Journal of Food Science and Technology (Iran)

Homepage:www.fsct.modares.ir

Scientific Research

Effect of temperature and storage time on bioactivity and physicochemical stability of containing hydrolyzed bee pollen protein nanovesicles

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ABSTRACT

In this study, the effect of temperature and storage time on the biological activity and the physicochemical stability of nanovesicles (specifically, liposomes and niosomes) containing bee pollen hydrolyzed protein obtained from alcalase and pepsin enzymatic hydrolysis was evaluated. The nanoliposomes were coated with 0.2% chitosan. DPPH radical scavenging power, ferric ion reducing power, ACE scavenging power, particle size, particle dispersion index, zeta potential, encapsulation efficiency and release rate of hydrolyzed proteins from nanovesicles during 28 days storage at both refrigerator and ambient temperature were investigated. The results from Dynamic Light Scattering (DLS) revealed s significant increase in the size of nanovesicles upon loading with hydrolyzed protein and coating with chitosan ($P<0.05$). Chitosan coated nanoliposomes had the highest amount of PDI. The zeta potential of nanovesicles reached the highest value by coating with chitosan. Chitosan coated nanoliposomes had the highest encapsulation efficiency. After a 28 day storage period, both coated and uncoated nanovesicles exhibited a substantial increase in size, ranging from 2 to 26 times larger than their initial sizes.Howeever, the encapsulation efficiency of nanonisomes and uncoated nanoliposomes showed the lowest and highest decrease, respectively. The values of the measured factors during storage at the refrigerator were significantly lower than ambient temperature ($P<0.05$). The decline in the antioxidant activities of nanovesicles was significantly prevented by loading hydrolyzed proteins and coating the nanovesicles with chitosan. The ACE inhibition was lower in the nanoliposomes as compared with the nanoniosomes. After 28 days, the ACE inhibition activity of the loaded in nanoliposomes without coating chitosan decreased slightly. These findings are of great importance for designing and developing nutritious foods containing hydrolyzed protein.

ARTICLE INFO

Article History:

Received: 2023/5/1
Accepted: 2023/8/12

Keywords:

Nanovesicles,

hydrolyzed protein,

bee pollen,

biological activity,

stability

DOI :10.22034/FSCT.20.142.31
DOR:20.1001.1.20088787.1402.20.142.3.3

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1- Introduction

Bioactive peptides are healthy, safe, low molecular weight, high activity and easily absorbed compounds. The characteristics of these peptides include the ability to bind to minerals, antimicrobial, antioxidant, anti-blood clotting, reducing blood cholesterol, anti-hypertensive, anti-allergenic and improving bioavailability. and anticancer [1]. Pollen, which is collected by honey bees, is considered as an important source of protein for the production of bioactive peptides, having 10 to 40% of protein [2]. Some bioactive compounds may undergo chemical degradation during transit from the digestive system and under the influence of acidic conditions or existing enzymes, so designing delivery systems with the ability to maintain, improve stability and target release of these compounds is necessary [3]. Liposomes and niosomes are one of the most common delivery systems for microencapsulation, protection and release of bioactive compounds due to the use of food compounds and components in their production formulation [4]. Liposomes, vesicles[1] are spheres in which the phospholipid molecules are organized as sheets and by joining the hydrophobic tails of the phospholipids to each other, they form a bilayer membrane that simultaneously has the ability to carry both types of hydrophilic substances (inside the vesicle) and hydrophobic substances (inside the bilayer membrane) . Niosomes are vesicles of non-ionic surfactants with layered microscopic structures that are prepared from the hydration of non-ionic surfactants with or without cholesterol in an aqueous environment and are important drug delivery systems [4]. Many researches have been done in the field of microencapsulation of bioactive peptides and their stability. Sarabandi et al. (2019), microencapsulated casein hydrolyzed with alcalase and pancreatin using nanoliposome. The average particle size and dispersity index, microencapsulation efficiency and zeta potential of nanoliposomes were in a suitable range. The samples kept at 4 degrees Celsius showed more stability and efficiency of microcoating than the samples kept at 25 degrees Celsius [5]. Hosni et al. (2019), stated that chitosan largely caused physicochemical stability and biological activity, as well as maintaining the maximum efficiency of microencapsulation in nanoliposomes carrying bioactive peptides obtained from carp protein hydrolyzed by alcalase enzyme [6]. Fulmer Correa et al. (2019) stated that the average particle size, antioxidant activity and zeta potential of nanoliposomes loaded with hydrolyzed sheep protein were maintained at their optimal value after 30 days [7].Poole and Yeganeh (2022) reported that the average particle size and dispersion index of nanoliposome particles containing peptides obtained from shrimp waste hydrolysis ranged from 228.9 to 436.7 nm and 0.389 to 0.453 nm, respectively. Microencapsulation efficiency and antioxidant activity were improved by adding chitosan to the surface of nanoliposomes [8]. Yousefi et al. (2021) loaded soybean proteins into liposomal nanocarriers. The microcoating process with the help of ultrasound did not lead to a decrease in the antioxidant activity of the trapped peptides and a decrease in the physicochemical stability of the nanovesicles [9]. Despite the research done on the microencapsulation of bioactive peptides, no other research has been done on the microencapsulation of bee pollen protein, except its microencapsulation with maltodextrin and viprotein and the investigation of its structure and stability [10]. Therefore, in this research, the effect of temperature and storage time on antioxidant and inhibitory propertiesACE And the physicochemical stability of nanovesicles (liposome and nisome) containing hydrolyzed protein of flower pollen with alcalase and pepsin enzymes was investigated.

2- materials and methods

In this research, bee pollen samples were obtained from an apiary located in the protected natural area of Ardabil province under the supervision of the Iranian Animal Science Research Institute. Bee pollen samples were collected by local beekeepers during 30 different days from June to September 2019.

[1]- Vesicles

1,1-diphenyl-2-picrylhydrazyl (DPPH)[2], potassium ferricyanide[3] Trichloroacetic acid[4] (TCA), ferric chloride,[5]HHL and angiotensin converting enzyme[6] (ACE, from rabbit lung) was purchased from Sigma Corporation (St. Louis, MO).

Alcalase enzyme (serine protease from*Bacillus licheniformis)*، Pepsin and pancreatin enzyme, soy lecithin, Tween 80, Span 60, cholesterol and chitosan (with medium molecular weight (190 to 310 kDa) and degree of distillation above 75%) were purchased from Sigma Company. All the mentioned materials and other chemicals used in this research were of laboratory grade purity.

2-1-Production of pollen protein isolate

First, the protein, moisture and ash values of pollen were measured according to the AOAC standard method [42]. To determine the total protein in the primary raw materials, a Keldahl machine (made in Germany, Behr, S3), the amount of ash was used by using an electric furnace (made in Germany, Nabertram, FX118-30), and the moisture content was placed in a 115 degree oven. Celsius was obtained for 24 hours. Based on this, the pollen used contained 5.8% moisture, 21.2% protein and 2.8% ash. Flower pollination was defatted using hexane (with a ratio of 1 to 3) for 24 hours in an orbital shaker according to the method of Maqsoodlou et al. (2018). The degreased powder was mixed with distilled water at a ratio of 1 to 20 and the pH was brought to 10.5 with 1 normal sodium hydroxide at 25 degrees Celsius and mixed for one hour at 25 degrees Celsius. then at 4°C with rev*g*6000 were centrifuged for 20 minutes. The resulting Romand was separated and adjusted to pH 3.4 with 1 normal hydrochloric acid and kept at 25 degrees Celsius for 30 minutes. The resulting suspension at 4 degrees Celsius and*g*6000 was centrifuged for 20 minutes and the sediments were washed with 20 ml of distilled water and dried by a freeze dryer [11].

2-2-Production of hydrolyzed protein of flower pollen

Hydrolysis of pollen protein was performed according to the method of Maqsoodlou et al. (2018) and Maqsoodlou et al. (2019). Based on this, hydrolyzed protein of flower pollen, using 1.5% alkalase and enzymatic hydrolysis time of 4 hours (in 8pH and temperature of 50 degrees Celsius) and 2% pepsin and the duration of enzymatic hydrolysis is 2.5 hours (in 3pH and temperature of 37 °C) was prepared in Shikardar incubator. Finally, the enzymatic reaction was stopped at a temperature of 85 degrees Celsius for 10 minutes. Then in a refrigerated centrifuge with revg 4000, separation was done for 30 minutes, and after collection, the sample was dried with a freeze dryer [11 and 12].

2-2-1- Determining the degree of hydrolysis

The degree of hydrolysis was measured according to the method of Hoyle and Merritt (1994). Hydrolyzed protein suspension of flower pollen and trichloroacetic acid (0.44 M) were mixed in a volume ratio of 1:1 and incubated for 15 minutes at 4 degrees Celsius. Then, the mixture was centrifuged at 10000 rpm for 10 minutes. The amount of protein in Romand containing trichloroacetic acid was determined by the Bradford method. Finally, the degree of hydrolysis was determined using equation 1-2 [43]:

degree of hydrolysis (percentage) = protein (TCA + Romand) / protein (hydrolyzed protein suspension) x 100 (Equation 1-2)

2-2-2- Solubility test

To determine the solubility of hydrolyzed proteins, 200 mg of hydrolyzed proteins were dispersed in 20 ml of distilled water and pH

[2]- 1,1-diphenyl-2-picrylhydrazyl

[3]- Potassium ferricyanide

[4]- Trichloroacetic acid

[5]- N-hippuryl-Lhistidyl-L-leucine hydrate

[6]- Angiotensin-converting enzyme

The mixture was adjusted to 1 to 12 using sodium hydroxide or 1N hydrochloric acid. The solution was centrifuged at 10,000 g for 10 min. Then, the amount of protein in the supernatant solution was determined using the Bradford method and the percentage of solubility was determined based on the amount of dissolved protein on the total protein of the sample [5].

2-3 preparation of nanovesicles

The production of nanoliposomes and nanoniosomes was done using the thin film hydration method according to the method of Sarabandi et al. (2019) and Diskava et al. (2018) with some modifications. In order to produce nanoliposomes, complete dissolution of 0.09 g of lecithin, 0.01 g of cholesterol and 0.02 g of tween 80 in 10 ml of pure ethanol was done for 30 minutes with a magnetic stirrer. In order to produce Nisome, 50 mg of Tween 80 and 50 mg of Span 60 surfactant were mixed and dissolved in 10 ml of pure ethanol. The process of solvent evaporation and thin film formation was done using a rotary evaporator at 60 degrees Celsius and a rotation speed of 70 rpm. Then the balloon was placed in a desiccator for 16 hours to completely remove the solvent. The hydration of the thin film was done with 10 ml of hydrolyzed protein solution. then reducing the size and producing uniform nanovesicles using probe ultrasound (UP200H Hielsher, Germany)It was performed in 10 cycles (1 minute on and 1 minute off) at a frequency of 20 kHz and in an ice bath. Chitosan was used for liposome coating. Chitosan in concentration (0.2%W/V) It was prepared in 0.01 acetic acid solution and slowly and in drops with stirring (300 rpm), it was added to the nanoliposome solution [13, 5]. Finally, the treatments prepared for evaluation in this research were named as follows (Table 1):

Table1. Nanovesicle samples

Abbreviation	The name of the treatment	
N-PBS	Niosomes without hydrolyzed protein	1
N-HP	Niosomes containing protein hydrolyzed by pepsin enzyme	2
N-AH	Niosomes containing protein hydrolyzed by alcalase enzyme	3
L-PBS	Liposome without hydrolyzed protein	4
L-HP	Liposome containing protein hydrolyzed by pepsin enzyme	5
L-AH	Liposome containing protein hydrolyzed with alcalase enzyme	6
CH-L-HP	Liposome coated with 0.2% chitosan containing protein hydrolyzed with pepsin enzyme	7
CH-L-AH	Liposome coated with 0.2% chitosan containing protein hydrolyzed with alcalase enzyme	8

2-4 determination of particle size,PDI and zeta potential

After diluting the sample (with a ratio of 1:100 with distilled water), the average particle size, dispersion index and zeta potential (surface charge at the interface of droplets dispersed in aqueous solution) of produced nanoliposomes and nanoniosomes, using the deviceDLS[7] (Dynamic light scattering) model(Horiba SZ-100 V2.20, UK) was measured Z-average was presented as the hydrodynamic diameter of vesicles. All experiments were performed at a temperature of 25 degrees Celsius [5].

2-5 Determining microcoating efficiency

Micro-covering efficiency was calculated based on the method of Sarabandi et al. (2019). 2 ml of nano microcoated samples to an Amicon filter with a molecular weight of 30 kDa (molecular weight cutoff = 30 kDa, Millipore, UK) was transferred and then centrifuged at 2500 g for 10 minutes. The protein concentration in the solution passed through the filter (free or uncoated peptides) was determined according to the Bradford method. Bovine serum albumin (BSA) was used as the standard protein to draw the standard curve. The equation of the standard line was obtained as follows: (Equation 2-2)

$Y= 0.1216X + 0.4353$ $R^2= 0.97$
(Equation 2-2)

Finally, the efficiency of microencapsulation was calculated through the ratio of the protein concentration loaded inside nanoliposomes and nanoniosomes to the total protein available [5].

2-6 Effect of storage conditions on the characteristics of nanoparticles

2-6-1 The effect of storage at room temperature and 4 degrees Celsius on particle size and microcoating efficiency and release rate

In order to perform this test, according to the method of Sarabandi et al. (2019), 2 ml of each sample was transferred into a glass vial and kept at 4 and 25 degrees Celsius, respectively, at 4 degrees Celsius and the environment for 28 days. Then the particle size and microcoating efficiency were measured based on the methods mentioned above. The release rate of peptides after 1, 3, 7, 14, 21 and 28 days was determined based on the Bradford method and reported as a percentage [5, 14].

2-6-2 The effect of storage on the antioxidant properties of nanoparticles carrying hydrolyzed proteins

In order to determine the antioxidant properties of nanoliposomes and nanosomes carrying hydrolyzed proteins as well as hydrolyzed protein alone, on day 0 and day 28 of storage at 4 degrees Celsius, each sample was placed in a water bath at 100 degrees Celsius for 5 minutes to Complete release occurs. Then the antioxidant property was measured] 14 [.

2-6-2-1 property of free radical inhibitionDPPH

After mixing nanoliposomal and nanonisome samples carrying hydrolyzed proteins and also hydrolyzed protein, with 99.5% ethanol and solutionDPPH Ethanol with a concentration of 0.02%, the mixture was stirred and incubated for 60 minutes in the dark at room temperature. Reducing the amount of radicalsDPPH It was investigated at 517 nm and the rate of inhibition of this radical was expressed as a percentage based on equation 2-3 [12]:

(Equation 2-3) 100x (absorption of control/(absorption of sample-absorption of control)) = DPPH radical inhibition percentage

In a control sample, all steps were performed like the original sample, but distilled water was used instead of the original sample. Also in this test of BHT[8] was used as a positive control.

2-6-2-2 reducing power of iron ion

To measure the power of nanoliposomes and nanoniosomes carrying hydrolyzed proteins and

[7]- Dynamic light scattering

[8]- Butylated hydroxytoluene

hydrolyzed protein in iron reductionIII The method of Maqsoodlou et al. (2019) was used. For this purpose, 1 ml of each sample is mixed with 2.5 ml of 0.2 M phosphate buffer (6.6pH) and 2.5 of 1% potassium ferricyanide was mixed. The mixture was incubated at 50°C for 30 minutes, then 2.5 ml of 10% (weight-volume) trichloroacetic acid solution was added to it. The mixture was centrifuged at 1650 x g for 10 minutes and finally 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of a solution of 0.1% by weight (weight-volume) of iron chloride. . After 10 minutes of reaction, the absorbance of the resulting solution was read at 700 nm. The increase in absorption of the mixture indicated the increase in its regenerating power [12].

2-6-3 Checking the harness featureACE

This test was performed according to the method described by Jamdar et al. (2010) [15]. In this method, 1 ml of the compound N-(3-(2furyl)acryloyl)-L-phenylalanyl-glycyl-glycine[9] (dissolved in 50 µL of bufferTris-HCl ،5/7 pH) with 20 µl of the compoundACE and 200 microliters of nanoliposomal and nanonisome samples containing hydrolyzed proteins and hydrolyzed protein mixed with bufferTris-HCl, were properly mixed with each other and the intensity of absorption reduction at the wavelength of 345 nm for 2 minutes at room temperature was reported. Enzyme inhibition percentageACE, was calculated from the following equation: (Equation 2-4)

(Equation 2-4) 100x (absorption of control per minute / absorption of sample per minute - absorption of control per minute) = inhibition percentage

3- Results and discussion

3-1 degree of hydrolysis and solubility of hydrolyzed pollen protein

The degree of hydrolyzation of flower hydrolyzed protein by alcalase and pepsin was calculated as 56.34% ± 6.5%, 37.14% ± 2.4%, respectively. The higher degree of hydrolysis of protein hydrolyzed with alcalase enzyme compared to pepsin had two main reasons; Higher duration of alcalase enzyme hydrolysis as well as non-specific performance of alcalase enzyme compared to pepsin enzyme in breaking peptide bonds. Increasing the time of the process will prolong the activity of the enzyme and its effect on the substrate. As a result of breaking more peptide bonds, the length of the peptide chain is smaller, their molecular weight distribution decreases and the amount of free amino acids increases [5]. Alcalase, as a serine protease, has amino acids asparagine, histidine and serine in its active site. The non-specific action of alcalase enzyme in attacking amide bonds increases its efficiency in the progress of hydrolysis and the production of peptides with different chain lengths. On the other hand, the active site of pepsin enzyme contains a thiol (SH) group, and pepsin enzyme only breaks the adjacent bonds of amino acids tryptophan, tyrosine and phenylalanine, and narrower peptide chains are produced [2, 11].

As can be seen in Figure 1, the non-hydrolyzed protein of flower pollen showed the greatest drop in solubility at acidic pH (around 3-5). The results indicate the deposition of primary proteins and peptides with high molecular weight at the isoelectric point. But by increasing the pH of the medium to 6, the solubility of protein isolate increased significantly ($P<0.05$). At higher pHs, the solubility remained at the highest level. Mazloumi et al. (2020) and Sarabandi et al. (2019) also to the results

[9]- N-[3-(2-Furyl) acryloyl]- L-phenylalanyl-glycyl-glycine

They obtained a similar result [14 and 5]. The solubility of the hydrolyzates increased significantly at the isoelectric point. The reason for this problem is that, by increasing the hydrolysis process and reducing the molecular weight of peptides and producing amino acids and increasing the charged groups, the sensitivity to pH is reduced and the solubility is maintained in a wide range of pH. Also, enzymatic hydrolysis increases the solubility of proteins by breaking insoluble protein masses, producing smaller peptides, increasing the availability of hydrophilic groups and facilitating the reaction of hydrophilic amino acids with the aqueous medium. [2, 5 and 11].

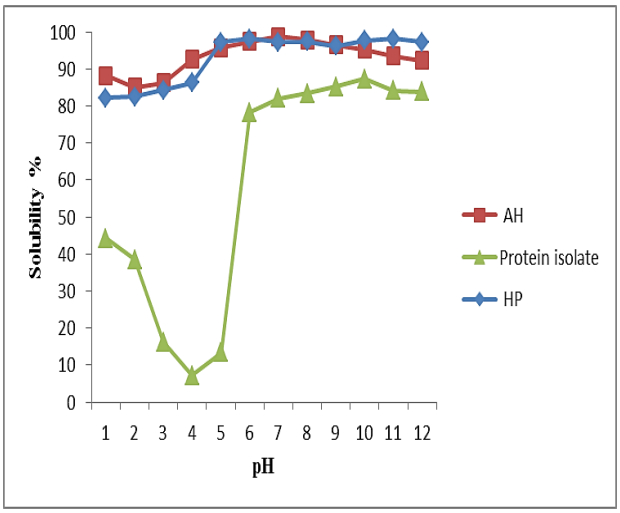

Fig.1. Solubility of protein hydrolyzates in different pH

3-2 determination of particle size,PDI and zeta potential

In this research, the effect of the type of hydrolyzed protein on the average particle size and disintegration index of nanoniosomes and nanoliposomes was investigated (Table 2). The results showed that the size of nanoniosomes increased significantly after loading with hydrolyzed protein and was affected by the type of hydrolyzed protein. In the case of nanoliposomes without chitosan coating, the increase in size was significant when loaded with hydrolyzed proteins ($P< 0.05$). In previous studies, the increase in the particle size of nanocarriers after loading with casein (Sarabandi et al., 2019); a hydrolyzed protein obtained from a type of rainbow fish.[10] (Ramazanzadeh et al., 2017), whey protein (Mohan et al., 2016), a type of protein drug (Barani et al., 2020) has also been reported [5, 16, 17, 18]. On the other hand, the coating of nanoliposomes with chitosan significantly increased the particle size. The level of these changes was affected by the type of hydrolyzed protein. For example, as shown in Table 2, chitosan coating of 2% for nanoliposome containing protein hydrolyzed by alcalase and pepsin significantly increased the particle size from 90.49 and 80.71 nm to 179.79 and 34.3 nm, respectively. increased by 160 nm ($P< 0.05$). The increase in the particle size of nanoliposomes was the result of the presence of the chitosan layer on the lipid monolayer membrane. This result was consistent with the results of Barani et al. (2020) and Mazloumi et al. (2020) [14, 18]. García Monerco et al. (2020) also stated that the use of compounds such as polyethylene glycol and chitosan to coat nanovesicles increased the stability of the system and increased their size [19]. However, in this context, Hosni et al. (2019) and Bang et al. (2011), stated that chitosan-coated peptide nanoliposomes were smaller than uncoated nanoliposomes. They stated the reason for the decrease in the size of nanoliposome particles with the increase in the concentration of chitosan coating is the contraction force caused by ionic attraction between liposome and chitosan components [6, 20]. Nanovesicles carrying peptides obtained from enzymatic hydrolysis with pepsin had smaller particle sizes than nanovesicles carrying peptides obtained from enzymatic hydrolysis with alcalase (Table 2). Due to the difference in hydrolysis conditions as well as the type of amino acids present in the active site of pepsin and alcalase enzymes, the molecular weight distribution in the hydrolyzed ones was different [12]. Based on this, the reason for the smaller size of the nanoparticles loaded with peptides from pepsin can be attributed to the easier placement of peptides with more uniform size in the structure of nanoliposome and nanonisome and the improvement of the order of the vesicle layer compared to larger and higher molecular weight peptides [21].

[10] - *rainbow trout*

On the other hand, Mohan et al. (2016) stated that the size of nanoliposomes has a positive correlation with the free amine group of peptides and the interaction between the functional groups of hydroxyl peptides [17]. Based on this, due to the breaking of more peptide bonds by alcalase than pepsin, there are more amino groups in the peptide solution obtained with alcalase enzyme, which has led to the larger size of nanovesicles loaded with it [22]. In this study, the dispersion index of nanoparticles was also between 0.258-0.389 (Table 1-4). The low value of this index indicates the uniformity and homogeneity of the particle size in the system [23]. The dispersibility index largely depends on the amount of surfactants and their ratio. For example, tween 80 with havingHLB [11] Above, it has a greater affinity for the blue phase; so that the formation of homogeneous vesicles becomes a problem [22]. Therefore, by using Span 60 along with Tween 80 in the present study, a balance was established to a large extent, which was consistent with the results of Tavano et al. (2011) [24]. In addition, Akbari et al. (2022) and Rostam Kalaei et al.HLB adjusts, the properties of the loaded vesicles can be controlled to achieve the optimal value in terms of size, dispersibility index, microencapsulation efficiency and stability [25 and 26]. The dispersion index of nanoniosomes, after loading with hydrolyzed proteins, changed significantly from 0.258 to 0.279 and 0.281 (for N-AH and N-HP, respectively)$P< 0.05$). Similarly, Mohan et al. (2016) showed that nanoparticles loaded with peptides had a higher dispersion index than nanoparticles without hydrolyzed protein [17]. This problem indicates that the nanoparticles were spread more uniformly than the loaded nanoparticles. by coating nanoliposomes loaded with 0.2% chitosan containing hydrolyzed proteins with alcalase; The sufficiency index increased significantly from 0.285 to 0.389 ($P< 0.05$) which was consistent with the results of Sarabandi et al. (2019) [5]. These changes indicate the instability and heterogeneous and non-uniform particle size distribution due to the accumulation and adhesion of particles to each other, after the loading of nanovesicles and also their coating [14]. In the research of Lee et al. (2015), a similar instability was observed in nanoliposomes loaded with hydrolyzed salmon protein after coating with different concentrations of chitosan [27]. However, García Monrica et al. (2020), who used polyethylene glycol to coat nanovesicles, stated that the use of this coating reduced the disintegration index [19].

Determination of zeta potential is a common method for determining the electrostatic properties of liposome and niosomes and is a useful indicator of the surface charge of particles and the colloidal repulsive forces between them and the physical stability of nanocarrier systems. High values of zeta potential by increasing the repulsion reactions and reducing the adhesion of liposome and niosome particles make them more stable [28]. As can be seen in Table 1-4, the zeta potential of nanoniosomes significantly changed from -17.78 mV to -13.8 mV after loading with pepsin hydrolyzed proteins.$P< 0.05$). Previous studies have reported a wide range of zeta potential values for peptide-carrying liposomal and niosomal nanoparticles, with very low values including -5.5 mV (Rosa Zavarez et al., 2014) and high values including -40.8 mV (Masquera et al., 2014) reported [23 and 29]. This wide range of zeta potential can be attributed to differences in the type, composition, and purity of the materials used (especially phospholipids in the case of liposomes), as well as differences in solution conditions (e.g.pH and ionic strength) attributed. It is generally known that the use of non-ionic surfactants in the structure of nanovesicles, due to the dipolar nature of the ethoxy groups in them, causes negative zeta potential [26]. In the present study, the zeta potential value of nanoliposome was -9.07 mV. This issue can be explained as follows: the hydroxyl group in the head of cholesterol forms a hydrogen bond with the choline group in the polar head of phosphatidylcholine, and the choline group with a positive charge is drawn into

[11]- Hydrophilic-lipophilic balance

the membrane, and the phosphatidyl group, which has a negative charge, is pushed to the surface of the membrane. and therefore the negative charge increases and causes electrostatic repulsion of particles [22]. In a similar study, the zeta potential of control and loaded nanoliposomes with hydrolyzed muscle protein and croaker fish by-products was reported as -5.8, -5.5 and -2.2 mV, respectively [23]. Zeta potential of nanoliposomes after protein loadingThe hydrolyzate obtained from alcalase and pepsin enzymes changed from -9.07 mV to -10.11 mV and 4.52 mV, respectively ($P< 0.05$). These results show that the composition of phospholipid and the reaction between phospholipid and intact peptides affect the surface charge of liposomes. Although the zeta potential is influenced by other factors such as particle composition, dispersion medium,pH and the ionic strength should be placed in the solution [22]. After coating nanoliposomes with a concentration of 0.2 percent chitosan, the zeta potential of nanoliposomes containing protein hydrolyzed by alcalase and pepsin enzymes changed from -10.11 mV to 20.21 mV and from 4.52 mV to 24.32 mV, respectively. Found ($P< 0.05$). In this context, Hosni et al. (2019) reported the zeta potential of nanoliposomes carrying peptides obtained from alcalase without chitosan coating and with chitosan coating of 0.5%, respectively -51.7 and +50 mV [6]. Hasibi et al. (2020) stated that the zeta potential of ±30 millivolts stabilizes nanodispersions through electrostatic repulsion. This amount of zeta potential makes it possible to prevent the formation of mass and the integration of particles [22]. However, the values of zeta potential in the present study are far from this value, which is probably related to the presence of Tween 80 in the formulation of nanovesicles. Nanovesicles that use Tween 80 in their formula have a low zeta potential due to the presence of a larger hydrophilic group and the creation of more hydrogen bonds in the functional groups of peptides [30]. The increase in the size of the particles during 28 days of storage, which can be seen in Figure 2-4, indicates the decrease in the stability of nanodispersions and the merging and sticking of particles to each other. In the present study, it was found that the size of liposome nanoparticles was smaller than niosome nanoparticles. Similarly, Rezvani et al. (2019) also announced that after one month of storage of nanoniosomes and nanoniosomes at refrigerator temperature, the size of nanoliposome particles increased less compared to nanoniosomes [31].

Table 2. Mean size, PDI, zeta potential, and microencapsulation efficiency of nanovesicles loaded with hydrolyzed pollen proteins

Encapsulation Efficiency (%)		Zeta-potential(mV)		PDI		Size (Z-average) (nm)		Samples
-----		-17.87 ± 1.27	g	0.258 ± 0.02	c	119 ± 2.14	It is	N-PBS
79.1 ± 1.81	It is	-13.8 ± 0.98	f	0.281 ± 0.01	b	126 ± 3.32	d	N-HP
75.3 ± 1.59	f	-17.1 ± 1.12	g	0.279 ± 0.01	b	187.5 ± 2.98	a	N-AH
-----		-9.07 ± 0.85	d	0.278 ± 0.02	b	73.116 ± 3.11	It is	L-PBS
84.6 ± 1.32	c	4.52 ± 1.19	c	0.271 ± 0.01	b	80.71 ± 4.51	f	L-HP
81.16 ± 1.83	d	-10.11 ± 0.95	It is	0.285 ± 0.03	b	90.49 ± 3.24	f	L-AH
93.08 ± 2.24	a	24.32 ± 1.59	a	0.277 ± 0.01	b	160.34 ± 4.31	c	CH-L-HP
90.02 ± 1.13	b	20.21 ± 1.15	b	0.389 ± 0.02	a	179.79 ± 5.12	b	CH-L-AH

The data are shown as mean ± standard deviation. Different letters in each column show the significant difference at the level of 0.05 in Duncan's tests.

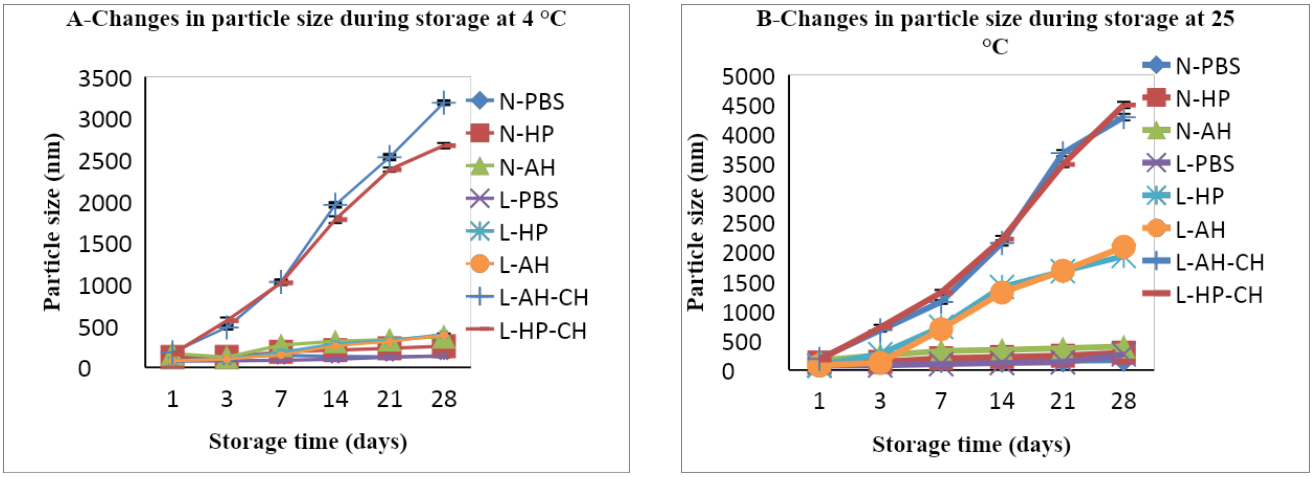

Fig 2. Changes in particle size of nanoliposomes and nanoniosomes during 28 days of storage at A) 4 °C and B) 25 °C

3-3 Determining microcoating efficiency

Microencapsulation efficiency is considered to be the main indicator of the efficiency of the structure of nanocarriers for preserving active compounds. In this research, the effect of the type of hydrolyzed protein and chitosan coating on the microencapsulation efficiency of nanoliposomes and nanoniosomes was investigated (Table 2). The microencapsulation efficiency of nanoniosomes loaded with peptides derived from pepsin (79.1%) was higher than the microencapsulation efficiency of nanoniosomes loaded with peptides derived from alcalase (75.3%).P< 0.05). Similarly, in the research conducted by Hasibi et al. (2020), nanovesicles such as niosomes and liposomes formulated with soy lecithin, Tween 80, Span 60, and cholesterol were used to encapsulate the antioxidant compound. The optimal formulation with the maximum encapsulation efficiency (72-75%) were nanovesicles formulated with lecithin and Tween 80 in the presence and absence of cholesterol [22]. Tawano et al. (2011) also showed that the proper ratio of Tween 80 and Span 60 is necessary for the proper release of antioxidant compounds from niosomes formulated with these compounds [24]. In uncoated nanoliposomes, nanoliposomes containing hydrolyzed protein produced with pepsin enzyme had a higher microencapsulation efficiency (84.6%) than hydrolyzed protein

produced with alcalase enzyme (81.16%).P< 0.05). This difference can be due to the better placement of peptides with smaller and more uniform molecular weight in the structure of nanoliposomes and nanoniosomes [12]. The values of the microencapsulation efficiency obtained from this research were more than the results reported by Weir et al. (2004), who performed the microencapsulation of nisin in the nanoliposome structure by the thin film hydration method and reported a microencapsulation efficiency of about 54% [32]. The efficiency of microencapsulation is related to the acyl chain length of Tween 80 used in the structure of nanovesicles. The hydrophobic end groups of Tween 80 cause the encapsulation of hydrophobic peptides and the polar polyoxyethylene group at the other end of Tween 80 has created hydrogen bonds with the functional groups of the peptides and increased the microcoating efficiency [32]. Other effective factors on the efficiency of liposome encapsulation include the nature of the active substance (lipophilic or hydrophilic); phospholipid nature (in terms of type and arrangement of fatty acids); ratio of phospholipid to active substance; Nanoliposome production method; concentration and type of stabilizer such as cholesterol and environmental conditions such as temperature,pH and ion power pointed out [31]. In addition, the coating of nanoliposomes with 0.2% chitosan led to an increase in the ability of vesicles to retain peptides from alkalase hydrolysis by 90.2% and pepsin by 93.03% (P< 0.05). The increase in microcoating efficiency with the coating of dehinanoparticles using chitosan was also proven in the research work of Hosni et al. (2019) and Barani et al. (2020) [6, 18]. In general, in this study, the microencapsulation efficiency of uncoated nanoliposomes (84.6% for pepsin and 81.16% for alcalase) was higher than nanoniosomes (79.1% for pepsin and 75.3% for alcalase). This result was consistent with the results of Mohammad and Fahmi (2020) and Hasibi et al. (2020) [22 and 34]. Although Bayandir and Tuxel (2010) links betweenHLB and microcoating efficiency suggested that nonionic surfactants with amtHLB The bottom leads to higher micro coverage [35]. However, in the present study, nanoliposomes, whose structure lacked Span60, showed a higher microencapsulation efficiency than niosomes. Probably, the low level of hydration in nanovesicles containing Span 60 leads to a decrease in their microencapsulation efficiency compared to nanovesicles containing Tween 80 [35]. In addition, with the presence of cholesterol, the lipid chains are stiffened and cholesterol prevents the rupture of the liposomal membrane by active compounds and increases the efficiency of microencapsulation [22].

4-3- The effect of storage conditions on the characteristics of nanoparticles

3-4-1-Effect of storage conditions on particle size

In this research, the effect of storage at 4 degrees Celsius and ambient temperature on the physical stability of nanoliposomes and nanoniosomes loaded with hydrolyzed proteins with alcalase and pepsin was investigated. The obtained results showed that the size of nanoniosomes loaded with peptides almost doubled after 28 days of storage at 4 degrees Celsius (Fig.2A). The trend of these changes in storage conditions at 25 degrees Celsius was almost similar to the changes in conditions of 4 degrees Celsius and there was no significant difference (Fig.2B). The average particle size of nanoliposomes loaded with pepsin and alcalase peptides increased by 2 and 5 times, respectively, after 28 days of storage at 4 degrees Celsius. But as in fig2B It can be seen, the size of hydrolyzed protein-free nanoliposomes loaded with peptides derived from pepsin and alcalase, after 28 days of storage at 25 degrees Celsius, increased to 3.5, 24.65, and 26, respectively, which is consistent with the results of Rezvani et al. (2019) and Sarabandi et al. (2019) were consistent [5 and 31]. The reasons for these results can be attributed to the effect of loaded peptides on the reduction of zeta potential, neutralization of surface charge and instability of particles. The smaller size and possibly higher surface energy of liposome nanoparticles and their greater tendency to reduce excess energy through aggregation can be the reason for the larger increase in the size of nanoliposomes compared to nanoniosomes [36]. The average size of nanoliposomes coated with chitosan, which

contained peptides hydrolyzed with alcalase and pepsin, increased to 17.5 and 16.5, respectively, after 28 days of storage at 4 degrees Celsius. As in Fig2BIt is known that after 28 days of storage at 25 degrees Celsius, the size of nanoliposomes coated with chitosan containing pepsin and alcalase peptides increased by 22.5 and 26.37, respectively. By comparing the slope changes of uncoated nanoliposomes and with 0.2% chitosan coating in Fig2AAnd2B It can be seen that the intensity of size increase for nanoliposomes coated with chitosan was less than that of uncoated nanoliposomes. This difference can be attributed to the protective effect of chitosan coating due to the increase in membrane thickness, increase in zeta potential, and creation of electrostatic repulsion between adjacent particles [27]. In the current study, the size of nanonisome particles was not affected by the storage temperature, but the size of nanoliposome particles was strongly affected by the storage temperature of 25 degrees Celsius. The stability of nanovesicles depends on the fluidity of the lipid membrane and temperature. At a temperature of 25 degrees Celsius, due to the high fluidity of the membrane, liposomes agglomerate and form a clot, which will result in an increase in the size of the particles, a wide and heterogeneous particle size distribution [37]. Among the samples produced in this research, the smallest change in particle size and the highest stability was related to the control nanoliposome and nanonisome samples (not loaded with active ingredient). These results show the effect of the active substance and its destructive reaction in the system, which leads to the instability of liposome and niosome structure and extensive changes in particle size, especially at high temperature. Cholesterol in optimal amounts prevents the mixing process by increasing the stiffness of the membrane and increasing the zeta potential and as a result increasing the electrostatic repulsion between the particles and increases the stability of the particle size [22].

3-4-2-Effect of storage conditions on release speed and microcoating efficiency

The efficiency of microencapsulation and release of nanoniosomes and nanoliposomes (with/without chitosan coating) containing peptides obtained from pepsin enzyme and peptides obtained from alkalase decreased and increased, respectively, after 28 days of storage at 4 degrees Celsius (Fig.3A and 4A). This decrease and increase in the efficiency of microcoating and the release rate were more significant in storage conditions at 25 degrees Celsius (Fig.3BAnd4B). The trend of changes in the release rate of nanoliposomes under temperature storage conditions in nanoliposomes loaded with peptides derived from alcalase (43.73% increase) was higher than nanoliposomes loaded with peptides derived from pepsin (30.19% increase) (Fig.4B).

In the research of Sarabandi et al. (2019), the effect of high temperature on increasing the release rate of peptides was reported [5]. More stability and less release of nanoliposomes at low temperature can be attributed to lower permeability of the membrane at low temperature, reduction of agglomeration as a result of lower molecular mobility.[12] and attributed the delay in the oxidative process of unsaturated fatty acids in phospholipids [38]. In the present study, the release rate (at 25°C and 4°C) in nanoniosomes and nanoliposomes loaded with pepsin-derived peptides was higher than alcalase-derived peptides. With the better loading of peptides with lower molecular weight and more uniform size from pepsin in the nanoliposome and nanonisome structure, compared to larger peptides with higher molecular weight, the order of the vesicle monolayer is probably improved and leads to an increase in the strength and stability of the nanonisome and nanoliposome structure. became [12]. In the present study, with the passage of time, the rate of release in nanoliposomes has occurred more than in nanoniosomes, which is consistent with the report of Rezvani et al. (2019) [31]. Due to the smaller size of liposomes, probably the reduction of the vesicle size has led to a significant increase in the curvature of the membrane and as a result, a weaker closure of the components in the vesicle layers [39]. In general, the greater stability and less release of liposomal and niosome nanoparticles at low temperature can be attributed to the lower permeability of the membrane at low temperature, the reduction of agglomeration as a result of lower molecular mobility.[13] and attributed the delay in the oxidative process of unsaturated fatty acids in phospholipids [38]. By comparing the slope of the graph of changes in microcoating efficiency and release rate Nanoliposomes without coating and coated with 0.2% chitosan in Figure 3 and 4, it can be seen that the intensity of microcoating efficiency reduction for nanoliposomes coated with chitosan was less than that of uncoated nanoliposomes. The reason for that was the protective effect of the chitosan coating through increasing the thickness of the membrane and preventing the leakage of microcoated materials [27]. In this study, the smaller initial size of nanoliposomes produced with these peptides compared to the initial size of nanoniosomes

[12]- Low molecular mobility

[13]- Low molecular mobility

probably caused the release rate in nanoliposomes to be higher than in nanoniosomes, which is consistent with the results of Razvani et al. (2019) [31].

3-4-3 The effect of storage on the antioxidant properties of nanoparticles carrying hydrolyzed proteins

In this research, the effect of loading hydrolyzed proteins from alcalase and pepsin in nanoniosomes, nanoliposomes and chitosan coating (0.2 wt/volume) on their antioxidant properties was investigated (Figures 5 and 6). Based on the findings, the antioxidant activity of the hydrolyzed protein was influenced by the type of enzyme. Radical scavenging activityDPPH And the reducing power of iron ion in the hydrolyzates obtained from pepsin was higher than the hydrolyzates obtained from alkalase ($P< 0.05$). By carrying out the hydrolysis process by pepsin and alcalase enzymes and breaking most of the peptide chains, the production of peptides capable of donating electrons to reduce trivalent to bivalent iron ions increases [2]. The presence of hydrophobic free amino acids and peptides containing aromatic amino acids and electron donation to free radicals causes them to become stable [11]. The difference in the amount of antioxidant activity of pepsin and alcalase hydrolysates after being loaded into nanoniosomes was also evident. However, the antioxidant activity was slightly reduced after being loaded into nisomes and liposomes, which was probably due to the lower concentration of hydrolyzates in the measured nanovesicles solution compared to the pure hydrolyzates. However, Hasibi et al. (2020) reported that the antioxidant activity of phenolic compounds loaded in nanoliposomal and nanosomal structures increased compared to pure phenolic compounds, and the reason for this was the increased solubility of those compounds after microencapsulation [22]. However, chitosan coating significantly improved the antioxidant activity in nanoliposomes ($P< 0.05$). So that in nanoliposomes coated with chitosan containing proteins hydrolyzed by alcalase and pepsin, more than 91 and 88% of radical inhibition activity, respectively.DPPH And 93 and 96% of the reductive power of iron ion was preserved, which was consistent with the results of Sarabandi et al. (2019) and Ramzan Zadah et al. (2017) [5, 16]. In this context, Hosni et al. (2019) announced that the higher antioxidant effect in nanoparticles coated with chitosan was due to the antioxidant effect of chitosan [6]. Free radical scavenging activityDPPH In the present study, it was higher than the values obtained for nanoliposomes loaded with casein peptides (about 37-48%) by Sarabandi et al. (2019) [5]. As shown in Figures 5 and 6, the antioxidant power of hydrolyzed proteins decreased significantly after 28 days of storage, but this decrease was significantly lower in the samples loaded in nanonisomes and nanoliposomes.$P< 0.05$). In addition, in nanoliposomes coated with chitosan, 0.2% of radical scavenging activityDPPH There was no significant difference on the 28th day compared to the first day. Therefore, chitosan coating significantly improved the preservation of the bioactive compounds loaded in the nanoliposome and their antioxidant activity ($P< 0.05$) which was consistent with the results of Sarabandi et al. (2019), Mazloumi et al. (2019), Hasibi et al. (2020) [5, 14 and 22].

3-5 Checking the harness featureACE Nanoliposomes and nanoniosomes

In this research, the effect of loading hydrolyzed proteins from alcalase and pepsin in nanoniosomes, nanoliposomes and chitosan coating (0.2 wt/volume) on the inhibitory properties.ACE They and the changes of this characteristic were examined after twenty-eight days of storage (Figure 7). in containment activityACE Pepsin hydrolyzates (91.49%) and alkalase hydrolyzates (87.07%) showed a significant difference.$P<0.05$) which was due to the difference in hydrophobic-hydrophilic characteristics of peptides, molecular weight and amino acid sequence in the resulting peptides.

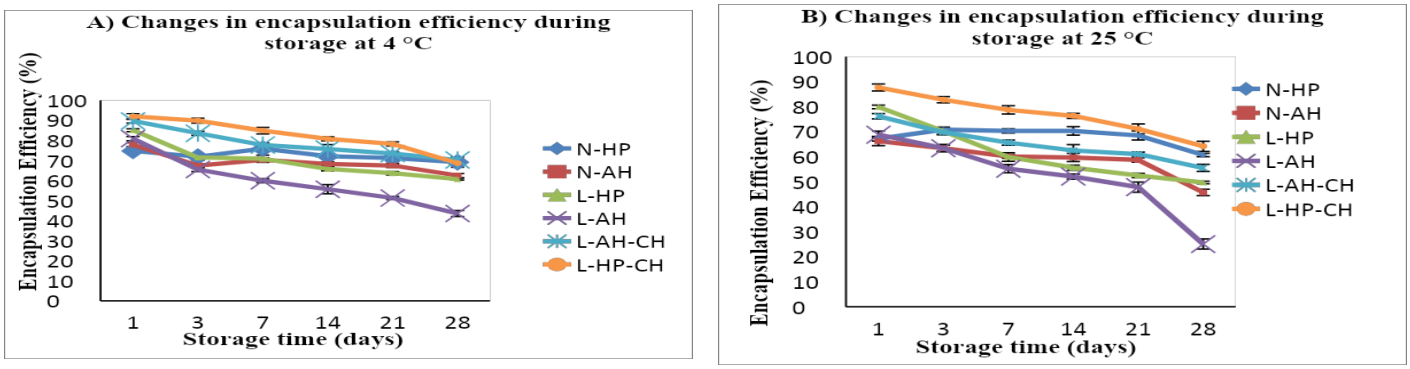

Fig. 3. Changes in Encapsulation Efficiency (%) of nanoliposomes and nanoniosomes during 28 days of storage at A) 4 °C and B) 25 °C

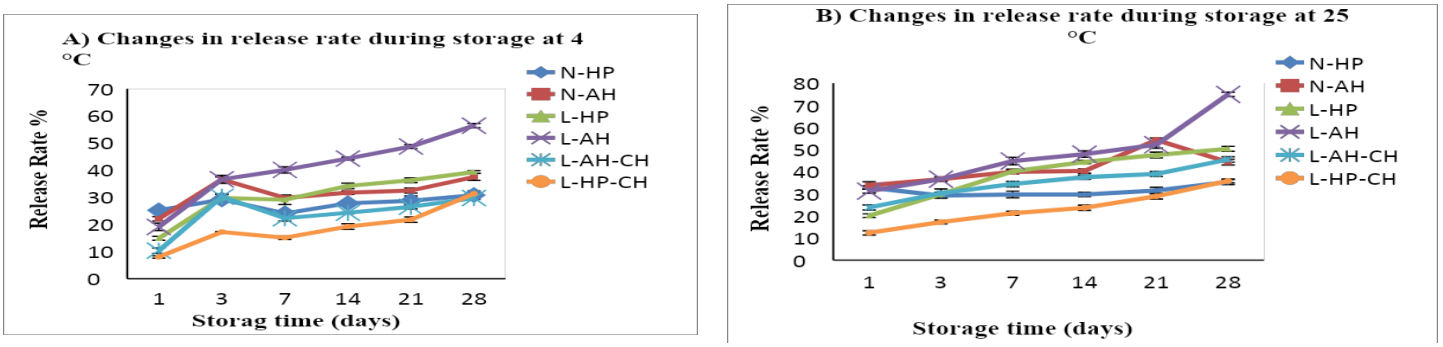

Fig. 4. Changes in release rate (%) of nanoliposomes and nanoniosomes during 28 days of storage at A) 4 °C and B) 25 °C.

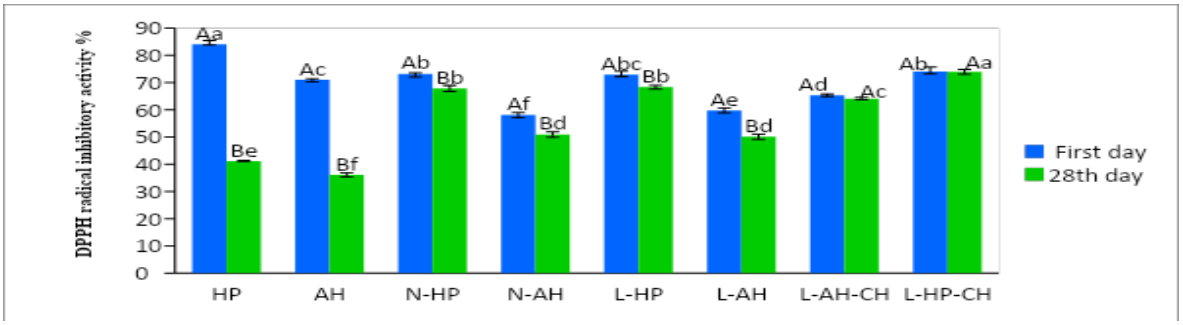

Fig. 5. DPPH radical scavenging activity of nanovesicles on the first day and the 28th

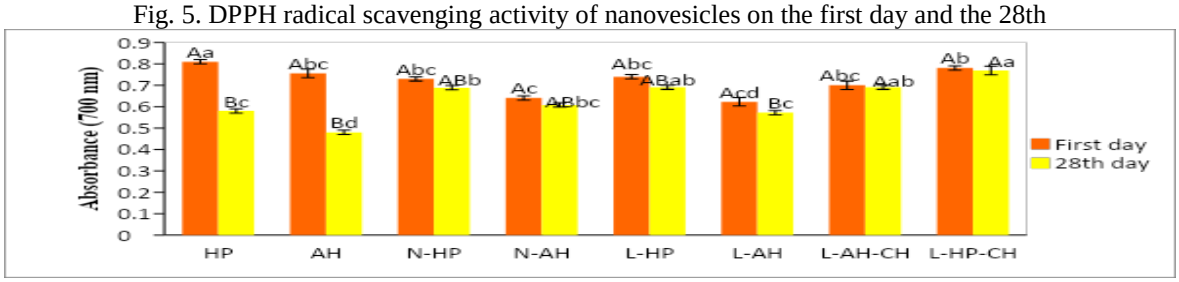

Fig. 6. Reducing power of nanovesicles on the first day and the 28th day (similar lowercase and uppercase letters respectively indicate the absence of significant differences between the treatments and in each treatment on the first day and the 28th day of storage

According to the researches of Maqsoodlou et al. (2018) and Maqsoodlou et al. (2019), glycine and proline were the two main hydrophobic amino acids present in the peptides identified in alcalase hydrolysates, and glycine and alanine were the two main hydrophobic amino acids present in the peptides identified in pepsin hydrolysates. inhibitory activity agentACE They were [11 and 12]. After loading hydrolyzates in nanovesicles, inhibition activityACE significantly decreased ($P<0.05$) which was consistent with the results of Fulmer Correa et al. (2019) [7]. However, Aguilar Toala et al. (2022) in a research work stated that peptides derived from a type of collagen, after micro-encapsulation in the nanoliposome structure, inhibit activity.ACE kept 100% [41]. The amount of inhibition activityACE It was significantly lower in nanoliposomes than in nanoniosomes ($P<0.05$) which was consistent with the results of Rezvani et al. (2019) [31]. Comparing the results of the biological activities of loaded nanovesicles and the results ofDLS showed that proper particle size distribution alone cannot lead to proper chemical stability of nanovesicles. In this context, different compositions of vesicles play an important role. Unsaturated fatty acids in the liposome structure led to more penetration of the liposome membrane than niosomes, which resulted in less liposome biological activity. By coating nanoliposomes with 0.2% chitosan, there is a significant decrease in inhibition activityACE was not observed. In fact, these results indicated that under the effect of the chitosan protective coating, the peptides were completely microcoated, without reducing their biological activity, which was in line with the results of Hosni et al. (2019) [6]. After 28 days, inhibition activityACE Alcalase and pepsin hydrolyzates decreased significantly ($P<0.05$) (Figure 7). During this time in the activity of containmentACE Alcalase and pepsin hydrolyzates loaded in 0.2% chitosan-coated nanoliposomes were observed to decrease by 13% and 15%, respectively, which was in line with the results of Rezvani et al. (2019) [31]. Probably the gradual release of peptides containing hydrophobic amino acids that are responsible for inhibitionACE among the fatty acids in the structure of nanoliposomes, this activity has been reduced in nanoliposomes. In addition, chitosan coating significantly preserves bioactive compounds loaded in nanoliposomes and inhibits activityACE It was found in them that it was consistent with the results of Hasibi et al. (2020) [22].

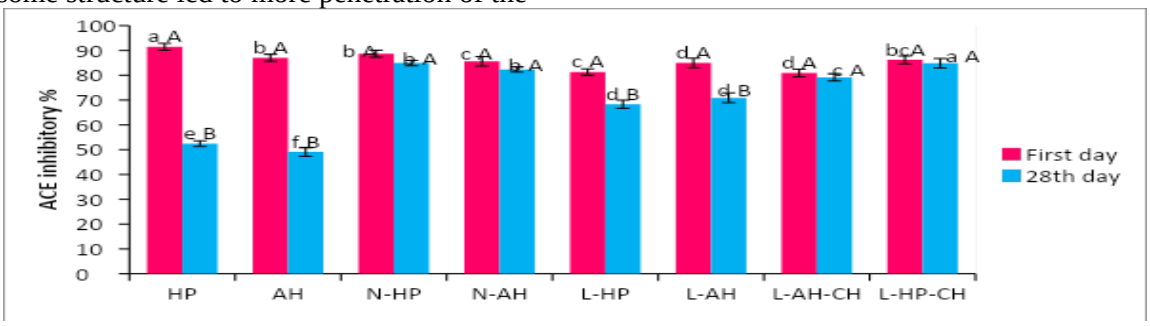

Fig. 7. ACE inhibitory activity of nanovesicles on the first day and the 28th day (similar lowercase and uppercase letters respectively indicate the absence of significant differences between the treatments and in each treatment on the first day and the 28th day of storage)

4 - Conclusion

In this research, the possibility of producing two different types of nanovesicles as an enrichment compound containing hydrolyzed pollen protein was investigated. The results showed that the size, zeta potential, particle size distribution and microencapsulation efficiency of nanovesicles increased significantly by coating with chitosan. After 28 days, the size of nanovesicles increased. The microcoating efficiency of uncoated nanonisomes and nanoliposomes showed the lowest and highest decrease, respectively. Nanovesicles showed more stability during storage at 4°C. By loading hydrolyzed proteins in nanovesicles and coating them with chitosan, reducing the antioxidant activity and inhibitingACE They were prevented during the 28 days of storage. Therefore, it was found that both of these nanosystems can be used as an enrichment compound in food systems in terms of physicochemical stability and preservation of biological activity, but liposomal nanosystems containing chitosan coating worked more efficiently. Of course, nanonisome systems were also competitive with it and placed in second place. Since the use of dangerous and non-food solvents (such as chloroform) in the preparation of nanocarriers prevents their use in the food industry, the production methods presented in this research can be a promising strategy in food processes to improve and enhance the health properties of food, maintain stability in To suggest the duration of storage and bioavailability of food products containing hydrolyzed protein of flower pollen.

5- Thanksgiving

I hereby express my gratitude to the fund for supporting the country's researchers.Iran National Science Foundation: INSF) who provided financial support for the project with code 98024390, we announce.

6- Resources

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مجله علوم و صنایع غذایی ایران

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مقاله علمی_پژوهشی

اثر دما و زمان نگهداری بر فعالیت زیستی و پایداری فیزیکوشیمیایی نانووزیکول‌های حاوی پروتئین هیدرولیز شده‌ی گرده زنبور عسل

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اطلاعات مقاله

تاریخ های مقاله :

تاریخ دریافت: ۱۴۰۲/۲/۱۱

تاریخ پذیرش: ۱۴۰۲/۵/۲۱

کلمات کلیدی:

نانووزیکول‌ها،

پروتئین هیدرولیز شده،

گرده زنبور عسل،

فعالیت زیستی،

پایداری

DOI: 10.22034/FSCT.20.142. 31

DOR:20.1001.1.20088787.1402.20.142.3.3

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چکیده

در این پژوهش، اثر دما و زمان نگهداری بر فعالیت زیستی و پایداری فیزیکوشیمیایی نانووزیکول‌های (لیپوزوم‌ها و نیوزوم‌ها) حاوی پروتئین هیدرولیز شده‌ی گرده زنبور عسل حاصل از آنزیم آلکالاز و پپسین ارزیابی شد. از کیتوزان ۰/۲ درصد برای پوشش‌دهی نانولیپوزوم‌ها استفاده گردید. قدرت مهار رادیکالDPPH، قدرت احیاکنندگی یون آهن ، قدرت مهار آنزین مبدل آنژیوتنسین (ACE) ، اندازه ذرات، شاخص بس‌پاشیدگی، پتانسیل زتا، بازده ریزپوشانی و میزان رهایش پروتئین‌های هیدرولیز شده از نانووزیکول‌ها در مدت ۲۸ روز نگهداری در دمای ۴ و ۲۵ درجه سانتی‌گراد بررسی‌شد. نتایج DLS نشان داد اندازه نانووزیکول‌ها با بارگیری با پروتئین هیدرولیزشده و پوشش‌دهی با کیتوزان افزایش معنی‌دار یافت ($P<0.05$). نانولیپوزوم‌های حاوی پوشش‌کیتوزان بیشترین‌مقدار PDI (۰/۳۸۹) را داشتند. پتانسیل‌زتای نانووزیکول‌ها با پوشش‌دهی با کیتوزان، به بیشترین مقدار (۲۴/۳۲میلی‌ولت) رسید. نانولیپوزوم‌های حاوی پوشش‌کیتوزان بیشترین بازده ریزپوشانی (۹۳/۰۸ درصد) را داشتند. بعد از ۲۸ روز اندازه نانووزیکول‌های با پوشش و بدون پوشش، ۲ تا ۲۶ برابر افزایش یافتند. میزان بازده‌ریزپوشانی نانونیوزوم‌ها و نانولیپوزوم‌های بدون پوشش به ترتیب کمترین و بیشترین کاهش را نشان دادند ($P<0.05$). مقادیر فاکتورهای اندازه‌گیری شده در طول نگهداری در دمای ۴ درجه سانتیگراد، به طور معنی-داری کمتر از دمای ۲۵ درجه سانتیگراد بود ($P<0.05$). با بارگذاری پروتئین‌های هیدرولیزشده در نانووزیکول‌ها و پوشش-دهی‌آنها با کیتوزان از کاهش فعالیت ضداکسایشی‌شان در طول مدت ۲۸ روز نگهداری جلوگیری شد. میزان فعالیت مهارACE در نانولیپوزوم‌ها به‌طور معنی‌داری کمتر از نانونیوزوم‌ها بود ($P<0.05$). بعد از گذشت ۲۸ روز در فعالیت مهار ACE هیدرولیزشده‌های بارگذاری شده در نانولیپوزوم‌های بدون پوشش کیتوزان کاهش جزیی مشاهده گردید. این یافته‌ها برای طراحی و توسعه غذاهای سلامتی‌بخش حاوی پروتئین هیدرولیز شده از اهمیت بالایی برخوردار است.