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One-pot nanoparticulation of potentially bioactive peptides and gallic acid encapsulation

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ABSTRACT

Whey protein isolate was hydrolyzed to an *in vitro* antioxidative hydrolysate, followed by transglutaminase-induced cross-linking and microemulsification in an oil phase. The obtained microemulsion was then dispersed in a gallic acid-rich model wastewater which caused gallic acid transportation into internal nanodroplets. Whey peptides were consequently gelled, yielding nanoparticles. Electrophoresis showed that β-lactoglobulin and low molecular weight peptides were cross-linked by transglutaminase. Protein hydrolysis and subsequent enzymatic cross-linking increased the ζ-potential value. Microscopic investigation indicated that most particles were non-spherical. Non-cross-linked and cross-linked peptides underwent a form of heat-triggered self-assembly in the dry state, while nanoparticles did not show such behavior. Peptide crystallites size was increased by cross-linking and acid-induced particle formation. The latter also caused a reduction in intensity of C–H stretching and C–N bending peaks in infra-red spectrum. Gallic acid release from particles to simulated gastrointestinal fluids was through diffusion from swollen particles, and reached almost 70% release.

1. Introduction

Multiple bio-functionalities of whey protein hydrolysate (WPH), including anticardiovascular diseases activity, and immunomodulatory, antioxidative, antihypertensive, antidiabtic and antiallergenicity effects, (Madureira, Tavares, Gomes, Pintado, & Malcata, 2010; Urista, Fernandez, Rodriguez, Cuenca, & Jurado, 2011) have persuaded researchers to employ WPH in diverse products. In addition to formulation applications, whey protein-based peptides are promising for the formation of nutraceutical-carrying particles. Recently, for example, Bagheri, Madadlou, Yarmand, and Mousavi (2014) successfully generated potentially bioactive caffeine-loaded particles from WPH via an antisolvent addition method.

Cold gelation is customarily used for fabrication of particles from globular proteins at ambient temperature by (micro) emulsification-internal gelation procedure (Sadeghi, Madadlou, & Yarmand, 2014). In this method, a globular protein is heat-denatured at pH values far from its isoelectric point, followed by water-in-oil emulsification and in situ gelation through either ions or pH-alteration (Alting et al., 2004; Martin & de Jong, 2012). In spite of the abovementioned desirable bio-functionalities, WPHs are much less capable of forming a gel network than whey proteins. The drawback is due most probably to the inferior association and lattice-building competence of peptides compared to proteins. This has limited the value of WPH as a carrier-forming ingredient.

Transglutaminase-induced cross-linking of denatured whey proteins can reinforce the structure of the resultant cold-set hydrogels through catalyzing acyl transfer reactions between the γ-carboxyamide group of glutamine residues and ε-amino groups of lysine (Gaspar & de Goes-Favoni, 2015; Eissa, Bisram, & Khan, 2004). Abaee and Madadlou (2016) indicated that the time required for gelation initiation decreased dramatically by enzymatic cross-linking of whey proteins.

Phenolic compounds are common organic contaminants found in the wastewaters of some food industries such as pickled vegetables, fish processing, meat canning, and oil refining units, ranging from one to several hundred milligrams per liter (Chiaiese et al., 2011; Edalatmanesh, Mehrvar, & Dhib, 2008). In addition to the environmental pollution concerns, phenolic compounds recovery from wastewaters can provide a resource for a class of nutraceuticals that are being included within health-promoting products.

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One of the interesting and efficient techniques for removal of soluble species from aqueous solutions is the emulsion liquid membrane (ELM). In this method, a water-in-oil (W/O) emulsion is dispersed in the wastewater intended for decontamination through continuous mixing (Mortaheb, Amini, Sadeghian, Mokhtarani, & Daneshyar, 2008). The solute of interest is conveyed from the external phase (wastewater) across the membrane oil phase by surfactant reverse micelles or a specific carrier into the internal aqueous phase, where it is concentrated over time. Lately, Garavand and Madadlou (2014) used ELM and microemulsion liquid membrane (MLM) extractors for recovery of phenolic compounds from a model pistachio processing wastewater. They found that MLM was more efficient compared to the conventional ELM extractor. Sadeghi et al. (2014) used the aqueous nanodroplets of a W/O microemulsion as template to form nanoparticles by gluconic acid-induced cold gelation of whey proteins isolate (WPI).

The aim of this work was to combine the advantages of phenolic acids recovery by the MLM technique and microemulsion-based nanoparticle fabrication technique. For this purpose, an *in vitro* antioxidative WPH was microemulsified in an oil phase, followed by dispersing the W/O microemulsion at an external water phase (a model wastewater rich of gallic acid) which caused peptide-rich droplets transformation to peptidic nanoparticles due to (gallic) acid-induced cold gelation. The generated particles not only carried gallic acid but were also potentially bioactive by themselves. Peptides were cross-linked enzymatically by transglutaminase before microemulsification to enhance the acid-induced gelation property of WPH, and to reinforce the structure of generated particles. Accordingly, a one-pot procedure was designed by which gallic acid was recovered from a model wastewater using a MLM extractor and encapsulated within WPH particles. The characteristics of the microemulsion and those of generated particles were assessed.

2. Materials and methods

2.1. Materials

Whey protein isolate (DI-9224) with 92% (w/w) protein content was kindly gifted by Arla Food Ingredients (Viby J, Denmark). The enzyme transglutaminase (activity $\geqslant$ 1500 U/g), pepsin (activity $\geqslant$ 2500 U/g), pancreatin (4 $\times$ USP (United States pharmacopeia)), gallic acid, sorbitan monooleate (Span 80), β-mercaptoethanol and O-phthaldialdehyde (OPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and purchased from Fisher BioReagent (New Jersey, USA).

2.2. Preparation and cross-linking of WPH

The WPI powder was dissolved in distilled water (50 mg mL^{-1}) along with sodium azide (0.2 mg mL^{-1}) as antimicrobial agent. The protein solution was stirred at 500 rpm for 120 min at 25 °C and stored overnight at 4 °C to ensure complete hydration. Afterwards, the solution was heated at 80 °C for 5 min and quickly cooled to room temperature (25 °C) under trapping water. The pH value of the protein solution was adjusted to 2.0 with 2 M HCl and pepsin was added at a substrate-to-enzyme mass ratio of 160:1. The pH was controlled and kept constant during the hydrolysis. The solution was then shaken at 100 rpm for 30 min at 37 °C. The pH adjustment was done during the hydrolysis. Subsequently, pepsin was inactivated by adjusting the pH of protein hydrolysate to 8.0 with 2 M NaOH and heating at 90 °C for 15 min. The hydrolysate was then rapidly cooled to 30 °C under running water (Adjonu, Doran, Torley, & Agboola, 2013). The degree of hydrolysis (DH) was determined by assaying free amino groups content by the OPA method as described by Nielsen, Petersen, and Dambmann (2001). Cross-linking of the WPH solution by transglutaminase was performed at the optimized condition outlined by Eissa et al. (2004) for whey proteins. Peptidic solution was incubated with transglutaminase (800 μL/100 g peptidic solution) at pH 8.0 and 50 °C for 20 h while being shaken at 120 rpm. The enzymatic reaction was stopped by heating at 60 °C for 15 min followed by cooling down the solution to 4 °C in an ice bath (Báez, Moro, Ballerini, Busti, & Delorenzi, 2011).

2.3. Reducing power assay

The *in vitro* antioxidant activity of samples was measured by reducing power method described by Yildirim, Mavi, and Kara (2001). Briefly, 1 mL of diluted samples (20 mg mL^{-1}) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (10 g L^{-1}) and then solutions were incubated at 50 °C for 20 min. Then, 2.5 mL trichloroacetic acid (100 mg mL^{-1}) was added and the mixture was centrifuged at 6000*g* for 10 min (refrigerated centrifuge model 8k, Sigma, SciQuip Ltd., Newtown, United Kingdom). Afterwards, 2.5 mL of the centrifugal supernatant was mixed with 2.5 mL distilled water and 0.5 mL ferric chloride (1 mg mL^{-1}) and the absorbance was recorded at wavelength of 700 nm. The higher the absorbance, the stronger was the reducing power (Liu & Zhong, 2013).

2.4. Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted following the method described by Laemmli (1970) with 5–25% polyacrylamide gradient gel. Diluted WPI, WPH and cross-linked WPH solutions (5 mg mL^{-1}) were mixed with a buffer solution (25% glycerol, 2% SDS, 10% β-mercaptoethanol, 0.125 M Tris, pH 6.8) and heated for 5 min at 90 °C. Five μL of each sample per lane was loaded onto the gel with a protein molecular weight standard marker (~11–245 kDa) (Sinna Clon, Tehran, Iran). The protein bands were fixed and stained using a solution of 0.1% Commassie Brilliant Blue R-250.

2.5. Gallic acid extraction

The organic membrane phase of the MLM extractor was prepared by mixing Span 80 and sunflower oil at equal masses and was kept overnight (12 h) in a closed glass container at 30 °C to acquire a homogenous and clear blend. Then, the cross-linked WPH solution (50 mg mL^{-1}) was added drop wise (each time 50 μL) to the organic phase up to a final mass ratio of 4:96% (w/w) while stirring continuously at 500 rpm at 35 °C. The obtained water-in-oil microemulsion was transparent and macroscopically homogenous. Subsequently, the microemulsion was dispersed within the model wastewater (distilled water containing gallic acid at concentration of 200 mg L^{-1}, pH 3.77 ± 0.01 as external aqueous phase) at 1:3 ratio (v/v) and thoroughly stirred at 400 rpm for up to 10 min at 40 °C. Transportation of gallic acid from the exterior aqueous phase by surfactant reverse micelles into the internal peptide-rich aqueous phase converted the microemulsified nanodroplets to cold-set particles. Fig. 1 schematically illustrates the particle formation procedure. By the event of particles generation, the W/O microemulsion destabilized and split-up readily by centrifugation (Sorvall supper T21, Newtown, CT, USA) at 4000*g* for 5 min to an upper oil phase, a middle gel phase and a lower gallic acid-depleted aqueous phase The gel phase was washed three times with fresh petroleum ether to remove the rest of the oil/surfactant and then twice with double distilled water. Peptidic particles were recovered from wash water by centrifugation (5415C;