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.

Keywords: Whey protein microgel; Hydrogel; Cross-linker; Citric acid.

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

43 fabricated of protein nanoparticles (Garti & McClements, 2012; Hoare & Kohane, 2008). Whey
44 protein microgels (WPMs) are spherical particles of aggregated protein molecules with diameters
45 of up to several hundred nanometers and relatively low polydispersity. The microgels are formed
46 via heating of whey protein solutions at a narrow pH range between 5.7–5.9 (Schmitt et al.,
47 2010).

48 The existence of free carboxyl and amino groups in protein molecules gives possibility to tune
49 their chemical and functional properties by a variety of means including pH manipulation, and
50 ion-mediated, as well as, other chemicals-induced cross-linking (Garti & McClements, 2012).

51 Cross-linking is a common approach to improve the thermal and enzymatic digestion stability of
52 protein assemblies (Bagheri, Yarmand, Madadlou & Mousavi, 2014; Wang, 2013). Whey
53 proteins have been cross-linked with various agents, such as glutaraldehyde, transglutaminase
54 and citric acid. Glutaraldehyde progresses protein chains cross-linking through formation of an
55 imine bond (C=N) between aldehydic groups of glutaraldehyde and amine groups of proteins. In
56 spite of being highly efficient, the cytotoxicity of glutaraldehyde limits its application in the field
57 of food science and drug delivery (Mitra, Sailakshmi, Gnanamani & Mandal, 2013; Pal, Paulson
58 & Rousseau, 2009). The enzyme transglutaminase is one of zero-length cross-linkers that induce
59 a covalent link directly between two potential groups without incorporation of any extrinsic
60 atoms. As a result, elements become slightly closer after conjugation (Wong & Jameson, 2012).

61 Citric acid is a poly functional monomer, readily available, inexpensive and nontoxic metabolic
62 product that is involved in Krebs cycle (Franklin & Guhanathan, 2014). Molecular composition
63 of the spacer arm of this cross-linker provides valuable functionality such as balancing the
64 hydrophilicity of polymer network, participating in hydrogen bonding interaction within the
65 network and improving pH-responsive swelling behavior of protein based gels due to increasing

number of carboxyl groups (Gyawalia 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 CaCl₂. 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, $10 \text{ }\mu\text{L}$ 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 sec^{-1} .

2.8. Microstructure of bulk hydrogel samples

Small pieces of bulk gels (1 cm³) 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.

2.9. Water-holding capacity (WHC) of hydrogels

The WHC of bulk gel samples was determined using a centrifugation procedure (Kuhn, Cavallieri & Cunha, 2011). Small pieces of bulk hydrogels (1–1.5 g) were gently cut with a sharp blade and weighed. The gel pieces were placed in 15 mL centrifuge tubes and centrifuged (Universal 320, Hettich Zentrifugen, Germany) at room temperature at 1500×g for 10 min. The WHC was expressed according to the following equation:

$$WHC = 100 \times \left[1 - \left(\frac{\text{water}_{\text{released}} (g)}{\text{water}_{\text{gel}} (g)} \right) \right] \quad (\text{Eq. 1})$$

Where water_{released} is the amount of water in grams released from hydrogel after centrifugation and water_{gel} is the amount of water in intact hydrogel (before centrifugation). The standard deviation was less than 5%.

3. Result and discussion

3.1. Chemical interactions

Figure 1a-d shows the FTIR spectra of native WPI, conventional, post-cross-linked and pre-cross-linked microgels. The amide I vibration is sensitive to secondary structural changes that occur on unfolding and aggregation (Barth, 2007). The band at 1657 cm^{−1} in the amide I region for native WPI is attributed to the vibration of C=O stretching in α-helix structures (Jackson & Mantsch, 1995). Displacement of the band to a lower wavenumber (1637 cm^{−1}) with a shoulder

156 at $\sim 1620\text{ cm}^{-1}$ for conventional (not cross-linked chemically) microgel indicates α -helix to β -
157 sheet interconversion (Geara, 1999) and manifests the heat-induced aggregation of protein
158 molecules. The band at 3293 cm^{-1} in the spectrum of native WPI is assigned to hydrogen bonded
159 N–H stretching. For conventional microgels, this band disappeared and another band is observed
160 at 3414 cm^{-1} which is assigned to non-hydrogen bonded N–H stretching. As well, the existence
161 of a shoulder in vicinity of 3450 cm^{-1} in the spectrum of conventional microgel sample could be
162 an indication of free N–H groups (Stuart, 2004). These alterations in comparison with the
163 spectrum of native WPI suggest that hydrogen bonds destructed considerably due to heat-
164 induced microgel formation. In the spectrum of post-cross-linked microgel sample, the band due
165 to C=O stretching of amide I shifted to 1648 cm^{-1} which is assigned to unordered structure
166 (Jackson & Mantsch, 1995). It is known that unfolded protein shows a broad amide I band
167 centered near 1650 cm^{-1} that is characteristic of unordered structure. In contrast, aggregated
168 protein often shows a band near or below 1620 cm^{-1} which is characteristic of intermolecular β -
169 sheets (Barth, 2007). Therefore, it is concluded that whey protein molecules unfolded upon
170 heating in presence of citric acid and α -helix content decreased in favor of random coil structure.
171 The hydrogen bonded N–H stretching vibration wavenumber (3293 cm^{-1}) did not change in the
172 spectrum of this sample (i.e. post-cross-linked microgel) compared with native WPI and in
173 contrast to conventional microgel sample. This suggests that citric acid diminished the
174 aggregation of unfolded protein units to β -sheets. The band intensity however decreased. It has
175 been mentioned that the number of similar groups in a molecule influences the relative strength
176 of the corresponding absorption band (Kalsi, 2004) and the relative intensity of one band
177 compared with another provides information on the amount of a specific functional group, in
178 addition to type of the group (Socrates, 2001). Accordingly, the decreased intensity of the band

at 3293 cm^{-1} is attributed to the cross-linking reaction between carboxyl groups of citric acid and NH groups of whey proteins which decreased the number of NH groups. The width of the band at 3293 cm^{-1} also became broader owing likely to hydrogen bonds formation by OH groups of included citric acid molecules in WPMs composition. Miller (2003) mentioned that hydrogen bonding broadens X–H bands.

In the FTIR spectrum of pre-cross-linked sample, the C=O stretching vibration of amide I that is assigned to α -helix structure is observed at 1658 cm^{-1} in accordance with native WPI. Therefore, prolonged citric acid cross-linking of whey proteins before heat-induced microgel formation preserved proteins native secondary structure and prevented unfolding. In contrast with conventional microgel but in similarity to post-cross-linked sample, the wavenumber (3293 cm^{-1}) of hydrogen bonded N–H stretching vibration did not change (Figure 1d) for the pre-cross-linked microgel sample compared with native WPI. This probably reflects the decreased extent of proteins aggregation and β -sheet formation. It has been proposed (Wang, 2013) that cross-linking may enhance steric hindrance and attenuate hydrophobic attraction between proteins through limiting the exposure of hydrophobic patches by heat. We argue that a higher number of citric acid molecules included in the composition of pre-cross-linked sample as bridging agents because width broadening and decrease in intensity of the band at 3293 cm^{-1} is more pronounced for this sample compared with the post-cross-linked counterpart. The amide II absorption (1480–1575 cm^{-1}) derives mainly from N–H bending (60%) and C–N stretching (40%) vibrations. An increase in intensity of amide II band is observed in the spectra of two cross-linked microgels in comparison with the spectrum of conventional microgel (Figure 1). It can be caused by cross-linking reaction, since reaction of carboxyl groups of citric acid with amino groups of whey proteins leads to formation of further C–N groups.

3.2. SDS-PAGE analysis of microgels

SDS-PAGE patterns of microgel samples are represented in Figure 2. Conventionally-made microgel showed the most intensive bands for both β -lactoglobulin (MW= 18.4 kDa (Zhong, Wang, Hu & Ikeda, 2013)) and α -lactalbumin (MW=14.1 kDa (Zhong et al., 2013)). The lower molecular weight band corresponding to α -lactalbumin became very vague for post-microgelification cross-linked microgel and disappeared for pre-microgelification cross-linked microgel. Also, the intensity of the band corresponding to β -lactoglobulin decreased slightly for the latter. The more extensive monomeric band disappearance in SDS-PAGE pattern of pre-microgelification cross-linked sample suggests more intensive citric acid-mediated cross-linking of this sample, as compared with post-microgelification cross-linked microgel.

3.3. Size of microgels

AFM images of microgels are shown in Figure 3. Most microgel particles were more or less roughly spherical with size distribution ranges of 150–300, 80–130 and 100–150 nm for conventional, post-cross-linked and pre-cross-linked samples, respectively. Given that native whey protein molecules self assemble inherently into 8 nm superstructures (Purwanti et al., 2011), size analysis results by AFM images confirm heat-triggered and pH-architected aggregation of proteins in all three samples. In the case of pre-cross-linked sample, formation of intramolecular amide linkages between two of carboxyl groups of citric acid and free amine groups of a protein chain, improved proteins heat stability. This in turn preserved proteins native α -helical structure (as revealed by the FTIR results) and limited the extent of hydrophobic (dispersion van der Waals forces) interactions amongst heat-treated proteins. Therefore, less intensive aggregation of proteins occurred, resulting in smaller particles (compare the size of WPMs). The average size of pre-cross-linked microgels was slightly greater than that of post-

microgelification counterpart, which implies formation of more carboxyl-mediated intra and intermolecular cross-linkages in the former. In post-microgelification cross-linking procedure, citric acid molecules could not enter into whey protein particles structure as extensive as pre-microgelification cross-linking mode to form intra and intermolecular cross-linkages. Citric acid molecules however surrounded unfolded protein units and decreased intermolecular β -sheet formation which limited protein aggregation and resulted in smaller particles. Bagheri et al. (2014) cross-linked heat-treated whey proteins by transglutaminase or citric acid and fabricated particles from cross-linked proteins via alcohol addition. The enzymatically cross-linked whey proteins yielded smaller particles than acid cross-linked counterpart which was attributed to the zero-length character of the transglutaminase-induced cross-linkages. This is in line with the observations of the present study that higher number of cross-linkages formed by a non-zero-length cross-linker (citric acid) led to the formation of larger microgel particles.

3.4. Textural properties of bulk gels

The complex modulus (G^*) defines overall stiffness of a sample (Miri, 2011). Figure 4 shows the G^* of the bulk hydrogels fabricated of different WPMs over a frequency range from 10^{-2} to 10 Hz. The G^* of the hydrogels prepared by both types of cross-linked microgels were greater than that of the hydrogel fabricated of non-cross-linked microgels. It is hypothesized that smaller cross-linked microgels which had higher specific surface areas and more contact points among particles formed well-established and integrate gel networks resulting in firmer textures. Purwanti et al. (2011) suggested that aggregate size is one of the most important factors affecting the final gel strength due to the influence on particle contact points. It is also important to consider the role of unreacted carboxyl group of the citric acids included in composition of cross-linked WPMs. Those groups could interact electrostatically with Ca^{2+} ions, resulting in

increased ion-mediated cross-linking of WPMs compared to conventional (not cross-linked chemically) counterpart. This would strengthen the gel texture. Higher number of cross-linkages formed by citric acid in pre-cross-linked sample than post-microgelification counterpart may account for the firmer texture of the bulk hydrogel fabricated of the former (Figure 4) in spite of having a slightly larger particle size. This contradiction reveals that $\text{COO}^- - \text{Ca}^{2+} - \text{COO}^-$ interactions between calcium ions and unreacted carboxyl groups of citric acid bridges played a significant role in gel strength dictation.

Large deformation textural analysis results (Table 1) are in accordance with those of oscillatory test and indicate that fracture force was the highest for the hydrogel made of pre-cross-linked particles. The bulk hydrogel made of conventional microgels had the lowest fracture force and G^* ; however, resisted up to higher frequencies against the applied oscillatory shear before that G^* descended (Figure 4). On the other hand, the bulk hydrogel made of pre-cross-linked microgels underwent G^* descend soon. These suggest that the texture of the hydrogel made of pre-cross-linked microgels was more brittle than that of the hydrogel made of conventional microgels. A high cross-linking density results in greater mechanical strength, as well as reduces the mobility of (bio)polymer chains, causing the polymer network to be relatively brittle (Hillel, Shah, Elisseff & Hopkins, 2007). Accordingly, extensive cross-linking of protein chains in the pre-cross-linked WPMs made the gel network less flexible compared with other samples and resulted in a more brittle gel.

3.5. Gel microstructure

All gel samples had sponge-like structure of connected cells in freeze-dried state (Figure 5a-c). The gel fabricated of conventional microgels possessed a thinly connected network of tiny cells with many pores and fissures. Whilst, microstructure of the gel fabricated of post-cross-linked

microgels had cells with thick walls and less pores. Microstructure of the gel made of pre-cross-linked microgels was denser than that of the gel from conventional microgels. Given that pre-microgelification cross-linked particles were smaller than the conventionally-made microgel particles, Donato, Kolodziejczyk, and Rouvet (2011) also reported that smaller particles lead to denser networks. In conclusion, decreased microgel size and increased Ca^{2+} -mediated interconnections in citric acid cross-linked samples caused formation of less porous and more connected networks. This is consistent with changes of gel firmness.

3.6. Water-holding capacity of gels

The ability of gels to retain water under force (centrifugal force in this case) is related to the pore size of gel network and the polymers characteristic. A gel network with small pores holds water tightly by capillary forces (Kuhn, Cavallieri & Cunha, 2010; Vardhanabhuti, Foegeding, McGuffey, Daubert, & Swaisgood, 2001). The hydrogel fabricated of conventional microgels with a more porous microstructure allowed water to flow out more easily under force resulting in less WHC (see Table 1). The gel fabricated of pre-cross-linked microgels showed greatest water-holding capacity due both to small pore size of the gel network and greater number of sites available to interact with water. These sites were free hydroxyl groups of citric acid residues that included into the composition of WPMs. The gel fabricated of post-cross-linked WPMs that had the smallest particle size showed less WHC than the gel formed of pre-cross-linked particles. This shows that less water-binding sites existed in the composition of post-cross-linked WPMs than the pre-microgelification counterpart.

4. Conclusion

The employment of citric acid instead of hydrochloric acid in microgelification process of whey proteins yielded smaller particles with contribution of citric acid as intra and intermolecular

cross-linker. The stage of cross-linking i.e. before or after heat-triggered microgel formation influenced the size distribution range and flow behavior of microgels solution. The bulk hydrogel made of pre-cross-linked microgels had a dense microstructure and the highest firmness and water holding capacity compared with those made of conventional and post-cross-linked microgels. These superiorities were attributed to the existence of enormous number of COO^- – Ca^{2+} – COO^- electrostatically formed bridges between unreacted carboxyl groups of citric acid molecules that cross-linked protein chains by their other two carboxyl groups, as well as, to more hydrogen bond-forming sites available to interact with water. This study showed that due to the formation of covalent cross-linkages in the structure of whey protein microgels by a non-toxic reagent, bulk hydrogels with **modified** mechanical and structural properties can be fabricated with an easily scale-upable process. The **modification of the properties of bulk whey protein hydrogel by cross-linking of the forming units i.e. microgels may introduce an edible delivery vehicle with a more tuned release behavior of the loaded cargo.**

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Table 1. Effect of cross-linking on the properties of microgel particles and corresponding bulk hydrogels.

Table 1

Microgel type	Microgel Size (nm)	Microgel structure	Hydrogel firmness (N)	Hydrogel WHC (%)	Hydrogel microstructure
Conventional	150–300	Intermolecular β -sheets	0.77±0.01 ^c	75.6±2.42 ^c	Less dense, thinner walls with many pores
Post-cross-linked	80–130	Unordered	0.80±0.02 ^b	81.7±2.23 ^b	Less dense, thicker walls with less pores
Pre-cross-linked	100–150	α -helix	0.97±0.01 ^a	84.3±2.16 ^a	Dense network, walls with less pores

Means within the same column with different superscripts differ significantly (P <0.05).

Figure captions

Fig. 1. FTIR spectra of native WPI (a), and conventional (b), post-cross-linked (c), and pre-cross-linked (d) microgels.

Fig. 2. SDS-PAGE patterns of microgel solutions. Lane 1= conventional, Lane 2= post-cross-linked, and Lane 3= pre-cross-linked microgels.

Fig. 3. AFM images of conventional (a), post-cross-linked (b), and pre-cross-linked (c) microgels.

Fig. 4. Complex modulus of whey protein gel samples fabricated of conventional ($\circ$), post-cross-linked ($\blacksquare$), and pre-cross-linked ($\blacktriangle$) microgels.

Fig. 5. SEM micrographs of whey protein gel samples fabricated of conventional (a), post-cross-linked (b), and pre-cross-linked (c) microgels.

Figures

Fig.1.

Fig.2.

Fig.3.
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Fig 4.

Fig. 5.