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Effect of transglutaminase and acidification temperature on the gelation of reconstituted skim milk

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ABSTRACT

Effects of transglutaminase (TG), acidification temperature and total milk solids level on the acid gelation of skim milk were investigated. Despite similar acidification kinetics, TG-treated milk acidified at ≥35 °C showed differences in elastic modulus (G') with acidification time, particularly by inhibiting the formation of the peculiar shoulder ($G'_{shoulder}$) observed at an early stage of gelation in the control milk. Regardless of the milk solids content, the $G'_{shoulder}$ was absent in both types of milk at 30 °C. However, control milk above 2.5% (w/w) milk solids showed the $G'_{shoulder}$ at 45 °C. $G'_{shoulder}$ is proposed as the transition from the first increase in G', due to aggregation of soluble protein complexes at the early stage of acidification, to the second, due to further aggregation of casein micelles (and aggregated soluble complexes) as acidification progresses. The $G'_{shoulder}$ was absent in acidified TG-treated milk due to the lack of soluble protein complexes containing caseins.

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1. Introduction

An acid milk gel is a three-dimensional network of interconnected milk protein aggregates spanning throughout the serum of milk (Bonanomi, Sandkuhler, Sefcik, Morari, & Morbidelli, 2004; Urbicain & Lozano, 1997). The formation of acid milk gels is crucial in the dairy industry as it is the initial step of the production of commonly consumed dairy products such as yoghurt (Lucey, 2002). During the gelation of milk by acidification, the nature of the molecular interactions and the arrangement of macromolecular matrices govern the gel's final properties. These interactions and arrangements are dependent on the starting materials, and the processing conditions during manufacture (e.g., heat treatment, pH, and temperature). Variation of these parameters during manufacture can allow manipulation of the gel matrix so that desired gel properties are achieved.

Transglutaminase (TG; R-glutaminyl-peptide:amine γ-glutamyl-transferase; EC 2.3.2.13) (Gauche, Vieira, Ogliari, & Bordignon-Luiz, 2008; Ikura, Kometani, Yoshikawa, Sasaki, & Chiba, 1980; Lauber, Henle, & Klostermeyer, 2000; Law, 2010; Zhu, Rinzema, Tramper, & Bol, 1995) induces intra- and inter-molecular crosslinks between proteins (Lauber et al., 2000) by catalysing acyl-transfer reactions (Arrizubieta, 2007). In the crosslinking reaction, TG links the γ-carboxyamide group of peptide-bound glutamine residues (acyl donors), with the primary ε-amino groups of peptide-bound lysine residues (acyl acceptors) (Nonaka et al., 1989), and the resultant covalent bond formed is called a ε-(γ-glutamyl)-lysine isopeptide bond (de Jong & Koppelman, 2002). TG can also catalyse amine incorporation and deamidation reactions. The amine incorporation reaction occurs for other ε-amino groups which act as acyl acceptors. Deamidation occurs when primary amines are absent, where water acts as the acyl acceptor (Zhu et al., 1995). It is considered that traditionally made acid milk gels are weak gels, as they are stabilised predominately by weak non-covalent interactions (Schorsch, Carrie, & Norton, 2000). The introduction of covalent bonds into milk by the treatment with TG modifies the structure of the acid milk gels, and TG-treated acid milk gels are found to have stronger gel strength, lower permeability and much finer microstructure than TG-untreated milk gels (Faergemand & Qvist, 1997; Faergemand, Sorensen, Jorgensen, Budolfsen, & Qvist, 1999; Lorenzen, Neve, Mautner, & Schlimme, 2002).

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During acid gelation, the acidification temperature impacts the gelation kinetics and the characteristics of the final gels formed, particularly the rheological behaviour and degree of syneresis. Elevated temperatures (e.g., ≥35 °C) are often used in yoghurt manufacture to speed up the acidification rate and reduce manufacturing time, since higher temperatures can increase the gelation pH and decrease the gelation time (Lee & Lucey, 2003, 2004; Lucey, Tamehana, Singh, & Munro, 1998a; Lucey, Teo, Munro, & Singh, 1997a; Vasbinder, Rollema, Bot, & de Kruif, 2003). However, several authors have reported that, when milk is acidified at elevated temperatures (≥35 °C), a peculiar behaviour in the elastic modulus (G') versus gelation time curve occurs, where a shoulder in the elastic modulus G' ($G'_{shoulder}$) during the early stage of gelation is observed (Anema, 2009a; Ercili-Cura et al., 2013; Horne, 2003; Hyun et al., 2011; Koutina & Skibsted, 2015; Lee & Lucey, 2004, 2006). This was also observed in the case of the acidification of protein dispersions containing a mixture of micellar caseins and whey proteins (Famelart, Tomazewski, Piot, & Pezennec, 2003, 2004; O'Kennedy & Kelly, 2000). Anema (2009b) found that $G'_{shoulder}$ was not observed on the gelation profile at 30 °C; whereas Lucey et al. (1998a) and Ercili-Cura et al. (2013) reported its occurrence at 30 °C.

Two main explanations have been offered in the literature to explain the presence of the $G'_{shoulder}$. The first explanation is the transition from the first increase in G' due to the weakening of intramicellar casein–casein interactions induced by colloidal calcium phosphate (CCP) solubilisation at the early stage of acidification, to the second increase in G' due to enhanced casein–casein interactions when the acidification progresses (Anema, 2009a; Ercili-Cura et al., 2013; Horne, 2003; Koutina & Skibsted, 2015; Lee & Lucey, 2004). The second explanation is the transition from the first increase in G' involving aggregation of complexes containing denatured whey proteins and caseins, to the second increase in G' involving casein–casein interactions as acidification progresses (Famelart, Tomazewski, Piot, & Pezennec, 2004, 2003; Lucey, Munro, & Singh, 1998b; O'Kennedy & Kelly, 2000).

As well as pre-treatment of milk with TG, increasing the total milk solids level before acidification also improves the textural and sensory attributes of acid milk gels (Lee & Lucey, 2010; Tamime, 1980). An increase in total solids content can be obtained by fortifying the milk with milk solids such as skim milk powder, micellar casein, milk protein isolate and sodium caseinate (Peng, Serra, Horne, & Lucey, 2009), or through concentrating the milk by membrane filtration (Biliaderis, Khan, & Blank, 1992). All of these are alternatives to the use of hydrocolloids such as gelatin and starch to address quality concerns such as poor gel texture and syneresis (Lorenzen et al., 2002; Peng et al., 2009). When the total solids concentration of an acid milk gel is increased, the viscosity (Guirguis, Broome, & Hickey, 1984; Wacher-Rodarte et al., 1993) and the final gel firmness and elasticity (Anema, 2008a; Gastaldi, Lagaude, Marchesseau, & Tarodo de la Fuente, 1997) increase. Increasing the milk solids content from 10% to 30% (Harwalkar & Kalab, 1986), and adding whey protein isolate or sodium caseinate, also reduce whey separation of acid milk gels (Isleten & Karagul-Yuceer, 2006).

Although acid gels from TG-treated milk have been previously studied, studies on the combined effects of TG, acidification temperature and total solids content, and studies explaining the existence of the $G'_{shoulder}$ that occurs during the early stage of gelation, remain limited. Therefore, this study investigated the effects of acidification temperature (30, 35, 40 and 45 °C) and total milk solids (2.5, 5, 10, 15, 20, 25 and 30%, w/w) on the formation and rheological properties of acid milk gels with and without TG treatment.

2. Materials and methods

2.1. Materials

Low-heat skim milk powder, with composition of 33.5% protein; 3.7% moisture; 7.8% ash; 0.9% fat; and 54.5% lactose (as provided by the manufacturer) was kindly donated by Westland Co-Operative Dairy Company Limited (Hokitika, New Zealand). TG (TG-BW-MH) with specific activity of 634 ± 2 U g^{-1} was gifted by Ajinomoto (Kuala Lumpur, Malaysia), and was used without further purification. The specific TG activity was determined using the hydroxamate method by Sigma-Aldrich Co. (1994), based on the method of Folk and Cole (1966). All the chemicals used were of analytical grade. Sodium azide (0.02%, w/w) was added to Milli-Q water (resistivity at 18.2 MΩ cm) used in sample preparation to prevent bacterial growth.

2.2. Preparation of milk samples

The steps during sample preparation are shown in Fig. 1. Low-heat skim milk powder was reconstituted with Milli-Q water to obtain stock skim milk samples with solids concentrations of 2.8, 5.5, 11.1, 16.6, 22.1, 27.6 and 33.2% (w/w). Mixtures were gently stirred for 2 h using a magnetic stirrer to ensure thorough dispersion and reconstitution of the milk powder and were subsequently divided into two equal fractions. One fraction was treated with TG solution (95 U g^{-1} of milk protein), and the other fraction had Milli-Q water added and is referred to as TG-untreated. The pH of the milk fractions with and without TG was adjusted to 6.70 by the addition of 1 M HCl or 1 M NaOH. Milk fractions had final milk solids concentrations of 2.5, 5, 10, 15, 20, 25 and 30% (w/w) and were incubated at 30 °C for 15 h. The samples were heat-treated at 70 °C for 15 min to inactivate the TG. Samples prepared with and without TG were divided into 30 g portions and acidified with glucono-δ-lactone (GDL) to lower the pH below the isoelectric point (pI, 4.6) of caseins. GDL was added at a level of 0.2 g g^{-1} milk solids.

2.3. Preparation of sodium caseinate

Sodium caseinate was isolated from 10% (w/w) reconstituted milk, based on an adaption of the method of Lucey, Srinivasan, Singh, and Munro (2000). Skim milk was acidified to pH 4.60 ± 0.01 (isoelectric point of caseins) at 20 °C by drop-wise addition of 2 M HCl. The serum was discarded and the casein curd was poured into cheesecloth, washed five times with Milli-Q water and then dewatered for 10 min. The washed curds were removed from the cheesecloth and dispersed in a 1:3 mixture with Milli-Q water. The pH of the mixture was then increased to 6.80 ± 0.01 using 2 M NaOH to resolubilise the proteins in the curd. The prepared sodium caseinate solution was left overnight at 4 °C to ensure full hydration and solubilisation. The solution was divided into small portions and transferred to 50 mL centrifuge tubes, frozen at −80 °C in an ultra-low temperature upright freezer and lyophilised using a FreeZone Plus 12 freeze dryer (Labconco Corporation, Missouri, USA) at −83 °C for 72 h.

2.4. Isolation of milk serum fractions

Acidified milk samples were placed in 30 mL Nalgene centrifuge tubes (Oak Ridge Style 3119, Thermo Scientific, New York, NY, USA) and centrifuged at 38,000 ×g for 60 min at 20 °C in a Sorvall RC 6 Plus Superspeed Centrifuge (Thermo Fisher Scientific, Waltham, NC, USA) with a Fiberlite fixed angle rotor (F21-8x50y, Thermo Fisher Scientific). The supernatant (milk serum) was removed from

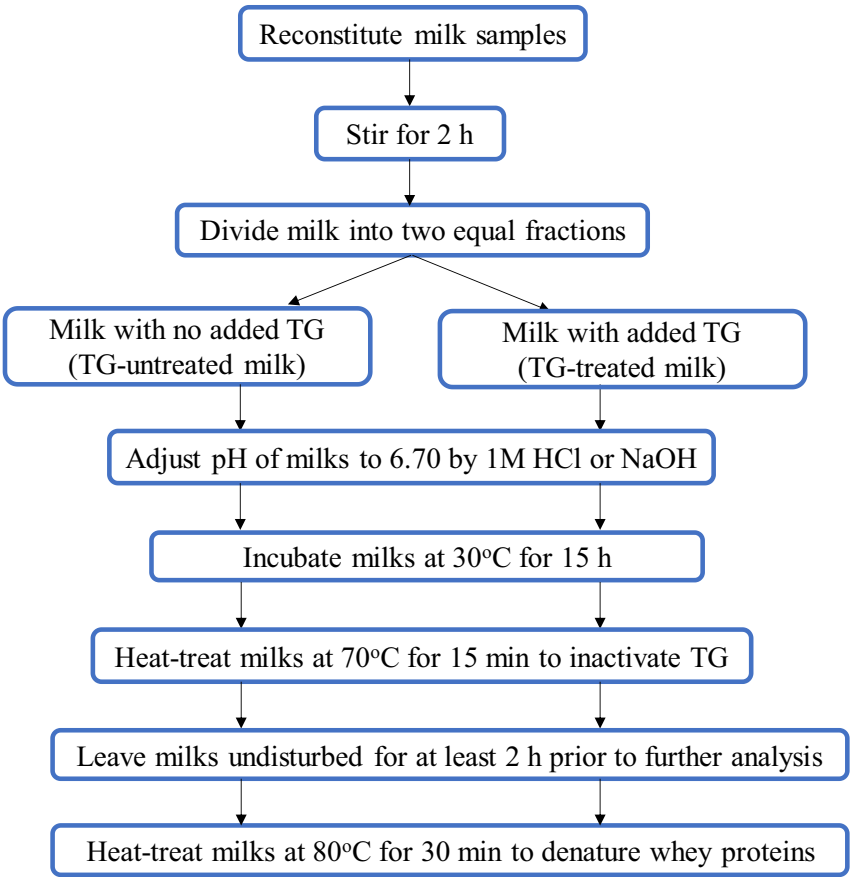

Fig. 1. Flowchart of steps during sample preparation; details can be found in Section 2.2.

the pellet material (casein micelles). The centrifuged supernatants were used as they are.

2.5. Rheological measurements

Dynamic oscillatory rheology was performed to measure the elastic or storage modulus (G') and the viscous or loss modulus (G'') of milk samples during and after acid gelation. An MCR 302 stress-controlled rheometer (Anton-Paar, Graz, Austria) fitted with a cup and bob geometry, consisting of two coaxial cylinders (CC27) (bob diameter: 26.5 mm; length: 48 mm; cup inner diameter: 27.5 mm) was used for all measurements. Approximately 13 mL of sample was poured into the cup and the surface of the sample was covered with a thin layer of soya oil to prevent evaporation. The rheology measurement profile consisted of a time sweep measurement followed by a frequency sweep measurement. The time sweep was initialised by loading the acidified sample into the measuring geometry at 25 °C, then increasing the temperature to the desired value at a rate of 2.5 °C min^{-1}. Acidification temperatures are 30, 35, 40 and 45 °C. Once the target temperature was reached, the measurement of G' and G'' was automatically initiated by the Start Rheoplus software (Anton-Paar). G' and G'' values were recorded as a function of time, each minute for 300 min, at a constant frequency of 1 Hz and a constant applied strain of 0.5%. At the end of the time-sweep measurement, the temperature was equilibrated at 25 °C for 30 min. The frequency sweep was performed by varying the frequency from 0.01 to 10 Hz at a constant strain of 0.5% whilst maintaining the temperature at 25 °C.

The observed $G'_{shoulder}$ is equivalent to the maximum in the tangent of the phase shift angle (δ) (tan δ_{max}) (ratio of the viscous modulus (G'') to G') noted in some publications (Lee & Lucey, 2010; van Marle & Zoon, 1995). Tan δ initially increases after reaching the gel point until tan δ_{max} is achieved (as seen as the first increase in G' and the shoulder region in the $G'_{shoulder}$), after which it decreases again as acidification progresses (as seen as the second increase in G' in the $G'_{shoulder}$). The value of $G'_{shoulder}$ is obtained as the midpoint between the tan δ after reaching the gelation pH and the tan δ_{max}. $G'final\ at\ 25^{o}C$ is the final gel strength of TG-treated and TG-untreated milks after a gelation time of 5 h, obtained from the value of G' at 1 Hz during frequency sweep at 25 °C, as a function of temperature.

Two additional rheological experiments were carried out to elucidate the presence of $G'_{shoulder}$. The first experiment involved adding 0.5% (w/w) freeze-dried sodium caseinate into the TG-treated milk after heat treatment at 80 °C for 30 min. The acidification of heated (80 °C for 30 min) TG-treated milk containing 0.5% (w/w) sodium caseinate at 45 °C was then monitored by small dynamic oscillatory measurements as per Section 2.5.

The second experiment involved monitoring the gelation of a suspension containing the micellar fraction of heated (80 °C for 30 min) TG-treated milk and the serum fraction of heated (80 °C for 30 min) control milk during acidification at 45 °C. The suspension was prepared by centrifuging heated control and TG-treated milks to obtain their pellet (micellar) and serum fractions as in Section 2.4, then taking the micellar fraction of the TG-treated milk and resuspending it in the serum fraction of the control milk.

During the rheological measurements, the pH of the milk samples was measured in situ by dipping a micro pH electrode (Model Z451, Schott, SI Analytics GmbH, Mainz, Germany) connected to a pH meter (Lab860, Schott, SI Analytics GmbH) into the milk samples.

2.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Proteins in milk and serum samples heated at 80 °C for 30 min, and those in unheated milk serum samples, were characterised by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions in the presence of 2-mercaptoethanol with a Mini-PROTEAN tetra cell (Bio-Rad Technologies, Inc., Richmond, CA, USA). SDS-PAGE was performed following the method of Laemmli (1970) using 12% acrylamide resolving gel containing 0.01% (w/v) sodium dodecyl sulphate and 4% acrylamide stacking gel. Milk samples were mixed with Laemmli buffer (Bio-Rad Technologies, Inc.) and 2-mercaptoethanol in ratio of 1:1.9:0.1 (by vol), then heated at 100 °C for 3 min before loading into the wells (loading volume: 10 μL). The gels were run at a constant voltage of 110 V for 4 h with a premixed electrophoresis buffer (pH 8.30) (Bio-Rad Technologies, Inc.), followed by staining using 0.1% (w/v) Coomassie brilliant blue R-250 for 24 h. The stained gel was destained using 40% (v/v) methanol, 10% (v/v) glacial acetic acid and 50% (v/v) Milli-Q water.

2.7. Statistical analysis

A two-tailed paired sample T-test at a 5% significance level ($P < 0.05$) was carried out to investigate the effect of TG treatment on the gelation pH, the final gel strength after 5 h of gelation, and the distribution of minerals [Ca^{2+}, Mg^{2+} and inorganic phosphate (P_i)] during acidification. Details of the quantification of Ca^{2+}, Mg^{2+} and P_i are displayed in Supplementary material Fig. S1. The SPSS Statistics version 22 software (IBM Corp., New York, NY, USA) was used to perform the statistical analyses.

3. Results and discussion

3.1. pH changes during gelation of acidified milks at different temperatures

Acid gels from control and TG-treated milk (10%, w/w) were formed by slowly decreasing the pH of the milks using GDL (Fig. 2). Despite the presence of TG-induced crosslinks, the pH profile and rate of acidification were similar for both types of milk at 30, 35, 40 and 45 °C (note that, for clarity, only curves obtained at 30 and 45 °C, are shown in Fig. 2). The acidification rate increased with increasing temperature. This is due to the increase in the rate of GDL hydrolysis, shifting the equilibrium reaction in favour of the formation of more gluconate ions and protons, thus decreasing the pH at a faster rate (Anema, 2008a). This finding was consistent with that of Anema (2008b), who showed a decrease in the acidification time as the temperature increased from 25 to 40 °C. Lucey et al. (1998a) also found that the pH dropped at a faster rate at 42 °C compared with 30 °C during GDL-induced or bacterial acidification of milk. In addition to the similar pH change between control and TG-treated milks, the partition of minerals (Ca^{2+}, Mg^{2+} and P_i) were also similar ($P < 0.05$) (Supplementary material Fig. S1).

3.2. Effect of acidification temperature on the gelation profiles of acid milk gels

Although there were similar pH profiles and acidification rates at the temperatures studied (Fig. 2), the change in G' as a function of time for 10% (w/w) control (Fig. 3A) and TG-treated (Fig. 3B) milks, differed depending on the temperature of gelation. The differences in the change in G' over time between both types of milk was previously ascribed to the differences in the casein micelle structure before acidification and the temperature-dependent micellar structural changes during acidification (Anema, 2008a; Vasbinder et al., 2003). Before acidification, native casein micelles are stabilised by the balance between hydrophobic and electrostatic interactions, and the presence of CCP (Anema & Li, 2000; Horne, 1998; Liu & Guo, 2008; McMahon & Oommen, 2008). The latter is linking the caseins through phosphoserine residues, neutralising the charges between caseins, and favoured attractive interactions (Horne, 1998; Walstra, 1990). On top of these forces to maintain the micellar integrity, the casein micelles after TG treatment also contained covalent ε-(γ-glutamyl)-lysine isopeptide bonds between caseins (de Jong & Koppelman, 2002). The caseins in TG-treated milk were susceptible to crosslinking in the order of κ-casein >β-casein >α_s-casein, as shown in our previous study (Lam et al., 2018), indicating that TG crosslinking occurred both at the surface and in the core of the casein micelles (Hinz, Huppertz, & Kelly, 2012; Hinz, Huppertz, Kulozik, & Kelly, 2007; Huppertz & de Kruif, 2007a,b; Lam et al., 2018; Sharma, Lorenzen, & Qvist, 2001; Smiddy, Martin, Kelly, de Kruif, & Huppertz, 2006).

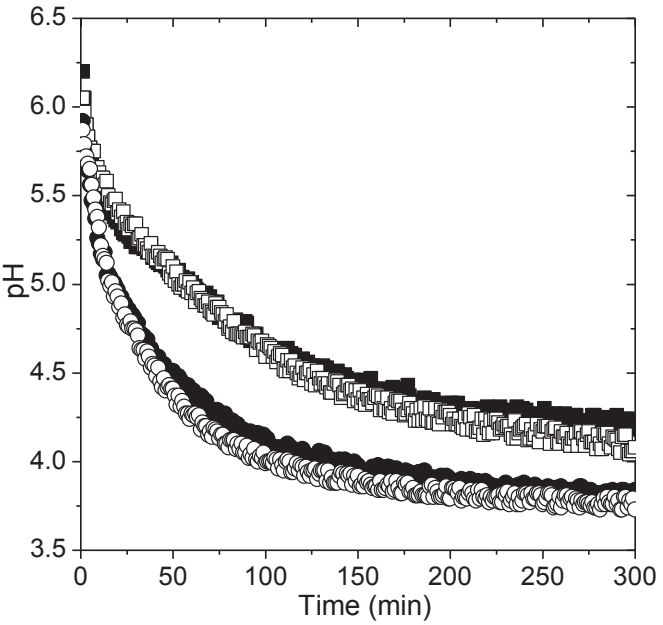

Fig. 2. pH as a function of time for 10% (w/w) heated (80 °C for 30 min) skim milks untreated (solid symbols) and treated with TG (open symbols), and acidified with 2% (w/w) GDL at different acidification temperatures. Temperatures of acidification are 30 °C (■, □) and 45 °C (●, ○).

When acidified at 30 °C, the change in G' for control and TG-treated milks during acid gelation was similar (Fig. 3A,B). They both experienced a lag period in G', but after reaching a critical pH value, the gelation pH, e.g., at pH 5.28 ± 0.01 for control milk and pH 5.21 ± 0.01 for TG-treated milk, the G' values of both milks started to increase, followed by a uniform increase in G' as acidification progressed. Acidification at elevated temperatures (≥35 °C), however, induced different gelation profiles between the control and TG-treated milk (Fig. 3A,B). While a lag period in G' at the beginning of acidification was seen for both types of milk, as the pH was lowered, the control milk did not increase uniformly in G' as seen at 30 °C. Instead, a shoulder region occurred in the G' profile at the early stage of acidification, induced by the transition from the first increase in G' at the time of gelation, to the second increase in G' following acidification (Fig. 3A). This shoulder region in the G' profile is referred to as $G'_{shoulder}$. Anema (2009b) also reported a $G'_{shoulder}$ in the G' profiles when pre-heated (80 °C for 30 min) milk was acidified with GDL at 35, 40 and 45 °C, but not at 30 °C; whereas Ercili-Cura et al. (2013) and Lucey et al. (1998b) reported observations of $G'_{shoulder}$ at 30 °C. For pre-heated yoghurt gels

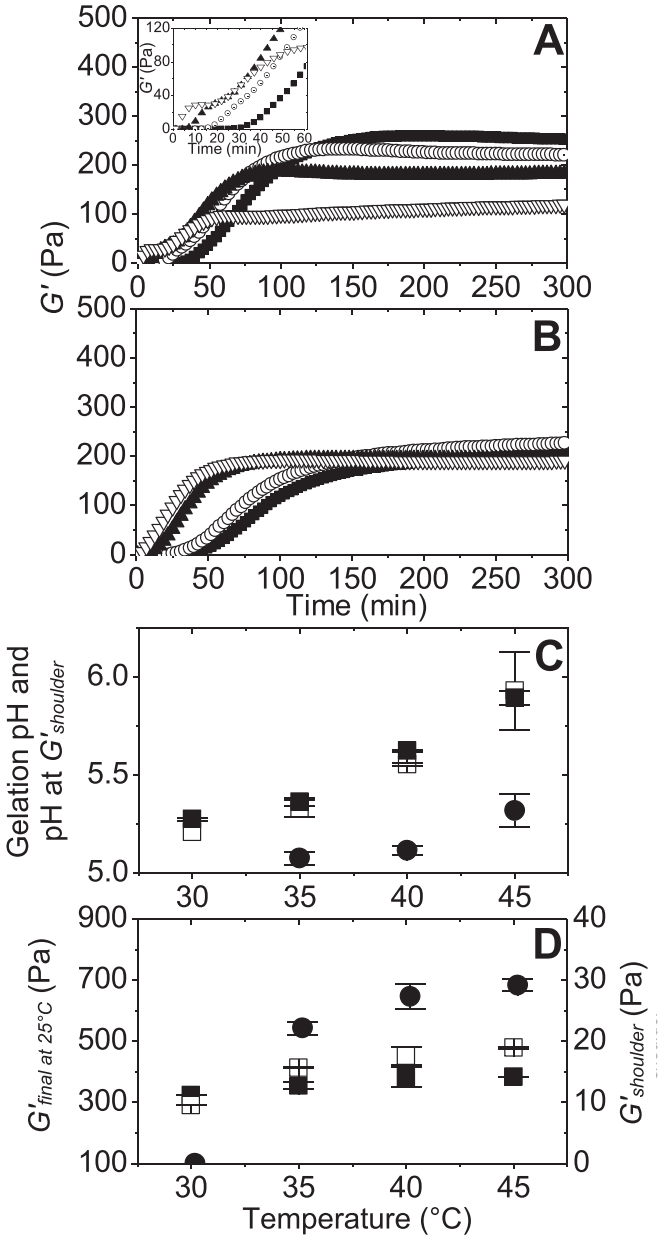

Fig. 3. G' as a function of gelation time for 10% (w/w) heated (80 °C for 30 min) skim milks (A) untreated and (B) treated with TG, and acidified with 2% (w/w) GDL at different gelation temperatures. Temperatures of gelation are: 30 °C (■), 35 °C (○), 40 °C (▲), and 45 °C (▽). Inset in (A): values of G' in (A) plotted for the first 60 min. (C) Gelation pH as a function of gelation temperature for 10% (w/w) skim milks untreated (■) and treated (□) with TG, heated at 80 °C for 30 min, and acidified with 2% (w/w) GDL; and pH at $G'_{shoulder}$ as a function of gelation temperature for 10% (w/w) heated skim milk untreated with TG (●) and acidified with 2% (w/w) GDL. (D) $G'_{final\ at\ 25°C}$ (the G' obtained at 1 Hz after gelation for 5 h during frequency sweep at 25 °C) as a function of gelation temperature for 10% (w/w) heated (80 °C for 30 min) skim milks untreated (■) and treated (□) with TG, and acidified with 2% (w/w) GDL; and $G'_{shoulder}$ as a function of gelation temperature for 10% (w/w) heated (80 °C for 30 min) control skim milk (●) and acidified with 2% (w/w) GDL. In (C) and (D), error bars correspond to standard deviations of triplicate measurements.

prepared with cultures, Lee and Lucey (2004, 2006) demonstrated the appearance of a $G'_{shoulder}$ on the gelation profiles at 40, 44 and 45.7 °C, but not at 32 and 38 °C. This peculiar behaviour of a $G'_{shoulder}$ was also observed in pre-heated suspensions containing micellar casein isolate and whey protein isolate, acidified with GDL at 40 °C (O'Kennedy & Kelly, 2000), or with cultures at 42 °C (Famelart et al., 2003, 2004). The $G'_{shoulder}$ in the control increased with the increase in acidification temperature (Fig. 3D). In comparison, TG-treated milk did not show a $G'_{shoulder}$ when acidified at ≥35 °C (Fig. 3B), in accordance with the findings of Ercili-Cura et al. (2013), who showed that, regardless of the acidification temperature (20, 30 or 40 °C), during the formation of TG acid crosslinked gels, the $G'_{shoulder}$ was absent from the gelation profiles. In addition, no $G'_{shoulder}$ was observed on the acid gelation profile of the mixture containing micellar casein and whey protein isolate in simulated milk ultrafiltrate incubated with TG and subsequently acidified at 40 °C (Mounsey, O'Kennedy, & Kelly, 2005). The presence and absence of the $G'_{shoulder}$ in the gelation profiles of control and TG-treated milk, respectively, at elevated temperatures will be further discussed in Section 3.4.

The gelation pH, i.e., the pH at which proteins start to aggregate as seen from the first increase in G', increased as a function of increased temperature for both control and TG-treated milks (Fig. 3C). There was no significant difference ($P < 0.05$) in the gelation pH between both types of milk at all the temperatures studied. For instance, gelation pH was 5.28 ± 0.01 at 30 °C and pH 5.90 ± 0.03 at 45 °C for control milk, whereas a pH of 5.21 ± 0.01 at 30 °C and a pH of 5.93 ± 0.14 at 45 °C were measured for TG-treated milk. These values indicate that, at a given temperature, the presence of TG-induced crosslinks did not influence the pH at which protein aggregation starts. Previous studies also showed that an increase in the acidification temperature resulted in an onset of gelation at higher pH values (Lee & Lucey, 2003, 2004, 2006; Anema, 2009a; Banon & Hardy, 1992; Ercili-Cura et al., 2013; Haque, Richardson, & Morris, 2001; Heertje, Visser, & Smits, 1985; Laligant, Famelart, Paquet, & Brulé, 2003; Lucey, Teo, Munro, & Singh, 1997a; Lucey, Tamehana, Singh, & Munro, 1998c). The pH at $G'_{shoulder}$ for control milk also increased as a function of increasing acidification temperature (Fig. 3C). These results indicate that increasing the acidification temperature accelerated the process of protein aggregation towards the formation of a gel network for both types of milk.

The final gel strength at the end of 5 h of gelation (G'_{final}) decreased with increasing acidification temperature for acid gels from control milk (Fig. 3A). This is in agreement with previous published results on different types of acid milk gels (Arshad, Paulsson, & Dejmek, 1993; Laligant et al., 2003; Lee & Lucey, 2003, 2004, 2006; Lucey, van Vliet, Grolle, Geurts, & Walstra, 1997b; Lucey et al., 1998a). In contrast, for acid gels from TG-treated milk, G'_{final} was independent of the acidification temperature (Fig. 3B). G'_{final} is influenced by factors such as the number and strength of bonds within the casein micelles (intra-micellar bonds) and on the spatial distribution of particles (inter-micellar bonds) in the gel network (Roefs, de Groot-Mostert, & van Vliet, 1990; Roefs & van Vliet, 1990), and the structural rearrangements of the gel network (intra-particle, inter-particle and cluster rearrangements) (Mellema, Walstra, van Opheusden, & van Vliet, 2002).

During and after gelation, different types of structural rearrangement can occur sequentially. These include: (1) intra-micellar rearrangement; (2) inter-micellar rearrangement; (3) inter-cluster rearrangement (or coarsening of the gel structure); and (4) syneresis, or expulsion of whey (Mellema et al., 2002). Intra-micellar rearrangement can also result in the dissociation of caseins into the serum during acidification. Inter-micellar rearrangement is aggregation of micelles into small clusters, resulting in increased in contact area between them and increased density of the aggregates. The clusters can then undergo inter-cluster rearrangement to further aggregate to form strands. The strands are stretched and gel pores are developed. When the strands are too extensively stretched, they become thinner and eventually result in breakage due to contractional forces within the gel. This leads to syneresis,

shrinkage of the gel and excretion of whey after gelation (Mellema et al., 2002).

Intra- and inter-micellar rearrangements can occur during the early stage of gelation. At higher temperatures, the hydrophobic interactions that drives intra-micellar rearrangement is stronger (Mellema et al., 2002). Fig. 4 shows the SDS-PAGE profiles of the milk samples with and without TG treatment, indicating that all κ-casein and β-casein and most of the α_S-caseins were cross-linked after TG treatment. For control milk, the destabilisation of casein micelles and the dissociation of caseins to the serum during acidification could possibly be the main causes for intra-micellar rearrangements. When the temperatures are high, stronger intra-micellar hydrophobic interactions between caseins within the micelles results in the shrinkage of casein micelles, with more compacted structures, reduced regions of contact and reduced inter-micellar bonding (Lee & Lucey, 2004; Renan, Arnoult-Delest, Pâquet, Brulé, & Famelart, 2008; Roefs & van Vliet, 1990; Zoon, van Vliet, & Walstra, 1988). Due to less inter-micellar bonds, micelles have higher mobility and thus inter-micellar rearrangements probably occur more rapidly and extensively during gelation, which could possibly lead to the formation of dense clusters. The dense clusters may undergo rapid subsequent inter-cluster rearrangements to form a gel network that is less rigid, less continuous and less homogeneous, where some aggregates in the dense clusters do not contribute to the rigidity of the gel (Zoon et al., 1988). This could explain why at higher acidification temperatures, the G'_{final} of control milk gel is smaller. In contrast, the G'_{final} of the gel from TG-treated milk was insensitive to acidification temperature possibly because the TG-induced isopeptide crosslinks between caseins inhibited the dissociation of caseins during acidification (Schorsch et al., 2000; Vasbinder et al., 2003). This may hinder intra-micellar interactions and the subsequent rearrangements that occur during gelation even at high temperatures (Ercili-Cura et al., 2013). As compared to the control milk (Fig. 4, lane 2), TG extensively crosslinks caseins (Fig. 4, lane 3). Previous studies have shown that extensively cross-linked casein micelles do not dissociate on removal of CCP or disruption of hydrophobic interactions and hydrogen bonds (Smiddy., Martin, Kelly, de Kruif, & Huppertz, 2006). Hence, the aforementioned intra-micellar changes occurring during acidification of control milk are unlikely to happen during acidification of TG-treated milk.

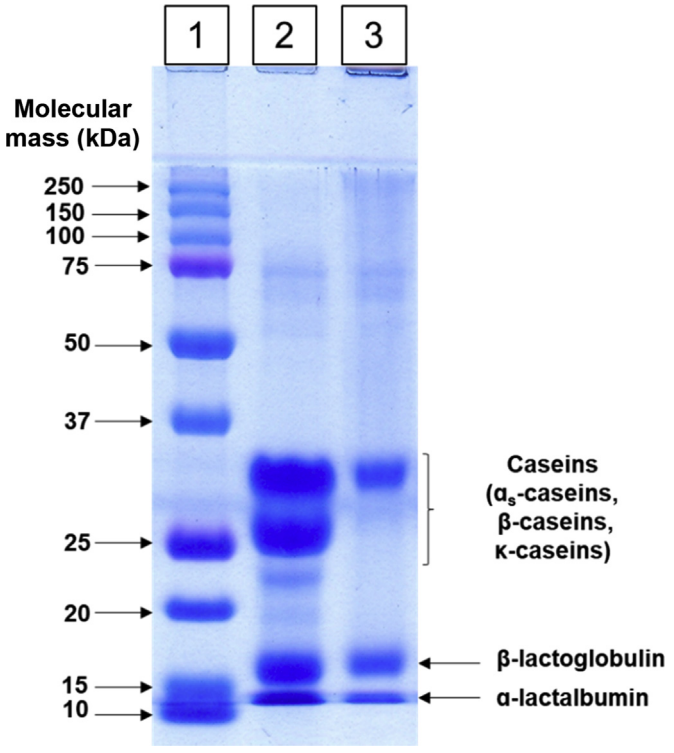

Fig. 4. Reducing SDS-PAGE electrophoretograms of 10% (w/w) control milk (lane 2) and TG-treated milk (lane 3) after heat treatment at 80 °C for 30 min. Molecular mass markers are in lane 1.

The final gel strength of control and TG-treated milks after a gelation time of 5 h, obtained from the value of G' at 1 Hz during frequency sweep at 25 °C ($G'_{final\ at\ 25°C}$), as a function of temperature, is shown in Fig. 3D. The $G'_{final\ at\ 25°C}$ values for both types of milk gel (with and without TG) in Fig. 3D were noticeably higher than the G' observed at the end of 5 h of gelation at the acidification temperature (G'_{final}) in Fig. 3A,B, due to the consolidation of the acid milk gel network upon cooling at 25 °C (Haque et al., 2001). The $G'_{final\ at\ 25°C}$ of control milk increased when the acidification temperature was increased from 30 to 40 °C (Fig. 3D). Above 40 °C, it plateaued at ~380 Pa. As for the TG-treated milk, $G'_{final\ at\ 25°C}$ increased as a function of temperature from 30 to 45 °C. For instance, the $G'_{final\ at\ 25°C}$ of TG-treated milk was 291 ± 1 Pa at 30 °C and 478 ± 3 Pa at 45 °C. These results suggest that, the higher the acidification temperature, the greater the gel network consolidation during the cooling process at 25 °C (Haque et al., 2001), resulting in higher $G'_{final\ at\ 25°C}$ values for both types of milk.

3.3. Effect of total solids content on the gelation profiles of acid milk gels

The change in G' during acidification of control and TG-treated milk was also influenced by the total solids content (Fig. 5). When the total solids content was increased by raising the milk concentration from 2.5% up to 30% (w/w), $G'_{shoulder}$ was absent in both control and TG-treated milks at an acidification temperature of 30 °C (Fig. 5A,B). However, when acidified at 45 °C, control milk above 2.5% (w/w) showed the $G'_{shoulder}$ during the early stage of gelation (inset in Fig. 5C).

When the total solids content is increased, the $G'_{final\ at\ 25°C}$ of acid milk gels made at 30 °C increased for both control and TG-treated milks (Fig. 6). This is in agreement with previous studies which all showed that increasing the total solids content of milk increased the firmness of acid gels (Anema, 2008b; Gastaldi et al., 1997; Kristo, Biliaderis, & Tzanetakis, 2003; Pereira, Matia-Merino, Jones, & Singh, 2006). These increases are attributed to the increase in the protein concentration resulting in more protein complexes involved in the interconnection in the protein network (Anema, 2008b) and more inter-micellar bonds for the enhancement of interactions between micelles in a given area (Gastaldi et al., 1997). The $G'_{final\ at\ 25°C}$ of the control and TG-treated milk, as a function of increasing milk solids level, was not significantly different ($P < 0.05$) (Fig. 6), suggesting that at 30 °C the TG-induced crosslinks did not significantly affect the $G'_{final\ at\ 25°C}$. Similar to 10% (w/w) milk gels (Fig. 3D), the $G'_{final\ at\ 25°C}$ values of acid milk gels with varying milk solids content were substantially higher than those measured at the acidification temperature at the end of gelation time (G'_{final}) (Fig. 5), due to gel network consolidation upon cooling to 25 °C. At elevated temperatures, e.g., 45 °C, the $G'_{shoulder}$ increased as a function of increasing milk concentration for control milk (Fig. 6).

3.4. Mechanism of formation of $G'_{shoulder}$ at the early stage of gelation

As mentioned previously, two mechanisms have been postulated to explain the presence of the $G'_{shoulder}$ (Anema, 2009a; Ercili-

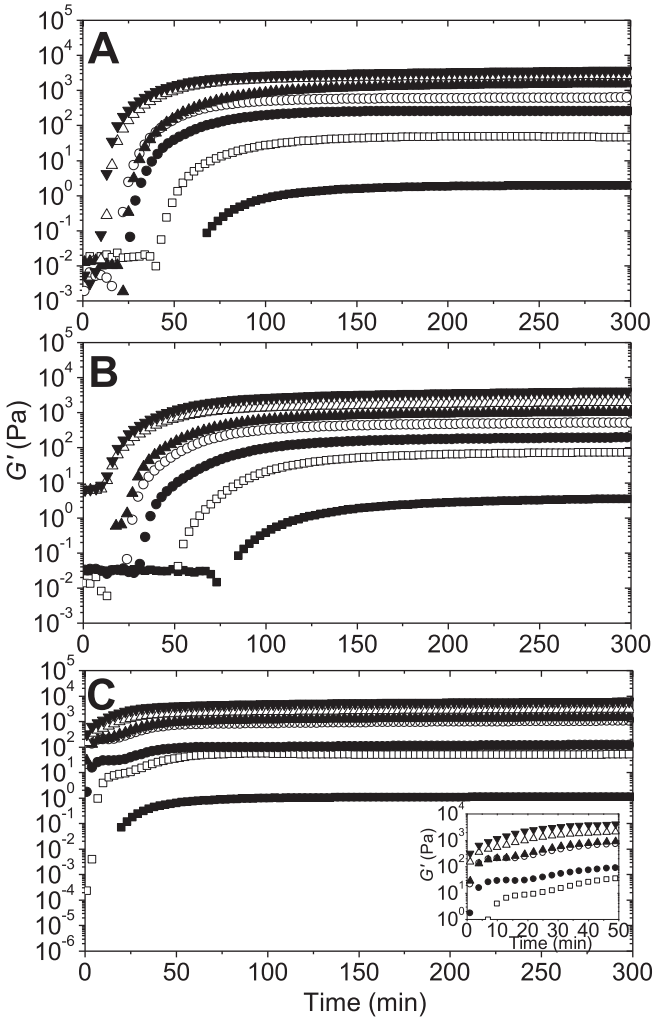

Fig. 5. G' values as a function of gelation time for heated (80 °C for 30 min) skim milks containing GDL, (A) untreated with TG and (B) treated with TG, with different total solids concentrations at a gelation temperature of 30 °C; and (C) heated control skim milk, with different total solids concentrations and acidified with GDL at a gelation temperature of 45 °C. Inset in (C): values of G' in (C) plotted for the first 50 min. Skim milk concentrations are (w/w): 2.5% (■), 5% (□), 10% (●), 15% (○), 20% (▲), 25% (△), and 30% (▼).

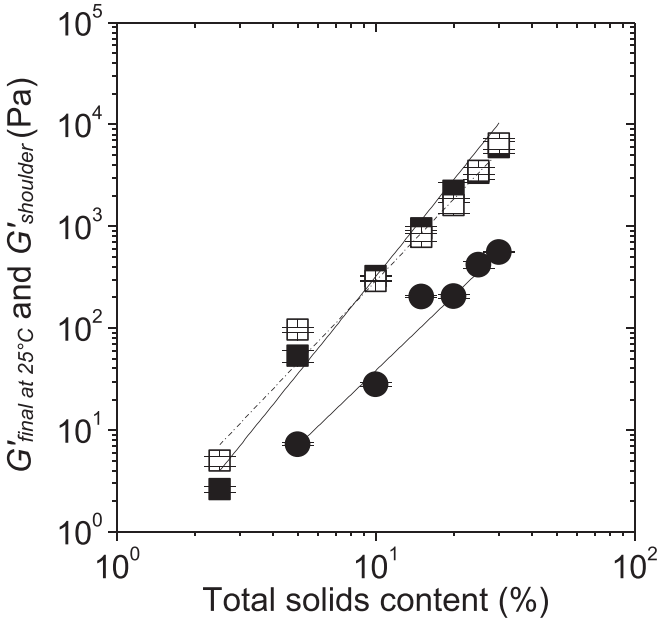

Fig. 6. $G'_{final\ at\ 25°C}$ values (the G' obtained at 1 Hz after gelation for 5 h during frequency sweep) as a function of total solids content (%, w/w) for milks untreated (■) and treated (□) with TG, and acidified with GDL at 30 °C: (●) corresponds to $G'_{shoulder}$ as a function of total solids content for control milk acidified with GDL at 45 °C. Lines represent power law fitting of data points. Error bars correspond to standard deviation of triplicate measurements.

Cura et al., 2013; Famelart et al., 2004, 2003; Horne, 2003; Koutina & Skibsted, 2015; Lee & Lucey, 2004; Lucey et al., 1998b; O'Kennedy & Kelly, 2000), but there are disagreements between the two explanations. Although there were clear differences between the two proposed mechanisms, both explanations suggested that $G'_{shoulder}$ could be a result of two gelation phases, with both explanations agreeing that the second phase was most likely due to casein–casein interactions.

The $G'_{shoulder}$ in explanation 1 was proposed to be due to the solubilisation of CCP weakening the casein–casein interactions (Anema, 2009a; Ercili-Cura et al., 2013; Horne, 2003; Koutina & Skibsted, 2015; Lee & Lucey, 2004). In other words, this explanation suggests that $G'_{shoulder}$ is induced when the release of CCP results in notable changes in the micellar structure. As mentioned earlier, there is no difference in the change in pH during acidification (Fig. 2) and no significant difference ($P < 0.05$) between the distribution of Ca^{2+}, Mg^{2+} and P_i (minerals that make up the CCP) between the micellar and aqueous phase of milk with and without TG treatment during acidification (results shown in Supplementary material Fig. S1). Thus, the fact that mineral solubilisation is the same for both types of milk indicates that TG creates a stable protein framework from which CCP is removed by acidification. Huppertz and de Kruif (2008) also reported that nanogel particles with a TG-crosslinked casein network maintained their structural integrity after the removal of micellar calcium phosphate. Therefore, given the results for TG-treated milks, the presence of $G'_{shoulder}$ in control milk cannot be due solely to the solubilisation of CCP.

Knowing that TG-treatment of milk inhibited the formation of $G'_{shoulder}$ in the gelation profiles by withstanding the notable micellar structural changes after CCP solubilisation, further experiments were performed to investigate the effect of interactions of proteins on the presence of $G'_{shoulder}$ (explanation 2). SDS-PAGE was used to give an indication of the protein content (caseins and whey proteins) in the sera of control and TG-treated milks following heat treatment at 80 °C for 30 min, and in the serum of unheated milk (Fig. 7). It was assumed that the micelle-bound protein complexes formed during heat treatment would be sedimented along with the casein micelles upon centrifugation. Hence, the amount of denatured whey proteins associated with casein micelles during heat treatment in both types of milk could be determined by comparing the whey protein bands of the heated control (Fig. 7; lane 3) and TG-treated (Fig. 7; lane 4) milk sera with that of the serum from unheated milk (Fig. 7; lane 2) in the SDS-PAGE profile. Both milk sera without (Fig. 7; lane 3) and with (Fig. 7; lane 4) TG treatment contained lower amounts of soluble whey proteins than in the unheated milk serum (Fig. 7; lane 2), indicating the presence of heat-induced micelle-bound protein complexes in these samples. However, in comparison to the serum from heated control milk (Fig. 7; lane 3), soluble caseins were barely detectable in the serum from TG-treated milk (Fig. 7; lane 4), possibly due to the cross-linking by TG. These TG-crosslinked caseins could be represented by the high molecular mass band at the top of the gel (red arrow on lane 4; Fig. 7). These results suggest that the control milk contained micelle-bound protein complexes and soluble protein complexes containing whey proteins and caseins, whereas in the TG-treated

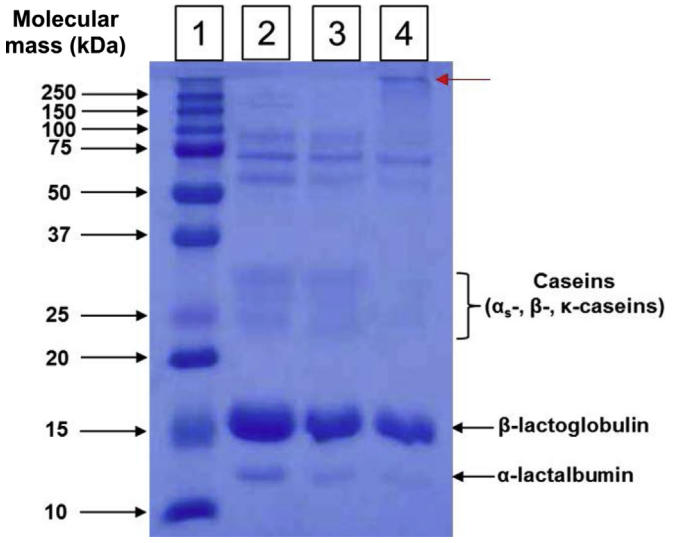

Fig. 7. Reducing SDS-PAGE electrophoretograms of 10% (w/w) unheated milk serum (lane 2), and control (lane 3) and TG-treated (lane 4) milk sera after heat treatment at 80 °C for 30 min. Red arrow on lane 4 represents TG-crosslinked proteins. Molecular mass markers are in lane 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

milk, the soluble protein complexes containing caseins without TG-crosslinks were absent.

To demonstrate the role of soluble caseins in the $G'_{shoulder}$, two exploratory experiments were carried out (details in Section 2.6). In the first experiment, without the addition of sodium caseinate, as expected, $G'_{shoulder}$ was not observed in the TG-treated milk when acidified at 45 °C (Fig. 8, white triangles). However, the presence of 0.5% (w/w) sodium caseinate induced a $G'_{shoulder}$, indicating that the presence of caseins in the serum was important for the existence of the $G'_{shoulder}$ in the gelation profile (Fig. 8, black triangles). In the second experiment, after acidification under similar conditions as indicated above, the $G'_{shoulder}$ was discernible in the heated TG-treated milk pellet resuspended in the serum of the heated control milk (Fig. 8, white circles). This is due to the presence of heat-induced soluble protein complexes containing caseins from the control milk serum fraction. These results confirm the importance of soluble caseins in inducing the $G'_{shoulder}$ when acidification temperatures are high (≥35 °C).

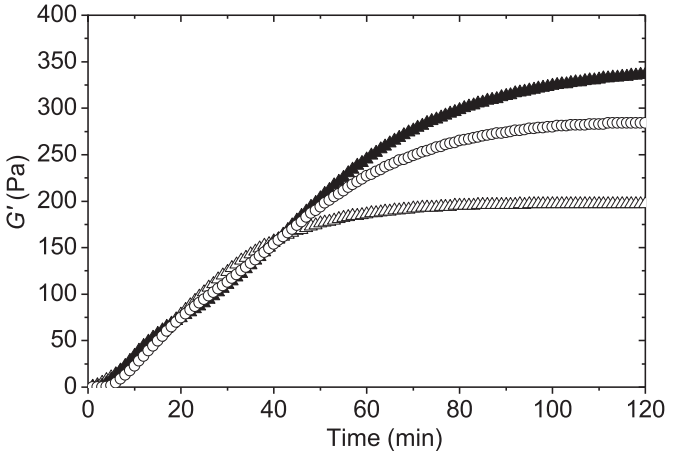

Fig. 8. G' values as a function of gelation time for 10% (w/w) heated (80 °C for 30 min) TG-treated skim milk containing 0% (△) and 0.5% (w/w) (▲) added sodium caseinate, and modified suspension prepared by resuspending the TG-treated milk micellar fraction into the control milk serum fraction (○). All samples were acidified with 2% (w/w) GDL at 45 °C.

Both these experiments indicated that soluble caseins played a role in the presence of $G'_{shoulder}$ when milk was acidified at elevated temperatures (≥35 °C). This supported the mechanism (explanation 2) suggested by Lucey et al. (1998b), O'Kennedy and Kelly (2000), and Famelart et al. (2003, 2004), postulating that the $G'_{shoulder}$ could be due to the presence of casein–casein, whey protein-casein and whey protein-whey protein soluble complexes in heated milks. Upon acidification, these soluble complexes would aggregate, resulting in the first increase in G' then, when the pH decreases further, the casein micelles (and the aggregated soluble complexes) would start to aggregate, further resulting in the second increase in G'. The result of these two increases in G' will be the presence of $G'_{shoulder}$. Finally, because $G'_{shoulder}$ was observed only at high acidification temperatures, this would suggest that the first increase in G' involving the soluble complexes is due to hydrophobic interactions (Haque & Kinsella, 1988).

4. Conclusions

The rheological properties of milk during and after acid gelation, were influenced by the action of TG, acidification temperature and the milk total solids content. Despite the similar acidification kinetics for both control and TG-treated milks, their gelation profiles were different depending on the acidification temperature. At 30 °C, the change in G', gelation pH and gel firmness (G'_{final}) were relatively similar between both types of milk. However, at elevated temperatures (≥35 °C), the $G'_{shoulder}$ was observed for control milk, but not for TG-treated milk. The $G'_{shoulder}$ was proposed to be the result of the transition of the first increase in G' due to the aggregation of soluble protein complexes at the early stage of acidification, followed by the second increase in G' due to further aggregation of casein micelles and aggregated soluble complexes as acidification progressed. TG-treated milk did not show a $G'_{shoulder}$ during acidification due to the lack of soluble protein complexes containing caseins. Increasing the milk solids concentration did not induce $G'_{shoulder}$ in both control and TG-treated milks during acidification at 30 °C. Nevertheless, when acidified at 45 °C, the $G'_{shoulder}$ was only found in control milk above 2.5% (w/w) solids during the early stage of gelation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.01.006.

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