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Gelation by bioactives: Characteristics of the cold-set whey protein gels made using gallic acid

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ABSTRACT

A bioactive molecule, gallic acid (GA), was used to form cold-set whey protein gels. This method enables exploitation of the bioactive compound per se to gel whey proteins. Also, citric acid was used to crosslink whey proteins before gelation by GA. Compared with phosphoric acid (PA), gelation by GA prevented formation of intermolecular β-sheets and resulted in a porous microstructure. Also, the GA-induced gel had a lower firmness and water-holding capacity (WHC), but a higher swellability in pepsin-free simulated gastric fluid. Citric acid pre-crosslinking decreased the proportion of α-helix structures in favour of β-turns in the GA-induced gel. It also increased the size of ordered structures (α-helix and β-sheets), caused formation of larger protein aggregates and increased gel WHC. The PA-induced gel initially underwent a lower in vitro peptic disintegration; however, the disintegration of the PA-induced gel rapidly increased, surpassing those of GA-induced gels.

1. Introduction

Whey proteins are highly nutritious and exert physiologically appreciated effects such as fat loss (Lockwood et al., 2017), and rapid muscle gain (Wróblewska et al., 2018). In addition, they can be processed to the particles possessing technologically important properties such as stabilisation of foams (Schmitt, Bovay, & Rouvet, 2014), as well, oil-water (Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks, 2014) and water−water (Nguyen, Nicolai, & Benyahia, 2013) emulsions. Moreover, whey proteins are capable of forming cold-set (Hongsprabhas, Barbut, & Marangoni, 1999) and heat-induced (Luo, Borst, Westphal, Boom, & Janssen, 2017) hydrogels. Whey protein hydrogels are convenient matrices for encapsulation and subsequent stimulus-responsive delivery of bioactives, vitamins, probiotics and antioxidants (Gunasekaran, Ko, & Xiao, 2007).

In contradiction to the heat-induced method, cold-set gelation of whey proteins allows encapsulation of heat-labile ingredients. For this purpose, whey protein solutions are at first heat-denatured at non-gelling condition, i.e., at pH values far from the isoelectric point (pI) of the protein and at low ionic strengths. Then, the solutions are cooled down to ambient temperatures and supplemented with the bioactive cargo of interest. Finally, gelation is induced via screening the electrostatic repulsive forces among the proteins by addition of a salt and/or adjustment of pH towards the pI of the protein (Bryant & McClements, 1998).

Generally speaking, cold-set gels are more homogenous than the corresponding heat-set gels at the same pH value and salt content. Additionally, the salt-induced gels show higher resistance to fracture than heat-set counterparts (Nicolai, Britten, & Schmitt, 2011). Currently, research is actively ongoing to fabricate, improve, and exploit whey protein cold-set hydrogels as food and cargo delivery systems. For instance, recently it was shown that the addition of cysteine at different steps of the gel formation process, as well post-gelation heat treatment of whey protein cold-set gels improves their viscoelastic properties (Lavoisier, Vilgis, & Aguilera, 2019).

It is known that the acidulant type, i.e., either mineral acids such as phosphoric acid (Vardhanabhuti, Khayankan, & Foegeding, 2010) or organic acids, for instance gluconic acid (Britten & Giroux, 2001) influences the viscoelastic characteristics of acid-induced gels. In seek of new cold-set gelation techniques, citric acid, a multi-carboxylic organic acid, was used as the gelling agent of whey proteins at pH $\geq$ 7.0 and in the absence of ionic gelators. Carboxylic acid groups of citric acid react with free amino groups in proteins through a nucleophilic substitution reaction in the presence of an alkali, causing protein crosslinking and finally gel formation at

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sufficiently high concentrations of citric acid and protein (Abaee, Madadlou, & Saboury, 2017).

Phenolic acids, which are found in plants and flowers, show antioxidative, antiproliferative, antibacterial (Pires et al., 2018) and many other biologically beneficial activities. They can be recovered from food processing waste streams and exploited at formulation of health-promoting functional products (Garavand & Madadlou, 2014; Nourbakhsh, Madadlou, Emam-Djomeh, Wang, & Gunasekaran, 2016,b). Gallic acid (3,4,5-trihydroxybenzoic acid, $C_6H_2(OH)_3COOH$, $pK_a = 4.41$ at 298 K) is a monocarboxylic phenolic acid and has antioxidant, anti-inflammatory, antifungal and anti-tumor properties (Cláudio et al., 2012). The direct addition of phenolic acids such as gallic acid into food is challenging because of the phenolics susceptibility to enzymatic browning, bitter taste and astringency (Rawel, Kroll, & Hohl, 2001). Encapsulation of phenolic acids, e.g., within alginate-tragacanth hydrogel beads prepared using an ionic gelation method (Apoorva, Rameshbabu, Dasgupta, Dhara, & Padmavati, 2020), and whey protein particles prepared through internal gelation of emulsion droplets by cations (Jia, Dumont, & Orsat, 2016) is considered an efficient way to protect phenolics from degradation and mask their unpleasant taste. From this perspective, gallic acid has been encapsulated into zein fibres (Neo et al., 2013) and matrices of chitosan, β-cyclodextrin and xanthan (da Rosa et al., 2013).

In addition to gallic acid encapsulation by proteins, gallic acid can be used to modify the functional and molecular characteristics of whey proteins. Thermal denaturation of whey proteins increases the binding of proteins to gallic acid and other phenolic compounds (KılıçBayraktar, Harbourne, & Fagan, 2019). Binding takes place mostly by hydrophobic interactions but also hydrogen bonding (Cao & Xiong, 2017a) and can decrease the thermal transition temperature of the native form of β-lactoglobulin (Cao & Xiong, 2017b). Gallic acid binding has also implications on the cold-set gelation of preheated whey proteins. It accelerated the glucono-δ-lactone-induced gelation of skim milk and increased the storage modulus (G′) of the resulting gel (Harbourne, Jacquier, & O'Riordan, 2011). A merit of gallic acid as a bioactive compound is that it can cause, per se, the gelation of thermally denatured whey proteins. We showed that gallic acid recovery from the exterior aqueous phase into the interior aqueous phase of transient water-in-oil-water double emulsions induced the gelation of heat-denatured whey proteins or whey protein-derived crosslinked peptides present in the interior phase (Nourbakhsh, Madadlou, Emam-Djomeh, Wang, Gunasekaran, et al., 2016). The use of biologically active phenolic acids to gelify whey proteins is advantageous as it enables cold-set gelation of whey proteins by the compound to be encapsulated. However, scarce information is available in the literature on the chemical structure, textural properties and technological functionality of bulk whey protein hydrogels induced by gallic acid.

In the present communication, we report the secondary structural and textural properties of cold-set whey protein gels induced by gallic acid in comparison with those made using phosphoric acid. As well, citric acid which is a safe-to-use and inexpensive substance was applied for crosslinking of whey proteins (Abaee et al., 2017; Xu, Shen, Xu, & Yang, 2015) prior to gallic acid-induced gelation for tuning the characteristics of the protein gels.

2. Material and methods

2.1. Materials

Whey protein isolate (WPI) with at least 90% protein content was kindly gifted by Arla Food Ingredients (Viby J, Denmark). Sodium hydroxide (NaOH), citric acid monohydrate, hydrochloric acid (HCl), phosphoric acid and sodium azide were purchased from Merck (Darmstadt, Germany). Pepsin and gallic acid were procured from Sigma Aldrich (Wicklow, Ireland).

2.2. Preparation of whey protein hydrogels

WPI solution (105 mg mL^{-1}) was prepared by stirring WPI in distilled water for 2 h; sodium azide was added as antimicrobial agent (100 ppm) and the solution was stored at 4 °C for 20 h. Then, the pH of the solution was adjusted on 8.5 by 5 M NaOH and the solution was heated in a closed tube at 85 °C for 15 min, while being stirred vigorously. After heat treatment, the WPI solution was rapidly cooled in an ice bath and its pH was decreased to 7.0 by 5 M HCl. Subsequently, the solution was divided into three volumes to make (i) citric acid pre-crosslinked and gallic acid-induced (CiGA-induced) gel, (ii) gallic acid-induced (GA-induced) gel, and (iii) phosphoric acid-induced (PA-induced) gel. The CiGA-induced gel was prepared by at first supplementing the heat treated WPI (pH 7.0) with citric acid (at pH 7.0 adjusted using 5 M NaOH) at a final citric acid concentration of 15 mM, storage at 40 °C for 4 h (Abaee et al., 2017) and finally charging with gallic acid (10 mg mL^{-1}). The two non-crosslinked gel samples (i.e. GA-induced and PA-induced) were prepared by addition of 5 M HCl (at pH 7.0; pH was adjusted by a comparable volume of 5 M NaOH used in preparation of the crosslinked sample) instead of citric acid to the heat treated WPI, storage at 40 °C for 4 h and lastly charging with either gallic acid (10 mg mL^{-1}) or 1 M phosphoric acid. The final concentration of phosphoric acid was 26 mM. After addition of gallic acid or phosphoric acid, the samples were stored at 4 °C for 24 h. The final pH of all samples was 5.1 ± 0.1.

2.3. Infra-red spectroscopy of whey protein gels

Fourier transform infra-red (FTIR) spectra of whey protein gels were collected using a FTIR spectrometer (Spectrum one; PerkinElmer, MA, USA). The gel samples were freeze-dried (Dena vacuum, Tehran, Iran) and the FTIR spectra were recorded at a wavenumber range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} in transmission mode.

The Fourier self-deconvolution technique was carried out on the amide I region to investigate the secondary structure of whey proteins. To perform the deconvolution, the OMNIC program (Version 6.0, Boston, MA, USA) was used according to the methods of Fourier self-deconvolution (Yang, Yang, Kong, Dong, & Yu, 2015) with a half-bandwidth of 39.5 cm^{-1} and a resolution enhancement factor of 2.8 cm^{-1}. To distinguish the overlapped bands from each other, second-derivatives spectra of the amide I bands (1600−1700 cm^{-1}) were calculated. A Gaussian curve-fitting method was performed to show the secondary structure peaks of each component. The peak area of every fitted bands was used to calculate the percentage of various secondary structures, using OriginPro software (Version 93E, MA, USA).

2.4. X-ray diffraction (XRD)

The XRD patterns of whey protein gels were taken using a X-ray diffractometer (EQUINOX 3000, Inel, France). The gel samples were freeze-dried and irradiated with Cu K-α radiation in the 2θ range of 5°−110° at 50 kV tube voltages and 1.0 mA tube current.

2.5. Gel microstructure

The microstructure of different whey protein hydrogels was observed using a scanning electron microscope (Madell Technology Corporation KYKY-EM 3200, USA) at 10,000, 5000× and 2500 × magnifications and acceleration voltage of 26 kV. The gels

were cut into small pieces and freeze dried, then coated with a thin layer of gold before imaging.

2.6. Gel firmness and strength

The strength and firmness of hydrogel samples were determined using a texture analyser (M 350-10 CT, Testometric, Lancashire, UK). Hydrogel samples (13 mm × 30 mm) were prepared in cylindrical tubes and then penetrated by a cylindrical stainless steel probe (diameter of 7.5 mm) at a constant speed of 60 mm min^{-1} for up to 75% of gel height. The maximum penetration force defined as the force required to rupture gel, was expressed as gel strength.

For the compression test, cylindrical shape samples with diameter of 13 mm and height of 30 mm were compressed uniaxially by a stainless steel plate up to 75% of the gel height at a constant speed of 60 mm min^{-1}. The gel compressive stress (σ_c) was calculated by the following equation (eq. (1)):

$$(\sigma_c) = \frac{F}{A} \quad (1)$$

where F is the peak force (N) and A is the cross-sectional area (m^2) of gel sample.

2.7. Water-holding capacity (WHC)

The WHC of gels was determined by a centrifugation technique. Samples were prepared in cylindrical tubes and then centrifuged at 1000×*g* at 10 °C for 10 min. The gel weight (g) was measured before centrifugation and after centrifugation. The WHC was determined according to the equation (eq. (2)):

$$\mathrm{WHC}\left(\%\right) = 100 \times \left[1 - \left(\frac{\text{water released (g)}}{\text{water gel (g)}}\right)\right] \quad (2)$$

where water released is the amount of water which leaves gel after centrifugation and water gel is amount of water in gel before centrifugation (Kuhn, Cavallieri, & da Cunha, 2010).

2.8. Swelling and in vitro digestion experiments

Water uptake of gels was measured following the method of Maltais, Remondetto, and Subirade (2009). Gel samples prepared in cylindrical tubes were taken out of the tubes and gel surface was dried by a paper towel, then the gel was weighed and immersed into a simulated gastric fluid (SGF) without enzyme at 37 °C. The SGF composed of 7.0 mL of 37% HCl and 2.0 g of sodium chloride dissolved in 1000 mL of distilled water at the final pH of 1.2. Periodically, the sample was removed from the medium, gently wiped and re-weighed. The following equation (eq. (3)) was used for determining swelling amount:

$$\%\ \text{swelling} = [(W_t - W_0)/W_0] \times 100 \quad (3)$$

where Wt is the gel weight (g) at time t and W_0 is the initial gel weight (g).

The in vitro digestion of gel samples was also measured using the SGF. For this purpose, SGF was supplemented with pepsin (3.2 g L^{-1}) and used for in vitro gastric digestion of WPI hydrogels. Small pieces of samples with determined weights were exposed to SGF up to 2 h at 37 °C by shaking at 100 rpm. The digestion fluid was centrifuged at 1500 ×g and the absorbance of whey protein in supernatant at 280 nm was measured to determine the concentration of released protein. A WPI standard curve ($R^2 = 98.2\%$) was used for concentration determination. Gel degradation extent was calculated as (eq. (4)):

$$\text{Degradation}\ (\%) = \frac{\textit{released protein}}{\textit{total protein}} \times 100 \quad (4)$$

where released protein is the amount of protein which was released into the SGF and total protein is the amount of protein within the gel matrix.

2.9. Statistical analysis

All experiments were conducted at three replications. Statistical analysis was performed using IBM SPSS 16 (IBM SPSS Inc., Chicago, IL, USA). The means ± standard deviations were subjected to one-way analysis of variance, and any significant difference among the means (at least three repetitions) was found by employing compare means and Duncan's tests at 5% significance level.

3. Results and discussion

3.1. Protein conformation in gel state

FTIR spectroscopy was conducted to determine changes in the secondary structure of whey proteins as influenced by crosslinking with citric acid and gelation using either gallic acid or phosphoric acid. Fig. 1 indicates the FTIR spectra of different WPI gel samples. The amide I band (~1650 cm^{-1}) corresponds to C=O and C—N stretching vibrations and the amide II band (~1550 cm^{-1}) is related to N—H bending and C—N stretching vibrations (Barth, 2007). Bands in amide I region represent α-helixes, 3_{10}-helixes, random coils (1645–1666 cm^{-1}), β-sheets (1620–1643 and 1689–1697 cm^{-1}) and β-turns (1666–1687 cm^{-1}) (Grewal, Huppertz, & Vasiljevic, 2018).

The wavenumber of amide I band was different in the PA-induced and GA-induced gels (Fig. 1); the amide I band had a lower wavenumber in the PA-induced gel (at 1617 cm^{-1}) compared with the GA-induced and CiGA-induced gels (at 1633 cm^{-1}). As a general rule, an inverse relationship exists between hydrogen bonding and the wavenumber of stretching vibrations (Barth, 2007; Vahedifar, Madadlou, & Salami, 2018) and as the amide I band stands for stretching vibrations, it is concluded that PA caused a higher extent of hydrogen bonding than GA in the protein structure. Amide I deconvolution indicated that gelation using PA or GA had remarkably different influences on the β-sheet structure (Table 1). Heat treatment of whey proteins disrupts the α-helix and β-sheets structures and creates new β-sheet arrangements in β-lactoglobulin (Eissa, Puhl, Kadla, & Khan, 2006). Gelation by PA caused formation of intermolecular β-sheets, whereas the GA-induced gel had a significant content of intramolecular β-sheets (Table 1).

Based on the interpretation of amide I band position (associated with hydrogen bonding) and estimation of the secondary structures (by deconvolution of amide I band), it is concluded that intermolecular β-sheet formation proceeded by hydrogen bonding (in the PA-induced gel) and gelation by GA prevents the intermolecular β-sheet arrangement. Phosphoric acid exists in the forms of acid (H_3PO_4) and phosphate anions ($H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}) in aqueous solutions. Because the phosphate anions are kosmotropic they interact with water rather than the non-polar segments of proteins and are preferentially excluded from the solvation shell of the hydrophobic domains. This stimulates hydrophobic interactions

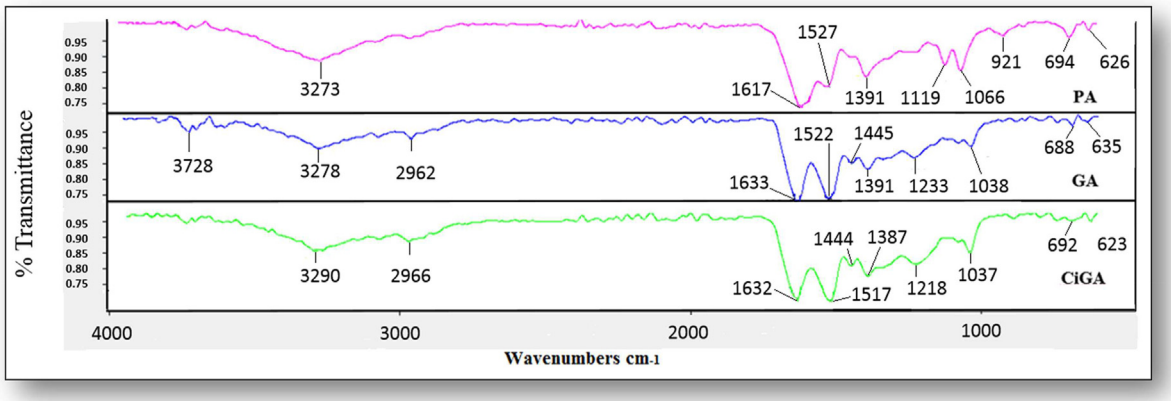

Fig. 1. FTIR spectra of phosphoric acid-induced (PA), gallic acid-induced (GA), and citric acid pre-crosslinked and gallic acid-induced (CiGA) whey protein gels.

Table 1
Secondary structure distribution (calculated from the FTIR spectra at amide I region) of whey protein gels.[a]

Structural element	Area (%)			Wavenumber (cm^{-1})
	PA	GA	CiGA	
Intramolecular β-sheet	–	77.52	77.3	1620–1643
Random-coil and helical	23.57	14.16	8.46	1645–1666
β-Turn	7.84	8.31	14.22	1666–1687
Intermolecular β-sheet	68.58	–	–	1614–1618

[a] Abbreviations are: PA, phosphoric acid-induced gels; GA, gallic acid-induced gels; CiGA, citric acid pre-crosslinked and gallic acid-induced gels.

between proteins (Moelbert, Normand, & De Los Rios, 2004), resulting in an increased aggregation of denatured whey proteins, which consequently enhances the hydrogen bond-mediated intermolecular β-sheet structuring. In contrast, gallic acid directly binds to heat denatured whey proteins by hydrophobic interactions (Cao & Xiong, 2017a). Such interactions could reduce intermolecular hydrophobic interactions between proteins and consequently decrease/prevent the hydrogen bond-mediated intermolecular β-sheet structuring. In accordance with changes in amide I band, the position of the amide II band in the GA-induced gel (1522 cm^{-1}) was lower than that in the PA-induced gel (1527 cm^{-1}), indicating a lower contribution of N—H bending vibration to the protein backbone conformation (Barth, 2007) when gelation was achieved using gallic acid.

The incorporation of gallic acid into whey protein structure is confirmed by a shift in the position of the band at 1066 cm^{-1} in the spectrum of PA-induced gel to 1037 cm^{-1} in the spectra of GA-induced and CiGA-induced gels. The band at 1037 cm^{-1} has been assigned to oligomeric and dimeric gallic acid structures, resulting from bonding between stretching vibrations of C—C and C—O groups of gallic acid (Neo et al., 2013).

Pre-crosslinking of heat-denatured whey proteins prior to GA-induced gelation did not influence the intramolecular β-sheet content of gel. However, it decreased the helical and random coil structures in favor of β-turns (Table 1). The α-helix form is dense and more ordered than β-turns. Crosslinking with involvement of N atoms in α-helices destroyed the hydrogen bonds that are responsible to keep the helical structures, resulting in alternation of helix structures to β-turn structures (Turasan, Barber, Malm, & Kokini, 2018). The effect of pre-crosslinking is as well observed by displacement of the band at 3278 cm^{-1} in the spectrum of the GA-induced gel to 3290 cm^{-1} in the spectrum of CiGA-induced gel. The band at about 3278 cm^{-1} stands for hydrogen bonded N—H stretching in whey proteins (Farjami, Madadlou, & Labbafi, 2015) and the displacement indicates that the free NH_2 of whey proteins crosslinked with the COOH groups of citric acid. The position of the amide II band (N—H bending and C—N stretching) also displaced from 1522 cm^{-1} to 1517 cm^{-1} as a consequence of citric acid pre-crosslinking prior to GA-induced gelation (Fig. 1).

3.2. XRD pattern of whey protein gels

The XRD patterns of different whey protein hydrogels are shown in Fig. 2. Crystalline fractions have a series of sharp peaks, while the amorphous fractions possess a broad background pattern. All whey protein hydrogels were characterised by two sharp peaks at 2θ of about 9.5° and 19.5°, which stand for α-helix and β-sheet structures, respectively (Zhao et al., 2015). The relative intensity (RI) of peaks was calculated by dividing the absolute intensity of a given peak by the absolute intensity of the strongest peak of every sample; the result is expressed in percentage. The most intense peak has a RI of 100% (Inoue & Hirasawa, 2013). The peak standing for β-sheet structure had a higher RI (i.e., 100%) than the peak standing for α-helix structure (Table 2), which is in agreement with the FTIR results (Table 1). It is known that the diffraction intensity is directly related to crystal size: the bigger the size of a crystal, the higher the diffraction intensity (Yu et al., 2015). Accordingly, β-sheet structures were the major ordered (crystal-like) component of the secondary structure of whey protein gels, irrespective of the acidulant type and pre-crosslinking before acid-induced gelation.

The RI of the α-helix structure was higher for the PA-induced gel than for the GA-induced and CiGA-induced gels (Table 2). This result is also in agreement with the FTIR deconvolution results (Table 1). The crystals size is inversely related to diffraction angle (Yu et al., 2015). As the diffraction angle of both ordered structural components (i.e., α-helix and β-sheet structures) decreased due to pre-crosslinking with citric acid it is deliberated that crosslinking increased the size of the ordered structures.

3.3. Microstructure

SEM micrographs of different WPI gels formed by either PA or GA are displayed in Fig. 3. The gel made using PA had a highly

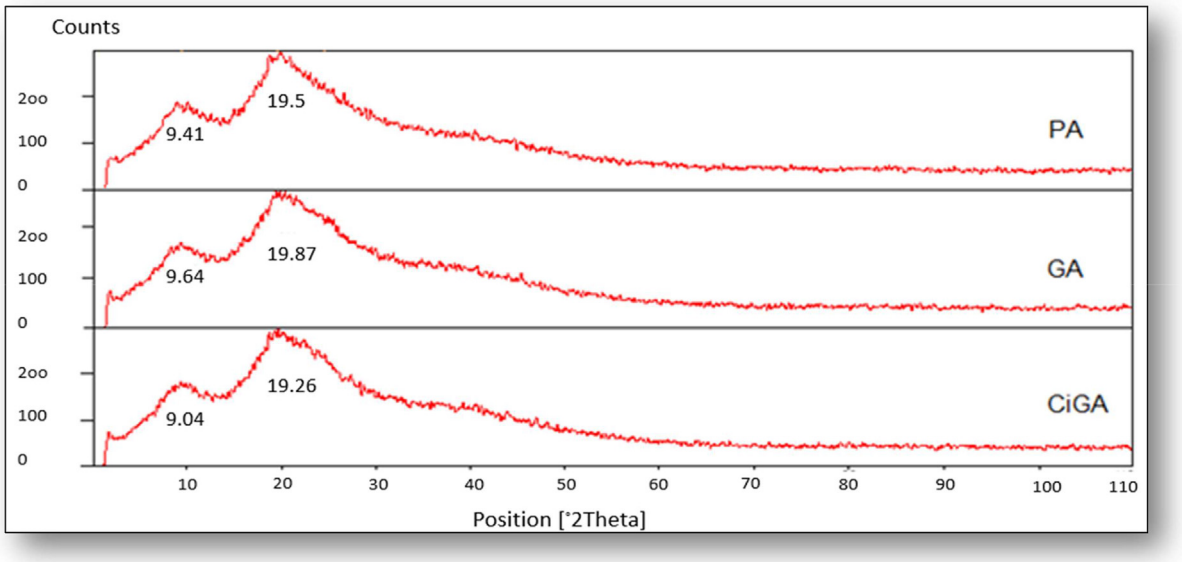

Fig. 2. X-ray diffraction pattern of phosphoric acid-induced (PA), gallic acid-induced (GA), and citric acid pre-crosslinked and gallic acid-induced (CiGA) whey protein gels.

Table 2
XRD parameters (peak diffraction angle and relative intensity) of whey protein gels.[a]

Structural element	Peak diffraction angle (2Θ/◦)			Relative intensity		
	PA	GA	CiGA	PA	GA	CiGA
α-helix	9.41	9.64	9.04	64.13	54.84	53.01
β-sheet	19.5	19.87	19.26	100	100	100

[a] Abbreviations are: PA, phosphoric acid-induced gels; GA, gallic acid-induced gels; CiGA, citric acid pre-crosslinked and gallic acid-induced gels. Relative intensity (RI) of XRD peaks was calculated by dividing the absolute intensity of each peak by the absolute intensity of the strongest peak of the same sample.

compact microstructure without visible pores at any magnification, whereas the gel made using GA (without pre-crosslinking) had a porous microstructure consisting of many interlinked small aggregates. This shows that different acidifying agents caused formation of dissimilar protein gel networks, which we find correlated with the gels intermolecular β-sheet content. The PA-induced gel had a significantly higher intermolecular β-sheet structure compared with the higher intramolecular β-sheet structure of the GA-induced gel (Table 1). A correlation between the content of intermolecular β-sheet structure and protein aggregation has also been reported for heat-induced gluten gels (Wang et al., 2017).

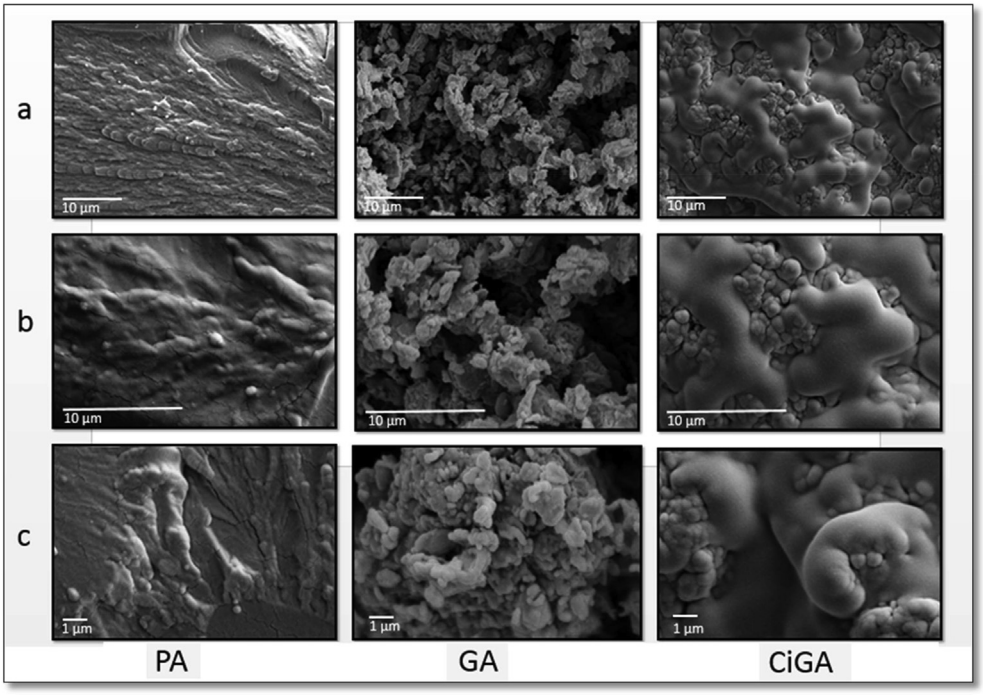

Fig. 3. SEM images of phosphoric acid-induced (PA), gallic acid-induced (GA), citric acid pre-crosslinked and gallic acid-induced (CiGA) whey protein gels at a) 2500 ×, b) 5000 ×and c) 10,000 × magnification. The scale bars in (a) and (b) represent 10 µm and in (c) represent 1 µm.

Provided that the final pH values of all gel samples were comparable (5.1 ± 0.1), the difference between the microstructural features of the gels was most probably caused by the acidification rate rather than acidification extent. The gel formation process of acid-induced cold-set whey protein gels consists of two distinct steps: the initial formation of gel network until gelation point and the succeeding structuration of the resulting gel through chemical bonds rearrangement. The acidification rate influences the second step (Cavallieri & da Cunha, 2008). Due to the difference between the pK_a values of phosphoric acid and gallic acid, pH reduction up to the pI by phosphoric acid is faster than gallic acid. Hence, the GA-induced gel probably stayed for longer duration in the metastable (bonds rearrangement) state than the PA-induced gel, which rapidly reached the ultimate gel network structure. At lower acidification rates and longer gel formation processes greater counts of bonds rearrangements and thus structural organisation take place (Cavallieri & da Cunha, 2008). It is believed that the more reordering/restructuring of protein aggregates in the GA-induced gel favoured the formation of intramolecular β-sheets (by hydrogen bonding) and smaller aggregates, interlinked together (probably by hydrophobic interactions) compared with the PA acidification that favoured formation of the enormously large protein aggregates amassed by intermolecular β-sheets.

Pre-crosslinking of whey proteins with citric acid before gelation by gallic acid had a significant impact on gel microstructure. In contrast to the microstructure of the non-crosslinked counterpart, the pre-crosslinked gel (i.e., CiGA) had a closed microstructure consisting of large protein aggregates in all three SEM magnifications. Pre-crosslinking increases the molecular weight of proteins, causing formation of large aggregates. In addition, pre-crosslinking might influence the surface hydrophobicity of proteins by disrupting proteins α-helix structure in favor of β-turns (Table 1). While, β-turn content and surface hydrophobicity are not related, a reduction in α-helix content increases the surface hydrophobicity of globular proteins (Wang et al., 2011). The concurrent formation of larger aggregates (Fig. 3) and reduction of α-helix structures (Table 1) due to pre-crosslinking indicates that citric acid-mediated pre-crosslinking prior to GA gelation increased the extent of hydrophobic interactions, resulting in fused (closely packed) network of large aggregates in the CiGA-induced gel.

3.4. Gel strength

The textural attributes including the compressive stress and penetration force of different gel samples are reported in Table 3. The compressive stress of the PA-induced gel was significantly higher than those of the GA-induced and CiGA-induced samples. However, the penetration force of the former was not significantly different from those of the latter gels. It is argued that the extensively compact microstructure accounts for the firmer (i.e., higher compressive stress) texture of the PA-induced gel (see Table 3).

Pre-crosslinking with citric acid did not significantly influence the textural attributed of the GA-induced gel (Table 3). However, a non-significant reduction of both of the textural indices (compressive stress and penetration force) is observed as a consequence of pre-crosslinking. Though pre-crosslinking resulted in larger aggregates with a more closely packed network (Fig. 3), it could also include the carboxylic acid spacer arms between proteins (Farjami et al., 2015; Xu et al., 2015). The inclusion of the crosslinker spacer arms within protein aggregates might cause distortions (as evidenced by α-helix content reduction, Table 1) at molecular level, which counterbalanced the closely packed arrangement of protein aggregates at supramolecular level (gel state). This not only prevented gel hardening but also slightly decreased final gel firmness.

Table 3
Textural and functional properties of phosphoric acid-induced (PA), gallic acid-induced (GA), and citric acid pre-crosslinked and gallic acid-induced (CiGA) whey protein gels.[a]

Gel sample	Compressive stress (kPa)	Penetration force (N)	WHC (%)
PA	1.233 ± 0.011[a]	1.741 ± 0.173[a]	99.72 ± 0.47[a]
GA	0.578 ± 0.060[b]	1.516 ± 0.066[a]	60.54 ± 0.37[c]
CiGA	0.506 ± 0.047[b]	1.284 ± 0.35[a]	72.59 ± 2.3[b]

[a] WHC, water-holding capacity. Means within the same column with different superscript letters differ significantly ($p < 0.05$).

3.5. Gel WHC

The WHC of hydrogels is of outmost importance, since it determines the gels functionality in technological applications and during storage. The gel made by PA had a significantly higher WHC than the GA-induced and CiGA-induced samples (Table 3). Water retention of hydrogels is related to their structure, the pore size of gel network, and characteristics of polymers. Water is hold tightly within the gel networks with small pores by capillary forces (Vardhanabhuti et al., 2001). As shown in Fig. 3, the PA-induced gel had an extensively compact microstructure, which enabled the network to immobilise water within the near vicinity of whey proteins rather than allow it to accumulate at discrete serum pools over a long gelation process. The water collected within serum pools is more easily squeezed out than that in proteins vicinity.

Citric acid crosslinking of whey proteins prior to gallic acid gelation resulted in a higher WHC. It is noteworthy that not all of the carboxylic acid groups of citric acid get involved in protein crosslinking (Xu et al., 2015). The presence of non-reacted carboxylic acid groups in the structure of crosslinked proteins could increase the WHC. In agreement to our observation, Farjami et al. (2015) reported that citric acid crosslinking of whey proteins increased the WHC of hydrogels made of protein microgels and attributed the result to inclusion of the non-reacted carboxylic acid groups in the microgels structure.

3.6. Gel swelling

The water uptake by gels causes gel swelling. Proteins are ionisable, so that they can imbibe water in considerable amounts by ion–dipole interactions. Swelling is an important parameter in the case of controlling the release rate of cargos from hydrogels (Göpferich, 1996). The swelling extent of the PA-induced and GA-induced hydrogels increased throughout the first 90 min (Fig. 4), which is ascribed to entry of water into protein lattice and its subsequent binding to proteins. The GA-induced gel had a higher swelling extent at every given time; however, the swellability of the PA-induced gel increased at a higher rate than the GA-induced gel: an increase of ~3.25 fold for the PA-induced gel compared with ~2.75 fold for the GA-induced over a period of 2 h. The GA-induced gel had a much more porous microstructure (Fig. 3) than the PA-induced gel. The porous structure of the GA-induced gel could enable entry of higher volumes of water into the gel network once the gel was immersed in SGF. Nonetheless, inclusion of GA which is hydrophobic into the gel structure lowered the water uptake rate, when compared with the highly ionisable PA.

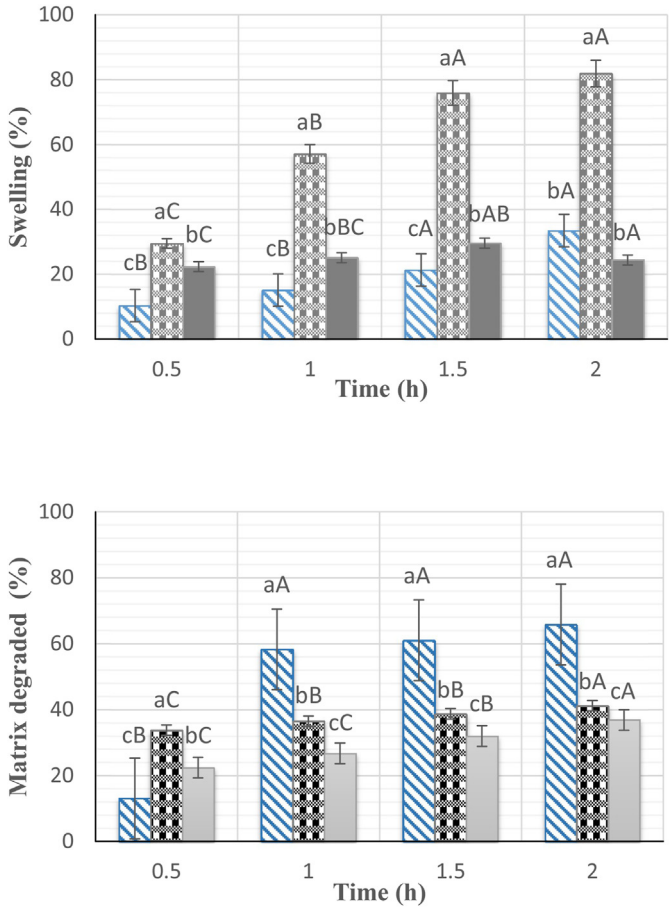

Fig. 4. Swelling in pepsin-free simulated gastric fluid and in vitro peptic degradation of whey protein gels at 37 °C; Gel samples included phosphoric acid-induced (▨), gallic acid-induced (▩), and citric acid pre-crosslinked and gallic acid-induced (■). Lower case letters indicate significant difference ($p < 0.05$) between samples at an identical time; upper case letters indicate significant difference for a given sample over time.

Pre-crosslinking with citric acid decreased the swelling extent of the GA-induced gel in pepsin-free SGF. As well, the swellability of the CiGA-induced gel did not change over longer immersion times. Hence, though citric acid pre-crosslinking increased the WHC of the GA-induced gel (Table 3), it reduced gel swellability. In the current study WHC was determined at a higher pH value (~5.1) than swellability (pH ~1.2). At higher pHs carboxylic groups are deprotonated (i.e., COO^-), while they become extensively neutralised when pH is reduced to a value of 1.2. On the contrary, free amino groups increasingly become positively charged (i.e., NH_3^+) when pH decreases. Based on these facts we concluded that the difference between the WHC of the GA-induced and CiGA-induced gels was rather governed by the presence of non-reacted COO^- groups involved into gel network during crosslinking; whereas, the difference between swellability of the gels was dependent on the presence of free NH_2 groups. The COO^- groups could increase WHC by retaining water in gel network at a higher pH (5.1) but became neutralised in acidic condition of SGF (COO^- protonates to COOH). On the other hand, free NH_2 groups of proteins were consumed due to crosslinking; therefore, less NH_3^+ groups were present in the structure of the CiGA-induced gel in the acidic condition. NH_3^+ groups could strongly bind to the imbibing water and swell the gel.

3.7. In vitro gastric digestibility

The simulated gastric digestion of hydrogel samples was assessed in SGF containing pepsin to investigate and understand the structural alterations of hydrogels as food components in physiological terms. All three types of the acid-induced WPI gels degraded in the SGF by pepsin (Fig. 4). The PA-induced gel had a lower degree of degradation at the first 30 min but then degraded rapidly to a higher extent within the next 30 min (total time of 1 h) compared with the GA and CiGA-induced gels. The significantly lower water uptake (swellability) of the PA-induced gel (Fig. 4) during the first 30 min might cause a lesser entry of pepsin into the gel structure and hence resulted in the initially lower degradation extent, compared with the GA and CiGA-induced gels. However, it is concluded that after pepsin diffusion into gel network (which took place in the first 30 min), molecular interactions (not supramolecular organisation in the gel state) mainly determined the degradability rate. GA interacted with whey proteins via hydrophobic forces, which could decrease pepsinolysis. Pepsin is known to preferentially cleave the peptide bonds which include hydrophobic amino acids. Therefore, GA interaction with the hydrophobic amino acid residues of whey proteins decreased the peptic degradability. Pessato et al. (2018) showed that interaction of whey proteins with tea and coffee phenolic compounds increased the resistance of proteins to the gastrointestinal digestion. Considering the proteins secondary structure (Table 1), it can be elucidated that intermolecular β-sheets (found in the PA-induced gel) were more prone to pepsinolysis than intramolecular β-sheets (found in the GA and CiGA-induced gels). As expected, citric acid crosslinking of whey proteins prior to gelation with GA decreased the peptic degradation extent at any given time. Crosslinking reduced pepsin diffusion in to gel network (lower swellability, Fig. 4) and interconnected proteins with exotic (not occurring naturally) amide bonds.

4. Conclusion

We showed how a biologically active compound, gallic acid, can be exploited for gelation of whey proteins. Fig. 5 schematically illustrates the interactions between gallic acid and whey proteins, in comparison with those between phosphoric acid and whey proteins, as well as, it shows how citric acid crosslinking before gallic acid gelation influences gel microstructure.

This document indicates that some bioactive cargos can be exploited as the gelling agent. The GA-induced gel underwent a lower extent of disintegration in the simulated gastric fluid compared with the PA-induced gel. The disintegration extent of the GA-induced gel further decreased by pre-crosslinking whey proteins before gelation. These observations indicate that GA holds premises as a gelling agent to modulate proteins gastric digestibility. Nonetheless, the release behaviour of gallic acid from the protein gel network, as well as the influence of crosslinking on allergenicity of whey proteins remains to be explored by performing additional studies, which concern intestinal digestions, cellular uptake, and inflammatory assessments.

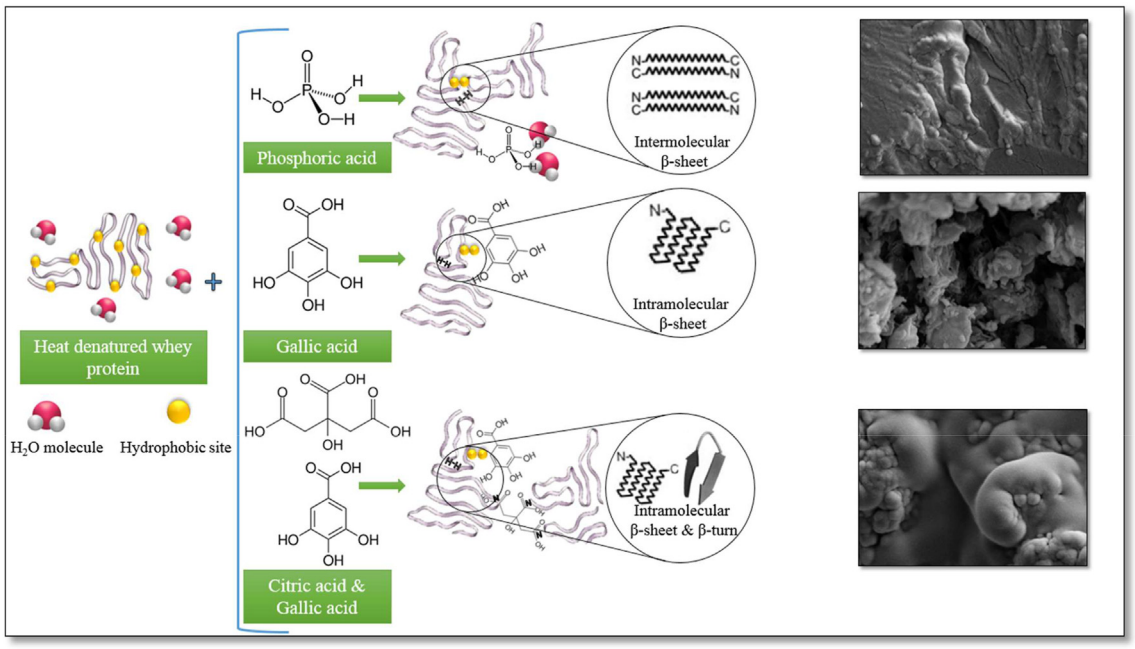

Fig. 5. Schematic illustration of interactions in whey protein gels and their microstructural images formed by different acidulants.

Declaration of competing interest

The authors of the manuscript declare no conflict of interest.

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