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Extended Abstract

The Production and Evaluation of Nanoliposomes Containing Bioactive Peptides Derived from Fish Wastes Using the Alkalase Enzyme

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Abstract

In recent years, several studies have focused on the production of bioactive peptides from fish waste due to its beneficial effects on human health. Bioactive peptides with low molecular weight require protective methods to increase gastrointestinal stability and active absorption, controlled release and optimized efficacy during oral delivery. The aim of this study was to develop an oral phospholipid nanoliposomal system incorporated with bioactive peptides derived from fish protein hydrolysate (FPH) of common carp (*Cyprinus carpio*) in chitosan coating (0.05, 0.1, 0.5% w/v) by the alkalase enzyme. The results showed that chitosan coating greatly improved the stability of nanoliposomes. The average particle size was in the range of 339-459 nm with a zeta potential of -51.7 to +50 and a poly dispersity index (PDI) of 388- 0.487 in nanoliposomes. The Encapsulation Efficiency (EE%) values were significantly influenced by changes in the concentration of chitosan and the maximum EE% (86± 2.65) was observed in the nanoliposome coated with 0.5% chitosan. Studying the releasing rate of the peptide in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) showed the effect of coating on the stability of peptides in simulated biological environments. Also, the evaluation of antioxidant activity by DPPH and ABTS tests showed that there was a high activity of radical scavenging activity in nanoliposomes in chitosan-coated and non-coated samples. The results of this study showed that encapsulation of bioactive peptid in the liposomal system could be a useful approach for direct application of peptides with antioxidant potential in food products.

Keywords: Antioxidant Activity, Bioactive Peptide, Chitosan, Nanoliposome, Release

Introduction

Bioactive structural compounds derived from marine resources with anti-bacterial, antioxidant, anticancer, low blood pressure potential can be used to produce functional food to enhance the level of consumer health and improve the shelf-life of food products. Newly, much consideration has been paid to bioactive peptides extracted from fish hydrolyzed proteins (FPH) as functional components in the diet and in pharmaceuticals (Nasri *et al.*, 2013);

(Galla, Pamidighantam, Akula, & Karakala, 2012). The alkalase enzyme, has a high potential for hydrolysis of protein sources with relatively low cost among other enzymes (Diniz & Martin, 1996). The FPH has a good solubility in a wide range of pHs and usually sustains under heating process without deposition. In addition, it improves textural and emulsifying properties, freshness, water holding capacity and stability of food products (Taheri, Sabeena Farvin, Jacobsen, & Baron, 2014). Antioxidative peptides have also been extensively investigated as potential biopreservatives in food technology (Lassoued *et al*., 2015; Nalinanon, Benjakul, Kishimura, & Shahidi, 2011; Razali, Amin, & Sarbon, 2015; Vignesh, Haq, Devanathan, & Srinivasan, 2011). Bioactive peptides may be denatured and lose their bioactivities in the acidic environment of the stomach; proteolytic enzymes in the stomach and intestine will also degrade small peptides; and as discussed above, unpalatable bitterness of hydrophobic peptides also hinders the application of protein hydrolysates into functional food products (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). Encapsulation can be expressed as a key technique to package sensitive ingredients in the form of micro and nano-particles within different wall materials, control the release of valuable compounds, lower flavor loss during storage and raise the stability (Ghorbanzade, Jafari, Akhavan, & Hadavi, 2017). The use of lipid-based carrier systems such as liposomes (i.e. nanometric surfactant micelles) has been studied in enhancing the bioavailability and biostability of food-bioactive constituents. However, lipid-based delivery systems are not suitable due to instability an acidic gastric environment of bile salts and lipase (Page & Cudmore, 2001). Many attempts have been made to overcome the stability problem of liposomes. Mucoadhesive polymer systems, like chitosan, are the most promising approach for enhancing liposomal delivery of bioactive peptides orally (Hosseini, Ramezanzade, & Nikkhah, 2017). Therefore, according to the high amount of fish waste in the country, as well as the production potential of bioactive compound from these materials, the purpose of this study was to examine the possibility of producing and evaluating nanoliposomes containing antioxidant bioactive peptides to overcome the challenges of using these valuable compounds in the food and pharmaceutical industries.

Material and methods

In order to obtain the FPH, the common carp (*Cyprinus carpio*) byproduct were subjected to a hydrolysis reaction alcalase protease for obtaining protein hydrolysates (Ovissipour *et al*., 2009). Degree of hydrolysis (DH%) in the sample with 20% trichloroacetic acid (TCA) was evaluated according to Ovissipour *et al*. (2009). Hydrolysis (DH%) in the samples was 43.5±0.21%.

For preparation of nanoliposomes, Protein Hydrolysate (10 mg/mL) and phospholipid/cholesterol were hydrated in distilled water and heated to 80-60 °C with glycerol. Crude liposomes with different ratios (0.05, 0.1 and 0.5% w/v) of chitosan solution was mixed at room temperature for 1 h at about 200 rpm. The liposomal solution was sonicated and stored at room temperature for the next analysis (Rasti, Jinap, Mozafari, & Yazid, 2012).

Mean particle size, size distribution (Polydispersity Index; PDI) and zeta potential of nanoliposome were determined using a laser particle size distribution analyzer (Malvern Instruments, UK) at 25 °C.

The encapsulation efficiency was defined as the ratio of encapsulated FPH to free FPH *x* 100 as determined by using the Bicinchoninic Acid (BCA) Protein Assay Kit (Li, 2014). In order to assess the stability and protective effects imparted by the CH-coating layer, coated and uncoated liposome formulations were incubated in simulated-gastric-fluid (SGF) and simulated-intestinal-fluids (SIF) (Agrawal, Harde, Thanki, & Jain, 2013). A volume of 20 μL

of coated and uncoated liposome formulations was diluted to 1 mL in micro centrifuge tubes with both simulated fluids and incubated at 37 °C with continuous shaking. Incubation times of up to 2 h for SIF, and up to 4 h for SGF were used. At each interval, the FPH content was estimated following the BCA Protein Assay. DPPH free radical scavenging capacity of the samples was measured according to a modified method of (Taheri *et al*., 2014) and ABTS radical scavenging activity was determined using the procedure proposed by (Wang, Li, Chi, Zhang, & Luo, 2012) to evaluate Antioxidant activities of nanoliposomes.

Results and discussion

The size distribution of nanoliposome containing FPH was shown in table 1. Mean diameter and poly dispersity index (PDI) of nanoliposomes were in range 334.5 to 459.1 nm and 0.387 to 484.1 respectively, which is similar to (Rasti *et al*., 2012) which reported size range of liposome. The results showed that the particle size of nanoliposomes were decreased by increasing the chitosan concentration. This shrink force is produced by the ionic interaction between the CH-coating and the loaded-liposomes. As CH concentration increases, the shrink force also grows, which leads to further size reduction (Bang *et al*., 2011). Zeta potential is a physical property of particles in suspension that can be used to optimize suspension stability. In the present study, the mean zeta potential of nanoliposome was varied (-51.7 mV up to +50) in the uncoated and coated nanoliposom with 0.5% chitosan concentration. It increased rapidly to above +50 mV regardless of the initial phospholipid content. This change in zeta potential was perhaps caused by the ionic attraction between positively charged chitosan amino groups and the negatively charged liposome surface, indicating the successful coating of CH onto liposome surface.

The encapsulation efficiency (EE) of FPH in the uncoated and coated liposomes with different concentration of chitosan was shown in Table (1), the addition of CH concentration increased the EE for all three for all formulation. It was found that Lower encapsulation efficiency in uncoated nanoliposomes can be attributed to the damage caused by ultrasound-centrifugation. This results were in agreement with (Drusch *et al*., 2012).

Table 1. Effect of different levels of chitosan (0.01, 0.05, 0.5%) on physical properties of nanoliposomes

	Zeta potential	PDI	particle size (nm)	EE%
Uncoated nano-liposoms	-51.67 ± 6.51	0.44 ± 0.01	459.10 ± 0.46	57.33 ± 6.11
Coated with 0.05% CH	-32.00 ± 2.00	0.39 ± 0.00	429.30 ± 2.08	60.00 ± 6.24
Coated with 0.1% CH	30.67 ± 1.15	0.48 ± 0.00	384.90 ± 4.36	75.00 ± 3.61
Coated with 0.5% CH	50.33 ± 2.52	0.42 ± 0.01	334.50 ± 4.14	86.00 ± 2.65

Mean particle size, polydispersity index (PDI), encapsulation efficiency (EE %) and zeta potential (mV) values of nanoliposomes (Mean ± Standard Deviation).

The in vitro release profiles obtained with different formulations are shown in Fig. (1) and (2). All treatments displayed a similar release profile. At each time interval, the SPH release rates were significantly reduced by the addition of the CH-coating layer (P<0.05). The initial burst release within the first 30 min was likely due to desorption of the absorbed SPH from the liposome surface. The gradual release after 1 h of digestion was more likely due to the diffusion of the FPH through the coating layers via the hydrocarbon portion of the membrane and the pores within the membrane (Kuboi, Shimanouchi, Yoshimoto, & Umakoshi, 2004). Generally, uncoated liposomes are relatively stable in acidic environments (Li, 2014). CH-coated liposomes were found to be more stable in both SGF and SIF than uncoated liposomes, perhaps attributable to the formation of a robust protective coating layer by strong electrostatic attraction between the chitosan and the surfaces of the liposomes, preventing the exposure of liposomes to the external environment.

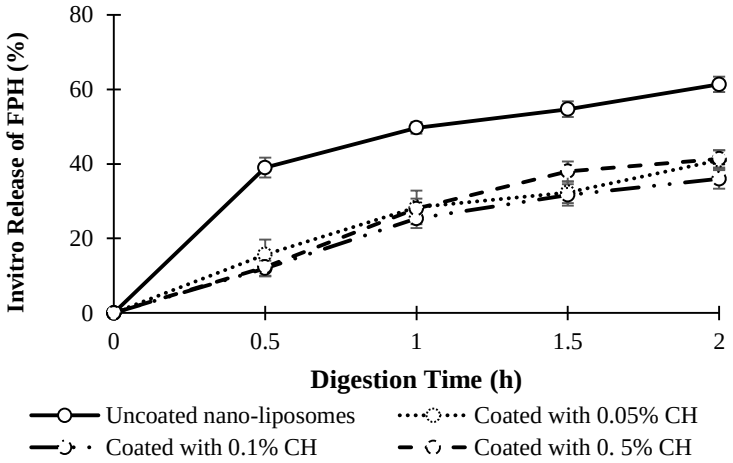

Fig. 1. Invitro release of FPH in uncoated and coated nanoliposomes in CH (0.05, 0.1 and 0.5%) in SGF

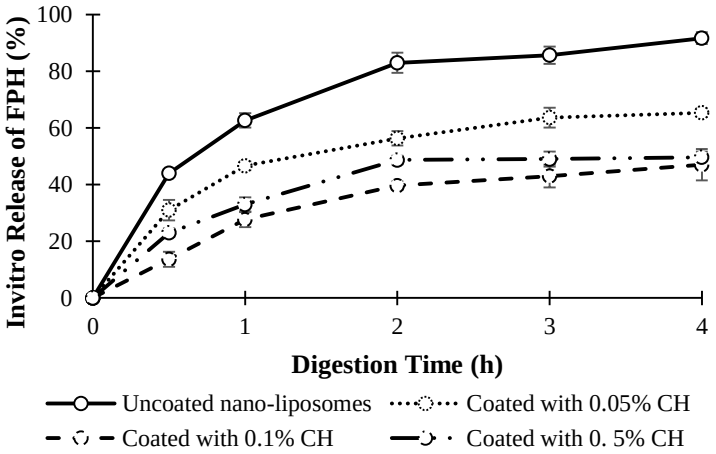

Fig. 2. Invitro release of FPH in uncoated and coated nanoliposomes in CH (0.05, 0.1 and 0.5%) in SIF

The results of this study clearly showed that the hydrolyzed protein obtained from the common carp byproduct showed an appropriate antioxidant activity against free radical scavenging tests (Fig. 4), which was in agreement with Hosseini *et al*. (2017). The antioxidant activity of FPH is significantly related to high levels of hydrophobic amino acids, which increases lipid solubility and thus increases anti-oxidant activity (Mosquera *et al*., 2014). According to the results, there was no significant difference between free radical inhibitory ability in free peptides and nanoliposomes containing peptide. The results indicate a positive correlation between chitosan levels and free radical inhibitory effects (Fig. 3 and 4). However, there was no significant difference in the treatment of free and nanoliposomal peptide.

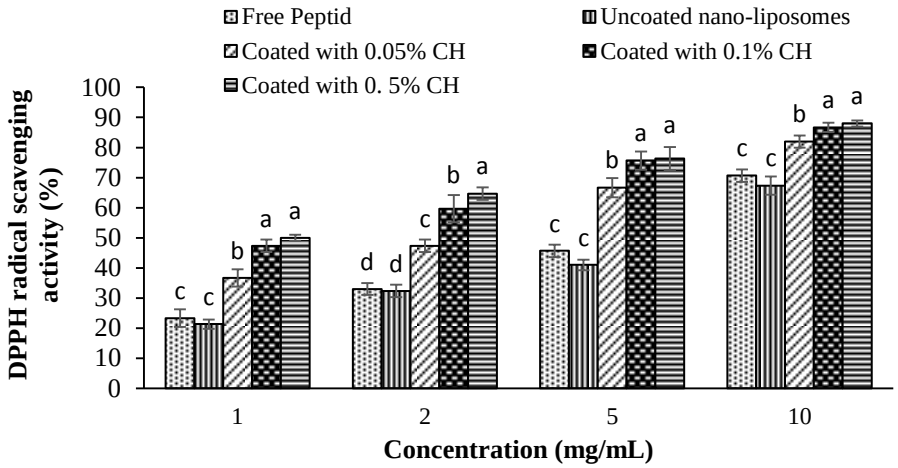

Fig. 3. DPPH radical scavenging activity of peptide nanoliposomes

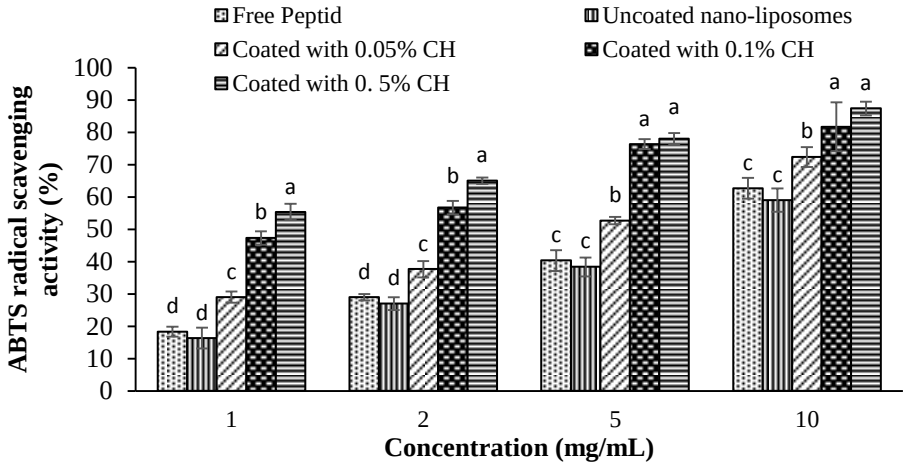

Fig. 4. ABTS radical scavenging activity of peptide nanoliposomes

Conclusion

The results showed that encapsulation of peptides as a bioactive compound in the liposome has the potential to overcome proteolytic degradation and the reaction with other ingredients in the food that is important in direct use of nanoliposomal peptides in foods.

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