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Potentially bioactive and caffeine-loaded peptidic sub-micron and nanoscalar particles

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ABSTRACT

Whey proteins were hydrolyzed with pepsin to antioxidant peptides, cross-linked by transglutaminase and then particulated through ethanol addition. Reducing power assay indicated that cross-linking enhanced the antioxidant activity of hydrolysate. Particles prepared from non-hydrolyzed protein, peptides and cross-linked peptides were all of bimodal size distribution comprised from sub-micron (>100 nm) and nanoscalar (<100 nm) populations. The enzymatic cross-linking resulted in generation of more monodisperse particles and increased the volume fraction of nano-sized population. Scanning electron microscopy revealed an almost spherical morphology for samples and Fourier transform infrared spectroscopy confirmed the cross-linking of peptides. Heat-scanning of samples by a differential scanning calorimeter indicated that cross-linking did not affect the thermal behavior of particles. However, a reinforcing effect on particles was suggested for cross-linking based on the *in vitro* tests carried out at various digestion media. Cross-linking slowed down the release rate of entrapped caffeine from particles in a simulated gastric fluid.

1. Introduction

Whey protein products are widely used for a variety of functional and biological purposes in food formulations (Foegeding, Davis, Doucet, & McGuffey, 2002). The hydrolysate of whey proteins may contain bioactive peptides with antihypertensive, antioxidant and antithrombotic properties (Madureira, Tavares, Gomes, Pintado, & Malcata, 2010). It has been shown that whey protein hydrolysates are much more antioxidative than their unhydrolysed counterparts, indicating the essence of hydrolysis for liberation of antioxidant peptides (Adjonu, Doran, Torley, & Agboola, 2013). Preheating of whey portions prior to enzymatic hydrolysis results in structural changes due to intra- and inter-molecular disulfide interchanges and denaturation of proteins. The latter may induce re-orientation of peptide bonds and exposure of more aromatic and hydrophobic residues, which can enhance the hydrolysis of some globular proteins, such as β-lactoglobulin (Adjonu et al., 2013). Although whey proteins have been widely investigated as carriers for nutraceuticals and drugs (Chen & Subirade, 2005; Gunasekaran, Ko, & Xiao, 2007) there is no report in the literature about the application of whey protein-originated peptides as carrier-forming bricks in drug delivery systems.

One of the most commonly used approaches to control the release of encapsulated substance from protein nanoparticles and improve their thermal stability is cross-linking (Fuguet, van Platerink, & Janssen, 2007). Compared to aldehyde containing cross-linkers such as glutaraldehyde, the enzyme transglutaminase is preferred for proteins because provides a totally non-toxic approach for reinforcement of protein assemblies and particles. This enzyme catalyzes an acyl-transfer reaction introducing covalent cross-linkages between γ-carboxyl groups of glutamine residues and ε-amino

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groups of lysine residues of protein substrates (Soares, Albuquerque, Francine, & Marco, 2004). Although transglutaminase has been extensively applied to modify the technological and functional properties of food proteins (Babiker et al., 1998; Clare, Gharst, & Sanders, 2008; Watanabe, Suzuki, Ikezawa, & Arai, 1994), information about its effect on degradation stability and the control release character of nutraceutical-loaded nanoparticles from whey protein hydrolysates is lacking. The main objective of the present study was therefore to fabricate vehicles for caffeine (as model hydrophilic core) from the peptides obtained through hydrolysis of whey proteins and then cross-linked by transglutaminase. This is the first report on drug- and/or nutraceutical-loaded antioxidant peptidic particles. It was expected that enzymatic digestion of whey protein isolate with pepsin would result in a bioactive hydrolysate which was subsequently particulated through adding the antisolvent ethanol in order to encapsulate caffeine. The enzymatic cross-linking of hydrolysate prior to desolvation aimed to reinforce the structure of generated particles and preserve their integrity during incubation within a simulated gastric fluid.

2. Materials and methods

2.1. Materials

The lactose- and fat-free whey protein isolate (WPI) with 92% protein content was a commercial product kindly gifted by Arla Food Ingredients (Viby J, Denmark). The enzymes pepsin, trypsin and transglutaminase (activity $\geqslant$ 1500 U g^{-1}) were obtained from Sigma–Aldrich (Wicklow, Ireland). Ascorbic acid, ferric chloride, potassium ferricyanide, trichloroacetic acid, phosphate-buffered saline, sodium hydroxide, hydrochloric acid, sodium azide and absolute ethanol were purchased from Merck (Darmstadt, Germany). Caffeine was obtained from FTZ JC YUJIE international Inc. (Qingdao Shandong, China).

2.2. Preparation of peptidic particles

Whey protein isolate powder (10.5 g) was dissolved in 210 mL double distilled water containing 0.2 mg mL^{-1} sodium azide (giving a 5% w/v protein solution), and stirred at 500 rpm at room temperature (25–27 °C) for 2 h. The solution was allowed to hydrate at 4 °C overnight and then pre-heated at 80 °C for 15 min followed by quick cooling to the room temperature.

Hydrolysis of whey protein solution was carried out with pepsin following the method by Adjonu et al. (2013). To commence the peptic hydrolytic process, the pre-heated whey protein solution was adjusted to 2.6 with 2.0 M HCl. The enzyme pepsin was then added to ensure the pepsin to whey protein ratio of 1:40. The hydrolysis was performed for 12 h while shaken at 100 rpm throughout the hydrolysis process to avoid sedimentation of whey proteins. The solution was maintained at pH 2.6 with 2.0 M HCl during the entire hydrolysis period after which it was adjusted to pH 7.0 with 2.0 M NaOH. The hydrolysate was then heated at 90 °C for 15 min to inactive the enzyme pepsin and immediately cooled down to ambient temperature in an ice bath.

To prepare the enzymatically cross-linked peptides, the whey protein hydrolysate was supplemented with transglutaminase (70 μL g^{-1} protein) and incubated in a sealed container at 37 °C for 72 h while shaken at 120 rpm. A control non-cross-linked sample was also prepared by ageing a whey protein hydrolysate without added transglutaminase at the same condition. After the enzymatic cross-linking, caffeine was dissolved in cross-linked protein hydrolysate at mass ratio of 1:15 of caffeine to hydrolyzed protein. In order to prepare particles, absolute ethanol was added continuously at rate of 1 mL min^{-1} to the cross-linked and non-cross-linked protein hydrolysates, stirring at 500 rpm, until the solutions became just turbid. The amount of added absolute ethanol was approximately 1.1 mL per mL of whey protein hydrolysate. The hydrolysates were adjusted to pH 9.0 with 2 M NaOH just before desolvation with ethanol to obtain smaller particles (Bagheri, Madadlou, Yarmand, & Musavi, 2013). The generated particles were separated by centrifugation at 18,000*g* (refrigerated centrifuge model RS-20IV, TOMY SEIKO Co., Ltd., Tokyo, Japan) at 5 °C for 15 min. After centrifugation, the supernatant was removed and the sedimented particles were vacuum-dried at 60 °C. In the case of caffeine-loaded cross-linked particles, supernatant was kept for determination of encapsulation efficiency as described later. Vacuum-dried particles were then stored at −80 °C until analyses.

2.3. Reducing power assay

The antioxidant activities of why protein isolate and whey protein hydrolysates either being cross-linked or not were determined by reducing power method according to the procedure of Sharma and Kumar (2013). In this method, reductant species in the sample reduce the Fe^{3+}/ferricyanide complex to ferrous and Fe^{2+} concentration was then monitored by measuring the amount of formed Perl's Prussian blue at 700 nm. Increased absorbance of the reaction mixture represented a stronger reducing power. Briefly, 1 mL of each sample at different concentrations (0, 5, 10 and 30 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}). The mixture was incubated at 50 °C for 20 min and then 2.5 mL trichloro acetic acid (100 g L^{-1}) was added. The mixture was centrifuged at 6000*g* for 10 min after which, 2.5 mL of the supernatant was mixed with 2.5 mL double distilled water and 0.5 mL ferric chloride (1 g L^{-1}). The absorbance of specimen was measured at 700 nm by a UV/Visible spectrophotometer (CE2502, Cecil Instrument Ltd., England). Ascorbic acid (at concentration range of 5–10 mg mL^{-1}) and double distilled water were employed as standard and blank, respectively. Reducing power activity of samples was compared to that of ascorbic acid and is expressed as percentage of reducing power. For this purpose, slope of the absorbance-concentration curve of each sample was divided to that of ascorbic acid curve (Adjonu et al., 2013).

2.4. Particle size

Freshly prepared particles were dissolved in double distilled water at ratio of 1:400 (w/v) and used for particle size and polydispersity measurements. Hydrodynamic diameter of samples was measured by a dynamic light scattering (DLS)