، درجه هیدرولیز آنزیمی، بازیافت پروتئینی، محتوای پروتئین هیدرولیز شده، حلالیت، ظرفیت امولسیون‌کنندگی، پایداری امولسیون، ظرفیت کف‌کنندگی، پایداری کف و فعالیت آنتی اکسیدانی در مقایسه با هیدرولیزات‌های تهیه شده توسط آنزیم فلاورزایم بودند. افزایش زمان هیدرولیز آنزیمی از ۳۰ تا ۹۰ دقیقه، منجر به افزایش حلالیت و کاهش ظرفیت امولسیون‌کنندگی، پایداری امولسیون، ظرفیت کف‌کنندگی و پایداری کف هیدرولیزات‌های پروتئینی تولیدی گردید (p<۰/۰۵). در کل، به دلیل فعالیت ضداکسایشی پروتئین جوانه بروکلی و بهبود قابل توجه آن پس از فرآیند هیدرولیز آنزیمی، به ویژه توسط آنزیم آلکالاز در زمان ۹۰ دقیقه، هیدرولیزات‌های پروتئینی تهیه شده به عنوان آنتی اکسیدان‌های طبیعی جهت حفظ کیفیت و ایمنی محصولات غذایی پیشنهاد می‌گردند.