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Effects of Transglutaminase-Induced Modification in the Presence of Oligochitosan of 1 kDa on the Structure and Gelling Properties of Caseinate

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Abstract

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Caseinate, transglutaminase (TGase), and an oligochitosan of 1 kDa were used to prepare a glycated and cross-linked caseinate (GC-caseinate), aiming to assess potential changes in both the structure and gelling properties of such caseinate. The results of Fourier transform infrared analysis revealed that only GC-caseinate contained saccharide portions in its molecules, evidencing TGase-induced caseinate glycation. Circular dichroism results showed that GC-caseinate possessed a more ordered secondary structure than caseinate. Other results also demonstrated that TGase-induced modification resulted in a lower gelation temperature of GC-caseinate (59°C vs. 68°C) and increased the final tan δ value (0.30 vs. 0.15) compared to caseinate during the development of acidified gels. In addition, the acidified gels from GC-caseinate were detected to have lower water holding capacity (0.720 vs. 0.781 g/g gels), expanded gel network, and larger pore sizes than those from caseinate. It is thus evidenced that the used TGase-induced modification could confer caseinate with ordered secondary structure, expanded gel network but lower water holding capacity.

Keywords: milk proteins; oligosaccharide; enzyme; glycation; cross-linking; property

Milk gels have both traditional and high commercial value within the dairy industry (Schorsch *et al*. 2000; Siamand *et al*. 2014). Besides renneting, acidification of milk is the most routine way to form milk protein gels. Acidifying milk using glucono-δ-lactone (GDL) can slowly form structured gel products (Lucey 2004). Two major steps are involved in the formation of the acidified milk gels. In the first step, a collapse of the hairy brush of the casein micelles occurs, and the colloidal calcium and phosphate are then dissolved out of the casein micelle as a result of the protonation of ionised phosphate groups (Gaygadzhiev *et al*. 2009). Micellar structure is thus profoundly altered but without dissociation. In the second step, at a pH value about 5.0, casein precipitates isoelectrically, or under quiescent conditions it forms rather fragile gels (Lucey 2004). The balance between attractive and repulsive forces within protein molecules results in gel formation (Havea 2006; Rocha *et al*. 2009). Both intrinsic (e.g. amino acid compositions, molecular weights, hydrophobicity, etc.) and extrinsic (protein concentrations, pH value, temperature, metal ions, and ionic strengths) factors all show impacts on gelation and physiochemical properties of protein gels.

During the last years, transglutaminase (TGase, EC 2.3.2.13) has been used to modify proteins via inducing protein cross-linking (between glutamine and lysine residues of the proteins) (Anema & Kruif 2012; Domagała *et al*. 2015). TGase-induced modification is able to form a more homogeneous gel network (Færgemand & Qvist 1997; Ercili-Cura *et al*. 2013), and thus improve water retention (Schorsch *et al*. 2000; Ercili-Cura *et al*. 2013) and texture (Ercili-Cura *et al*. 2010) of protein gels.

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Glycation is also able to impact gelling properties of proteins; for example, the Maillard-type glycation can enhance the water-holding capacity of dried egg white (Handa & Kuroda 1999) and gel strength of hen ovalbumin (Segura-Campos *et al*. 2011). In recent years, TGase is used to induce protein glycation (between the $-NH_2$ of amino-containing saccharides and the glutamine residues of proteins) (Jiang & Zhao 2010, 2011; Song & Zhao 2014a, b), besides the above-mentioned protein cross-linking. TGase thus shows a potential way to incorporate those amino-containing saccharides into proteins, which generates glycated and cross-linked proteins (GC-proteins). It has been demonstrated that both GC-caseinate and GC-soy protein were better in water binding and emulsion stability (Song & Zhao 2014a, b). In addition, when caseinate and an oligochitosan of 1 kDa are used for the preparation, the acidified gels prepared from GC-caseinate have shorter gelation time and enhanced textural indices (Song & Zhao 2013). However, other properties of the GC-caseinate are not assessed and reported at the present time.

In this study, structural changes and gelling properties of the GC-caseinate were assessed using both caseinate and cross-linked caseinate as two controls. Fourier transform infrared (FTIR) and circular dichroism (CD) analyses were used to reflect structural changes, while rheological analysis was used to monitor gel development during acidification. The gels were evaluated for their water-holding capacities, and also characterised for microstructure using scanning electron microscopy (SEM). The aim of this study was to verify the effects of the carried out glycation and cross-linking on the structure and gelling properties of caseinate.

MATERIAL AND METHODS

Material and chemicals. Caseinate was purchased from Sigma-Aldrich Co. (St. Louis, USA) with protein content of 90.7% (on dry basis). Oligochitosan, with a declared deacetylation degree about 75% and an average molecular weight of 1 kDa by the supplier, was purchased from Zhejiang Golden-Shell Biochemical Co. (Hangzhou, China). TGase was brought from Jiangsu Yiming Fine Chemical Industry Co., Ltd. (Qinxing, Jiangsu, China) with a measured activity of 92 units per gram (U/g). Other chemicals used were of analytical grade.

Preparation of GC-caseinate and acidified gels. GC-caseinate was prepared as previously described (Song & Zhao 2014a). The acidified gels were prepared as per the Koh *et al*. (2002) method with minor modifications. During the preparation, GDL granules were added into protein dispersions (40 g/kg, pH 7.0) at 0.2 g/g protein, and mixed thoroughly for 2 min at 25°C. This GDL addition was sufficient to induce a drop in pH to about 5 in approximately 2 h (Koh *et al*. 2002). The prepared gels were kept at 4°C for 12 h before evaluation.

Assay of water-holding capacity. Water-holding capacity (WHC) of the acidified gels was evaluated as per the Tsumura *et al*. (2005) method. Gel samples with known weights (W_1, g) were centrifuged at 800 *g* for 10 min at 4°C. The supernatants were collected, and weighed (W_2, g). WHC was calculated by the equation $[(W_1 - W_2)/W_1] \times 100$.

Fourier transform infrared spectroscopy, circular dichroism, and scanning electron microscope analyses. The analysed samples were prepared in KBr discs as per the Schneckenburger *et al*. (2012) method. FTIR spectra (400–4000 cm^{-1}) were recorded at a Spectrum One FT-IR spectrometer (Perkin Elmer Inc., Norwalk, USA), using 1 cm^{-1} resolution and an accumulation of 32 scans.

Secondary structure features of the analysed samples were assessed using a Jasco J-815 CD spectrometer (Jasco Corporation, Tokyo, Japan) at 25°C. Diluted protein samples (50 μg/ml in 10 mmol/l phosphate buffer, pH 7.0) were scanned at a wavelength range of 190–240 nm. Reported molar ellipticity was calculated as [θ] (deg cm^2/dmol) (Jiang *et al*. 2009).

SEM observation of the acidified gels was carried out as previously described (Haga & Ohashi 1984). Gel samples were fixed in 25 g/kg glutaraldehyde (0.1 mol/l phosphate buffer, pH 6.8) and 10 g/kg osmium tetraoxide, and then subjected to recommended dehydration. The samples were freeze-dried (–20°C) at a freeze-dryer (Flexi-dry™; FTS System Inc., Stone Ridge, USA), mounted, sputter-coated with gold-palladium and then observed at a Hitachi S-4300 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) at 20 kV.

Small-deformation oscillatory measurement. A Malvern Bohlin Gemini II rheometer equipped with parallel plates (60 mm diameter and 1 mm gap) was used to monitor the development of acidified gels. The protein dispersions were prepared at 40 g/kg, while GDL was added at 0.2 g/g protein. To prevent water evaporation and surface drying during the

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measurement, all samples were surrounded with silicone oil. Temperature ramps of the protein dispersions during acidification were enhanced from 25°C to 85°C at a fixed rate of 1°C/min, followed by holding at 85°C for 5 minutes. The dispersions were oscillated at a frequency of 1 Hz and at a strain of 1%, which was previously found in the linear viscoelastic region. Storage modulus (G') and loss tangent (tan δ = G''/G', G'', loss modulus) of the dispersions were recorded. Gelation temperature was assigned to the temperature point at which $G' \geq 1$ Pa for the gels (Ercili-Cura *et al*. 2010).

Statistical analysis. All experiments and analyses were carried out three times. All reported data were expressed as means or means ± standard deviations. Differences between the means of multiple groups were analysed by one-way analysis of variance (ANOVA) with Duncan's multiple range tests.

RESULTS AND DISCUSSION

Secondary structural features of GC-caseinate. FTIR spectra (Figure 1) show structural features of caseinate, cross-linked caseinate, and GC-caseinate. Three protein samples had similar profiles in most absorption peaks but showed a difference around 1100 cm^{-1} (as the arrow indicates). In total, GC-caseinate showed stronger absorption around 1100 cm^{-1} than caseinate and cross-linked caseinate. The absorption around 1100 cm^{-1} is used to reflect C–O stretching and O–H deforming vibration (Guan *et al*. 2006). It is thus suggested that GC-caseinate had more –OH than caseinate and cross-linked caseinate. This result proves an indirect fact: the oligochitosan of 1 kDa was covalently linked to the molecules of caseinate during the reaction. This result is supported by a previous result of HPLC analysis (Song & Zhao 2014a), in which only GC-caseinate (but neither caseinate nor cross-linked caseinate) was detected to have conjugated glucosamine at a level of 4.74 g/kg protein.

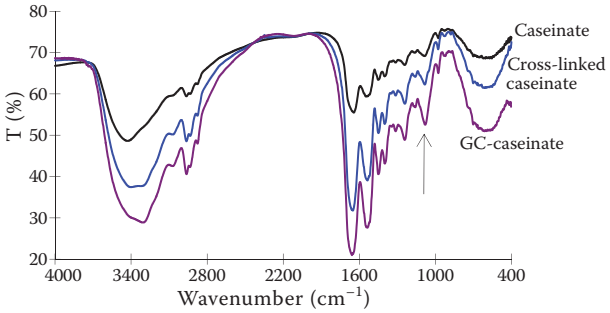

Figure 1. FTIR spectra of caseinate, cross-linked caseinate, and GC-caseinate

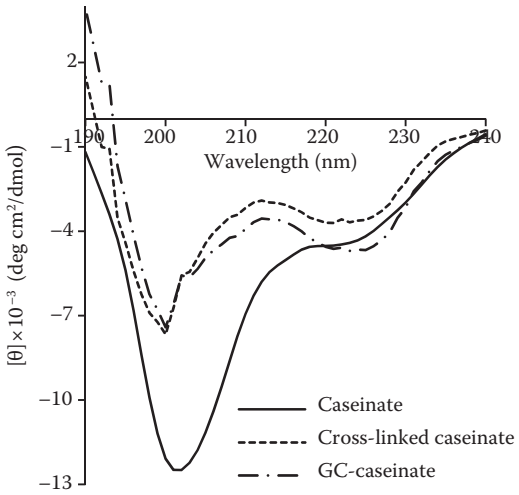

Figure 2. Circular dichroism spectra of caseinate, cross-linked caseinate, and GC-caseinate dispersed in 10 mmol/l phosphate buffer of pH 7.0

Results of CD analysis are shown in Figure 2. In comparison with caseinate, both cross-linked caseinate and GC-caseinate showed clearly weaker negative absorption around 200 nm, an indicator of the disordered secondary structure (Johnson 1990). These results point out that they both had the less open (i.e. more ordered) secondary structure than caseinate. This finding is reasonable. TGase-induced caseinate cross-linking would generate several intramolecular covalent bonds in the molecules; therefore, both cross-linked caseinate and GC-caseinate got more intra-interaction and thereby they exhibited the more ordered secondary structure than caseinate. It is well-known that caseins have an open secondary structure (Creamer *et al*. 1981), thus they will have maximum negative absorption around 200 nm (Johnson 1990) as the present analysis showed. Darewicz and Dziuba (2001) have found that random loops of β-casein during its reaction with glucose are partially converted into ordered conformation. This finding shows that protein glycation results in the ordered secondary structure, sharing the same conclusion with the present study.

Gelling properties of GC-caseinate. The obtained results (Figure 3A) indicate that GC-caseinate had a lower gel point temperature than caseinate and cross-linked caseinate (59°C vs. 68°C and 70°C). This result proves that the carried out modification

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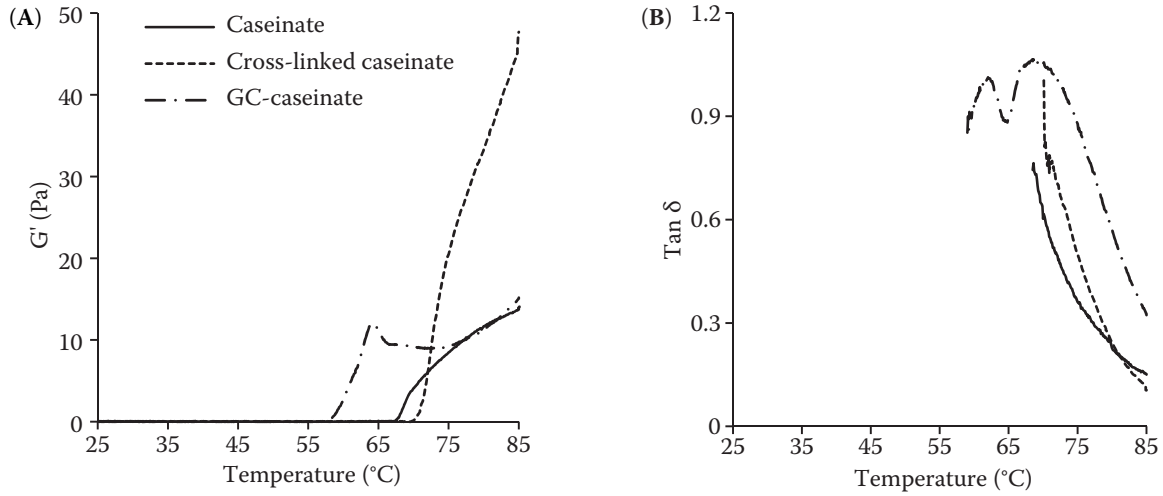

Figure 3. Monitored (**A**) storage modulus (*G'*) and (**B**) tan δ values of caseinate, cross-linked caseinate, and GC-caseinate dispersions during the development of acidified gels. Protein concentration and GDL addition used were 40 g/kg and 0.2 g/g protein, respectively. The temperature was increased at a rate of 1°C/minute

conferred GC-caseinate with faster gelation (i.e. GC-caseinate gelatinised at a lower temperature). Moreover, it was also observed that the GC-caseinate gels had the higher final tan δ value (0.30) than caseinate and cross-linked caseinate gels (0.15 or 0.10) (Figure 3B). Hydrogen bonds, belonging to the most important forces involved in the interaction of protein molecules, can take part in protein gelation (Schorsch *et al*. 2000). GC-caseinate contained saccharide groups in its molecules whilst neither caseinate nor cross-linked caseinate had any saccharide groups. It is reasonable that GC-caseinate was able to easily form acidified gels through the hydrogen bonds, reflected by the detected lower gelation temperature. Moreover, the higher final tan δ value also indicates that the gels would be formed quickly with larger pore sizes (Van Vliet *et al*. 1991; Ercili-Cura *et al*. 2013). Obviously, the result of the present analysis showing the final tan δ values of three protein samples gives a conclusion consistent with the two mentioned studies.

WHC and microstructural features of acidified gels. WHC of the three acidified gels were measured using a centrifugation method. The results given in Figure 4 demonstrate that the gels from GC-caseinate had the lowest WHC value (0.720 g/g gels), followed by those from caseinate (0.781 g/g gels) and cross-linked caseinate (0.844 g/g gels). These data indicate that TGase-induced cross-linking conferred higher WHC for the cross-linked caseinate gels, whilst TGase-induced oligochitosan conjugation and cross-linking led to lower WHC for the GC-caseinate gels. TGase treatment of skim milk can enhance WHC of the gels, due to decreased cluster size and increased homogeneity of the gel network (Ercili-Cura *et al*. 2013). The gels from cross-linked caseinate therefore had higher WHC than those from caseinate. However, why the gels from GC-caseinate showed lower WHC was not characterised in detail and thereby it would need additional evaluation (e.g. using SEM observation) to obtain extra evidence.

Results of the SEM analysis (Figure 5) reveal that the three gels had different microstructural features. In general, the gels from GC-caseinate had larger pore size and expanded gel network (Figure 5C), indicating

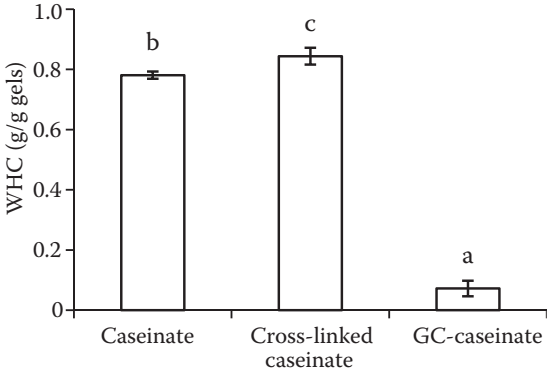

Figure 4. WHC values of the acidified gels from caseinate, cross-linked caseinate, and GC-caseinate. Different lowercase letters above the columns indicate that the mean values analysed by one-way analysis of variance (ANOVA) are significant differences ($P < 0.05$)

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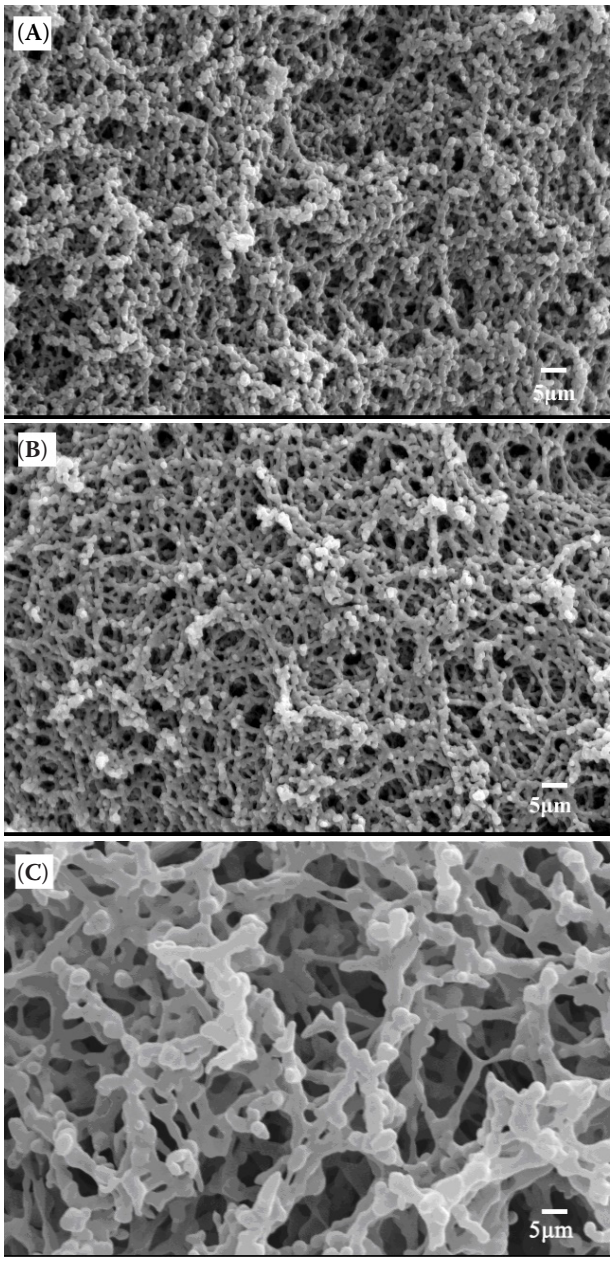

Figure 5. Microstructural features of the acidified gels from caseinate (A), cross-linked caseinate (B), and GC-caseinate (C). The gels were prepared at a protein concentration of 40 g/kg with GDL addition of 0.2 g/g protein

their structural instability. On the contrary, the other two gels showed smaller pore sizes (Figures 5A, B). The gels from cross-linked caseinate showed a more homogeneous network (Figure 5B). These results support that the gels from GC-caseinate and cross-linked caseinate should have the highest and lowest WHC, respectively. It has been proved that caseinate cross-linking results in a homogeneous gel network (Færgemand & Qvist 1997; Schorsch *et al*. 2000). More important, the gels with small pore size are suggested to be responsible for good WHC (Mao *et al*. 2001). All these mentioned results give scientific support to the results of the present analysis, evidencing lower WHC of the GC-caseinate gels.

CONCLUSION

In this study, the structure and gelling properties of a modified caseinate (glycated and cross-linked caseinate, GC-caseinate) induced by transglutaminase in the presence of oligochitosan were characterised. The modification conferred caseinate with saccharide conjugation and ordered secondary structure. GC-caseinate also had a lower gelation temperature during the development of acidified gels. The gels thus formed had an expanded gel network, larger pore size, and lower water holding capacity. It is thus concluded that this modification results in caseinate modified structure and gelling properties.

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