



Research Article

Physicochemical and antioxidant properties of chitosan-coated nanoliposome loaded with bioactive peptides produced from shrimp wastes hydrolysis

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Abstract

The aim of the present study in the first stage was encapsulation of peptides produced from hydrolysis of shrimp wastes (by neutrase enzyme) with combined coating of nanoliposome and chitosan in the form of four treatments (NP or peptide-carrying nanoliposomes, NP-CH-0.05, NP-CH-0.1 and NP-CH-0.5 or peptide-carrying nanoliposomes with coating of 0.05, 0.1 and 0.5% chitosan). In the next step, physicochemical and antioxidant properties of the treatments were investigated. The results showed that average particle size and particle dispersity index in different treatments varied from 228.9 ± 4.85 to 436.7 ± 1.08 nm and 0.389 ± 0.01 to 0.453 ± 0.01 , respectively. Zeta potential in nanoliposomes shifted to positive values with increasing chitosan concentration and from -62.59 ± 4.36 mV in NP reached to $+56.94 \pm 3.71$ mV in NP-CH-0.5. Encapsulation efficiency improved by adding chitosan to the surface of nanoliposomes and the highest amount was found in NP-CH-0.5 treatment ($94.12 \pm 3.73\%$). Evaluation of release profiles of treatments in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) showed that coating of peptides using nanoliposome and chitosan helped in protecting the structure of peptides and reduce their release rate significantly ($p < 0.05$). Chitosan-coated nanoliposomes showed more antioxidant activity than NP treatment. Also in chitosan-coated treatments, with increasing chitosan concentration, antioxidant activity of the treatments increased significantly ($p < 0.05$). According to the results, coating bioactive peptides with combined coating of nanoliposomes and chitosan is a suitable technique to protect and increase the efficiency of peptides.

Keywords: Bioactive peptides, Nanoliposomes, Chitosan, Physicochemical properties, Antioxidant activity

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Introduction

With increase in per capita consumption in Iran and export of shrimp, centers are established in north and south of the country for shrimp processing and packaging. A large amount of waste containing protein (head, shell, and ...) is produced daily in these centers, which can be turned into high-value-added products if used optimally. One of these products is hydrolyzed proteins (or bioactive peptides). These proteins are result of chemical (acidic and alkaline treatments) or biochemical (hydrolysis by commercial enzymes, such as alcalase, protamex, neutrase, pepsin, papain, etc) hydrolysis of the wastes.

Bioactive peptides are specific protein components that are inactive in structure of major protein (Sarmadi and Ismail, 2010). These peptides have 2 to 20 amino acids and a molecular weight of fewer than 6,000 Daltons (Park *et al.*, 2001). Bioactive peptides exert certain physiological effects (antioxidant, antihypertensive, immune system booster, and cholesterol-lowering) after entering human body (Sun *et al.*, 2004; Jia *et al.*, 2010). These peptides are also widely used in the food industry as antioxidants, antimicrobials, foaming agents, and emulsifiers.

Bioactive peptides must be active before entering the bloodstream and during digestion and absorption to exert their physiological effects (Segura-Campos *et al.*, 2011). Because these peptides may be denatured and break down in the digestive system (stomach and intestines) under acidic and enzymatic conditions, it is necessary to

use methods to protect them; methods that protect the structure of bioactive peptides lead to high efficiency of absorption in the gastric and intestinal mucosa and improve liveability of the peptides (Rekha and Sharma, 2010). Also, use of bioactive peptides for various purposes in food requires methods that, in addition to protecting the structure of the peptides, improve their efficiency.

One of the best methods used to protect bioactive compounds against adverse conditions is encapsulation. Encapsulation technology is to trap solids, liquids, or gases in capsules that release their contents with controlled speed and under special conditions. Steps of this process are formation of a wall around the bioactive peptide, ensuring that the compounds do not leak out, and lack coverage of undesirable compounds. One type of lipid carrier used to encapsulate bioactive compounds, such as bioactive peptides, is liposome. Liposomes are colloidal vesicles composed of polar lipids (phospholipids) that form bilayer spherical structures in presence of water molecules (Ghorbanzade *et al.*, 2017). Due to their amphiphilic properties, liposomes can encapsulate hydrophilic, hydrophobic, and amphiphilic compounds. This property allows liposomes to encapsulate polar and nonpolar amino acids of protein hydrolysate (bioactive peptide). In addition to bioactive peptides, other hydrophilic compounds, such as small molecules and ions (salts and sugars), biopolymers (proteins and

polysaccharides), and particles (fat droplets, nanoparticles, and probiotics) can be incorporated into internal aqueous phase of liposomes. For example so far, in various studies, various hydrophobic (vitamin A, D, and E, carotenoids, coenzyme Q10) and hydrophilic (vitamin C, Iron, and calcium) compounds are finely encapsulated in liposome-based systems (Xia *et al.*, 2006; Tan *et al.*, 2013).

Liposomes and nanoliposomes are structurally, chemically, and thermodynamically identical. But because nanoliposomes are smaller than liposomes, they provide more surface area and increase solubility of the encapsulated material. Nanoliposomes are superior to liposomes in terms of controlled release of materials and precise delivery to target areas. Nanoliposomes also have more colloidal stability and make less turbidity (Rasti *et al.*, 2012).

Lipid-based delivery systems (such as nanoliposome) are not stable in the digestive system (due to acidic conditions and the presence of lipase enzymes) and are degraded (Page and Cudmore, 2001). The most effective way to increase stability of lipid-based delivery systems, such as nanoliposomes, in the digestive system is to coat them with chitosan. Chitosan is a biopolymer that in nature is found in cell walls of fungi of the class Zygomycetes, in the green algae *Chlorella* sp., yeast and protozoa, as well as, insect cuticles, and especially in exoskeleton of crustaceans. Chitosan is a deacetylated derivative of chitin, the

second abundant polysaccharide in nature after cellulose (Alishahi, 2012). This biopolymer is a hydrophilic, biocompatible, and biodegradable polymer of low toxicity (Guo *et al.*, 2003). Cationic groups of chitosan and anionic groups of phospholipids in nanoliposome are connected by electrostatic reactions and a layer of chitosan will be formed around the nanoliposome. This chitosan layer helps increasing the stability of nanoliposomes and improving the absorption efficiency of the product in the digestive system. Various studies used chitosan to increase stability of nanoliposomes and reported positive results (Henriksen *et al.*, 1994; Guo *et al.*, 2003; Li *et al.*, 2015; Ramezanzade *et al.*, 2017; Zhou *et al.*, 2018). In some studies, protective effect of combination of nanoliposome and chitosan on bioactive peptides after entering the digestive system is confirmed (Hsieh *et al.*, 2002; Hasani *et al.*, 2019).

In this research in the first stage, bioactive peptides are produced from shrimp wastes using biochemical (by neutrase enzyme) hydrolysis technique. Then these peptides are encapsulated using nanoliposome and chitosan. Finally, the physicochemical and antioxidant properties of the product are investigated.

Materials and methods

Shrimp wastes were prepared from one of the processing centers of this aquatic in Golestan province and after about three hours, along with the cold chain, it was transferred to the fishery products

processing laboratory of Sari Agricultural Sciences and Natural Resources University. The enzyme used in this study was the microbial enzyme neutrase which was made by Novozyme company and kept at 4°C until start of the experiment. NaOH, medium molecular weight chitosan (degree of deacetylation, 75-85%), bovine serum albumin (BSA), ferrozine, trichloroacetic acid, pepsin, potassium phosphate, potassium ferricyanide, ferric chloride, FeSO₄-EDTA, cholesterol, glycerol, butylated hydroxyanisole (BHA) and HCL were purchased from the German company Merck. Phospholipid (lyophilized) prepared from bovine lecithin with a purity of more than 99% were purchased from Sigma company.

Production of protein hydrolysate (SWPH¹)

In order to produce protein hydrolysate, 100 g of the waste sample was first placed in a 500 ml erlenmeyer. Then 200 ml of phosphate buffer with pH=7.4 was added. Next, the solution was placed in a water bath at 85°C for 20 minutes to inactivate internal enzymes of the waste tissue. After this time, it was allowed to cool at room temperature. In the next step, 30 Anson unit of Neutrase enzyme was added. The solution was immediately transferred to a shaker incubator at 50°C and left in this position for 60 minutes to perform hydrolysis process of the proteins. After these times in order to stop the hydrolysis reaction,

erlenmeyer was exposed to 95°C for 15 minutes. After this period and cooling, the solution was centrifuged at 10°C for 20 minutes at 8000 rpm and the supernatant was dried using a freeze-dryer (Guerard *et al.*, 2002; Ovissipour *et al.*, 2010).

Degrees of hydrolysis (DH)

After the end of the hydrolysis process, trichloroacetic acid (TCA) 20% solution was added to the supernatant in equal proportions and the resulting solution was centrifuged at 6700 rpm at 4°C for 10 minutes. The nitrogen in the new supernatant was then measured by Biuret method (Layne, 1957). Finally, degree of hydrolysis of the process was calculated using the following equation (Hoyle and Merritt, 1994). Bovine serum albumin was used as standard protein to draw standard curve and obtain the equation of spectrophotometer:

$$DH (\%) = (\text{Nitrogen in TCA } 10\% / \text{Total nitrogen of the sample}) \times 100$$

Preparation of nanoliposomes

SWPH (with a degree of hydrolysis of 32.97±1.64%) was passed through ultrafiltration (EMD, Millipore, Merck KGaA, and Darmstadt) to reach peptides with a molecular weight less than 3 kDa. The SWPH (10 mg/ml) and phospholipid/cholesterol were hydrated in distilled water and heated with glycerol to 60 to 80°C. Using 2 M sodium hydroxide (shaking the mixture at 60°C for one hour in a shaker), the pH

¹ Shrimp waste protein hydrolysate

of the mixture was raised to 7.3. Crude liposomes were homogenized under high pressure and mixed with different ratios of chitosan (0.05, 0.1 and 0.5 w/v) solution at room temperature for one hour at 2000 rpm (chitosan solution was prepared by dissolving chitosan in 1% acetic acid).

The resulting solution was sonicated at 25°C using a sonicator (with prop 200 UPS). The nanoliposomes were exposed to nitrogen gas at ambient temperature and stored for subsequent experiments (Rasti *et al.*, 2012; Ojagh *et al.*, 2022): Table 1 presents the research treatments.

Table 1: Research treatments.

Treatment number	Symbol	Contents of treatments
1	NP	Uncoated nanoliposomes (no chitosan)
2	NP-CH-0.05	loaded nanoliposomes coated with 0.05% (W/V) of chitosan
3	NP-CH-0.1	loaded nanoliposomes coated with 0.1% (W/V) of chitosan
4	NP-CH-0.5	loaded nanoliposomes coated with 0.5% (W/V) of chitosan

Physicochemical properties

Particle size and particle dispersity index

Particle size and particle dispersity index of nanoliposomes after diluting the sample to 10 times with buffer (phosphate-buffered saline) were measured by dynamic light scattering method using Zetasizer (Malven, Nano ZS, England).

Determination of zeta potential

This index in nanoliposomes measured using a Zetasizer. The nanoliposomes were diluted 10-fold using phosphate buffer. Zeta potential was then evaluated at a scattering angle of 173°C and a helium-tungsten wavelength of 633 nm.

Encapsulation Efficiency (EE)

In order to measure free peptides, 1 ml of the nanoliposome sample was passed through an Amicon filter (with a molecular weight of 10 kDa) and then centrifuged at 3500 rpm for 10 minutes. The filtered solution was used to determine the amount of free protein by Bradford (1976) method. Finally, encapsulation efficiency index was obtained using the following equation (Li *et al.*, 2015):

$$(\%) \text{ Encapsulation Efficiency} = \frac{(\text{total protein} - \text{free protein})}{\text{total protein}} \times 100$$

Release of nanoliposomes in SGF¹ and SIF²

In order to evaluate protective effect of nanoliposomes and chitosan, the resulting products were incubated in similar condition of stomach and

¹ Simulated Gastric Fluid

² Simulated Intestinal Fluid

intestine (Agrawal *et al.*, 2014). To make a stomach-like condition (SGF), 100 mg of pepsin was dissolved in 5 ml of distilled water (contains 0.35 ml of concentrated HCL). Then 100 mg of sodium chloride was added to the solution. The final volume of this solution was increased to 50 ml with distilled water. Next, pH of the solution was adjusted to 1.2 using HCL.

In order to make a condition similar to intestine (SIF), 340 mg potassium phosphate monobasic was dissolved in 10 ml of distilled water. Then 4 ml of 0.2 M sodium hydroxide solution and 500 mg of pancreatin were added to the prepared solution. The final volume and pH of the solution were increased to 50 ml and 6.8, respectively.

Twenty μ l of the sample of uncoated and chitosan-coated nanoliposomes were diluted to a volume of 1 ml. Then these solutions were mixed with both simulated solutions in microcentrifuge tubes (by thermomixer, RQ, Gerhardt, Germany). Incubation time for stomach and intestinal environments was 4 and 2 hours, respectively. At regular intervals in both conditions, the amounts of hydrolyzed protein were measured by a BCA kit (Agrawal *et al.*, 2014; Hasani *et al.*, 2019).

Antioxidant activity

Ferric Reducing power activity assay

To evaluate reducing power of the samples, in the first stage, 0.5 ml of sample (2 mg/ml), 0.5 ml of phosphate buffer (pH 6.6), and 0.5 ml of 1% potassium ferricyanide were mixed and placed in an oven at 50°C for 20 min. In

the next step, 0.5 mL of trichloroacetic acid (10%) was added to the mixture and centrifuged at 3000g for 10 min. Then, 1 ml of the supernatant was mixed with 0.2 ml of 0.1% (w/v) ferric chloride and 1 ml distilled water. The mixture was incubated at room temperature (25°C) for 10 min. Finally absorbance of the mixture was read at 700 nm. BHA at 200 ppm was used as a positive control. Higher adsorption indicates greater reducing power of the samples (Mazloomi *et al.*, 2020).

Hydroxyl radical scavenging activity

In order to evaluate the hydroxyl radical scavenging power of different treatments, at first 0.9 ml of phosphate-buffered saline or PBS (0.2 M, pH 7.4), 0.5 ml of α -deoxyribose (10 mM), 0.2 ml of hydrogen peroxide (10 mM), 0.2 ml of sample solution (10 mg/ml) and 0.2 ml of FeSO₄-EDTA (10 mM) were mixed and then incubated at 37°C for 1 hour. To stop the reaction, 1 ml of 1% thiobarbituric acid and 3% trichloroacetic acid were added to the mixture. Then it was heated in boiling water for 15 min, then cooled in ice and absorbance was monitored at 532 nm. Finally, hydroxyl radical scavenging activity was calculated using the following equation. BHA at 200 ppm was used as a positive control:

Hydroxyl radical scavenging activity (%) = $(Abs_c - Abs_s / Abs_c) \times 100$

Metal-chelating activity

To measure metal-chelating activity of bioactive peptide and other treatment, at

first 1 ml of sample solution (40 mg/mL) was mixed with 3.7 ml of distilled water. The mixture was then reacted with 0.1 ml of 2 mM FeCl₂ and 0.2 ml of 5 mM 3-(2-pyridyl)-5,6 bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 20 min at room temperature. In the next step, absorbance of the mixture was recorded at 562 nm. Finally, metal-chelating activity of treatment was calculated using the following formula. To make the control, distilled water was used instead of the sample (Decker and Welch, 1990). BHA at 200 ppm was used as a positive control:

$$\text{Metal-chelating activity (\%)} = [(1 - A_{562} \text{ of sample}) / A_{562} \text{ of control}] \times 100$$

Statistical analysis

All tests were performed in three replications and the results were reported as average±standard deviation. Normality and homogeneity of data were assessed using Kolmogorov

Smirnov and Levene test. Data analysis was performed using SPSS₂₁ software and graphs were drawn using Microsoft Excel (2013 version). The data were analyzed by one-way analysis of variance (One-Way ANOVA) and differences among the means were evaluated by Duncan's test at a 95% confidence level ($p < 0.05$).

Results

Physicochemical properties

particle size

Figure 1 shows average particle size of nanoliposomes in different treatments. Particle size varied from 228.9±4.85 (uncoated nanoliposomes or NP) to 436.7±1.08 nm (nanoliposomes with a coating of 0.5% chitosan or NP-CH-0.05). As shown in Figure 1, with increasing amount of chitosan in nanoliposomes particle size increased significantly ($p < 0.05$).

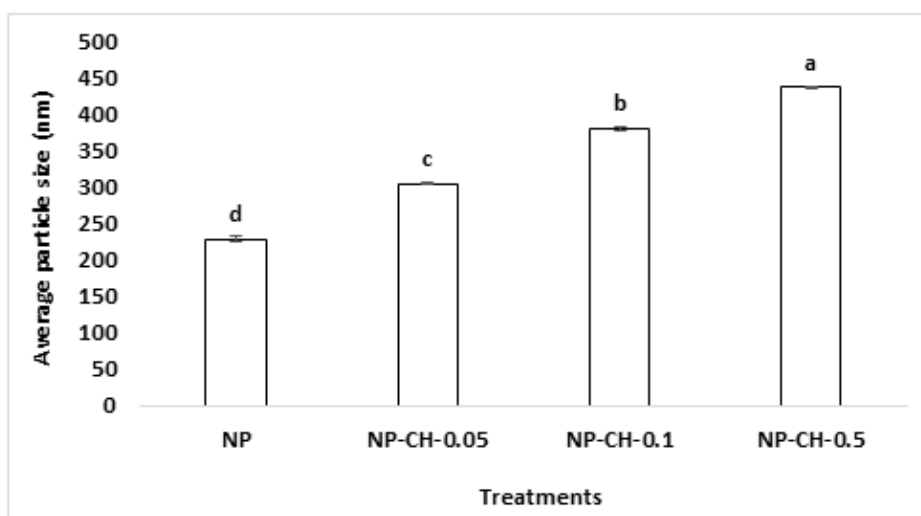


Figure 1: Particle size of different treatments. Different letters indicate significant difference among treatments ($p < 0.05$). Error bars represent standard deviation.

Particle dispersity index

Figure 2 shows particle dispersity index of research treatments. According to this figure, particle dispersity index varied from 0.389 ± 0.01 to 0.453 ± 0.01 in treatments. As shown in Figure 2, there was no significant relationship between

chitosan concentration in nanoliposomes and particle dispersity index. The lowest ($p<0.05$) particle dispersity index was related to NP (0.389 ± 0.01) and NP-CH-0.05 (0.391 ± 0.03).

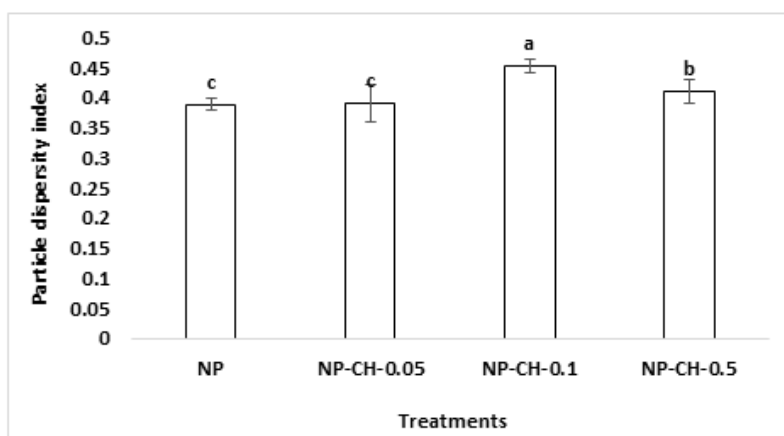


Figure 2: Particle dispersity index of treatments. Different letters indicate significant difference among treatments ($p<0.05$). Error bars represent standard deviation.

Zeta potential

Figure 3 shows zeta potential of nanoliposomes. As shown in this figure, zeta potential ranged from -62.59 ± 4.36 mV in uncoated nanoliposomes (NP) to 56.94 ± 3.71 mV in nanoliposomes containing 0.5% chitosan (NP-CH-0.5)

and all research treatments were significantly different in terms of this index ($p<0.05$). Also, with increasing chitosan concentration in nanoliposomes, zeta potential of particles increased significantly in a positive direction ($p<0.05$).

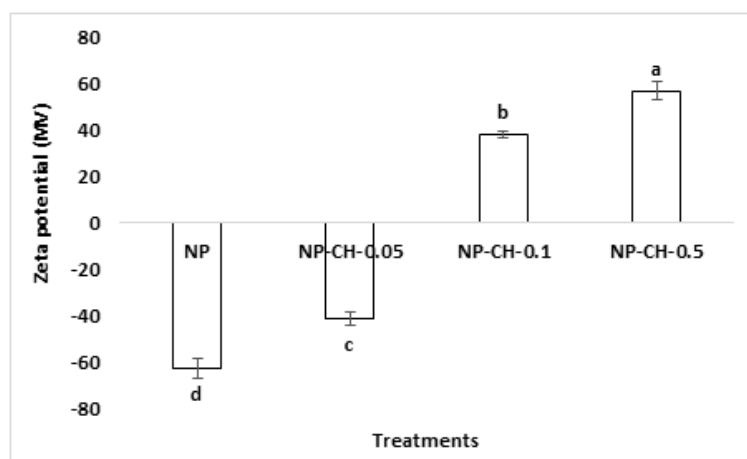


Figure 3: Zeta potential of different treatments. Different letters indicate significant difference among treatments ($p<0.05$). Error bars represent standard deviation.

Encapsulation Efficiency (EE)

Figure 4 shows encapsulation efficiency of different treatments. This index varied from $55.27 \pm 4.39\%$ (in NP) to $94.12 \pm 3.73\%$ (in NP-CH-0.5). Encapsulation efficiency in chitosan-coated treatments was significantly

higher compared to NP treatment (no chitosan coating), and also with increasing chitosan concentration in nanoliposomes, encapsulation efficiency increased significantly ($p < 0.05$).

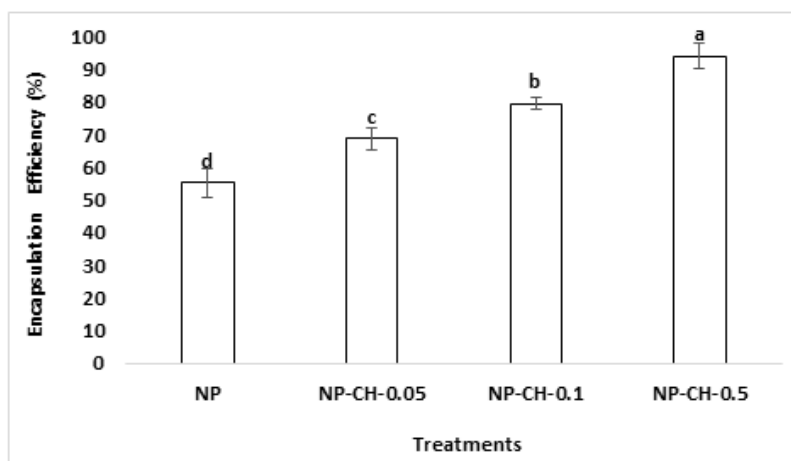


Figure 4: Encapsulation efficiency of different treatments. Different letters indicate significant difference among treatments ($p < 0.05$). Error bars represent standard deviation.

Profiles of release of peptides in simulated gastric fluid (SGF)

Figure 5 shows amounts of bioactive peptides released from the capsules over time in the simulated gastric fluid (SGF). In uncoated capsules (NP treatment), amount of peptide released after half an hour was $50.39 \pm 2.56\%$. This amount was 30.18 ± 0.89 , 28.96 ± 1.66 , and $10.34 \pm 2.18\%$ for NP-CH-0.05, NP-CH-0.1, and NP-CH-0.5 treatments, respectively. As shown in Figure 5, in all treatments after half an hour, release slope of peptides decreased. After 2 hours, the highest and lowest peptide release amounts were recorded in NP ($65.04 \pm 0.36\%$) and NP-CH-0.5 ($16.37 \pm 0.16\%$) treatments ($p < 0.05$). Peptide release profiles in NP-CH-0.05 and NP-CH-0.1 treatments were almost

similar over time ($p > 0.05$). The results showed that with addition of chitosan to nanoliposomes, release rate of peptides decreased significantly over time ($p < 0.05$).

Profiles of release of peptides in simulated intestinal fluid (SIF)

Figure 6 shows release profile of peptides in simulated intestinal fluid. According to this figure, in all hours the lowest peptide release was related to NP-CH-0.5 treatment ($p < 0.05$). Also, the highest amount of peptide release in all hours was recorded in NP treatment ($p < 0.05$). NP-CH-0.05 and NP-CH-0.1 treatments were not significantly different in terms of peptide release at different hours ($p > 0.05$). After 4 hours, the amount of peptide released from NP-

CH-0.5 treatment was $36.93 \pm 0.23\%$. These amount were reported as $49.85 \pm 0.56\%$, $51.38 \pm 0.64\%$, and $80.47 \pm 0.19\%$ for NP-CH-0.1, NP-CH-0.05, and NP treatments, respectively.

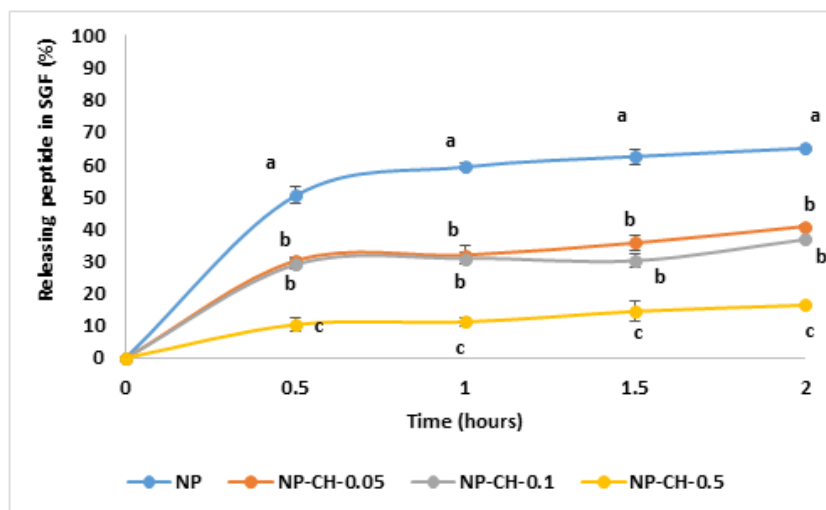


Figure 5: Amounts of peptides released from capsules at different times in SGF (peptide release profile). Different letters indicate significant difference among treatments ($p < 0.05$). Error bars represent standard deviation.

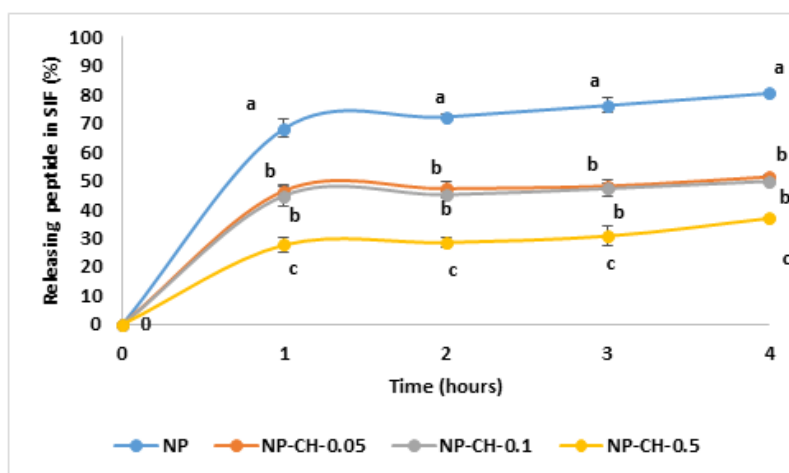


Figure 6: Amounts of peptides released from capsules at different times in SIF (peptide release profile). Different letters indicate significant difference among treatments ($p < 0.05$). Error bars represent standard deviation.

Antioxidant activity

Table 2 shows reducing power, hydroxyl radical scavenging activity and metal-chelating activity of different treatments. As can be seen in this table, these three indicators were not significantly different in free peptide and NP treatment ($p < 0.05$). According to the

results, nanoliposomes coated with chitosan had more antioxidant properties ($p < 0.05$) than nanoliposome without chitosan coating (NP treatment). In these treatments (nanoliposomes coated with chitosan), with increasing chitosan concentration, reducing power, hydroxyl radical scavenging activity and metal-

chelating activity increased significantly ($p<0.05$) and the highest amount of these three indicators was recorded in NP-CH-

0.5 treatment (Abs 0.947 ± 0.02 at 700nm, $79.12\pm 2.34\%$ and $33.42\pm 1.07\%$, respectively).

Table 2: Antioxidant activity of different treatments. Different letters in each column indicate significant difference among treatments ($p<0.05$).

Treatments	Reducing power activity (Abs at 700nm)	Hydroxyl radical scavenging activity (%)	Metal-chelating activity (%)
Free peptide	0.739 ± 0.02^d	42.21 ± 1.56^d	19.74 ± 0.92^d
NP	0.742 ± 0.08^d	41.96 ± 2.05^d	20.08 ± 1.44^d
NP-CH-0.05	0.861 ± 0.03^c	63.38 ± 0.99^c	25.81 ± 0.59^c
NP-CH-0.1	0.895 ± 0.01^b	64.05 ± 1.82^c	26.01 ± 0.63^c
NP-CH-0.5	0.947 ± 0.02^a	79.12 ± 2.34^b	33.42 ± 1.07^b
BHA	0.95 ± 0.04^a	92.68 ± 0.51^a	62.15 ± 1.86^a

Discussion

One of the most important physical properties of nanoliposome systems is average particle size. Particle size of nanoliposomes affects particle stability, encapsulation efficiency, and release rate of bioactive compounds. This index in treatments of the current study was at an ideal level compared to other studies. Da silva *et al.* (2014) reported an average particle size of phosphatidylcholine nanoliposomes containing antimicrobial peptides to be 150 nm. Particle size of liposomes in study of Hosseini *et al.* (2017) was 131.5 nm. Mean particle size of peptides, produced from common carp¹ hydrolysis, coated with nanoliposomes and chitosan varied from 333.9 to 533.9 nm (Ojagh *et al.*, 2022). Also, particle size of chitosan-coated nanoliposomes containing PF30 peptide fraction from rainbow trout skin gelatin was reported to be 169.2 to 234 nm (Ramezanzade *et al.*, 2017).

In the current study, with increasing concentration of chitosan in

nanoliposomes, particle size increased significantly. This finding is consistent with results of research by Ramezanzade *et al.* (2017) and Sadeghian *et al.* (2020). This result is due to strong bond between nanoliposome and chitosan, which forms a layer around the nanoliposome; therefore, particle size increases (Henriksen *et al.*, 1994). In another study, in contrast to the present study, particle size became smaller as concentration of chitosan in nanoliposome increased (Hasani *et al.*, 2019). The reason for the decrease in particle size is contraction force can be due to ionic resistance between peptide-carrier liposomes and chitosan-coated liposomes. So that when concentration of chitosan increases, contractile force increases and particle size becomes smaller (Bang *et al.*, 2011).

Particle dispersity index is an indicator of uniformity of the colloidal system. Higher values of this index indicate presence of large and non-uniform particles and masses in colloidal systems (Romero-Pérez *et al.*, 2010).

¹ *Cyprinus carpio*

Values recorded for this index in the present study showed that particles were evenly distributed. Particle dispersity index of nanoliposomes systems in other studies was also in the same range (Ramezanzade *et al.*, 2017; Hasani *et al.*, 2019; Sadeghian *et al.*, 2020; Ojagh *et al.*, 2022). Particle dispersity index was 0.25 in a study in which fish collagen-derived peptides were encapsulated using a liposomal system using relatively pure phosphatidylcholine (Mosquera *et al.*, 2014). Particle dispersity index in liposomal nanocarriers containing antioxidant peptides resulting from hydrolysis of muscle and wastes of fish using fluorzyme varied from 0.197 to 0.298 (Da Rosa Zavareze *et al.*, 2014). In other studies similar to the current research, there was no significant relationship between chitosan amount in coating and particle dispersity index (Ramezanzade *et al.*, 2017; Hasani *et al.*, 2019).

Zeta potential is the best indicator for determining surface electrical status of colloidal systems. Value of this index indicates the level of electrostatic and physical stability of nanocarrier systems. Reducing zeta potential difference below the critical value destroys charged double layer around the particles and, as a result aggregation of particles. When the total electric charge on particles increases, particles repel each other (no deposition of nanoliposomes) and the system becomes stable (Mozafari *et al.*, 2006). Particles with a zeta potential of more than +30 mV or less than -30 mV have the highest level of stability (Hasani *et al.*, 2019). In the current

research, with increasing chitosan concentration in nanoliposomes, zeta potential of particles increased from -41.3 mV (in NP-CH-0.05) to 56.94 mV (in NP-CH-0.5). In fact, with increasing chitosan concentration in the coating, this index moved towards positive values. The reason for this change is that chitosan is a cationic polysaccharide. This finding is consistent with findings of Guo *et al.* (2003), Li *et al.* (2015), Ramezanzade *et al.* (2017) and Ojagh *et al.* (2022). Increasing zeta potential of particles after coating of nanoliposomes using different amounts of chitosan increases the repulsive force between particles and thus reduces physical instability of the system. Also, chitosan layer around the nanoliposome reduces release rate and loss of loaded compounds (Da Rosa Zavareze *et al.*, 2014; Ramezanzade *et al.*, 2017).

Encapsulation efficiency is an important indicator of stability of encapsulated compounds. Because it indicates the ability of nanoliposome to prevent inner core from leaving. Presence of a chitosan layer around nanoliposomes increases encapsulation efficiency index by preventing peptides from leaving liposomal membrane (Li *et al.*, 2015). Increased encapsulation efficiency with increasing chitosan concentration in nanoliposomes was reported in the studies of Li *et al.* (2015), Hasani *et al.* (2019) and Ojagh *et al.* (2022) which is consistent with results of the current research. In another research, with increasing chitosan concentration from 0 up to 0.2 and 0.4%, encapsulation efficiency index also increased (68.5,

73.5, and 80.2%, respectively); but, with increasing chitosan concentration from 0.4 to 0.8 and 1%, this index had a decreasing trend, 55.2 and 46.1% (Ramezanzade *et al.*, 2017). The highest encapsulation efficiency index in the current study was $94.12 \pm 3.73\%$. This index was recorded 80.4 and 79.3% in liposomes containing hydrolyzed protein produced from muscle and by-products of fish¹ by Flavourzyme, respectively (Da Rosa Zavareze *et al.*, 2014). The maximum encapsulation efficiency in peptides, from Atlantic salmon² hydrolysis, coated with liposomes and chitosan was 71.3% (Li *et al.*, 2015).

One of the main objectives of the present study was to build a resistant capsule around bioactive peptides that, in addition to affect the release rate, maintain peptides in unfavorable environmental conditions of gastrointestinal tract, so that these peptides can exert their physiological effects after entering the blood. Results of the current research showed that nanoliposomes in combination with chitosan form capsule around peptides, these capsules remain stable in acidic and enzymatic conditions of gastrointestinal tract (SGF and SIF). Other studies that encapsulated bioactive peptides using nanoliposomes and chitosan and release them in SGF and SIF, reported similar results (Li *et al.*, 2015; Hasani *et al.*, 2019). Uncoated nanoliposomes are relatively stable in

acidic environments (Li *et al.*, 2015). But, in environments with a pH below 6.5, phospholipids are hydrolyzed and thus the structure of the nanoliposome is destroyed and its contents are released (Grit *et al.*, 1993). According to the results, presence of a chitosan layer around nanoliposome prevents its degradation in acidic environments. For this reason, in the current research chitosan-coated treatments released lower amounts of peptides (over time) compared to the uncoated treatment (NP). Lower stability of uncoated nanoliposomes compared to chitosan-coated nanoliposomes in simulated intestinal fluid was due to presence of pancreatin, phospholipase and esterase (Liu *et al.*, 2013). These enzymes destroy the structure of nanoliposomes by phospholipids hydrolysis, as a result, peptides are released.

Antioxidant properties were almost equal in free peptides and peptide-carrying nanoliposomes (without chitosan coating). This finding is consistent with results of Ramezanzade *et al.* (2017) and Hasani *et al.* (2019). However, in some studies, unlike the current research, encapsulation of bioactive peptides in form of nanoliposomes significantly reduced their antioxidant properties (Mazloomi *et al.*, 2020). The fact that encapsulation of bioactive peptides by nanoliposome did not affect antioxidant activity of bioactive peptides is considered a positive feature. Because with this

¹ *Micropogonias furnieri*

² *Salmo salar*

technique, peptides are protected from possible unfavorable conditions in the process of production and storage of food, while they can exert their antioxidant effects. Increasing antioxidant activity of peptides by adding chitosan, a substance with antioxidant properties, to loaded nanoliposomes indicates that this product can be potentially used as an antioxidant in various foods.

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