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Scientific Research

Nano-microencapsulation of bioactive peptides obtained from enzymatic hydrolysis of clover sprouts and evaluation of functional and antioxidant activity of peptides

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ABSTRACT

Enzymatic hydrolysis is one of the methods used to improve the functional and nutritional properties of food proteins. Considering the effect that hydrolyzed proteins have on the functional and health-giving properties of food, in this research, using Protamax and Bromelain enzymes, the protein obtained from the clover sprout was hydrolyzed and the effect of this process on the antioxidant properties (inhibition DPPH and ABTS free radicals activities) and their functional characteristics (solubility, foaming and emulsification) were investigated. Then the hydrolyzed protein was micro-encapsulated by nanoliposome and its characteristics were analyzed. According to the results, the sum of hydrophobic and aromatic amino acids for Protamax and Bromelain enzymes were 40.41, 37.91, 12.35 and 11.46, respectively. Also, among the enzymes, Protamax enzyme was able to produce hydrolyzable protein with a higher degree of hydrolysis, protein content, antioxidant properties and higher functional properties, and also increasing the hydrolysis time had a positive effect on the aforementioned characteristics. Therefore, the protein hydrolyzed by Protamax enzyme and time 60 minutes was covered by nanoliposome, the results related to the size of the particles carrying peptides equal to 93.64±3.37 nm, zeta potential equal to 42.1±1.38 8-mV and microcoating efficiency was 68.73±2.37%. The results of the present research showed nano protein can be a useful approach for direct application of clover sprout peptides with antioxidant capacity in food products.

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1. Introduction

During the last two decades, special attention has been paid to bioactive peptides obtained from food proteins. Bioactive peptides are special protein parts that have many biological properties such as antioxidant, antimicrobial, anti-hypertensive, anti-blood clotting, anti-cancer and control the activity of the immune system [1]. Bioactive peptides usually correspond to the main protein sequence and are mainly produced by chemical and physical methods. Among the chemical methods, we can mention alkaline, acid hydrolysis, plant, animal and microbial proteolytic enzymes [2]. Currently, the use of vegetable protein (clover sprouts) has become very important due to its high nutritional value, positive effects on the texture and reduction of yogurt wateriness, as well as its positive effect on improving the growth and survival of yogurt starters, especially probiotic bacteria during storage. is [3, 4]. Iranian clover with scientific name*Clover resupinatum* L. is an annual and autumn plant that is native to Asia Minor and Iran. Most of the studies conducted regarding the therapeutic effects of clover emphasize the phytoestrogenic properties of its isoflavones. In addition to estrogenic effects, the antioxidant, anti-inflammatory and anti-cancer effects of clover have been shown in the extracellular environment, cells and animal models [5, 6]. Due to its high nutritional value and suitable functional characteristics, nutritionists refer to the clover bud as a natural nutrient treasure and a source of human life [7, 8]. Clover sprout protein, which is rich in water-soluble and salt-water soluble proteins, has favorable functional characteristics that make it a useful combination for use in various food products. Emulsifying activity, high water retention ability, foaming and high solubility are among these characteristics [5, 9]. Clover bud protein contains natural proteins with high quality and high digestibility. The two most important amino acids in clover bud protein are cysteine and cisnine, which neutralize toxins in the body, and are also precursors of the body's strongest antioxidant, glutathione [10]. Mirsadeghi Darabi et al. (2022) studied the production of hydrolyzed protein of clover bud by Alcalase and Flowerzyme enzyme, they announced that the hydrolyzed protein of clover bud had high essential amino acids, Alcalase enzyme can hydrolyze protein with the degree of hydrolysis and properties. produce higher antioxidant and anticancer properties [5].

Since peptides are very active compounds, they may lose their beneficial effects due to contact with oxygen or food. Therefore, it is necessary to use methods to protect them in order to obtain the highest antioxidant activity. Microcoating of these compounds before using them in food or beverage systems is considered a suitable way to overcome these limitations. In addition, recently, improving the activity and effectiveness of nanoscale antimicrobial compounds compared to the traditional mode has attracted more and more attention [11, 12, 13]. Liposomes have been used as drug release systems in the human body for many years, and due to their ability to release hydrophilic and hydrophobic compounds, they have been of interest for a long time. Common lipids used in liposome production are phospholipids, especially phosphatidylcholine (lecithin) and phosphatidylethanolamine [14, 15]. Having special structural features, liposomes are capable of encapsulating hydrophilic compounds in the aqueous phase and hydrophobic compounds in their bilayer phospholipid part [15]. Tometry et al. (2020) announced that the microcoating of bioactive compounds with nanoliposomes improves the properties and preserves the antioxidant and antimicrobial properties of bioactive compounds [15].

Therefore, considering the richness of clover sprouts in terms of amino acid composition, in this research, it has been tried to use clover sprouts as a suitable protein source for enzymatic hydrolysis, and to study the functional antioxidant properties and health benefits of the resulting hydrolysates. Also, the possibility of producing and evaluating nanoliposomes carrying bioactive peptides to overcome the challenges in the way of using these valuable compounds in food and pharmaceutical industries was evaluated.

2- Materials and method

2-1 raw materials

Clover buds were obtained from Jovaneh Aftab Company, Iran, for the enzymatic hydrolysis of clover using Protamax enzymes (obtained from bacteria*Bacills subtilis*) and bromaline (extracted from pineapple fruit) were used. Enzymes and amino acid standards were purchased from Sigma Aldridge (USA). Bovine serum albumin, DPPH and ABTS reagents, ascorbic acid, hydrochloric acid and hydrochloric acid were purchased from Merck, Germany.

2-2 clover bud protein extraction

In order to prepare protein isolate, 100 grams of defatted clover sprouts were mixed in distilled water at a ratio of 1 to 10 (weight by volume) and the pH was adjusted to 11 using 1 M sodium hydroxide. Then it was stirred for 60 minutes at room temperature and at a speed of 500 rpm. The resulting alkaline extract was centrifuged at a rotational gravity of 1600 and a temperature of 4°C for 20 minutes. After collecting the supernatant, its pH was adjusted to 3 using 1 M hydrochloric acid, and the precipitated protein was separated by centrifugation. Finally, the resulting protein was dried in a freeze dryer and placed in plastic bags in the freezer at room temperature until hydrolysis. It was kept at 18°C [16].

2-3 Clover bud protein hydrolysis

In order to hydrolyze clover bud protein, protein was prepared at a ratio of 4% (weight-volume) in phosphate buffer 7 (optimal pH) for bromaline and protamax enzymes. Hydrolysis was performed at an optimal temperature of 50°C, enzyme concentrations of 1% and a time range of 10 minutes to 1 hour in a shaker incubator (model V-8480, South Korea) at 200 rpm. In order to deactivate the enzyme activity, the protein solution was again placed in a hot water bath with a temperature of 80°C for 15 minutes. In the next step, centrifugation was performed at 10,000 rotational gravity for 20 minutes. In order to produce hydrolyzed protein powder, the resulting supernatant was dried in a freeze dryer at a temperature of -20 degrees Celsius, a pressure of 40 millibars and a time of 48 hours (model 5509 FDB Operon, South Korea) and at a temperature of -20 degrees Celsius until use. was maintained [18].

2-3 degrees of hydrolysis and peptide chain length

The amount of hydrolysis was measured with the help of trichloroacetic acid (TCA). The basis of this method is to measure the ratio of proteins soluble in 10% trichloroacetic acid to all the proteins in the sample. For this purpose, 5 ml of the sample was mixed with 5 ml of 20% trichloroacetic acid and then centrifuged at 6700 rpm for 10 minutes. Then the amount of protein in the solution phas The measurement and amount of hydrolysis was calculated by equation 1 [19]:

Equation 1:

100x (the amount of protein dissolved in 10% trichloroacetic acid solution / the amount of protein in the sample) = degree of hydrolysis

Kristinsson and Rasco (2000) method was used to measure the approximate length of peptides obtained by hydrolysis with different enzymes. In this method, the degree of hydrolysis was used to calculate the chain length with the help of the following equation.

Equation 2: degree of hydrolysis / 100 = peptide chain length

2-4 Determining the amount of amino acids

The hydrolyzed protein powder was completely hydrolyzed for 24 hours at 110 degrees Celsius using 6 normal hydrochloric acid. Then, using phenyl isothiocyanate (PITC), amino acids were derivatized. Total amino acids were measured using a Smart line (Germany) HPLC device using a C18 column with a fluorescent detector (RF-530). [2].

2-5 antioxidant activity

For DPPH free radical inhibitory activity, 1 ml of different hydrolyzed protein treatments were added separately with 1 ml of 0.1 mM DPPH solution and the resulting mixture was shaken well and placed in a dark room for 15 minutes and then the samples were optically absorbed. It was read at a wavelength of 517 nm [20].

Equation 3:

100× (witness absorption / witness absorption - sample absorption) = DPPH free radical inhibition percentage

To measure the radical scavenging activity of ABTS, a solution of 7 mM ABTS in potassium persulfate was used according to the method of Alemán et al. (2011). 2.45 mM was prepared and kept for 16 hours at ambient temperature and in a dark place. After the desired time, dilution with distilled water was done until the absorbance of 0.7 at 734 nm wavelength was reached. Then, 20 microliters of the sample was

mixed with 980 microliters of diluted ABTS solution and incubated for 10 minutes in a dark place at 30°C. After the desired time, the absorption of the samples was read at 734 nm in order to compare different concentrations of ascorbic acid. The use and percentage of ABTS radical inhibition was calculated with the following formula [21].

Equation 4

100x (witness absorption / witness absorption - sample absorption) = ABTS free radical inhibition percentage

6-2 Measurement of the functional characteristics of hydrolyzates

2-6-1 Solubility

Solubility of hydrolyzed protein was performed using the method of Bera and Mukherjee (1989). One gram of the sample was mixed in 100 ml of water solution and its pH was adjusted to 10-2 using soda and 0.1 normal acid and then mixed for 60 minutes at room temperature. Centrifugation was performed for 20 minutes at a speed of 500 rpm, and the nitrogen content in the sample supernatant was determined using the Keldall method, and the percentage of soluble protein was calculated using equation (5) [22].

Equation 5:

100x (gram weight of prototype / gram weight of water of solid material dissolved in supernate) = Solubility index in water

2-6-2 Capacity and stability of emulsifier

50 ml of distilled water and 50 ml of rapeseed oil were added to 3 grams of sample and homogenized for 30 seconds with a homogenizer (APU500b, Dilkofnavar, Iran). Then, it was divided into 4 test tubes in equal amounts and centrifuged with a centrifuge at 2000 g for 5 minutes (Behdad, Iran). The emulsifying capacity was reported according to equation (6) below [23].

Equation 6:

Total volume / volume of emulsified part = emulsifying capacity (%)

10 milliliters of rapeseed oil was mixed with 30 milliliters of 1% protein solution and their pH was adjusted to five different pHs of 2, 4, 6, 8, 10, then it was homogenized with a homogenizer at a speed of 2000 rpm for one minute. Then he took 50 microliters from the liquid part of the bottom of the tube with a microsampler, which was done in 0 and 10 minutes. Then, the obtained samples were mixed with 5 ml of 1% sodium dodecyl sulfate at zero and ten minutes, and the absorbance of the diluted solution was read with a spectrophotometer at a wavelength of 500 nm [23].

Equation 7:

Time zero - time 10 minutes/ time zero x time 10 minutes = emulsifier stability (%)

2-6-3 Measuring the capacity and stability of foaming

To measure the foaming capacity, 20 ml of protein solutions with pH 5-8 were prepared and poured into a 50 ml graduated cylinder. Then it was stirred for one minute with a homogenizer at a speed of 10,000 rpm. The volume of the final mixture was read. The percentage of volume increase at zero time compared to the initial volume was considered as foaming capacity (Equation 8) [24].

Equation 8:

Sample volume before foam formation / sample volume at different times after foam formation - sample volume before foam formation = foaming capacity (%)

To calculate the stability of the foam, the volume of the foam was read at 0.5, 5, 10, 40 and 60 minutes after the formation of the foam. Then, using the above relationship, the percentage of floor volume remaining in each of the mentioned times was reported as an index of floor stability [24]. (equation 9)

Equation 9: $Floor\ stability = \frac{V0 \times 100}{\Delta V}$

100 x (the volume of the floor immediately after being hit / the volume of the floor after 60 minutes) = stability of the floor

After performing the above tests, the superior treatment was used for the next tests.

2-7 Preparation of microcoated peptide

2-7-1 Loading hydrolyzed clover bud protein in nanoliposomes

The liposome was produced using the thin layer hydration method and based on the method described by Liu et al. (2012) with some modifications [25]. The liposome production process included the dissolution of soy lecithin and cholesterol at a ratio of 9:1 in 10 ml of absolute ethanol. The process of evaporation and formation of a thin film at the end of the round-bottom balloon was carried out using a rotating evaporator with a speed of 60 rpm at a temperature of 60. To ensure the complete removal of the solvent, the circular balloon was kept in a desiccator at ambient temperature for 12 to 18 hours, then the lipid film It was dried with saline phosphate (7.4 pH) and rehydrated for 1 minute using a vortex. Then, 10 mg/ml of hydrolyzed protein was added to the resulting solution and stirred for 2 minutes using a rotary at 60°C, then stirred for 2 minutes at room temperature and cooled. The stirring process was repeated 3 times. Finally, the resulting solution is subjected to an ultrasonic probe of 70 power and 0.5 cycle for 10 steps and for 1 minute in each step.

In order to prevent the increase in temperature, the ultrasonic process was carried out under the condition of keeping nanoliposomes in cold water. Finally, the natoliposome produced with a lipid concentration (lecithin and cholesterol) of 10 mg/ml was kept in the refrigerator until the tests were performed.

2-7-2 Determination of particle size and zeta potential

For this purpose, each of the samples were first diluted using saline phosphate buffer at a ratio of 1:10. The particle size was measured as Z-average, also the electric charge of nanoliposomes was measured as zeta potential, specific electrical conductivity and electrophoretic movement using the same device.

2-7-3 efficiency of micro-covering

The microencapsulation efficiency of peptides obtained from enzymatic hydrolysis of quinoa protein was done using the method of Liu et al. (2015) with some modifications. In this way, in order to determine the amount of uncoated peptides in the liposome structure (proteins), a free amount of 1 ml of the liposome was prepared by centrifugation using an Amicon filter with a passing molecular weight of 10 kilodaltons and was centrifuged for 10 minutes at 3500 rpm. And the encapsulation efficiency of hydrolyzates was calculated using the following equation [26].

Equation 10

100 × (total protein amounts / unloaded protein amounts - total protein amounts) = microencapsulation efficiency (%)

2-8 Statistical evaluation

The experiments were conducted in three repetitions and in the form of a completely randomized design. Data analysis was done using SPSS 18 software. Comparison of treatment means was done with Duncan's multi-range test with a 5% error level. Graphs were drawn using Microsoft Excel 2013 software.

3. Results and Discussion

3-1 protein amounts in different treatments

The protein content of clover bud and isolated clover bud was 31.17±1.23% and 47.38±1.10%, respectively, and the protein values (Table 1) in different hydrolyzed protein treatments (time and different enzymes) were with 62.23-21.95 percent. According to the results of the hydrolyzed proteins produced in this research, the amount of protein is relatively high in all treatments. These treatments are rich in protein due to the presence of high amounts of soluble proteins during the production process, after separating the solid and insoluble parts of myofibril and stroma proteins by centrifugation. Therefore, the high content of crude protein in the studied hydrolyzed proteins makes them valuable protein supplements [27, 28].

2-3 Checking the values of the degree of hydrolysis and the length of the peptide chain

As the results related to the changes in the degree of hydrolysis (Table 1) show over time, the degree of hydrolysis increases with the increase in the duration of the reaction, in fact, the highest percentage of the degree of hydrolysis in relation to Protamax enzyme was in the period of 1 hour. Based on the results obtained from the effect of time on protein hydrolysis, as the reaction time increases, enzymatic hydrolysis begins with a fast phase and at this stage a large number of peptide bonds are broken [2, 29]. A comparison between commercial enzymes (such as Protamax enzyme) and plant proteolytic enzymes (such as bromaline) showed that enzymes of microbial origin increase the hydrolysis efficiency [17]. Protamax is a protease made from bacteria*Bacillus subtilis* It is widely used due to the production of protein with a high degree of hydrolysis in a short time and with less bitterness than other enzymes [18, 30].

The length of the peptide chain (Table 1) has an inverse relationship with the degree of hydrolysis, so that the length of the peptide chain by Protamax was less than that of bromaline, and with the increase in the hydrolysis time, the length of the peptide chain decreased significantly. Dorvaj et al. (2013) also announced that the hydrolyzed protein of Kilka fish by the microbial enzyme Promode had a significantly higher degree of hydrolysis and a shorter peptide chain length than the protein hydrolyzed by the bromelain enzyme [17].

Table 1. Effect of enzyme hydrolysis type and time of protein hydrolyzed on protein content, degree of hydrolysis and peptide chain length.

Enzyme		Protein content (%)	Degree of hydrolysis (%)	Peptide chain length
Type	hour (min)			
Protamax	10	64.59±0.50[It is]	14.21±0.72[d]	7.04±0.35[a]
	30	77.69±0.61[c]	23.69±0.79[c]	4.22±0.14[b]

	60	95.21 ±1.15[a]	35.17±1.59[a]	2.84±0.12[d]
Bromelain	10	62.23±0.55[f]	12.53±0.84[d]	8.00±0.56[a]
	30	74.62±1.08[d]	22.03±0.05[c]	4.53±0.01[b]
	60	90.58±0.45[b]	31.59±0.83[b]	3.16±0.08[c]

Averages with the different letters (in each columns) indicate that there is significant difference at the P< 0.05.

3-3 amino acid composition

According to the results (Table 2), it was found that both hydrolyzed proteins are rich in essential and non-essential amino acids and have the ability to provide more than the amounts required by humans, and only phenylalanine amino acids were limiting [31]. Hydrophobic amino acids (phenylalanine, proline, methionine, alanine, leucine, isoleucine, tyrosine, valine) and aromatic amino acids (phenylalanine, histidine, tryptophan, tyrosine), responsible for most of the functional and biological properties of hydrolyzed proteins, such as antioxidant activity, They are anti-inflammatory, anti-cancer and reduce sugar and blood pressure [32, 33]. Considering their high amounts in two types of production hydrolysis, we can expect their bioactive effects in the host [34]. The sum of hydrophobic and aromatic amino acids was 40.41, 37.91, 12.35 and 11.46 for protamax and bromaline enzymes, respectively. The highest amounts of essential amino acids for Protamax enzyme and bromaline were leucine 7.04% and 7.05%, the highest amounts of non-essential amino acid for Protamax enzyme and bromaline were aspartic acid 18.97%, 18.55%.

Purwin et al. (2015) stated that the highest amount of essential amino acid for red clover protein is leucine (5.35%), and the highest amount of non-essential amino acid is aspartic acid (12.08%) [35]. Mirsadeghi Darabi et al. (2022) in connection with the hydrolyzed protein of clover bud by alcalase and florezyme enzyme, the highest amounts of non-essential amino acids for alcalase and florezyme enzyme aspartic acid 18.99%, 17.58% and the highest essential amino acid amounts for 78.78% and 7.12% for alcalase enzyme and leucine flowerzyme [5]. According to the studies, amino acid values are different for clover species, but in all the mentioned species, the highest amino acid values were related to aspartic acid.

Table 2: Effect of enzyme hydrolysis type (at 60 min) of protein hydrolyzed on amino acid composition

Amino acid (g 100 g^{-1})	Protamax	Bromelain	FAO/ WHO, 1990
Threonine[a]	6.44	6.79	3.4
Valine[a]	5.99	5.66	3.5
Methionine[a]	0.39	0.47	
Isoleucine [a]	4.14	4.05	2.8
Leucine[a]	7.04	7.05	6.6
Phenyl alanine[a]	5.88	4.99	6.3
Histidine [a]	2.48	2.97	1.9
Lysine[a]	6.59	6.77	5.8
Arginine[a]	5.79	5.11	
Glycine	4.10	4.78	

Aspartic acid	18.97	18.55	
Glutamic acid	10.21	10.05	
Serine	5.11	6.05	
Alanine	5.79	5.11	
Tyrosine	3.99	3.68	1.1
Cysteine	0.44	0.45	
Proline	7.11	6.98	
Total amino acid	99.63	99.39	
LET[b]	40.41	37.91	
AAA[c]	12.35	11.46	

[a] Essential amino acids

[b] Combined total of hydrophobic amino acids (HAA)= alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, methionine and cysteine

[c] The total amount of aromatic amino acids (AAA) (phenylalanine, histidine, tryptophan and tyrosine)

3-4 antioxidant properties

Hydrolyzed proteins from different times showed different inhibitory activity. DPPH radical scavenging activity (Chart 1) increased with increasing hydrolysis time. Also, the antioxidant activity of Protamax enzyme was higher than Bromaline enzyme only at the end of the storage period. So that the protein hydrolyzed by Protamax enzyme with a hydrolysis time of 60 minutes had the highest antioxidant activity (87.62%). In total, the results have shown that both enzymes lead to the digestion of peptide bonds by breaking the bonds between hydrophobic amino acids and aromatic amino acids with other amino acids, and it is believed that the phenyl group at the remaining end of the peptide chain has the ability to inhibit radicals and It is an antioxidant [36]. It is also believed that the destruction of the natural structure of proteins due to enzymatic hydrolysis will lead to the opening of the structure and exposure to active groups of amino acids that can react with free radicals [37]. It has been proven that there is a direct relationship between the ability of radical and antioxidant inhibition and the hydrogenation ability of amino acids. The change in the length of peptide chains with the passage of time of hydrolysis also has a significant effect on the antioxidant power, and low molecular weight peptides have stronger antioxidant activity [36, 37].

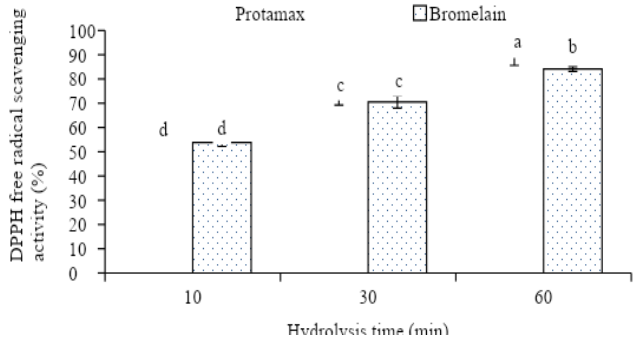

Figure 1: The DPPH free radical savaging activity of clover sprouts protein hydrolysates

Different letters in the same times showed a significant difference at $p<0.05$.

Also, the results have shown that the antioxidant activity of hydrolyzed proteins and peptides depends on the type of protease used for hydrolysis, degree of hydrolysis, molecular weight, sequence of amino acids and the structure of hydrolyzed peptides and proteins [2, 38].

Another method of measuring antioxidant activity is evaluating the ABTS radical scavenging power, which is one of the very good methods to check the antioxidant activity of electron-donating compounds and compounds that stop oxidation chain reactions by inhibiting lipid peroxyl radicals. . Hydrolyzed proteins from different times showed different inhibitory activity. ABTS radical scavenging activity (Chart 2) increased with increasing hydrolysis time. The protein hydrolyzed by Protamax enzyme and bromelain with a hydrolysis time of 60 minutes had the highest antioxidant activity. Hydrolyzed proteins have peptides that are electron donors and can react with free radicals and convert them into more stable compounds. The result of this action will be to stop oxidation chain reactions [39].

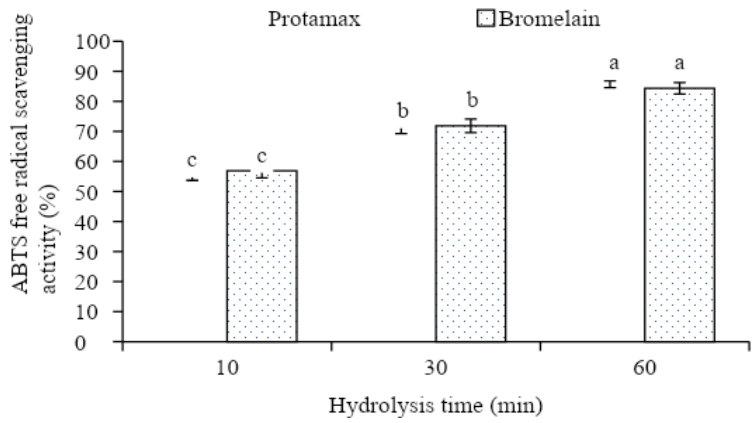

Figure 2: The ABTS free radical savaging activity of clover sprouts protein hydrolysates
Different letters in the same times showed a significant difference at $p<0.05$.

In fact, changes in antioxidant activity over time have been influenced by changes in the length of the resulting peptide chains with increased hydrolysis. The results of studies by many researchers show that peptides with a higher degree of hydrolysis have shown higher antioxidant activity [5, 27].

3-5 functional properties

3-5-1 Solubility

Solubility is usually considered as one of the most important functional properties of protein. Proteins are used in the food industry, they need high solubility, and other functional properties of protein such as foam, emulsion and gelation properties depend on initial solubility [40]. The solubility of the hydrolyzed protein (Chart 3) by different enzymes at the same time did not have any significant differences, but the solubility values increased with the increase of the hydrolysis time. So that the hydrolyzed protein had the highest solubility at the time of hydrolysis of 60 minutes. Therefore, increasing the hydrolysis time and degree of hydrolysis had a positive effect on the protein solubility values. Fan et al. (2020) reported that the higher solubility of proteins may be due to the presence of smaller peptides with a hydrophilic nature compared to healthy (intact) proteins, as it was stated that the length of the peptide chain is inversely proportional to the degree of hydrolysis and the time of hydrolysis [41]. . Yathisha et al. (2021) also announced the solubility of hydrolyzed protein of ribbon fish (*Lepturacanthus Savala*) increased with increasing hydrolysis time and degree of hydrolysis [33].

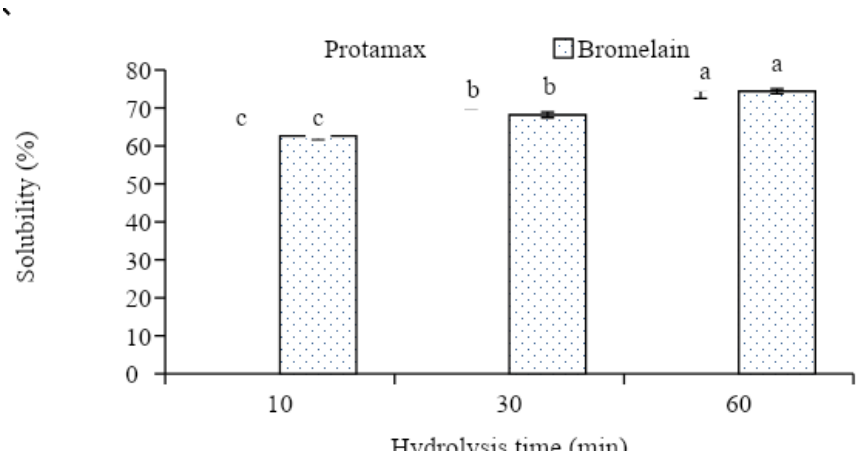

Figure 3: The solubility of clover sprouts protein hydrolysates. Different letters in the same times showed a significant difference at $p<0.05$.

3-5-2 Emulsifying properties

The emulsifying properties of protein-containing products are due to the presence of soluble and insoluble proteins as well as other compounds, such as polysaccharides. Proteins can form and consolidate an emulsion by reducing the surface tension of oil droplets and creating electrostatic repulsion on the surface of oil droplets [42]. The protein should have good solubility in water and be able to rapidly migrate and adsorb at the oil-water interface during homogenization. Emulsion stability depends on protein-protein interactions to form a strong viscoelastic layer at the interface [40]. The results related to emulsion stability (Chart 4) and emulsion capacity (Chart 5) were consistent and these two parameters were not significantly different by different enzymes at the same time. But with the increase in hydrolysis time, the emulsion capacity values increased. As an effective indicator in the hydrolysis process, the emulsification of proteins is affected by the length of the peptide chain and molecular weight, and the emulsification capacity increases with the decrease of the molecular weight. Gumus et al. (2017) stated that there is a direct relationship between the activity There is emulsification and peptide length, shorter peptide length can improve protein emulsification properties [42]. This finding may have potential application in functional foods.

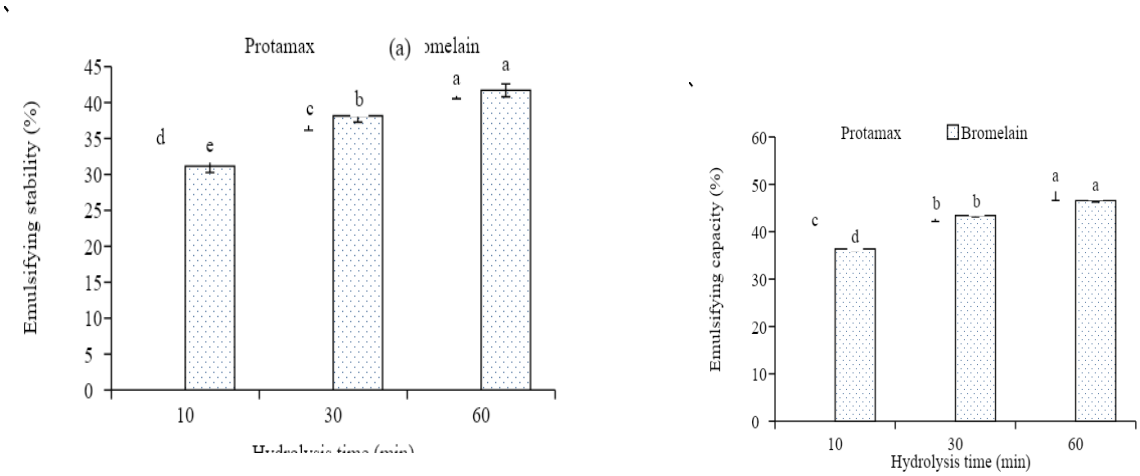

Figure 4: The emulsifying stability (a) and capacity (b) of clover sprouts protein hydrolysates
Different letters in the same times showed a significant difference at p<0.05.

3-5-2 Foaming properties

Proteins cause foam in flours due to their high surface activity. Soluble proteins can reduce the surface tension between the layer surrounding the liquid and the air bubbles, thus preventing the accumulation of bubbles. On the other hand, protein molecules can open and react with each other and form a multi-layer protein film that increases the flexibility of the air-water surface, as a result of which the breaking of air bubbles becomes harder and a stronger foam is formed [40]. The values of foam capacity (diagram a5) and stability of foam (diagram b5) were consistent with each other, foaming protein hydrolyzed by Protamax enzyme was higher at all hydrolysis times. There was a significant difference in the amount of foaming capacity between different times (P<0.05). With the increase in hydrolysis time, the amount of foaming capacity increased, so that the protein hydrolyzed by Protamax enzyme with a hydrolysis time of 60 minutes had the highest foaming capacity and foam stability. had Various researches have investigated the effect of enzymatic hydrolysis process and hydrolysis time on the foaming capacity and stability of protein hydrolyzates. Factors such as the solubility of proteins in different pH conditions, the degree of hydrolysis of the type of peptides, the ability to form a film are some of the factors affecting these indicators [43, 44]. In this way, the effect of increasing the degree of hydrolysis under the influence of the process time and the type of enzyme is effective in reducing the length of the peptide chain and their performance in creating and stabilizing the foam. The results of this research are similar to the findings of Jamdar et al. (2010) who stated that in enzymatic hydrolysis, by reducing the molecular weight and increasing the flexibility of peptides, it facilitates the formation of interfacial membrane and the production of foam [45].

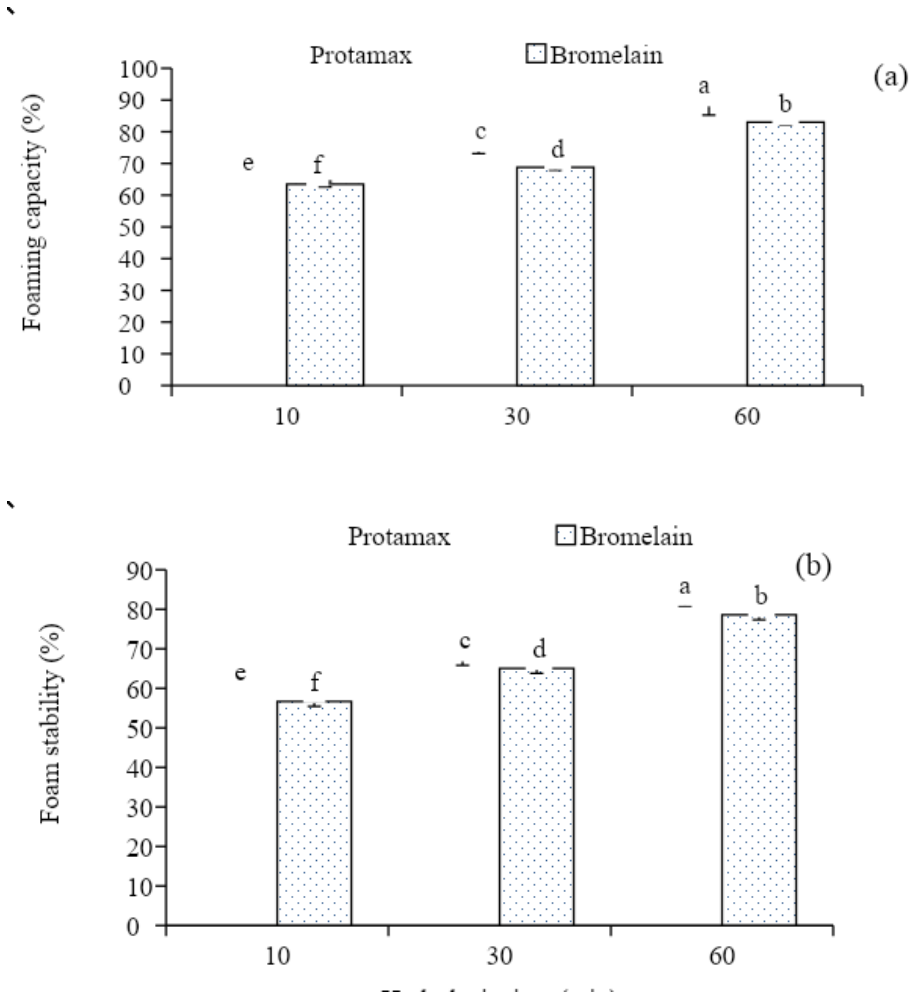

Figure 5: The foaming capacity (a) and stability (b) of clover sprouts protein hydrolysates
Different letters in the same times showed a significant difference at $p<0.05$.

3-6 review of nanolipozyme tests

3-6-1 Particle size

Particle size is an important and influencing factor on stability, microcoating efficiency and release of bioactive compounds. Due to the effects of particle size on the stability and also the release capacity of compounds enclosed in the core, this index has been of great interest [46, 47]. The results related to the size of peptide carrier particles in the present study were equal to 93.64 ± 3.37 nm. which indicates the appropriate size of nanoparticles. Da silva et al. (2014) reported the average particle size of nanoliposomes containing antimicrobial peptides to be 150 nm, and Hosseini et al. (2017) also reported the particle size of liposomes containing fish gelatin to be 131.5 nm [48, 49], which is almost consistent with the results of the current research. and the slight difference observed in the results can be due to the difference in sonication time and also the compounds participating in the dispersion [47].

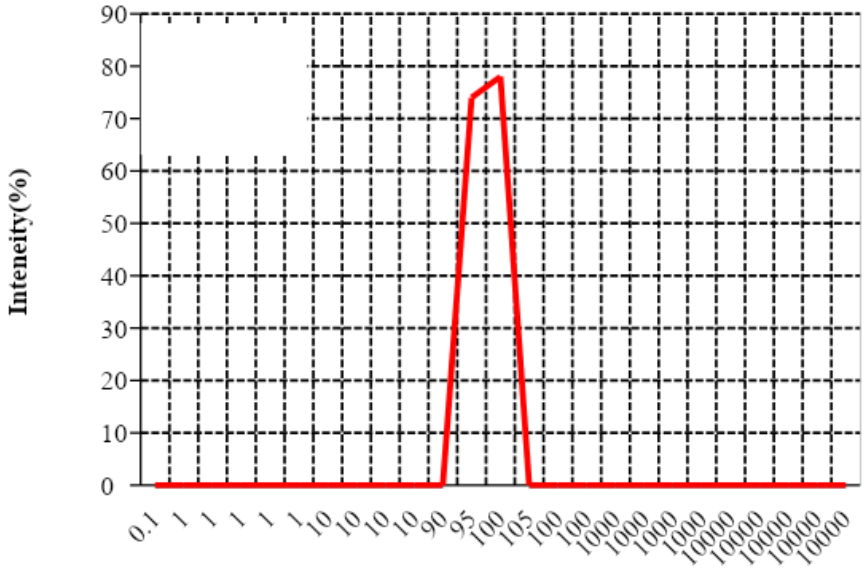

Figure 8. The curve of particle size and the dispersion of nanoparticles.

3-6-2 Zeta potential

Zeta potential is the best indicator to determine the surface electrical state of colloidal systems. Because it shows the amount of accumulation of charge in the immobile layer and the intensity of adsorption of opposite ions to the surface of the particle, and as a result, the amount of electrostatic stability [46]. Reducing the zeta potential difference below the critical value causes the charged double layer around the particles to collapse and aggregate the particles. Particles whose zeta potential is less than +30 mV or more than -30 mV have the most stability [12]. The zeta potential in the present study was equal to -8.42±1.38 mV, according to the results, liposomes containing peptides have a high zeta potential, which indicates the role of electrostatic repulsion force in the stability of nanoliposomes and preventing their aggregation over time. When the total charge on the particles increases, the particles repel each other and overcome the agglomeration. Zeta potential measurement is useful in controlling the aggregation and sedimentation of nanoliposomes, which are important factors in the stability of nanoliposomes [47, 50].

3-6-3 microcoating efficiency

Microencapsulation efficiency is one of the important factors in determining the stability of encapsulated compounds, because it indicates the ability of nanoliposome to prevent the release of the inner core. The efficiency of microcoating in the present study was equal to 68.73±2.37%. Microcoating efficiency is an effective factor on the physicochemical properties of nanoliposome such as oxidative stability, physical stability, morphology and release rate. In general, there is a view that the stability of compounds increases with increasing efficiency, and to achieve optimal conditions, the microcoating efficiency should be increased [46, 47]. These results are almost similar to the results of Hasani et al. (2019). They declared the microencapsulation efficiency of nanoliposomes carrying bioactive peptides obtained from fish waste to be 57.33% [47].

4 - Conclusion

The results related to the characteristics of hydrolyzed protein showed that among Protamax enzyme and bromelain, Protamax enzyme can produce hydrolyzed protein with higher degree of hydrolysis, protein recycling, antioxidant property and functional properties, and also increasing the hydrolysis time had a positive effect on the mentioned characteristics. Also, the current research showed that the nanoliposome was able to load the hydrolyzed protein of the clover bud well and have a suitable particle size, stability and microencapsulation efficiency. Since the microencapsulation of peptides in liposome overcomes proteolytic destruction, which is important in the direct use of nanoliposomes carrying bioactive peptides in food. Therefore, hydrolyzed nano protein can be used as a natural antioxidant to prevent lipids from spoiling.

5- Resources

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مقاله علمی_پژوهشی

نانو ریزپوشانی پپتیدهای زیست‌فعال حاصل از هیدرولیز آنزیمی جوانه شبدر و ارزیابی فعالیت عملکردی و آنتی‌اکسیدانی پپتیدها

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چکیده

هیدرولیز آنزیمی یکی از روش‌های مورد استفاده برای بهبود ویژگی‌های کاربردی و تغذیه‌ای پروتئین‌های غذایی است. با توجه به تأثیری که پروتئین‌های هیدرولیز شده بر خواص عملکردی و سلامت بخشی مواد غذایی دارند، در این پژوهش با استفاده از آنزیم‌های پروتامکس و برومالین پروتئین به دست آمده از جوانه شبدر هیدرولیز و تأثیر این فرایند بر خواص آنتی اکسیدانی (مهارکنندگی رادیکال‌های آزاد DPPH و ABTS) و ویژگی‌های عملکردی (حلالیت، کف کنندگی و امولسیون) آن‌ها بررسی شد. سپس پروتئین هیدرولیز شده توسط نانولیپوزوم ریز پوشانی و ویژگی‌های آن بررسی شد. با توجه به نتایج، مجموع اسیدهای آمینه آبگریز و آروماتیک برای آنزیم پروتامکس و برومالین به ترتیب ۴۰/۴۱ و ۳۷/۹۱، ۱۲/۳۵ و ۱۱/۴۶ بوده است. همچنین از میان آنزیم‌ها، آنزیم پروتامکس توانست پروتئین هیدرولیزی با درجه هیدرولیزاسیون، محتوای پروتئینی، خاصیت آنتی اکسیدانی و عملکردی بالاتری تولید کند و افزایش زمان هیدرولیز تاثیر مثبتی بر روی ویژگی‌های مذکور داشت. بنابراین پروتئین هیدرولیز شده توسط آنزیم پروتامکس و زمان ۶۰ دقیقه توسط نانولیپوزوم ریز پوشانی شد، نتایج مربوط به اندازه ذرات های حامل پپتیدها برابر با ۳/۳۷±۹۳/۶۴ نانومتر، پتانسیل زتا برابر با ۱/۳۸±۸/۴۲- میلی‌ولت و راندمان ریزپوشانی برابر با ۲/۳۷±۶۸/۷۳ درصد بوده است. نتایج تحقیق حاضر نشان داد استفاده از نانو پروتئین، می‌تواند رویکردی مفید در جهت کاربرد مستقیم پپتیدهای جوانه شبدر با قابلیت آنتی اکسیدانی در فراورده‌های غذایی باشد.