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Characteristics of the bulk hydrogels made of the citric acid cross-linked whey protein microgels

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ABSTRACT

A non-toxic, biocompatible and generally recognized as safe (GRAS) chemical, citric acid, was employed for pH adjustment and cross-linking of whey proteins either before or after their microgelification by heat. A conventionally-made microgel sample was also fabricated by using hydrochloric acid for pH adjustment on 5.8 and subsequent heating at 85 °C. Size approximation of microgel particles from atomic force microscopy images indicated that chemical cross-linking resulted in smaller particles (80–130 nm and 100–150 nm for post- and pre-microgelification cross-linked samples, respectively) compared with conventional counterpart (150–300 nm). Based on Fourier transform infrared spectroscopy results, it was concluded that prolonged citric acid cross-linking of whey proteins prior to microgelification caused extensive cross-linking of protein units and preserved proteins α-helical structure. The pre-cross-linked microgels formed firmer (higher fracture stress and complex modulus) calcium-induced cold-set bulk gel with higher water-holding capacity and denser microstructure compared with conventional and post-cross-linked microgels. These superiorities were attributed to existence of higher number of carboxyl residues in the structure of the pre-cross-linked microgels. The alteration in the properties of the final bulk hydrogels via cross-linking of the microgel building-units can be exploited for modulating and controlling the release behavior of loaded cargo within hydrogel, resulting in a more tuned application of gels as edible delivery systems.

1. Introduction

Biopolymer hydrogels are hydrophilic polymer networks with the ability of retaining large amounts of water. Hydrogels are ideal matrices for protection and controlled release of bioactive ingredients such as antioxidants, vitamins, bioactive peptides and probiotics and can be fabricated of protein nanoparticles (Garti & McClements, 2012; Hoare & Kohane, 2008). Whey protein microgels (WPMs) are spherical particles of aggregated protein molecules with diameters of up to several hundred nanometers and relatively low polydispersity. The microgels are formed via heating of whey protein solutions at a narrow pH range between 5.7 and 5.9 (Schmitt et al., 2010).

The existence of free carboxyl and amino groups in protein molecules gives possibility to tune their chemical and functional properties by a variety of means including pH manipulation, and ion-mediated, as well as, other chemicals-induced cross-linking (Garti & McClements, 2012). Cross-linking is a common approach to improve the thermal and enzymatic digestion stability of protein assemblies (Bagheri, Yarmand, Madadlou, & Mousavi, 2014; Wang, 2013). Whey proteins have been cross-linked with various agents, such as glutaraldehyde, transglutaminase and citric acid. Glutaraldehyde progresses protein chains cross-linking through formation of an imine bond (C=N) between aldehydic groups of glutaraldehyde and amine groups of proteins. In spite of being highly efficient, the cytotoxicity of glutaraldehyde limits its application in the field of food science and drug delivery (Mitra, Sailakshmi, Gnanamani, & Mandal, 2013; Pal, Paulson, & Rousseau, 2009). The enzyme transglutaminase is one of zero-length cross-linkers that induce a covalent link directly between two potential groups without

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incorporation of any extrinsic atoms. As a result, elements become slightly closer after conjugation (Wong & Jameson, 2012). Citric acid is a poly functional monomer, readily available, inexpensive and nontoxic metabolic product that is involved in Krebs cycle (Franklin & Guhanathan, 2014). Molecular composition of the spacer arm of this cross-linker provides valuable functionality such as balancing the hydrophilicity of polymer network, participating in hydrogen bonding interaction within the network and improving pH-responsive swelling behavior of protein based gels due to increasing number of carboxyl groups (Gyawali et al., 2010; Saito et al., 2007). Bagheri et al. (2014) cross-linked whey proteins either by the enzyme transglutaminase or citric acid and prepared small and stable particles through desolvating the cross-linked whey proteins. Wang (2013) improved the thermal stability of whey proteins by a combination of preheating and microbial transglutaminase cross-linking.

In addition to heat-induced disulfide bridges (Schmitt et al., 2010) it is of interest to set chemicals-mediated cross-linkages in whey protein microgels structure in order to manipulate the properties of both microgels and resulting hydrogels. The objective of the present work was therefore to investigate the influences of cross-linking of whey proteins with citric acid either before or after microgelification (microgel formation) on size, morphology and chemical properties of WPMs. Mechanical and microstructural properties of hydrogels formed from the cross-linked microgels were also investigated.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) with 90% protein content was donated by Arla Food Ingredients (Viby J, Denmark). Calcium chloride ($CaCl_2$), citric acid monohydrate, hydrochloric acid (HCl) and sodium azide were purchased from Merck (Darmstadt, Germany). Distilled water was used throughout the study.

2.2. Preparation of protein microgel dispersions

WPI was dissolved in distilled water at 40 mg mL^{-1} concentration and sodium azide (0.1 mg mL^{-1}) was added as antimicrobe. The protein solution was stirred at 25 °C for 2 h. This was followed by incubation of protein solution at 4 °C for 12 h in order to assure protein hydration. To obtain conventional microgel, pH of the protein solution was adjusted on 5.8 with 6 M HCl and heated in a water bath without stirring at 80 °C for 15 min. After heat treatment, the sample was rapidly cooled to room temperature by tap water and incubated at 37 °C for 48 h. To prepare post-microgelification cross-linked sample (post-cross-linked microgel), citric acid was added at ratio of 0.63 mg mL^{-1} to whey protein solution to adjust pH on 5.8. The solution was then heated immediately in a water bath at 80 °C without stirring for 15 min and rapidly cooled to room temperature. Cross-linking was performed by annealing the microgel solution at 37 °C for 48 h. For preparation of pre-microgelification cross-linked sample (pre-cross-linked microgel), pH of the protein solution was adjusted onto 5.8 with citric acid and incubated at 37 °C for 48 h. After cross-linking, the solution was heated in a water bath at 80 °C without stirring for 15 min and rapidly cooled to room temperature. The resulting cross-linked and conventional (not cross-linked chemically) microgels were freeze-dried.

2.3. Preparation of cold-set bulk hydrogels of WPMs

Freeze-dried microgel samples were rehydrated (12% w/v) by dissolution in distilled water. The solutions were stored overnight at 4 °C to promote complete protein solubilization. Gelation was induced at 30 ± 1.0 °C by adding 100 mM $CaCl_2$. The gels were held overnight at ambient temperature until analyses.

2.4. Fourier transform infrared (FTIR) spectroscopy

In order to investigate the alterations occurred in functional groups of whey proteins due to cross-linking process, FTIR spectra of microgels were acquired with a Perkin Elmer 2000 FT-IR spectrometer (Perkin Elmer Co., MA, USA) from 450 to 4000 cm^{-1} wavenumber range. Microgel solutions were dried and mixed with potassium bromide powder and then pressed into a tablet. The spectra of samples were plotted using OPUS software (Bruker Optics Inc, Billerica, USA).

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The formation of citric acid-mediated cross-linkages in WPMs structure was followed by non-reducing SDS-PAGE. The resolving and stacking gels contained 12% and 4% of acrylamide, respectively. Microgel solutions (1 mg mL^{-1}) were diluted with SDS-PAGE sample buffer and then heated in a boiling water bath for 5 min. After centrifugation at 10,000 rpm for 10 min, the resultant mixtures were loaded into the wells and electrically separated. The gel was stained with Coomassie Blue.

2.6. Protein microgels topography

Fresh protein microgel solutions were diluted in distilled water with inherent pH of 5.8 to the final concentration of 0.1 mg mL^{-1}. For atomic force microscopy imaging, 10 μL of the diluted dispersion was spread on a glass slide and left to air dry at 25 °C. The specimens were scanned using an atomic force microscope (NanoWizard, JPK Instrument AG, Germany). The size distribution in the microgel samples was determined by analysis of AFM images using SPIP™ image processing software version 6.3.3. (Image Metrology, Denmark).

2.7. Texture of bulk hydrogel samples

The complex modulus (G^*) of hydrogel samples was measured on a rheometer (Malvern Instruments Ltd., Worcestershire, UK) using a parallel plate geometry (diameter 25 mm, gap 2 mm). Specimens were oscillated at frequency of 0.01–10 Hz (constant strain of 1%) and at temperature of 30 °C.

The firmness of hydrogel samples was determined by measuring the maximum force needed to penetrate a distance of 5 mm using a texture analyzer (M350-10CT, Testometric, Lancashire, UK). The gels with 30 mm diameter and 10 mm height were penetrated with a cylindrical stainless steel probe (diameter 13 mm) at a constant speed of 0.25 mm s^{-1}.

2.8. Microstructure of bulk hydrogel samples

Small pieces of bulk gels (1 cm^3) were cut into cylinders, frozen at −48 °C and dried to sublimation (FREEZE-DRIER Zibrus, VaCo5, Germany) at pressure of −2.5 bar for 24 h. The dried samples were gently broken and coated with a thin layer of gold. Micrographs at 5000× magnification were taken using a field emission scanning electron microscope (Mira 3-XM, Tescan, USA) operating at 10 kV.