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Effect of temperature and shear stress on properties of nanovesicles carrying hydrolyzed pollen protein

Atefe Maqsoudlou [a,*], Alireza Sadeghi Mahoonak [a], Hossein Mohebodini [b]

[a] Department of Food Science and Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[b] Department of Animal Sciences, University of Mohaghegh Ardabili, Ardabil, Iran

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ABSTRACT

In this study, the effect of storage (65 °C, 24 h) and shear rates (2-100 s^{-1}, 10 min) on the particle size, polydispersity index (PDI), encapsulation efficiency (EE), Angiotensin-converting enzyme (ACE) and DPPH radical inhibition, iron ion reduction ability and flow behavior of nanovesicles containing hydrolyzed bee pollen (produced by alcalase and pepsin) were evaluated. Nanoliposomes were coated by chitosan (0.2 % w/w). After storage at 65 °C, the size of the nanovesicles raised 2–2.5 folds while EE showed 5–8 % decline. ACE and DPPH radical inhibition activity were also decreased. After applying the shear stress, PDI was increased significantly ($P < 0.05$), while EE of the nanoniosomes showed a drastic decline ($P < 0.05$). Nanoliposomes and nanoniosomes exhibited decline in the ACE inhibitory and antioxidant activity. At low shear rates Newtonian-like and weak pseudo-plastic behavior of the chitosan-coated nanoliposomes were observed. The flow index (n), (k) and (R^2) indicated the suitability of power law model for describing the flow characteristics.

1. Introduction

Bioactive peptides are healthy and safe compounds with low molecular weight. These peptides enjoy the capability of attaching to minerals and antimicrobial, antioxidative, anti-coagulation, blood cholesterol-decreasing, anti-hypertension, anti-allergic, improved bioavailability, and anti-cancer features (Sarmadi and Ismail, 2010).

Pollen composition include proteins, lipids, carbohydrates, vitamins and minerals, and have been described to contain important amounts of bioactive compounds, being considered as a functional and/or nutraceutical food (Almeida et al., 2016; Fadiran et al., 2018). The pollen collected by honey bees contains 10–40 % protein and can be considered a significant source of protein for the production of bioactive peptides (Arruda et al., 2013).

Maqsoudlou et al. (2018) and Maqsoudlou et al. (2019) indicated that hydrolysis of pollen using alcalase, pepsin and trypsin in the optimum time and enzyme concentration resulted in generation of peptides possessing different sizes and potential bioactivities. The analysis of hydrolyzed protein by size exclusion chromatography (SEC) and then Reversed-phase high-performance liquid chromatography (RP-HPLC) showed that the hydrolyzed proteins contain fractions with high antioxidant and ACE-inhibitory activities. The results of peptide sequencing showed a number of 6–21 amino acids in length. Their research has demonstrated that hydrolyzed pollen protein can be used as functional ingredient in food formulations.

Some bioactive compounds may experience chemical degradation during the transfer through the digestive system under the influence of acidic conditions or enzymes. Therefore, the design of a delivery system capable of preserving, improving the stability and target release of these compounds is of crucial necessity (Liu et al., 2015; Maqsoudlou et al., 2020).

Nanotechnology is one of the modern sciences that deals with the synthesis of nanoparticles and has a tremendous impact on developing science. The significance of nanotechnology is in the fields such as

Abbreviations: PDI, polydispersity index; EE, encapsulation efficiency; ACEI, Angiotensin-converting enzyme inhibitory; DLS, dynamic light scattering; DPPH, 1,1-diphenyl-2-picrylhydrazyl; TCA, trichloroacetic acid; HHL, N-hippuryl-Lhistidyl-L-leucine hydrate; BHT, Butylated hydroxytoluene; ANOVA, analysis of variance; N-AH, nanoniosomes containing alcalase-hydrolyzed protein; N-PBS, nanoniosomes containing PBS; N-HP, nanoniosomes containing pepsin-hydrolyzed protein; L-PBS, nanoliposome containing PBS; L-AH, nanoliposome containing alcalase-hydrolyzed protein; L-HP, nanoliposome containing pepsin-hydrolyzed protein; CH-L-AH, chitosan-coated nanoliposome containing alcalase-hydrolyzed protein; CH-L-HP, chitosan-coated nanoliposome containing pepsin-hydrolyzed protein; Nvs-AH, nanovesicles containing alcalase-hydrolyzed proteins; Nvs-HP, nanovesicles containing pepsin-hydrolyzed peptides; HLB, hydrophilic lipophilic balance.

* Corresponding author.
E-mail address: atefe.maqsoudlou@gmail.com (A. Maqsoudlou).

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bioengineering applications, drug delivery, biosensors, target release of bioactive compounds and agriculture sector. Due to the small size of nano particles, it gives more developed characteristics to the materials synthesized by nanoparticles compared to the macro size particles (Ringu et al., 2022). Nanoliposomes and nanoniosomes are among the most common delivery systems for encapsulation, protection, and release of bioactive compounds due to the use of food compounds and components in their production formulation (Mozafari et al., 2008). Liposomes are spherical vesicles in which phospholipid molecules are organized in regular planes. Upon joining the hydrophobic tails of phospholipids to each other, a double-layered membrane is formed which can simultaneously carry both hydrophilic (inside the vesicle) and hydrophobic (inside the bilayer membrane) substances. As one of the important drug delivery systems, niosomes are vesicles of non-ionic surfactants with layered microscopic structures formed by the hydration of non-ionic surfactants with or without cholesterol in an aqueous environment (Mozafari et al., 2008).

The permeability of a lipid layer is a function of its phase state with nonlinear dependence on thermodynamic variables such as temperature, pressure, or pH. Moreover, shear forces affect the phase state of vesicles and their membrane permeability.

Exposure of nanovesicles to shear force alters the melting temperature of the membrane lipid structures (Färber et al., 2022). Therefore, the shear force-induced changes in the melting temperature should be considered when designing bioactive compound-carrying liposomes. Färber et al. (2022) determined the permeability of vesicles composed of different phospholipid species under shear flow at different temperatures. They also measured the variations of the phase state of these vesicles under shear forces in the membrane. They found that the permeability of the membrane increased with the transformation of the gel phase to the liquid, and the maximum permeability of the membrane was observed near the main phase transition temperature. Saroglu et al. (2022) investigated the textural and rheological properties of liposome coated with xanthan gum and salep using steady shear and temperature sweep test. All the coated liposome samples showed non-Newtonian characteristics. By coating the liposome with xanthan gum and salep, the shear resistance of the liposome membrane and its temperature resistance were increased while the encapsulation efficiency was significantly maintained after applying temperature and shear stress.

As high temperature and shear forces affect the phase state of vesicles and the permeability of their membrane, it is necessary to assess the effect of different processes on the physicochemical characteristics and encapsulation efficiency of nanovesicles before their application in drug formulation, or for the enrichment of food and drink. This research, thus, examines the effect of temperature and shear stress on particles size, encapsulation efficiency, and rheological, antioxidant, and ACE inhibitory properties of nanovesicles (niosome, chitosan-coated liposome and uncoated liposome) containing hydrolyzed pollen protein with alcalase and pepsin enzymes.

2. Materials and methods

2.1. Materials, chemicals and reagents

In this study, honey and bee pollen samples were obtained from an apiary located in naturally preserved area of Ardabil Provence, under the supervision of Animal Science Research Institute (ASRI) of Iran. Bee pollen and honey samples were collected during 30 different days by local beekeepers from April until June in 2020.1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, N-hippuryl-Lhistidyl-L-leucine hydrate (HHL) and angiotensin-converting enzyme (ACE, from rabbit lung) have been purchased from Sigma (St. Louis, MO). Alcalase® 2.4L serine-protease from *Bacillus licheniformis*, Pepsin, Lecithin, Span60, Tween80 and Cholesterol, Chitosan (Medium Molecular Weight (190–310 kDa), degree of deacetylation above 75 %) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade. All chemical were ≤99 % pure and used as received.

2.2. Preparation of pollen protein isolates

Pollen and hexane were mixed at ratio of 1: 3 w/v. Defatting was carried out for 3 h in an orbital shaker. After removing the hexane in the vacuum oven, defatted powder was mixed with distilled water at the ratio of 1:20 w/v. In order to achieve maximum solubility of proteins, the *pH of mixture was increased to* 10.5 using NaOH. The mixture was blended at room temperature for 1 h. The resulting mixture was then centrifuged at the rate of 6000 g for 20 min. The supernatant (contain soluble proteins) was separated; in order to achieve protein precipitation, its pH was set to isoelectric point (3.4) using HCl (1 mol/L). It was then left at room temperature for 30 min. The distilled water was added to precipitate, and subject to centrifugation (4383×*g*, 10 min). The resulting supernatant was discarded, and the precipitate was retained for repeated washing using the same procedure at least 5 times, until the precipitate exhibited a gel-like texture. The desalted precipitate was redispersed in distilled water (*w/w* = 1:30), and the *pH was adjusted to* 7.0 using NaOH (1 mol/L) and freeze-dried to obtain the powder of pollen protein isolates (Maqsoudlou et al., 2018).

2.3. Production of hydrolyzed pollen protein

According to our previous works, the hydrolyzed pollen protein was prepared by enzymic hydrolysis using alcalase (1.5 % w/w to the substrate) for 4 h (*pH* 8.0, 50 °*C*) and pepsin (2 % w/w to the substrate) for 2.2 h of hydrolysis (*pH* 3.0, 37 °*C*) in a shaker incubator, which were called AH and HP respectively. The enzymatic reaction was finally stopped at 85 °*C* for 10 min. After centrifugation at 4000*g* for 39 min, supernatants were collected and freeze dried (Maqsoudlou et al., 2018, 2019).

2.4. Preparation of nanovesicles

The production of nanoliposomes and nanoniosomes was based on thin film hydration as reported by Sarabandi et al. (2019) and Diskaeva et al. (2018) with minor modifications. For the production of nanoliposomes, lecithin (0.09 g), cholesterol (0.01g), and Tween 80 (0.02 g) were dissolved in 10 mL of absolute ethanol for 30 min. The preparation of niosomes involved the dissolution of Tween80 (0.05 g) and Span60 surfactant (0.05 g) in 10 mL of ethanol. The resulting solutions were transferred to a round-bottom flask and the solvent evaporation process and the formation of a thin layer were performed using a rotary evaporator (Laborota 4002, Heidolph, Germany). For hydration of the thin layer and for the formation of the liposomes, 10 mL of the hydrolyzed protein solution (0.01 g/mL) was added to the film and vortexed. This process was repeated in 3 cycles. The particle size reduction was performed in 10 cycles (1 min on and 1 min off) using an ultrasound probe (UP200H, Hielsher, Germany) at the frequency of 20 kHz. Coating of the nanoliposomes was performed based on the method of Mazloomi et al. (2020) with some modifications. Chitosan was dissolved in 1 % acetic acid (v/v) at 0.2 % w/w for 16 h. Then a defined volume of chitosan solution to the same volume of liposome suspension was added gently (drop-wise) to the nanoliposome solution during stirring (300 rpm). All of the nanovesicles were kept at 4 °C until further tests.

2.5. Determination of size, PDI, and zeta potential

After sample dilution (1:100 using distilled water), the mean particle size, PDI, zeta potential of the nanovesicles were measured using DLS system (Horiba SZ-100 V2.20, UK) at 25 °C and 90° angle (Mazloomi et al., 2020).

2.6. Determination of EE

Measurment of EE was done as reported by Sarabandi et al. (2019). To this end, 2 mL from each of the nanovesicles was transferred to an Amicon ultra centrifugal filter (molecular weight cutoff = 30 kDa, Millipore, UK) and centrifuged (Universal 320 centrifuge, Hettich, Germany) for 10 min at 3000×*g* in order to determine the amount of non-encapsulated peptides in the nanovesicles structure. Then, the amount of the peptides passing the filter was determined based on the Bradford (1976) method and was used to determine the concentration of unloaded or free proteins. Finally, the encapsulation efficiency was calculated based on the percentage of the protein loaded into the nanovesicles divided by to the initial (total) protein concentration by equation (1):

$$EE\% = \left(\frac{CT - CF}{CT}\right) * 100 \qquad \text{equation1}$$

CT = total peptide concentration.

CF = concentration of the free peptide.

Bovine serum albumin (BSA) was utilized as the standard protein to plot the standard curve. The equation of the standard line was obtained as follows (Equation (2)):

$$Y = 0.1216X + 0.4353\ R^2 = 0.97 \qquad \text{Equation 2}$$

2.7. Subjecting nanovesicles to temperature and shear stress

According to the method described by Rezvani et al. (2019), 2 mL of each sample was transferred into a glass vial and kept at 65 °*C* for 24 h. To apply shear stress to the liposome and niosome samples, they were placed in a rheometer device where the shear rate reached from $2s^{-1}$ to $100s^{-1}$ in a 10-min interval (Heurtault et al., 2003). To find the rheological behavior of nanovesicles, their viscosity and shear stress were measured as a function of the shear rate (2-100 s^{-1}) for 10 min. Then their physicochemical and rheological properties were investigated.

2.8. Determination of size and EE

Particle size and nanovesicle encapsulation efficiency were measured after applying temperature and shear stress according to the method mentioned in sections 2.5 and 2.6.

2.9. Antioxidant properties

To determine the anti-oxidative properties of hydrolyzed protein-containing nanoliposomes and nanoniosomes, the samples were placed in the water bath at 100 °*C* for 5 min after application of shear stress and temperature to achieve complete release. The anti-oxidative features were then measured (Mazloomi et al., 2020).

2.9.1. DPPH radicals scavenging assay

According to Maqsoudlou et al. (2019), a volume of 100 μL of each nanovesicle sample was mixed with 500 μL of ethanol and 125 μL of DPPH solution (0.02 % in ethanol). The mixture was shaken vigorously and incubated in the dark. After 60 min, the absorbance was measured at 517 nm using a spectrophotometer. The decline in DPPH radical was examined at 517 nm. DPPH radical-scavenging activity was calculated as equation (3):

$$\text{DPPH radical scavenging activity } (\%) = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance control}}\right) * 100 \qquad \text{Equation 3}$$

The control was done in the same way, with the difference that distilled water was used instead of the sample. Butylated hydroxytoluene (BHT) was used as a positive control.

2.9.2. Ferric reducing power assay

The method of Maqsoudlou et al. (2019) was utilized to assess the ability of nanovesicles to reduce ferric ions. To this end, 70 μL of each sample was mixed with phosphate buffer (70 μL, 0.2 M, and *pH* 6.6), and potassium ferricyanide (10 mg/mL, 70 μL). The resulting mixture was incubated at 50 °*C* for 30 min. Then, TCA (100 mg/mL 70 μl) was added. The mixture was centrifuged at 1650 g for 10 min and the supernatant (140 μL) was finally mixed with 140 μL of distilled water and 28 μL of ferric chloride (1 mg/mL). The absorbance of the resulting solution was read at 700 nm after standing the mixture at room temperature for 10 min. A rise in the absorbance indicates an increment in the reducing power. Ascorbic acid was used as a positive control.

2.10. Determination of ACEI activity

At first each nanovesicle sample was placed in a 100 °C water bath for 5 min until complete release. Then the ACEI properties were measured (Mazloomi et al., 2020). The ACEI activities of the samples were assessed according to Maqsoudlou et al. (2018) method with minor changes. HHL and hydrolyzed pollen protein were used as substrate and inhibitor, respectively. The substrate solution was prepared by dissolving HHL in 100 mM sodium borate containing 300 mM sodium chloride (*pH* 8.3). The reaction was continued by mixing 100 μL of substrate with 25 μL inhibitor, 10 min of mixture incubation at 37 °*C*, adding the initiator (10 μL ACE solution, 100 mU/mL), 30 min of incubation at 37 °*C*. in the following, the reaction was completed by adding the terminator (HCL. 1N, 100 μL). The inhibitory activity was evaluated by the extraction of hippuric acid obtained from the ethyl acetate and dissolution in double distilled water (1 mL) followed by vacuum evaporation of the solvent and reading the absorbance of the solutions by spectrophotometer at 228 nm using the following equation (4):

$$ACEI\% = \left(\frac{A - B}{A - C}\right) * 100 \qquad \text{Equation 4}$$

In which A shows the absorbance in the presence of ACE, while B denotes the absorbance in the presence of ACE and inhibitor and C represents the absorbance in the absence of ACE. Double distilled water and Captopril (0.1 mg/mL) were considered as negative and positive controls, respectively.

2.11. Statistical analysis

Data analysis was carried out based on Duncan's mean comparison at the significance level of 5 % and one-way analysis of variance (ANOVA) as implemented in SPSS software (Ver. 20.0). All the measurements were conducted in triplicates and the results were expressed as mean ± standard deviation.

3. Results and discussions

3.1. Size and PDI measurements

In this research, the size of nanovesicles showed a significant increase after loading hydrolyzed protein and coating with chitosan (Fig. 1) ($P < 0.05$). The reason for this increase in size can be attributed

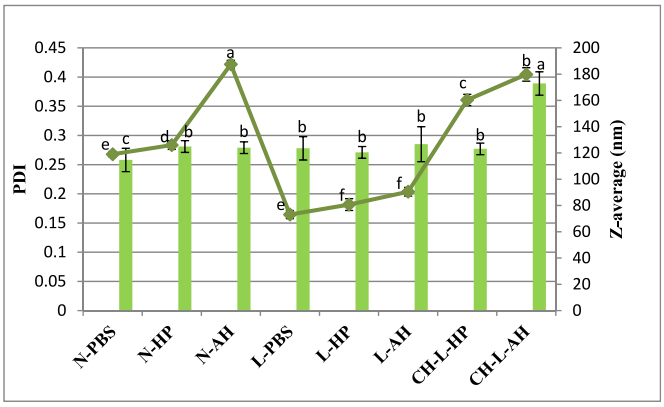

Fig. 1. Average particle size (Z-average), Polydispersity index (PDI) of nanovesicles loaded with hydrolyzed pollen proteins (Line and column charts, respectively). Similar letters in each chart indicate lack of significant difference for the samples.

to the formation of new hydrogen bonds between hydroxyl and amin groups of the peptides with the liposome and niosome wall. On the other hand presence of the hydrophilic peptides in the polar regions of the phosphatidylcholine can be caused the increasing the size of liposomes (Maqsoudlou et al., 2020; Mazloomi et al., 2020). Previous studies have also reported an increment in the size of nanocarriers after chitosan coating and loading casein (Sarabandi et al., 2019), rainbow trout hydrolyzed protein (Ramezanzade et al., 2017), whey protein (Mohan, McClements and Udenigwe, 2016) and a protein-type drug (Barani et al., 2020).

The nanovesicles containing pepsin-hydrolyzed peptides had smaller size compared to those containing alcalase-hydrolyzed peptides (Fig. 1) which can be attributed to the simpler loading of peptides with more uniform size in the structure of nanoliposomes and nanoniosomes and an improvement in the vesicle layer order compared to larger peptides with higher molecular weight (Zhiyu et al., 2015).

The PDI of the nanoniosomes showed a significant enhancement after loading of hydrolyzed proteins (Fig. 1) ($P < 0.05$). The peptide heterogeneity, based on the number of peptides within the hydrolysates, affects the uniformity of the niosomes dispersion. In particular, an increase in peptide number in the hydrolysates (decrease in peptide size) resulted in less uniformly dispersed niosomes. Therefore PDI was increased. Similarly, Mohan et al. (2016) reported significantly higher PDI of the peptide-loaded nanoparticles compared to nanoparticles with no hydrolyzed protein. The use of Span 60 and Tween 80 in the present study balanced HLB to a large extent. Akbari et al. (2022) also stated the possibility of preparing stable vesicles by using binary mixtures of surfactants (Tween and Span) which adjusted HLB. A significant rise can be seen in PDI by coating the alcalase-hydrolyzed protein-loaded nanoliposomes with 0.2 % chitosan ($P < 0.05$), suggesting unstable, heterogeneous, and non-uniform particle size distribution due to the accumulation and adhesion of particles (Mazloomi et al., 2020)

Table 1
Zeta potential of nanovesicles loaded with hydrolyzed pollen proteins.

Samples	Zeta-potential(mV)	
N-PBS	−17.87 ± 1.27	g
N-HP	−13.8 ± 0.98	f
N-AH	−17.1 ± 1.12	g
L-PBS	−9.07 ± 0.85	d
L-HP	4.52 ± 1.19	c
L-AH	−10.11 ± 0.95	e
CH-L-HP	24.32 ± 1.59	a
CH-L-AH	20.21 ± 1.15	b

Similar letters in each row indicate lack of significant difference for the samples.

3.2. Zeta potential measurements

Determination of zeta potential is a common approach for evaluating the electrostatic properties of liposome and niosomes as it offers a useful indicator of the surface charge of particles and the colloidal repulsive forces and the physical stability of nanocarrier systems (Da Silva Malheiros, Daroit, Brandelli, 2010). As seen in Table 1, the zeta potential of nanoniosomes showed a significant variation from −17.78 mV to −13.8 mV ($P < 0.05$) after loading of pepsin-hydrolyzed proteins. All of the niosomes showed negative zeta potential values, which could reflect adsorption of counter ions or preferential adsorption of hydroxyl ions at the niosomal surface (Mohan et al., 2016).

The zeta potential of nanoliposome was −9.07 mV. This trend can be explained as follows: the hydroxyl group on the head of cholesterol forms a hydrogen bond with the choline group on the polar head of phosphatidylcholine. The positively-charged choline group is drawn into the membrane while the phosphatidyl group with a negative charge is pushed to the surface of the membrane. Therefore, the negative charge increases, leading to electrostatic repulsion of particles (Hasibi et al., 2020). In a similar study, the zeta potential of control nanoliposomes and those loaded with hydrolyzed muscle protein was −5.8 and −5.5 mV, respectively (Zavareze et al., 2014). Zeta potential of nanoliposomes changed from −9.07 mV to −10.11 and 4.52 mV after loading with alcalase- and pepsin-hydrolyzed protein ($P < 0.05$), respectively. These results revealed that the phospholipid composition and the reaction between phospholipid and trapped peptides affect the surface charge of liposomes.

The peptides were incorporated into the phospholipid bilayers, with some of their charged groups exposed to the surrounding aqueous phase (Mohan et al., 2016; Mor et al., 2021). In addition, the presence of the alcalase-hydrolyzed protein within the liposomes would be expected to increase the electrostatic repulsion between them and thereby increase their aggregation stability. However this is contrary to decreases in surface charge magnitude when pepsin hydrolyzed protein were loaded in liposomes. The zeta-potential of liposomes containing the high molecular weight peptides of pepsin hydrolyzed protein, is significantly lower in magnitude compared to those of liposomes loaded with smaller peptides of alcalase hydrolyzed protein. An increase in the number of peptide molecules in the alcalase hydrolyzed protein (free amino groups) resulted in an increase in the magnitude of the anionic charge on the corresponding liposomes (Mor et al., 2021; Maqsoudlou et al., 2018).

After coating the nanoliposomes with 0.2 % chitosan, the zeta potential of nanoliposomes containing alcalase- and pepsin-hydrolyzed protein increased from −10.11 and 4.52 mV to 20.21 and 24.32 mV respectively ($P < 0.05$). In this context, Hasani et al. (2019) reported the zeta potential of −51.7 and + 50 mV for alcalase-hydrolyzed peptide--carrying nanoliposomes without and with 0.5 % chitosan coating.

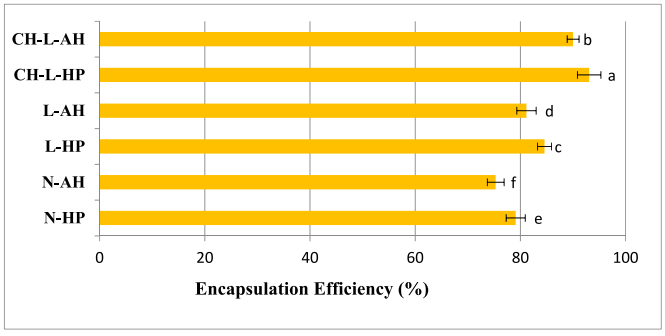

Fig. 2. Encapsulation efficiency of nanovesicles loaded with hydrolyzed pollen. Similar letters indicate lack of significant difference for the samples.

3.3. The encapsulation efficiency (EE)

In this research, the encapsulation efficiency of nanovisicles loaded with HP was better compared to the encapsulation efficiency of nanovisicles loaded with AH ($P < 0.05$) (Fig. 2). Such a difference can be assigned to the better loading of HP with lower and more uniform molecular weight than AH in the structure of nanovesicles. On the other hand, the presence of cholesterol in the structure of the liposome membrane stiffened the lipid chains and prevented the membrane from being broken by the active compounds and increased the efficiency of encapsulation compared to nanoniosomes, which was consistent with the theoretical and numerical results (Mohamad and Fahmy, 2020; Hasibi et al., 2020). The coating of nanoliposomes with 0.2 % chitosan enhanced the ability of vesicles to retain alcalase- and pepsin-hydrolyzed peptides by 90.2 % and 93.03 % ($P < 0.05$), respectively which was consistent with the results of Hasani et al. (2019) and Barani et al. (2020).

3.4. Effect of temperature on particle size and EE

One of the important characteristics of nanoliposomes is their transition temperature from solid gel state to liquid crystal (Tc), also called Krafft temperature. Krafft temperature refers to the temperature at which the lipid bilayers lose some of their initial arrangement and their fluidity increases due to the melting of non-polar parts and non-melting of polar portions (Roy et al., 2016). Roy et al. (2016) reported the main transition temperature of liposomes in the range of 15–65 °C.

In this study, nanonisomes and nanoliposomes showed an approximately 2-to-2.5-fold increment in their size after 1day of storage at 65 °C (Fig. 3). Sarabandi et al. (2019) reported a 4-fold increase in the size of nanoliposomes loaded with peptides obtained from enzymatic hydrolysis of casein with alcalase enzyme, after one day of storage 65 °C. The change in the size of the particles can be due to the state changes and phase transition of the gel structure of the vesicle membrane at this temperature and the increase in fluidity and as a result the acceleration of integration and aggregation processes (Li, Paulson & Gill, 2015).

Fig. 4 shows a significant decline in the encapsulation efficiency of nanoliposomes and nanoniosomes after one day of storage at 65 °C ($P < 0.05$). This decrease was lower in the chitosan-coated nanoliposomes due to the protective effect of chitosan. The decline in encapsulation efficiency indicates the release of loaded peptides from the membrane structure of nanovesicles as a result of the enhanced fluidity and permeability (Hasibi et al., 2020). The highest and lowest efficiency reduction occurred in niosomes and liposomes coated with chitosan, respectively. The lower reduction of efficiency in the encapsulation of liposome compared to niosomes can be assigned to the presence of cholesterol in liposomes and its absence in niosomes. The phospholipid-cholesterol interaction increased the membrane adhesion and shear hardness of the membrane (Roy et al., 2016). The stability increased by incrementing the hydrogen bonds between the hydroxyl group of cholesterol and the carbonyl group of phospholipid. In the liquid crystal phase, however, the addition of cholesterol limits the fluidity and mobility of acyl chains (Mohammadi et al., 2014).

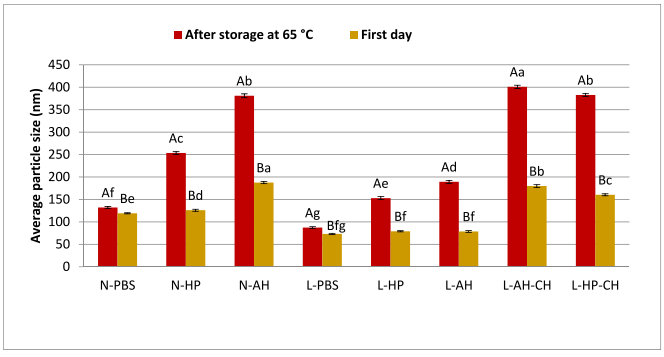

Fig. 3. The changes in average diameter of nanovesicles loaded with hydrolyzed pollen proteins during storage 65 °C.. (Similar uppercase and lowercase letters indicate lack of significant difference for each nanovesicles before and after treatment and between different samples stored at similar conditions, respectively.)

3.5. Effect of temperature on ACEI and antioxidant properties

Exposure of hydrolyzed protein-carrying nanovesicles to the temperature of 65 °C for one day caused a significant decrease ($P < 0.05$) in their ability to inhibit ACE and DPPH radical and their iron ion reduction (Figs. 5 and 6). Among the nanovesicles, chitosan-coated nanoliposomes and nanonisomes exhibited the lowest and highest reduction in activity, respectively i.e. 86.6 % and 19.72 % reduction in ACE inhibition activity (Fig. 5) and 5.05 % and 13.38 % reduction in DPPH radical inhibition activity (Fig. 6) respectively. Such a decrease in biological activity can be assigned to the decrease in encapsulation efficiency and the release of peptides from the membrane of nanovesicles due to the increase in their fluidity and permeability (Roy et al., 2016). In all cases, the reduction of ACEI and antioxidant activity in non-encapsulated hydrolyzed proteins was significantly more than their encapsulated counterparts. The better preservation of ACEI and antioxidant activity in liposomes compared to niosomes was due to the lower reduction in the encapsulation efficiency of liposomes compared to niosomes.

The presence of cholesterol in the membrane structure of liposome led to the better preservation of the membrane structure upon exposure to high temperature which prevented the release of bioactive peptides from the membrane (Mohammadi et al., 2014). By forming a protective layer on the membrane of liposomes, the chitosan coating provided more protection for the bioactive peptides enclosed in the membrane and prevented their exposure to high temperatures (Zhiyu et al., 2015).

3.6. Effect of shear stress on particle size and PDI

After applying the shear stress, the particle size of chitosan-coated nanoliposomes and loaded nanoniosomes increased significantly ($P < 0.05$) (Fig. 7). Shear stress significantly raised the PDI of chitosan-coated nanoliposomes and hydrolyzed protein-loaded nanoniosomes ($P < 0.05$) (Fig. 7).

Vesicles smaller than 100 nm obtained from lipids or surfactants (especially liposomes) require a shear stress of about 40 Pa for disruption of their membrane (Saxer et al., 2013). In the present research, as the shear rate was in the range of 0–100 s^{-1}, the maximum shear stress reached about 36 Pa. This stress level enlarged the niosome particle size which could be possibly due to the degradation of the niosomes larger than 100 nm and their adhesion. The liposomes with initial sizes smaller than 100 nm were less affected by this issue.

Chitosan-coated nanoliposomes showed lower enlargement. Smaller particles with non-spherical and heterogenous morphology were formed upon the destruction of the nanovesicles larger than 100 nm due to the shear stress. These destructed particles have higher energy level. The tendency of these particles to lose their excess energy through accumulation and adhesion resulted in the formation of larger particles. On the other hand, degradation of the structure of nanovesicles altered their surface charge and disrupted the electrostatic repulsion between the adjacent particles. Upon a decline in the electrostatic repulsion, the lateral interparticle forces promote their adhesion and the formation of larger particles, a rise in the particle size will result in the wide and nonhomogeneous size distribution (Bartelds et al., 2018). In the current research, the size distribution of the chitosan-coated nanoniosomes and nanoliposomes with enhanced mean particle size showed an increment.

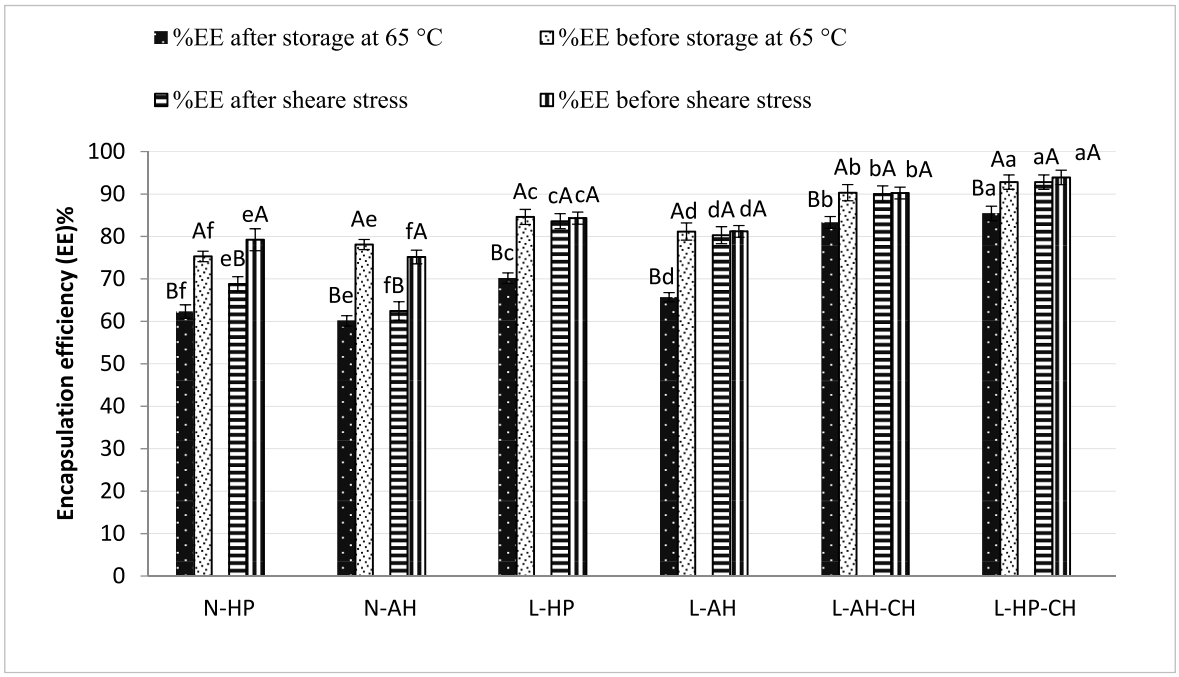

Fig. 4. The changes encapsulation efficiency of nanovesicles loaded with hydrolyzed pollen proteins after storage at 65 °C and shear stress (2-100 s^{-1}, 10 min). However, the samples under shear stress and high temperature were compared separately.. (Similar uppercase and lowercase letters indicate lack of significant difference for each nanovesicles before and after treatment and between different samples in similar conditions, respectively.)

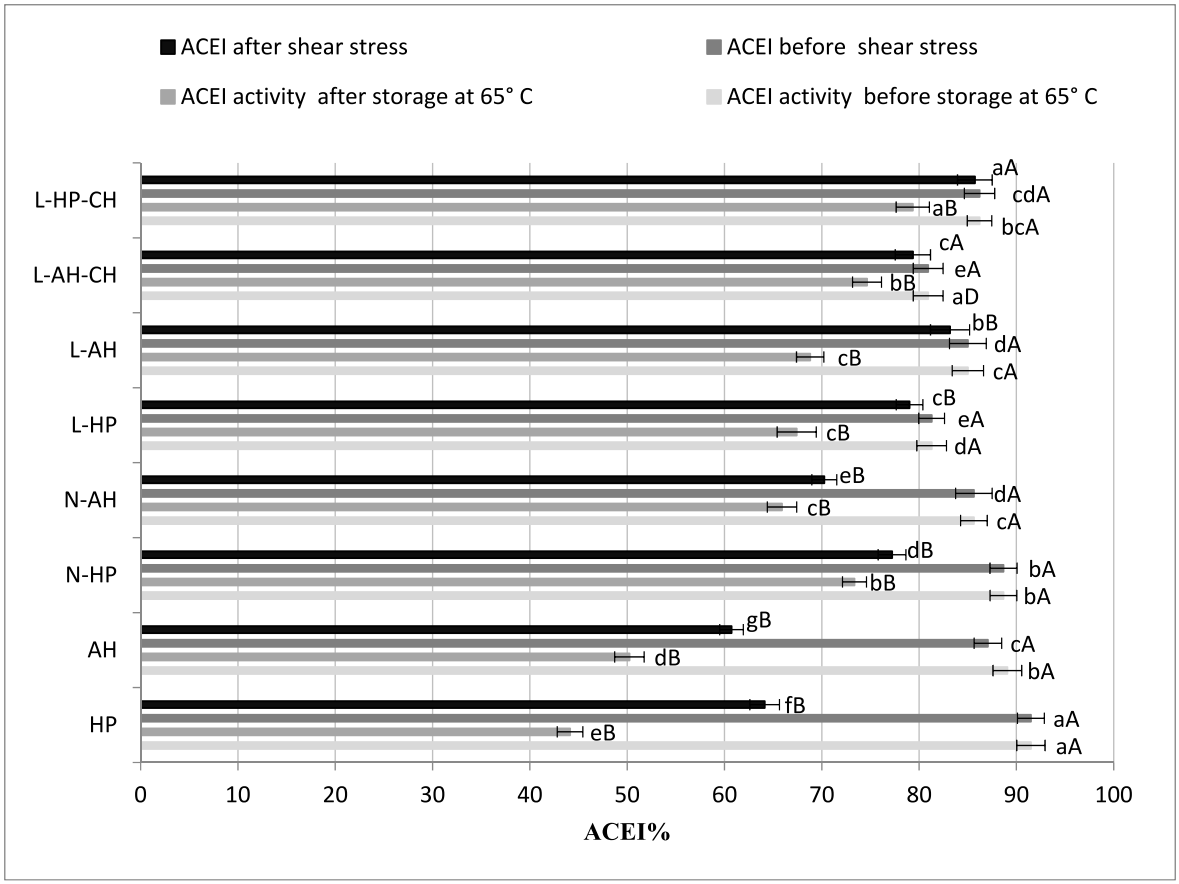

Fig. 5. Changes in ACEI activity of nanovesicles carring hydrolyzed pollen proteins before and after one day of storage at 65 °C and shear stress (2-100 s^{-1}, 10 min). However, the samples under shear stress and high temperature were compared separately. (Similar uppercase and lowercase letters indicate lack of significant difference for each nanovesicles before and after treatment and between different samples stored at similar conditions, respectively).

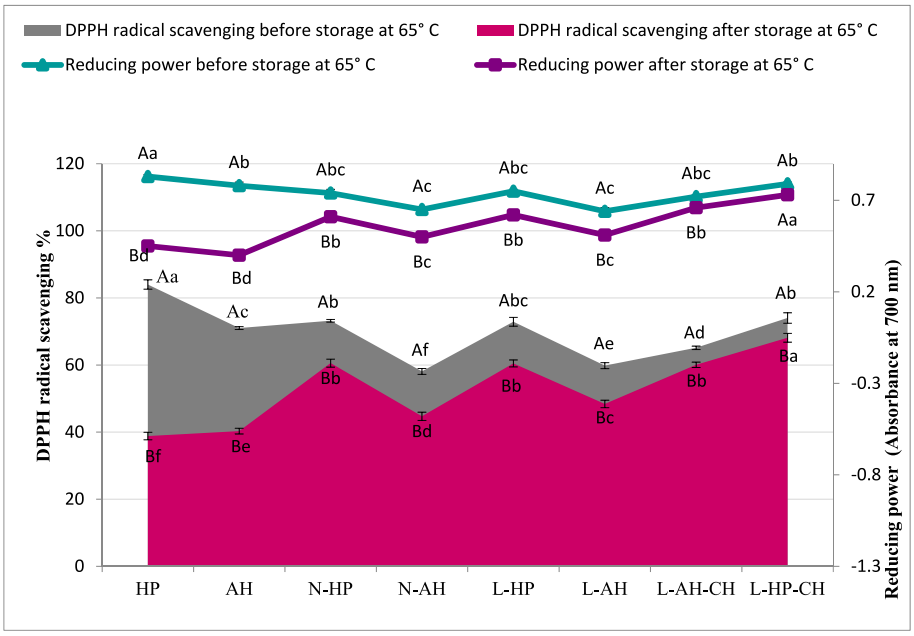

Fig. 6. Changes in DPPH rdical scavenging activity (Area chart) and reducing power (Line chart) of nanovesicles carring hydrolyzed pollen proteins before and after one day of storage at 65 °C
(Similar uppercase and lowercase letters indicate lack of significant difference for each nanovesicles before and after treatment and between different samples stored at similar conditions, respectively).

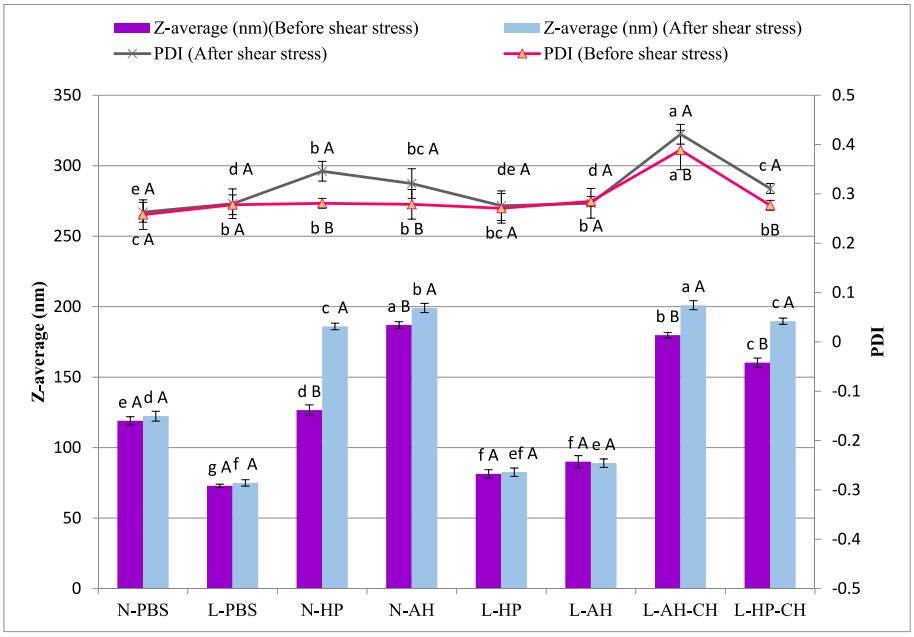

Fig. 7. The changes in size (column chart) and PDI (Line chart) of nanovesicles loaded with hydrolyzed pollen before and after shearing at room temperature (2-100 s^{-1} for 10 min).. (Similar uppercase and lowercase letters indicate lack of significant difference for each nanovesicles before and after treatment and between different samples at similar conditions, respectively.)

3.7. Effect of shear stress on encapsulation efficiency

As can be seen in Fig. 4, the encapsulation efficiency of nanoniosomes significantly declined by applying shear rate of 2–100 s^{-1} for 10 min ($P < 0.05$). The larger initial size of nanoniosomes compared to nanoliposomes led to the higher susceptibility of niosomes. The hydrolyzed proteins released and declined the encapsulation efficiency by the disruption of the membrane structure due to the shear stresses (Saxer et al., 2013). The presence of compounds such as lecithin and cholesterol in the structure of liposomes and their absence in the niosomes led to the lower flexibility and resistance of nanoniosomes against shear stress compared to nanoliposomes. With further destruction of their membrane structure, the encapsulation efficiency will decrease.

The presence of chitosan coating on the nanoliposome structure served as a protector and prevented damage to the membrane of nanoliposomes, so by maintaining the internal components of nanoliposomes, prevented the reduction of encapsulation efficiency. Wu et al. (2023) examined uncoated and sodium alginate-coated loaded

liposomes under shear rate of 0.01–100 s^{-1} at stable conditions. Their results showed an increment in the viscosity of liposomes with sodium alginate coating. Increase of the viscosity of the sample enhanced its stability during storage and prevented the loss of encapsulation efficiency. Increasing the viscosity of the system also reduced the mobility of the phospholipid molecular membrane of the liposome which slowed down the accumulation of liposomes, hence, improving its stability during storage (Wang et al., 2019).

3.8. Effect of shear stress on ACEI and the antioxidant properties of nanovesicles

Ten minute exposure to shear rates of 2–100 s^{-1} had a significant effect on the biological activity of nanonisomes and nanoliposomes ($P < 0.05$). As can be seen in Figs. 5 and 8, after 10-min exposure to shear rates (2- 100 s^{-1}), a significant decrease can be observed in ACE and DPPH inhibition and iron ion reduction of almost all samples, except the chitosan-coated nanoliposomes ($P < 0.05$). Nanoliposomes and nanonisomes showed the lowest and highest decrease in ACE inhibitory activity, respectively (Fig. 5). This also holds for the antioxidant activities, such that the lowest (2 %) and highest (10.08 %) decrease in DPPH radical inhibition activity were detected in nanoliposomes and nanonisomes, respectively (Fig. 8). In all cases, the reduction of ACEI and antioxidant activity in non-encapsulated hydrolyzed proteins was significantly higher than their encapsulated counterparts ($P < 0.05$).

The decrease in biological activity of hydrolyzed proteins encapsulated in nanoliposomes and nanoniosomes can be due to the decline in the encapsulation efficiency and the release of loaded peptides from the membrane structure of nanovesicles as a result of increased permeability induced by the application of shear stress. Chitosan coating prevented the release of bioactive peptides and their exposure to destructive factors and reduced antioxidant and ACEI activity of nanovesicles (Figs. 5 and 8). Moreover, the higher viscosity of chitosan-coated nanoliposomes reduced the mobility of the phospholipid molecular membrane and increased the stability of nanovesicles while preventing the release of bioactive compounds (Wang et al., 2019). The better preservation of ACEI and antioxidant activity in liposome compared to niosomes can be assigned to the presence of cholesterol and lecithin in the structure of liposome membrane and maintaining the resistance and flexibility of the membrane structure upon exposure to shear forces and the release of bioactive peptides from the membrane (Sun et al., 2022). In the current study, the initial size of nanoniosomes was larger than nanoliposomes, making the niosomes more susceptible to shear stress. By disrupting the membrane structure due to shear stress, hydrolyzed proteins leached out, decreasing the antioxidant and ACEI activity.

3.9. Effect of shear stress on the rheological properties of nanovesicles

To find out the rheological behavior of nanovesicles, the viscosity and shear stress were measured as a function of shear rate in a 10-min time interval at the shear rate range of 2–100 s^{-1} (Figs. 10 and 9 respectively). Fig. 10 shows a relatively linear relationship and the simplest flow behavior, i.e. Newtonian-like behavior. The viscosity-shear rate curve and flow index measurement (n) show weak pseudo-plastic behavior of nanoliposomes coated with 0.2 % chitosan at low shear rates. In these fluids, the slope of the shear stress-shear rate graph shows the viscosity, such that the slope of the curve increases by raising the viscosity of the fluid (Fig. 9). As a low-viscosity fluid, water is generally the dominant dispersion component of liposomal and

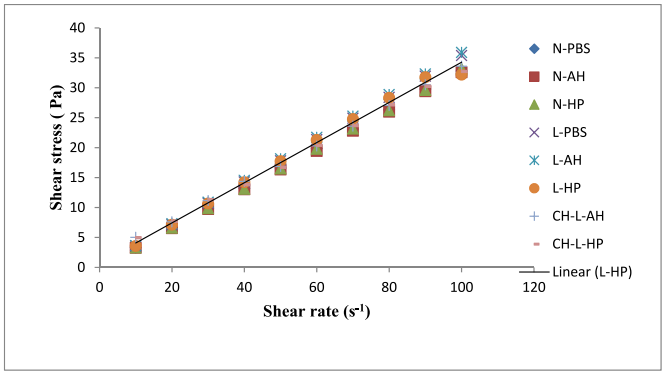

Fig. 9. The changes in shear stress of nanovesicles loaded with hydrolyzed pollen at shear rate of 2–100 s^{-1} for 10 min.

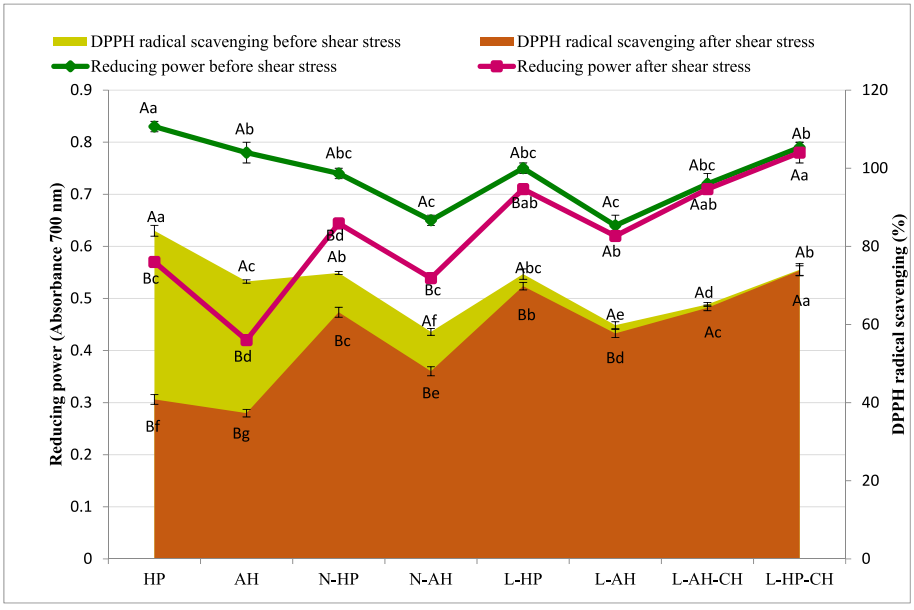

Fig. 8. The changes in DPPH radical scavenging activity (Area chart) and reducing power (Line chart) of nanovesicles loaded with hydrolyzed pollen before and after shearing at room temperature (2-100 s^{-1} for 10 min)
(Similar uppercase and lowercase letters indicate lack of significant difference for each nanovesicles before and after treatment and between different samples at similar conditions, respectively).

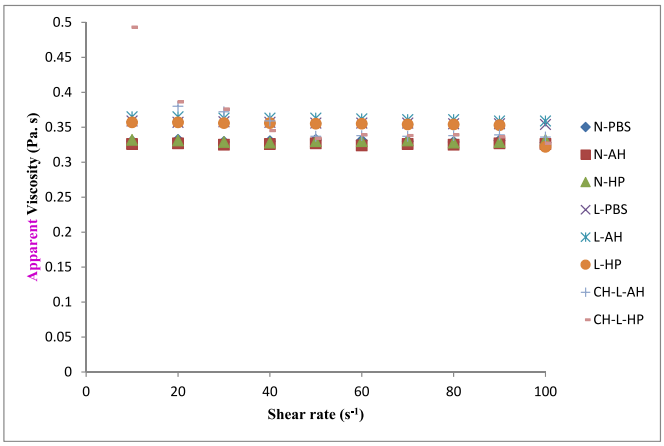

Fig. 10. The changes in viscosity of nanovesicles loaded with hydrolyzed pollen at shear rate of 2–100 s^{-1} for 10 min.

niosomal solutions. In these systems, the viscosity of the dispersed phase indicates the viscosity of the entire system (Pattnaik and Mishra, 2022). Bashiri et al. (2016) also obtained similar results. They prepared beta-carotene-carrying liposomal systems using phytosterol and enhanced the shear rate of 2–100 s^{-1} in 10 min. Their results showed a linear relationship and the simplest flow behavior, i.e. Newtonian-like behavior. The viscosity-shear rate diagram showed weak pseudoplastic behavior at low shear rates, confirming the flow index (n).

A remarkable decline in the viscosity of chitosan-coated nanoliposomes was observed in Fig. 10, which can be attributed to the friction and co-alignment of the dispersed phase particles and the dominance of the shear forces on the Brownian forces. This decline continued up to the shear rate of 50 s^{-1} beyond which, the viscosity showed no dependence on the shear rate as the rise in the rate caused not significant impact on the viscosity. This might be due to the changes in the Brownian rotational movement at lower shear rates. Therefore, the nominal viscosity was decreased (Chen et al., 2001). It can be assigned the higher viscosity of the chitosan-coated liposomes to the larger size and hydrodynamic volume of the coated liposomes. The viscosity of the nanoniosome and uncoated nanoliposome solutions was independent of the shear rate at all shear rates (Fig. 10) as the rise in the rate had no impact on their viscosity. Regarding the Newtonian and pseud-Newtonian behavior of the nanoliposome and nanoniosome solutions, it can be concluded that these solutions are dilute and their concentration did not reach the critical concentration. Therefore, they will not affect the rheological properties of the foods which are organoleptically favorable.

The power law is one of the models used to interpret the rheological behavior. Table 2 lists the values of the flow index (n) and consistency coefficient (k) obtained from the power law model, as well as the coefficient of explanation (R^2) for all the tested samples. The coefficient of explanation was above 95 % for all samples, indicating the suitability of the power law model for describing the flow characteristics of nanoniosomal and nanoliposomal solutions. In this context, Pattnaik and Mishra (2022) stated that an increase in the concentration of lecithin in the liposome formulation enhanced its viscosity. They also introduced the Herschel–Bulkley and power law models as the most appropriate models to interpret the rheological behavior of liposomes with high and low lecithin concentrations, respectively. The flow index decreased by coating nanoliposomes with 0.2 % chitosan, in other words, the flow behavior changed toward pseudoplastic state (Table 2). Similarly, Saroglu et al. (2022) investigated the rheological properties of nanoliposomes coated with xanthan gum-salep. They reported the non-Newtonian and shear-thinning behavior of the nanoliposomes coated with the mentioned gum under constant shear force.Gurbuz Çolak et al. (2022) also reported the non-Newtonian and pseudoplastically behavior of chitosan-coated amphotericin B-containing nanoliposomes upon exposure to shear forces at a constant shear rate.

Table 2
Rheological parameters obtained from power law model fitting.

Samples	(k) (Pa.s)	(n)	(R^2)
N-PBS	3.3089	0.9986	1
N-AH	3.2613	0.9995	1
N-HP	3.3093	0.9981	1
L-PBS	3.5916	0.9947	1
L-AH	3.6613	0.9927	1
L-HP	3.6275	0.9797	0.9985
CH-L-AH	4.5557	0.8461	0.9928
CH-L-HP	4.5386	0.8441	0.9941

Uncoated nanoliposome and nanoniosomes showed tendency to Newtonian behavior where the flow index is closer to one. Similarly, Wu et al. (2023) stated that the viscosity of collagen peptide-loaded liposomes was always constant and less than 2 Pa s under stable conditions and a shear rate of 0.01–100 s^{-1}, suggesting the Newtonian nature of these fluids. But the viscosity of liposomes coated with sodium alginate concentrations of 0.2–0.5 % decreased from about 26 Pa s to about zero with increasing the shear rate up to 100 s^{-1}, reflecting their non-Newtonian (shear dilution) behavior.

4. Conclusion

As high temperatures and shear stress affect the phase state of vesicles and the permeability of their membrane, it is necessary to evaluate the effect of different processes on the physicochemical features and encapsulation efficiency of nanovesicles before their application in drug formulation or enrichment of the foods and drinks. In this research, thermal and shear stress stability of two different types of nanovesicles were investigated as an enrichment composition containing hydrolyzed pollen protein. The results showed a 2- to 2.5-fold increment in the size of nanonisomes and nanoliposomes after 24 h of storage at 65 °C, while their encapsulation efficiency showed a 5–8 % decline. Chitosan-coated nanoliposomes and nanonisomes exhibited the lowest and highest decrease in ACE inhibition activity and DPPH radical inhibition, respectively. The PDI of chitosan-coated nanoliposomes and nanoniosomes showed a significant increase by applying shear rates of 2–100 s^{-1} for 10 min, ($P < 0.05$), while the encapsulation efficiency of the nanoniosomes showed a drastic decline ($P < 0.05$). The ability to inhibit ACE, DPPH radical, and iron ion reduction was significantly reduced in almost all samples, except chitosan-coated nanoliposomes ($P < 0.05$). Nanoliposomes and nanoniosomes respectively demonstrated the lowest and highest decline in the ACE inhibition and antioxidant activities. The investigation of the flow behavior of nanovesicles showed a relatively linear relationship and the simplest flow behavior, i.e. near-Newtonian behavior and weak pseudoplastic behavior of chitosan-coated nanoliposomes at low shear rates. Therefore, they will not affect the rheological properties of foods, which is organoleptically favorable in many cases. Therefore, the use of chitosan-coated nanoliposomes will offer greater physicochemical stability and better preservation of the biological activity of the encapsulated compounds in enrichment of food formulations at temperatures higher than 65 °C or shear stresses below 35–40 Pa.

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CRediT authorship contribution statement

Atefe Maqsoudlou: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alireza Sadeghi Mahoonak:** Writing – review & editing, Validation, Supervision, Methodology, Data curation, Conceptualization. **Hossein Mohebodini:** Writing – review & editing, Software, Resources, Project administration, Methodology, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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