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A novel technique for differentiation of proteins in the development of acid gel structure from control and heat treated milk using confocal scanning laser microscopy

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The incorporation of caseins and whey proteins into acid gels produced from unheated and heat treated skimmed milk was studied by confocal scanning laser microscopy (CSLM) using fluorescent labelled proteins. Bovine casein micelles were labelled using Alexa Fluor 594, while whey proteins were labelled using Alexa Fluor 488. Samples of the labelled protein solutions were introduced into aliquots of pasteurised skim milk, and skim milk heated to 90 °C for 2 min and 95 °C for 8 min. The milk was acidified at 40 °C to a final pH of 4·4 using 20 g glucono-delta-lactone/l (GDL). The formation of gels was observed with CSLM at two wavelengths (488 nm and 594 nm), and also by visual and rheological methods. In the control milk, as pH decreased distinct casein aggregates appeared, and as further pH reduction occurred, the whey proteins could be seen to coat the casein aggregates. With the heated milks, the gel structure was formed of continuous strands consisting of both casein and whey protein. The formation of the gel network was correlated with an increase in the elastic modulus for all three treatments, in relation to the severity of heat treatment. This model system allows the separate observation of the caseins and whey proteins, and the study of the interactions between the two protein fractions during the formation of the acid gel structure, on a real-time basis. The system could therefore be a valuable tool in the study of structure formation in yoghurt and other dairy protein systems.

Keywords: confocal scanning laser microscopy, acid milk gels, caseins, whey proteins, glucono-delta-lactone, heat treatment.

Acid milk gels form the basis of the structure of yogurt and many soft cheese varieties. When milk is acidified, colloidal calcium phosphate progressively becomes solubilized and released from the micelles, caseins dissociate as they lose their negative charge, and the ζ-potential increases from around −15 mV to about 0 (Guinee et al. 1993). The caseins thus form aggregates. All these physico-chemical changes allow the casein to reach a new equilibrium in the form of a gel network. Whey proteins become entrapped within the matrix of strands (Tamine et al. 2001). Acidulation can be achieved by fermentation or direct addition of acids. However, direct acidification at warm temperatures leads to instantaneous precipitation of the proteins. Glucono-δ-lactone (GDL) has been used by a number of authors (e.g. Harwalker & Kalab, 1981) to study acid gel formation, as the slow generation of gluconic acid results in a steady pH decrease and the generation of a true gel structure. The final pH is dependent on the initial concentration of the acidulant and the buffering capacity of the milk (Lucey & Singh, 1998).

Many processing parameters affect the texture of acid gels, such as milk heat treatment, milk protein addition, homogenisation, rate and temperature of acidification and final pH (Guinee et al. 1993; Lucey et al. 1997). A major aim of dairy manufacturers is to optimise texture of gels in the production of yogurt and soft cheeses. A better understanding of development of structure in those products would aid in the control of product quality. The role of heat treatment is particularly important. At temperatures above 80 °C, β-lactoglobulin and α-lactalbumin become denatured and bind to κ-casein on the surface of the casein micelles through interactions of the exposed –SH groups (Tamime & Robinson, 1999). This affects the

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gelling process and the properties of the resultant gel (Kalab et al. 1976). Disulphide interactions between whey protein aggregates have also been shown to play a role in acid induced gelation of heated milk (Vasbinder et al. 2004); while Guyomarc'h et al. (2003) have described the importance of both soluble and micelle-bound heat induced protein aggregates on gel formation.

Scanning electron microscopy and transmission electron microscopy have been invaluable in the study of acid gelation and explaining the properties of yogurts and other products (Kalab, 1979). However, confocal scanning laser microscopy (CSLM) is becoming more widely used in the study of food microstructure as it allows the observation of different components, without complex and potentially damaging sample preparation techniques. The technique allows 3D images of food structure to be built up, and, importantly to this study, permits the possibility of using different fluorescent labels to differentiate between different food components. Heertje et al. (1987) used the technique to study a number of different food systems including water globules in margarine, fats and proteins in Gouda cheese, mayonnaise structure and the rising of dough. Everett & Olson (2003) observed changes in fat globule structure during cheesemaking by CSLM. Hassan et al. (1995) used the method to investigate lactic acid bacteria in milk. Auty et al. (1999) studied protein aggregation in milk gels, including GDL systems, by CSLM in which the total protein fraction was labelled with Rhodamine B.

All the above features make confocal microscopy a very suitable method to study the gelation of milk. Several fluorescent probes have been described for protein staining in dairy systems, such as rhodamine B (in cheese at an excitation wavelength of 568 nm), nile blue (in dairy products at an excitation wavelength of 633 nm) and acid fuschin (in dairy gels) (Auty et al. 2001). However, the technique is limited when studying caseins and whey proteins together, as it is difficult to find fluorescent probes which will distinguish the two protein fractions. As one of the objectives of this study, it was necessary to find a labelling technique that would allow the distinction between the two major milk protein fractions. Fluorescent probes were identified which are specific to the proteins, are resistant to photobleaching, and do not have emission fluorescence spectra that overlap, so that the fluorescence emission of each probe can be viewed separately (Herbert et al. 1999).

In the current paper, the goal of the technique was to label the protein fractions with dyes whose emission spectra do not overlap, and observe the different proteins on different channels of the microscope. The images could then be superimposed in order to obtain a full picture of the sample, and simultaneously localize the caseins and the whey proteins during acid gelation of milk.

A second aim was to apply this novel technique to the study of the effects of heat treatment of milk on the behaviour of the caseins and whey proteins during acid gelation. Hence, gels prepared from control pasteurized (71·7 °C/15 s) skim milk, and from milk heated at 90 °C for 2 min and milk heated at 95 °C for 8 min, were compared.

Materials and methods

Milk and heat treatments

Pasteurized skimmed milk was obtained from a local dairy. Experiments using this milk with no further heat treatment are referred to as control. Heat treated samples were prepared by heating at 90 °C for 2 min or at 95 °C for 8 min, using a plate-type heat exchanger with appropriate holding tubes (Polartherm Ltd., Wakefield, WF2 7AS).

Protein isolation

The casein fraction used for labelling was extracted from raw milk obtained from the university farm. The milk was first centrifuged for 30 min at 2000 ***g*** to remove the fat. To extract and resuspend the casein, the method of Ford & Grandison (1986) was followed. This method was chosen as it had been demonstrated that micelles resuspended in their supernatant displayed rennet coagulation properties identical to the original milk, which indicates that their physical form would be similar to native micelles. Skim milk was centrifuged at 40 000 ***g*** (Sorvall RC5 Plus, Kendro Ltd., Bishops Stortford, CM23 5GZ) for 2 h and the resulting pellet was resuspended in the appropriate volume of milk ultrafiltrate by mechanical degradation and mixing for 5 min using a laboratory mixer (Model 17966, Silverson Machines Ltd., Chesham HP5 1PQ) followed by sonication for 2 min using a probe sonicator (MSE Soniprep 150, Labcaire Systems, Clevedon, BS21 6LH). The solution was again centrifuged at 40 000 ***g*** for 2 h after which time the resulting pellet was resuspended, using the Silverson mixer and probe sonicator, in a solution of 0·1m-sodium bicarbonate at a concentration of 2 mg ml^{-1} and left to equilibrate overnight at 4 °C.

The whey protein sample was Volactive 80 prepared from sweet whey (80% Protein, 10% lactose, 3% moisture, 2% ash, <1% fat; Volac International Ltd, Royston, SG8 5QX).

Protein labelling

Caseins and whey proteins were labelled separately with the fluorescent dyes Alexa Fluor 594 and Alexa Fluor 488 (Molecular Probes, Invitrogen Ltd, Paisley, PA4 9RF) respectively. The protein labelling kits included gel filtration purification columns, by which unincorporated dye is completely separated from the labelled protein. The dyes were chosen so that their absorbance and emission spectra were sufficiently different and hence could be used together. Alexa Fluor 594 labelled proteins have absorption

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and fluorescence emission maxima of 590 nm and 617 nm respectively, while Alexa Fluor 488 labelled proteins have maxima of 494 nm and 519 (Molecular Probes product information).

Study of the milk gelation system

The time of gelation was determined using two methods.

- Firstly, the time of gel formation was estimated visually: 200 mg GDL crystals (99% pure GDL, ADM Ringaskiddy, ADM Ingredients Ltd, Widnes, WA8 OYZ) were added to 10 ml skim milk at 40 °C, and mixed in a test tube in a water bath at 40 °C. The mixture was regularly checked for gelation. The time at which gel formation was first observed was recorded (clots formation).

  Changes in pH during gelation were monitored in a separate sample prepared as above. Sample pH was recorded every 10 min.
- Secondly, the viscoelastic properties of the acidifying product were recorded continuously as a function of time in a controlled strain rheometer (Bohlin CVOR, Malvern Instruments, Malvern, WR14 1XZ). 260 mg GDL were added to 13 ml skim milk at 40 °C. The rheometer was operated at an angular frequency (ω) of 1 Hz, as described by Guinee et al. (1997). Changes in the elastic modulus of the acidifying milk samples were recorded at 40 °C.

Real time gelation and CSLM

The confocal microscope used was an inverted motorised microscope (Leica DMIRE 2, Milton Keynes, MK5 8LP) used with the Leica TCS SP2 AOBS computer system. A (×63) glycerol objective was used. The microscope is equipped with an air-cooled ion laser.

The gels were prepared in 35 mm Iwaki tissue culture glass based (27 mm diameter) dishes (Bibby Sterilin, Stone, ST15 OSA). Seventeen microlitres of the pure labelled caseins and 17 µl of the pure labelled whey proteins (each containing approximately 1 mg ml^{-1} protein) were thoroughly mixed with 5 ml skim milk (control or heat treated as described above) at 40 °C, and 100 mg GDL powder was added with further mixing. Note that it was not practical to add the labelled proteins prior to heat treatment due to the quantities required.

The milk was left to acidify in the chamber of the microscope at 40 °C (±0·1 °C), and images were taken every 3 min, starting 10 min after the addition of GDL. The two fluorescent probes were consecutively excited with the laser in the same field of view, allowing separate location of each protein fraction on separate images. The separate images with caseins and whey proteins were superimposed in order to display simultaneously both protein fractions on the same image, the caseins appearing orange while the whey proteins appeared green.

Results and discussion

Distinction of caseins from the whey proteins

A major aim of this work was to enable the visualization of incorporation of caseins and whey proteins separately into the gel matrix in a dynamic study. Before this, it was necessary to validate the method by observing whether the individual labelled proteins could be distinguished in an acid gel.

A gel resulting from the acidification with GDL of control skim milk with added labelled caseins and whey proteins was studied with the confocal microscope, approximately 2 h after addition of GDL. The caseins (Fig. 1a) are shown in orange and the whey proteins (Fig. 1b) in green on the confocal images shown in Fig. 1. From these images, it can be seen that both caseins and whey proteins form the structure of the gel. The whey proteins can clearly be seen in the voids between the strands, but they also apparently form part of the matrix of the gel (Fig. 1a), suggesting interaction between caseins and whey proteins – also apparent on Fig. 1c. This may not have been expected, as the milk had not been heat treated after the addition of the labelled proteins, and hence heat-induced interaction of β-lg and α-la with the casein micelles would not have occurred. The nature of these interactions is not fully understood, although there is evidence from rheological studies that whey proteins have a negative effect on gel strength in unheated milk samples (unpublished data). Future studies will address this question.

Study of milk gelation

To study the 'real-time' development of gel structure by this method, milk inoculated with GDL was prepared from control and heat treated milk samples. The pH profile and onset of gelation were determined in a parallel experiment (Fig. 2). The onset of gelation determined visually for control samples was at 45 min after inoculation at pH 4·95. Gelation of heat treated samples was at 12 min for the lower heat treatment compared with 10 min for the more severe one.

Using the low-amplitude oscillatory rheometer, gelation time was considered as the time necessary for the gel to reach a G′ value of 0·5 Pa (Esteves et al. 2002). Again it was found that the gelation time was 45 min for control milk (Fig. 3) equivalent to pH 4·95, 10·5 min for the lower heat treatment (pH 5·5) and 9 min for the more severe one (pH 5·45). Hence there was good agreement between the visual and rheological methods of assessing gelation time.

The pH necessary to achieve gelation is lower if the milk has not been subjected to a heat treatment. Heertje et al. (1985) showed that at the same temperature of acidification (43 °C), the onset of gelation was at pH 5·5 for heated milk, whereas it was pH 5·1 for the unheated milk. This suggests that the maximum pH at which

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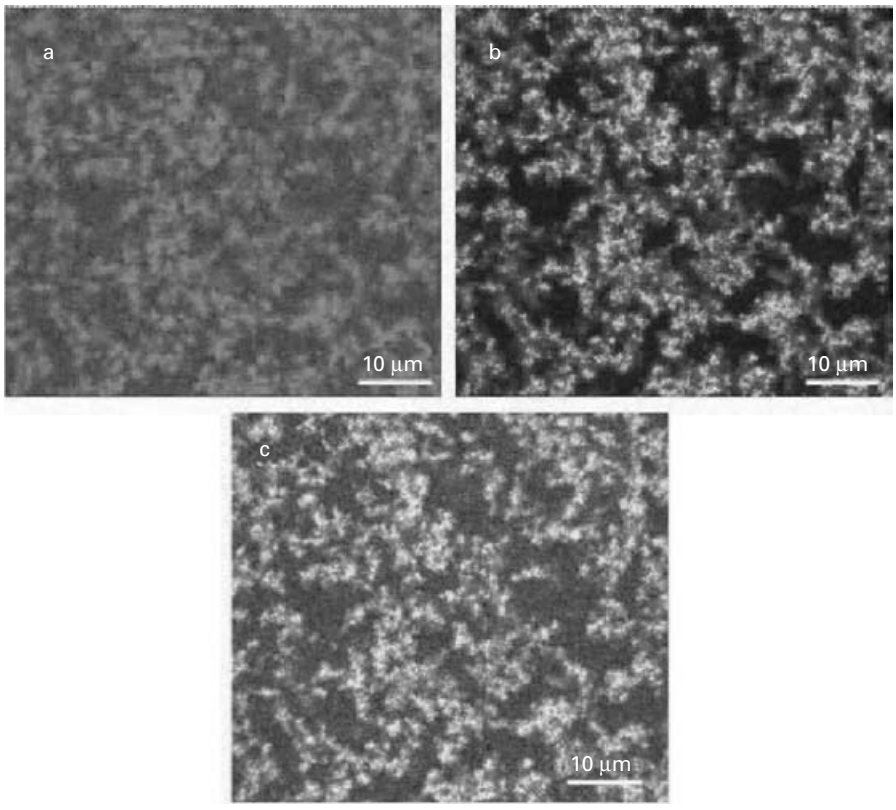

Fig. 1. Confocal scanning laser microscopy images of glucono-delta-lactone induced gelled skim milk containing labelled whey proteins and pure caseins. Fig. 1a was acquired using excitation at 488 nm (labelled whey proteins). Fig. 1b was acquired using excitation at 594 nm (labelled caseins). Fig. 1c is the result of the superimposition of image a and b. The black areas in the images are the aqueous phase of the system.

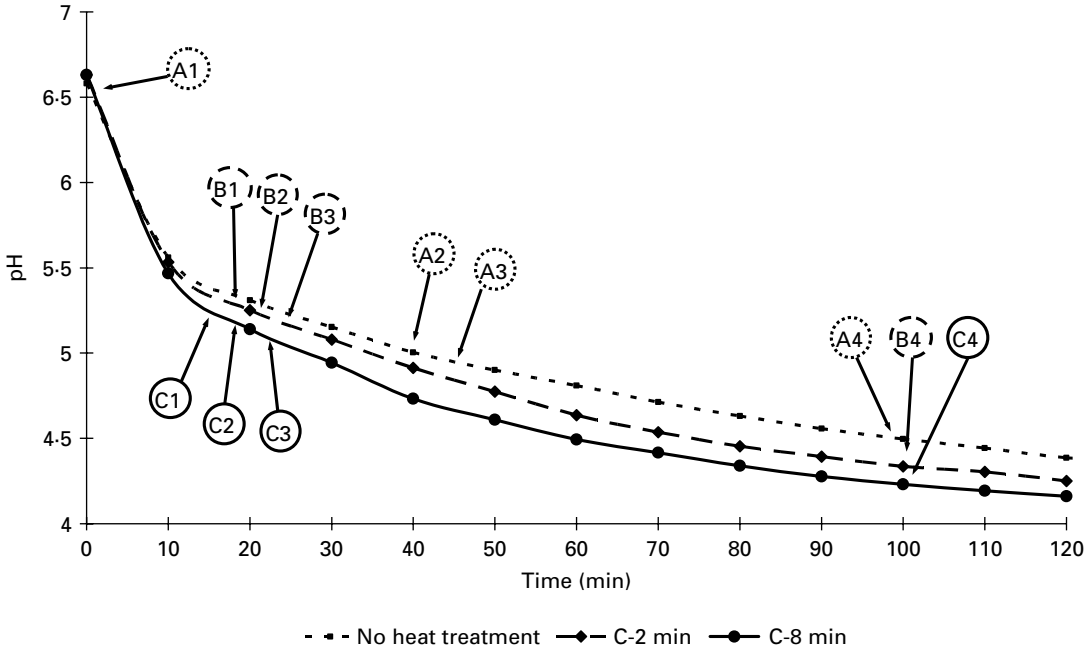

Fig. 2. Change in the pH profile of skim milk inoculated with glucono-delta-lactone (20 g/l) for (A) the control, (B) 90 °C-2 min and (C) 95 °C-8 min samples. The letters correspond to the confocal scanning laser microscopy images (Fig. 4).

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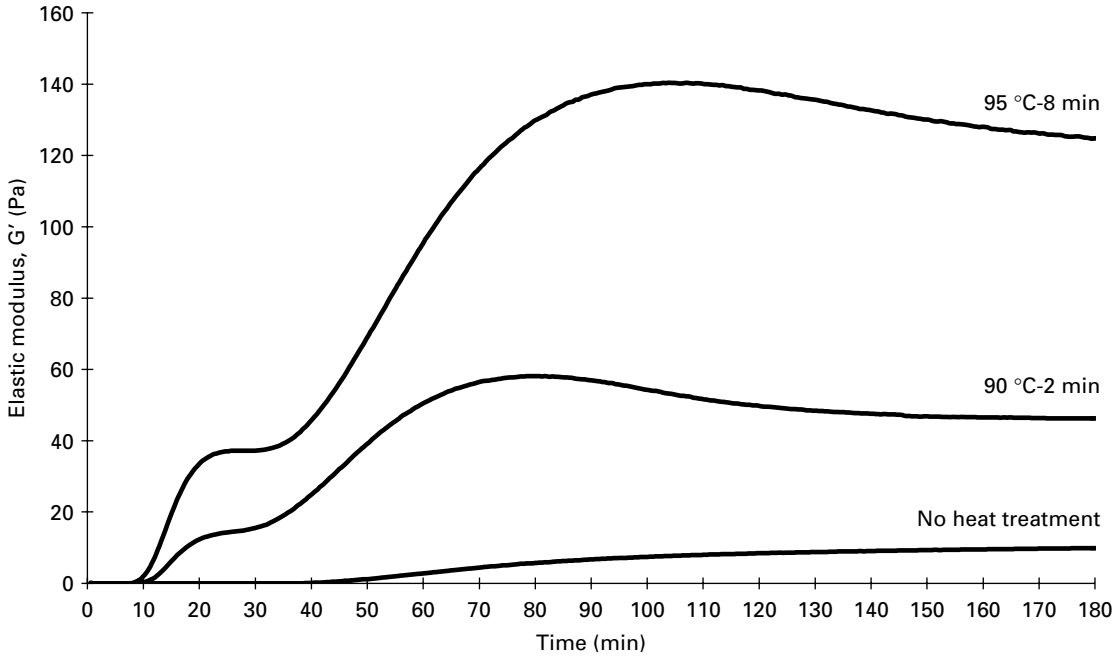

Fig. 3. Low-amplitude oscillatory rheometry data for glucono-δ-lactone coagulated skim milk showing the elastic modulus G′ (Pa) for the control, 90 °C-2 min and 95 °C-8 min samples.

development of structure can be seen microscopically, would also be lower and this was confirmed by the results below.

Study of microstructure during gelation

A series of images of exactly the same field was collected during gelation using both fluorescent probes. Figure 4 shows the sequence of CSLM images taken from the time-lapse animation during GDL-induced skim milk gelation for the control and heat-treated milks. The general appearance of the images is similar to those reported by Auty et al. (1999) using a single fluorescent label in GDL gels.

In the control milk, no distinct structure was visible until pH 5·3 was reached, although a fine dispersion of particles was observed (e.g. Fig. 4A1). These appeared orange and green, and corresponded to background fluorescence due to the caseins and whey proteins respectively. Harwalker & Kalab (1981) were able to observe structure formation in acid gels by electron microscopy at a higher pH (5·5), however these gels were formed at 70 °C or more. Images for the heat treated milks well above the pH of coagulation were very similar (not shown). The aggregation of casein (orange) structure in the pH range 5·3 to 5·0 is shown in Fig. 4A2. Gel formation occurred at pH 4·95, corresponding to Figs 4A2 & 4A3 (see also Fig. 2). At this point, the aggregates appeared to be composed largely of casein structure (very bright orange). The orange aggregates became brighter as they increased in size. The gaps between the orange aggregates have a green background colour and it can be assumed that they are filled with whey protein (green), the whey proteins being contained in the aqueous phase. At this stage, aggregates can only be seen on the casein channel, not on the whey protein channel. As acidification progressed, the whey proteins appeared to coat the gel structure, which until then was composed of mainly caseins. This is based on two observations: firstly, a structure, which matched the casein backbone started to become visible (not shown here) on the whey protein channel, and secondly, the gel matrix became greener in appearance (Figs 4A4 compared with 4A3). This last observation should however be treated with care as Alexa Fluor 594 (the casein label) seemed to be more sensitive to bleaching. Nevertheless, the images confirmed that the whey proteins were included in the structure when the pH was further decreased. This finding may be surprising in this unheated system, where interactions between caseins and the major whey proteins (β-lg and α-la) would not be expected. The nature of these interactions is uncertain.

For the milk heated at 90 °C for 2 min, little structure was visible around the pH of gelation (Fig. 4B1), but shortly afterwards, distinct stranded structure could be observed on both the whey protein and casein channels (Fig. 4B2). This indicates that both whey protein and casein are included in the structure of the gel from the point of gelation, as opposed to the control where only caseins were observed in the structure at the beginning of gelation. This is logical as interaction between whey proteins and caseins at this heat treatment is well established. As acidification progressed (Figs 4B3 & 4B4), the

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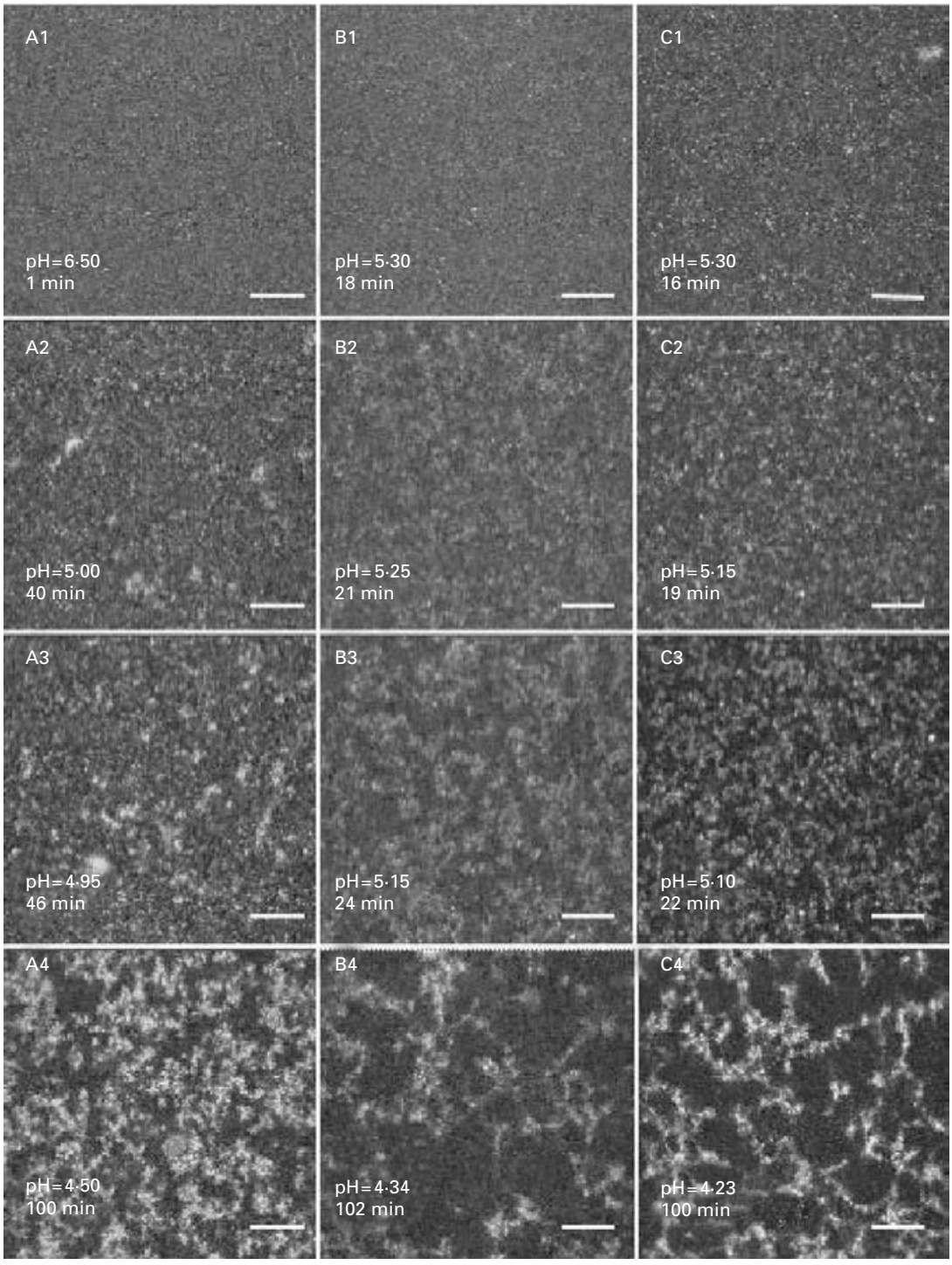

Fig. 4. Sequence of confocal scanning laser microscopy images of glucono-δ-lactone induced gelation of skim milk containing pure labelled caseins and whey proteins for (A) the control, (B) 90 °C-2 min and (C) 95 °C-8 min samples. The orange areas are caseins, the green areas are the whey proteins. The numbers refer to min after GDL addition. Images were acquired using 488 nm wavelength followed by 594 nm wavelength laser excitation. The black areas in the images are the aqueous phase of the system.

strand-like structure appeared to open out into a less dense structure. The final structure was continuous (Fig. 4B4) as opposed to the aggregated particles seen in the final control (Fig. 4A4).

With the samples heated at 95 °C for 8 min, little structure was observed at the point of gelation but a stranded structure was formed shortly afterwards (Fig. 4C2), which appeared denser than with the lower heat

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treatment. Again the structure opened out as acidification progressed, to form a continuous gel network (Fig. 4C4). The structure observed at the higher heat treatment was clearly redder than at 90 °C for 2 min, although structure could still be observed in the whey protein channel, indicating that some whey protein was present in the structure. This suggests that less whey protein contributes to the gel structure from the outset at the higher heat treatment, which could be attributed to the fact that at temperatures greater than 90 °C, the rate of whey protein denaturation is faster, which favours production of large aggregates (but still too small to be observed on CSLM) and hence diminishes interactions with the caseins.

A drawback of the current system is that, due to the small quantities of labelled protein available, it was impossible in practice to add sufficient quantities of labelled proteins prior to heat treatment of the milk. Therefore, we cannot be sure that the labelled whey proteins are behaving in exactly the same way as denatured whey proteins in the heated samples. However, the fact that differences in behaviour of labelled proteins were observed for the different heat treatments strongly suggests that the labelled proteins are actually giving a true indication of total casein and whey protein behaviour.

Microstructure and rheology

For all three milk gels the changes in the miscrostructure were accompanied by increases in the G′ up to the end of the experiment, 3 h after the GDL addition (Fig. 3). Aggregation and gel formation (Fig. 4) coincided with an increase in the elastic modulus. As the gel formation was complete (Figs 4A4, B4 & C4), the G′ reached a plateau. The heat treated milk clearly produced firmer gels in relation to the severity of the heat treatment (Fig. 3), which can be attributed to the more continuous stranded structures observed in Figs 4B4 & 4C4. With the heat treated samples, there was a clear step in the G′ curve (related to a maximum in tan δ) at around 20 min from addition of acidulant, which probably relates to the release of colloidal calcium phosphate (Lucey, 2002).

The casein and whey protein fractions used in this study can be assumed to contain the individual proteins in the same proportions as present in milk. The fluorescent labels are non-specific, binding covalently to primary amines of the proteins, and hence all the proteins in each fraction should be labelled to a similar extent. As the dye binding is through covalent bonds, migration of dyes from the protein or exchange between proteins should not occur in these types of experiment. In the current system, observation of the position of fluorescent dyes in the gel structure cannot be related with certainty to individual proteins. However, this approach could be further refined by labelling individual purified proteins (e.g. α-la and β-lg), and observing their behaviour during gelation.

Conclusions

Using individually labelled proteins, it is possible to observe gelation of milk acidulated with GDL, with specific attention to caseins and whey proteins, using CSLM. The method is easy to use and allows tracking of different proteins in static or dynamic systems, although quantification of the protein fractions is not currently possible. The appearance of an aggregate-type gel structure could be observed in unheated, acidified milk, while more continuous stranded-type structures were seen in heated milk. The mechanism of incorporation of the different proteins into the gel structure of the control milk appeared to be quite different to the heated samples. The model system described in this work could be a useful tool in the further study of the development of structure in dairy products such as yogurt and cheese or even other food products in which differentiation of two proteins is important (e.g. glutenin and gliadin in breadmaking).

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