Gelation of casein-whey mixtures: effects of heating whey proteins alone or in the presence of casein micelles

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Summary. The aim of the present work was to investigate the role of whey protein denaturation on the acid induced gelation of casein. This was studied by determining the effect of whey protein denaturation both in the presence and absence of casein micelles. The study showed that milk gelation kinetics and gel properties are greatly influenced by the heat treatment sequence. When the whey proteins are denatured separately and subsequently added to casein micelles, acid-induced gelation occurs more rapidly and leads to gels with a more particulated microstructure than gels made from co-heated systems. The gels resulting from heat-treatment of a mixture of pre-denatured whey protein with casein micelles are heterogeneous in nature due to particulates formed from casein micelles which are complexed with denatured whey proteins and also from separate whey protein aggregates. Whey proteins thus offer an opportunity not only to control casein gelation but also to control the level of syneresis, which can occur.

Keywords: Whey protein, casein micelle, gelation, heat treatment.

Milk gels are traditionally formed by acidification or renneting. Gelation induced by renneting is totally different from that induced by acidification as far as modification of the casein micelle is concerned: renneting affects mainly the κ-casein of the micelles (McMahon & Brown, 1984; De Kruif *et al.* 1992) whereas, during acidification, both the 'hairy brush' and the core (dissolution of the colloidal calcium phosphate) of the casein micelles are affected (Heertje *et al.* 1985; Roefs *et al.* 1985; Fox & Muhvihill, 1990).

When whey proteins and casein micelles are mixed together, the effect of heat treatment becomes quite important. Heating milk above 70 °C at natural pH, predominantly promotes the unfolding of whey proteins (Walstra & Jenness, 1984; Muhvihill & Donovan, 1987). β-Lactoglobulin molecules can therefore interact not only with themselves but also with the caseins: sulphydryl-disulphide reactions together with hydrophobic interactions and calcium bridging, take place and result in the co-polymerisation of binary and tertiary systems, containing β-lactoglobulin, α-lactalbumin and κ-casein. These interactions then subsequently affect interactions between casein micelles (Walstra & Jenness, 1984; Mottar *et al.* 1989; Muhvihill &

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Donovan, 1987). The understanding of the complex formation between κ-casein and whey proteins remains mainly descriptive; for example on heating to 90 °C for 15 min, filaments were observed by electron microscopy on the surface of micelles (Heertje *et al.* 1985). In the literature, papers reported the effect of conventional heat treatment of skim milk on acid or rennet gelation (Heertje *et al.* 1985; Mottar *et al.* 1989; Dalgleish, 1990; Singh *et al.* 1996) or the co-heat treatment of whey proteins with pure casein fractions (McKenzie *et al.* 1971; Doi *et al.* 1983; Noh *et al.* 1989).

The aim of the present work was to obtain further understanding of the effect of whey proteins on the gelation of micellar caseins. In particular the gelation behaviour of co-heated casein micelle/whey mixtures is compared with systems where whey proteins have been heated separately before addition to the casein micelles. For this purpose techniques such as rheology, microscopy and turbidimetry have been used to determine the effects of the heat treatment sequence on gelation kinetics and gel properties.

Materials and methods

Materials

Simulated milk ultrafiltrate (SMUF) was prepared according to the method of Jenness and Koops (1962). The whey protein powder used was a low heat powder, Bipro 95, provided by Davisco Food International (Le Sueur, USA). The proteins in the powder were shown, by size exclusion high performance liquid chromatography and acid precipitation, to be almost completely free of aggregates.

The casein micelles were prepared by INRA (Rennes, France) using a micro-filtration method (Schuck *et al.* 1994).

Preparation of protein dispersions and casein/whey mixtures

Micellar casein dispersions were obtained by dispersing the freeze-dried protein in SMUF while stirring with a paddle mixer for 30 min at 60 °C at a fixed concentration (90 g/kg). Whey powder was dispersed in SMUF at room temperature (20–22 °C) by stirring with a magnetic stirrer for 30 min to give a final concentration of 20 gl. The pH was then adjusted to 6·5. To obtain casein/whey samples, one volume of casein suspension was mixed with one volume of whey suspension to give mixtures containing 45 g casein/kg and 10 g whey protein/kg (which correspond to $\sim$ 15% skim milk dispersion).

Heat treatment of the suspensions

Two types of experiments were carried out. The first consisted of the co-heat treatment of whey protein with the casein micelles. Samples (45 g casein, 10 g whey protein/kg) were co-heated for 30 min at the temperatures studied (20, 60, 80, or 90 °C) and then cooled in ice to 5 °C. The samples were labelled 20/20, 20/60, 20/80 and 20/90, respectively. The second type of experiment consisted of heating the whey proteins alone for 30 min (for example at 80 °C), and then after cooling in ice, the whey solution was added to casein micelles and the mixture heated further at the temperatures specified above. After this treatment the samples were labelled 80/20, 80/60, 80/80 and 80/90, respectively.

GDL-induced acidification

Glucono-δ-lactone (GDL) was used as a slow acidulant to give a final pH of 4·7. A constant GDL concentration of 13 g/kg was used and acidification was performed at 20 °C.

Rheological properties

The storage modulus (G'), loss modulus (G'') and tanδ were determined *v.* time using a Physica Rheometer with a Couette cylinder (Z3 DIN) geometry. GDL was added to each sample at 5 °C and the mixture was stirred vigorously for 1 min before 15 ml were transferred to the rheometer. The sample was then heated from 5 to 20 °C at a heating rate of 1 deg C/min. A layer of mineral oil was used to prevent evaporation. Gels were oscillated at a frequency of 1Hz and measurements were taken every 2 min for 12 or 24 h. After gelation a frequency sweep was performed from 10 to 0·01 Hz at a deformation of 0·5 %.

Microscopy

Confocal Laser Scanning Microscopy (*CSLM*). A confocal scanning laser microscope (Biorad MRC 600; Hemel Hempsted, UK) was used to visualise the microstructure of the gels. Rhodamine B (0·01 g/kg) was added to the dispersions in order to stain the proteins and GDL was then added. A small quantity of the sample was placed into the cavity of a microscope slide, covered with a coverslip and stored for 24 h, at 20 °C. The slide was then placed on a temperature-controlled stage and observed using laser excitation at 488 nm.

Electron microscopy (*EM*). Samples were resin embedded using the following protocol: 2 mm² blocks of gel were excised with a razor, fixed for 4 h at 4 °C in phosphate-buffered saline (PBS; 1·47 mM-KH_2PO_4-1·68 mM-Na_2HPO_4-136mMNaCl-2·68 mM-KCl buffer, pH 7·2) containing 40 g paraformaldehyde and 0·5 glutaraldehyde/l, then dehydrated in ethanol series (50, 70, 90, and 100 %) for 30 min each, embedded in LR Gold (London Resin inc., UK): glycol methacrylate at 6:4 ratio for 4 d at 4 °C (fresh resin every 24h) and polymerised at 20 °C. under UV in dry (N_2) steam. Polymerised blocks were sectioned onto a nickel grid coated with collodion film. Sections were immunolabelled according to the protocol of Gagne & Miller (1987).The primary antibodies used were β-lactoglobulin rabbit IgG (Polysciences Inc. Warrington, Pasadena, USA) used at 1:3000 dilution and secondary antibodies were goat anti-rabbit conjugated with 10 nm colloidal gold (British Biocell, Cardiff, UK). The samples were examined using a JOEL 1220 transmission electron microscope at 80 KeV.

Syneresis

Syneresis was studied by measuring the amount of exuded liquid appearing on top of the sample after storage at 20 °C. The liquid was drained off and weighed. Each final percentage liquid loss was derived from the average of five repeat measurements. The error on the measurements was ± 3 %.

Turbidimetry

Turbidity spectra for casein micelle/whey dispersions were recorded between 700 and 800 nm on a spectrophotometer UV-2101 (Shimadzu, Scientific Instrument Inc., USA).

The turbidity (τ) of a suspension of particles is a measure of the reduction in intensity of the incident beam due to scattering. In regions far from the absorption peaks, the turbidity of the suspension is directly read in a spectrophotometer as optical density (OD),

$$\tau = 2{\cdot}303 \times \mathrm{OD}.l^{-1} \text{ (where } l \text{ is the length of the light path in the sample).}$$

The amount of light scattered by a particle in a given medium depends on several parameters, that can be separated into different contributions (Doty & Steiner, 1949):

$$\tau = \mathrm{H.Q.S.M.c},$$

H, Q and S are functions related to the optical constant, the intra- and inter-particle correction factors, respectively. M is the molecular weight (in g mol^{-1}) and c the concentration (in g cm^{-3}). H depends on the optical properties of both the solvent and particle and on the wavelength (λ).

In the present work, both the turbidity (τ) at 800 nm and the wavelength exponent $\mathrm{dlog}\tau/\mathrm{dlog}\lambda$ (between 700 and 800 nm) of the turbidity were measured. The derivative $\mathrm{dlog}\tau/\mathrm{dlog}\lambda$ can be written as follows:

$$\mathrm{dlog}\tau/\mathrm{dlog}\lambda = -4+\alpha_1+\alpha_2+\beta+\beta'$$

where α_1 and α_2 come from the wavelength dependence of the refractive index of the solution and the refractive increment of the solute. These are small negative correction factors (Cancellieri *et al.* 1974) and are neglected here. β is the wavelength dependence of the dissipation factor ($\mathrm{dlog}Q/\mathrm{dlog}\lambda$), which is a function of the size of the particle and can be directly estimated from the relation between Q and D/λ, D being the particle diameter (Cancellieri *et al.* 1974). For small particles, $\beta = 0$ and the wavelength dependence of the turbidity is close to the -4 predicted by the Rayleigh theory (Cancellieri *et al.* 1974). β' accounts for the wavelength dependence of S, and is given by $\mathrm{dlog}S/\mathrm{dlog}\lambda$.

RESULTS

Fig. 1 shows the acid gelation profiles for co-heated casein micelle/whey mixtures and also for mixtures in which the whey has been pre-heated separately. The results show that co-heat treatment of casein/whey mixtures above 60 °C imparts increased firmness to acid gels in comparison to gels made from unheated or low heated (below 60 °C) mixtures. At high temperature the gelation time is also significantly reduced (6 h against 21 h). Fig. 1 shows that for samples co-heat treated at high temperature, the pH of gelation was increased significantly (5·1 against 4·7). When whey proteins are pre-heated separately at 80 °C (sample 80/20) and added to casein micelles before acidification, gel formation begins as soon as the pH value starts to decrease from 6·0. This contrasts with the lag time shown for the 20/20, 60/20 or 20/80 samples. However the gel is very weak even at low pH values compared to the 20/80 or 80/80 samples. When the 80/20 sample is then co-heated (i.e. 80/80) a lag time becomes apparent in the gelation profile and gelation does not begin until the pH is close to 5·3. The gel firmness is also increased in comparison with the 20/80 sample.

These results highlight clear differences on acid gelation between the effects of pre-heat treatment of whey protein before addition to casein micelles compared to co-heat treatment of whey with casein micelles.

Examination of the gel structures by confocal microscopy and transmission electron microscopy show that four different types of structure are formed depending on the heat treatment sequence. For a sample co-heated above 60 °C, confocal microscopy shows that the network is much more dense (Fig. 2*b*) than for an

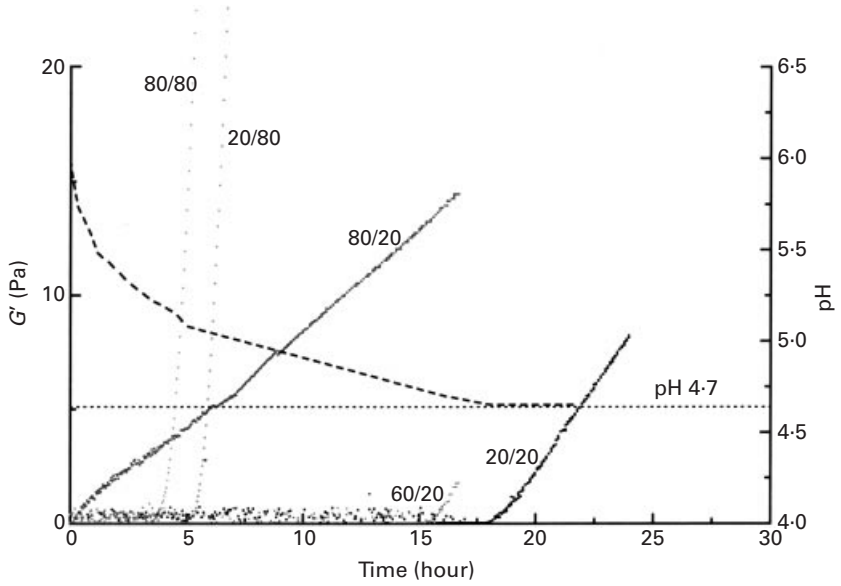

Fig. 1. Variation of G' with time after GDL addition (13 g/kg, 20 °C) to a micellar casein/whey protein dispersion (45 and 10 g/kg respectively). Whey proteins were pre-heated for 30 min at 20 °C (sample 20/20), 60 °C (sample 60/20) or 80 °C (sample 80/20) and then mixed with the casein dispersion at 20 °C. Co-heated 20/80 and 80/80 samples (Frequency 1 Hz) are shown for comparison. Dashed line: pH *v* time profile.

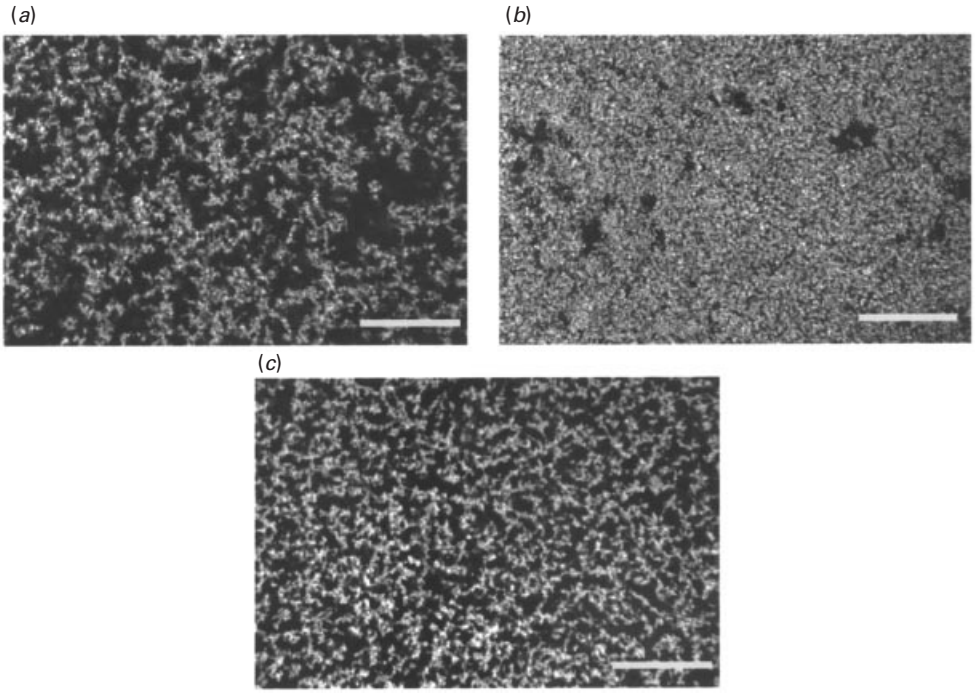

Fig. 2. Confocal micrographs, after GDL addition (13 g/kg, 20 °C), of micellar casein/whey protein dispersions (45 and 10 g/kg respectively) co-heated for 30 min at (*a*) 20 °C, (*b*) 80 °C or (*c*) with whey proteins pre-heated for 30 min at 80 °C. Scale bar: 50 μm.

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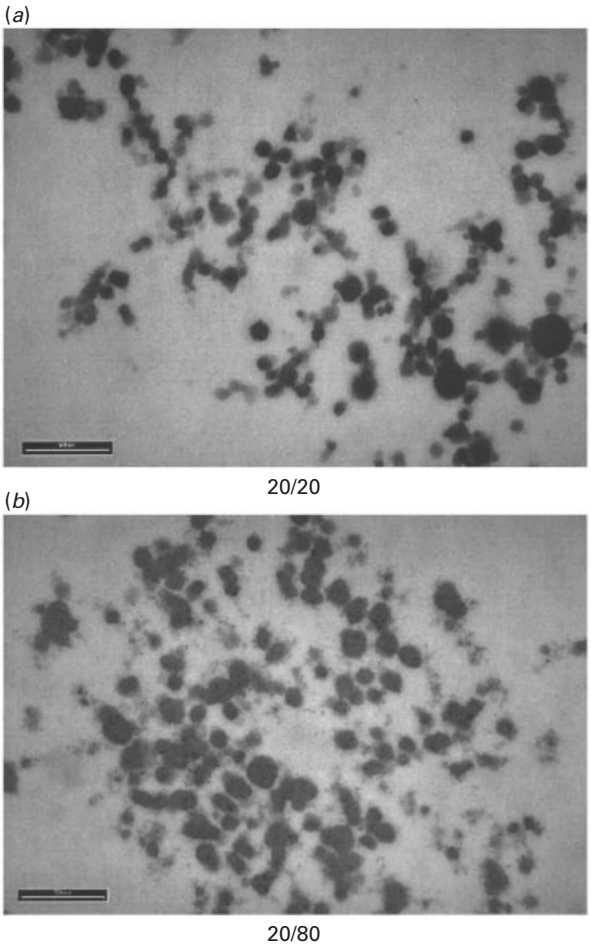

Fig. 3. Transmission electron micrograph after GDL addition (13 g/kg, 20 °C), of micellar casein/whey protein dispersions (45 and 10 g/kg respectively) heated for 30 min at (*a*) 20 °C and at (*b*) 80 °C. Scale bar: 500 nm.

unheated sample (Fig. 2*a*), while pre-heat treatment of whey at 80 °C leads to a network which is of intermediate density (Fig. 2*c*). These differences are highlighted in more detail by the electron micrographs shown in Fig. 3. The co-heated sample (20/80) shows the presence of appendages (denatured whey) on the surface of the micelles (Fig. 3*b*) while the unheated sample (Fig. 3*a*) does not. The binding of denatured whey to the micelle surface favours the formation of bridges between the casein particles leading to a narrow pored casein network. For samples in which the whey was pre-heated (e.g. 80/20) whey protein aggregates were more visible such that the gel was more like a particulated whey gel than a 'traditional' casein gel. When the pre-heated sample was co-heated further (80/80) a mixed network composed of casein micelles and whey aggregates was formed.

The syneresis behaviour of the gels is displayed in Table 1. Data obtained at 20 °C indicate that acid gels formed from co-heated whey protein/casein micelle mixtures are quite stable on storage, with a very low degree of syneresis ($\sim 5\%$ w/w water loss) which is independent of the heating temperature. By contrast, when pre-heated whey proteins are mixed with casein micelles, the gels formed are far more prone to

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Table 1. *Syneresis measurements (in % liquid loss) with time at 20 °C after GDL (13 g/kg) addition to micellar casein/whey protein dispersions (45 and 10 g/kg respectively). Samples 1–4 were heated after whey protein addition while samples 5–8 contained whey protein pre-heated for 30 min at 80 °C*

Sample	Temperature treatments, °C	Day 1	Day 2	Day 3	Day 4	Day 7
1	20/20	0·07	0·24	0·28	0·54	0·94
2	20/60	0·13	0·21	0·78	1·39	2·09
3	20/80	0·32	0·35	1·24	1·97	3·06
4	20/90	0·97	2·10	3·34	4·38	5·60
5	80/20	9·71	18·04	23·66	27·48	35·85
6	80/60	6·45	15·89	22·40	27·03	30·59
7	80/80	6·52	13·59	19·16	23·84	27·98
8	80/90	7·06	12·45	16·68	20·66	24·21

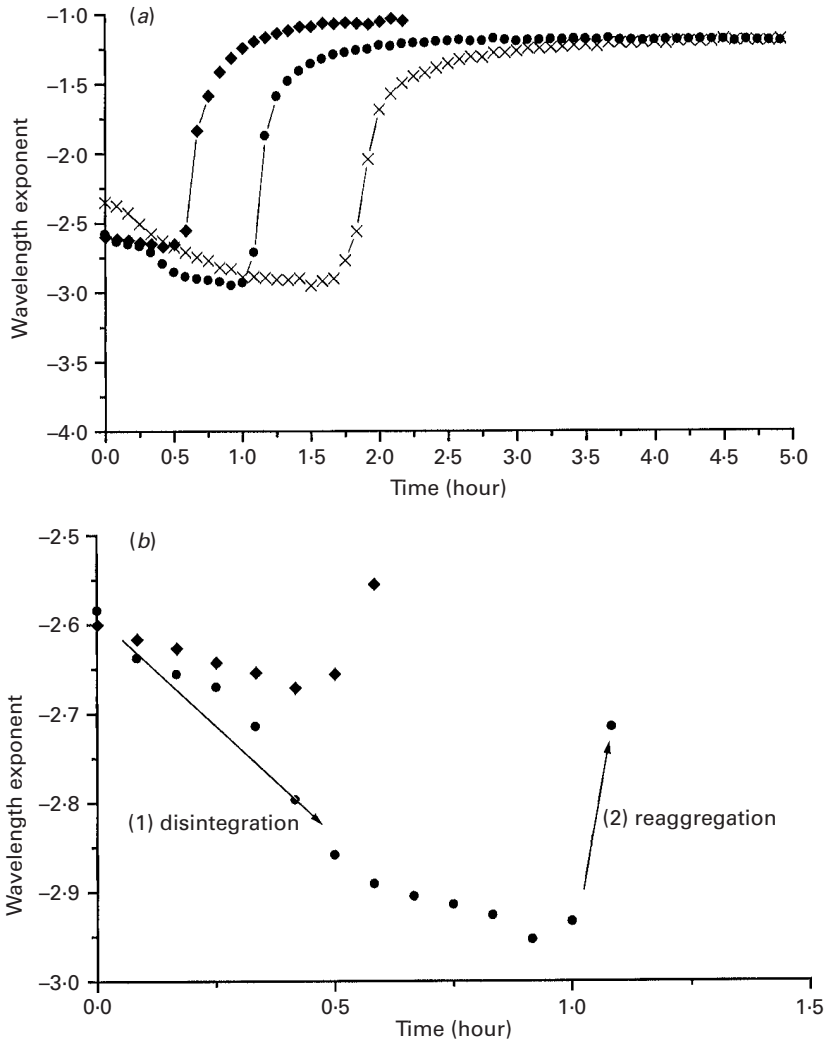

Fig. 4. (*a*) Wavelength exponent *v* time after GDL addition (13 g/kg, 20 °C), of micellar casein/whey protein dispersions (45 and 10 g/kg respectively; 1/4 diluted) heated for 30 min at 20 °C (●, sample 20/20) and at 80 °C (◆, sample 20/80). Data for a micellar casein dispersion are shown for comparison (×). (*b*) Enlargement of region before the transition (circa 1 h).

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syneresis (degree of syneresis ranges between 5 and 35% w/w water loss) and the extent of syneresis is clearly temperature dependent, with higher temperatures giving less liquid loss. The general trend for liquid loss is 20/20 < 20/80 < 80/80 < 80/20.

Turbidimetry measurements were carried out on more dilute dispersions to investigate the effect of whey protein addition on casein micelle structure. The protein concentrations studied were below 20 g/kg (to avoid saturation of the detector by the very turbid casein dispersions) which means that an infinite size network is not formed (the critical concentration for gelation was about 20 g/kg; data not shown), however, the first stages of aggregation can be followed by this method. The time course of the wavelength exponent was followed during acidification and the results are displayed in Figs. 4*a* & 4*b*. As time increased, the pH decreased so that a value close to 5·0 was obtained after 1 h. For the three samples (casein micelles, 20/20 and 20/80 casein/whey mixtures) illustrated in Fig. 4*a*, the curves can be decomposed into two steps. During the second step, the wavelength exponent increases quite significantly, indicating the formation of heterogeneities due to aggregation. As already demonstrated by the rheological data, these results show that aggregation occurs earlier for the sample which has been co-heated (20/80) compared to the unheated sample (20/20). It is important to highlight the difference in time scale compared with the rheological results (see Fig. 1) since for these experiments the protein concentrations are lower. Examination of the time-dependence of the wavelength exponent in more detail shows that in the first few minutes (Fig. 4*b*) there is a clear difference between the two samples. For the casein micelle dispersion alone and for the unheated 20/20 mixture, the wavelength exponent decreases significantly by 0·5 unit, indicating a decrease in the particle size. This decrease is far less pronounced when the mixture is co-heated at 80 °C (sample 20/80) hence in this case, the wavelength exponent remains constant. This indicates no decrease of the particle size before aggregation, suggesting that complexes formed between whey proteins and casein micelles on heating inhibit the dissociation of the micelles due to acidification.

Discussion

These results illustrate the effect of heat treatment, and particularly the heat treatment sequence of casein-whey mixtures, on subsequent formation of acid gels. Four types of gel structures can be formed as illustrated schematically in Fig. 5. These gel structures result from the changes occurring with successive heat treatments i.e. the change from system A (20/20) to B (20/80), system A (20/20) to C (80/20) and system C (80/20) to D (80/80). For unheated samples gel formation results mainly from aggregation of the casein micelles, with 'native' whey proteins acting only as 'inactive' filler (Fig. 5A). However, when the whey-casein mixture was co-heated at a temperature above 60 °C [system A (20/20) → system B (20/80)], a stronger gel is formed. This has a different structure from one made with unheated milk and is in agreement with previously published data (Kalab *et al.* 1976). According to Lucey *et al.* (1998*b*) heat treatment causes denatured whey proteins to complex with the casein micelle surface via κ-casein – whey protein sulphydryl interchange and the whey is then able to aggregate and form bridges between the micelles. This could explain why gelation occurs at a higher pH, because the isoelectric pH of the whey proteins is higher than that of the caseins (pI β-lactoglobulin = 5·3). Early views on the mechanism of acid gelation suggested that casein micelles aggregate as a result of charge neutralisation, leading to the

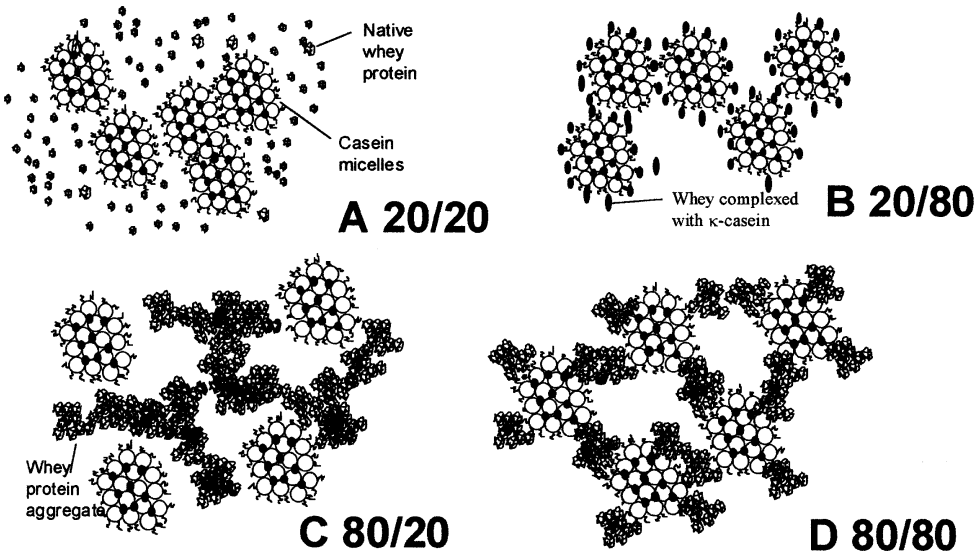

Fig. 5. Schematic representation of casein/whey protein acid gels. (A) casein/native whey protein (20/20); (B) casein/native whey protein after heating (20/80), heat treatment of mixture of both whey proteins and casein micelles; (C) casein/pre-denatured whey protein (80/20), heat treatment of only whey proteins; (D) casein/pre-denatured whey after heating (80/80), heat treatment of whey proteins and mixture with casein micelles.

formation of chains of micelles that are linked together to give a three-dimensional network (Davies *et al.* 1978). It is now thought that the process is more complex and involves partial disintegration of the casein micelles before aggregation (Heertje *et al.* 1985; Roefs *et al.* 1985; Fox & Muhvihill, 1990). This was confirmed by our turbidimetry results for an unheated casein micelle system, which showed an initial decrease in the wavelength exponent before the sharp transition due to aggregation. In contrast to the micellar casein system and the 20/20 casein/whey sample, the wavelength exponent for the 20/80 sample remained constant before the transition, suggesting that the formation of κ-casein/β-lactoglobulin complex prevents dissociation of the micelles and hence release of part of the β-casein molecules. The resultant gel consists of casein micelles with an average size, which was larger than for an unheated sample, connected via κ-casein/whey complexes formed on heating.

When they are heated alone whey proteins denature and self-aggregate to form particles mainly linked by disulphide bonds. These aggregated particles are unable to covalently attach to the κ-casein of the micelles when mixed at 20 °C, but they can associate with each other as the pH is slightly decreased to pH $\sim$ 5·8 [system A (20/20) $\rightarrow$ system C (80/20)]. For 80/20 and 90/20 samples, an instantaneous weak gel was formed which resembled a particulated whey protein gel rather than a 'traditional' casein gel. When the pH of these samples was reduced to a value that normally produces a strong casein gel only a weak gel was observed, and this is thought to be due to the particulate whey gel sterically hindering the formation of a casein network (Fig. 5C). These gels are 'heterogeneous' in nature, with clusters of casein micelles and a network essentially consisting of denatured whey protein. When these samples were then co-heated [i.e. system C (80/20) $\rightarrow$ system D (80/80)] a gel was formed consisting of a mixture of casein micelles and whey aggregates. This can be explained by the fact that the second heating step causes thermal reduction of the disulphide links within the whey aggregates allowing subsequent interactions

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with κ-casein on the surface of the casein micelle, and this in turn leads to a stronger gel. The heat treatment of the system (80/20) prior to gelation (80/80) caused a partial reversal back to the gelation mechanism of the whey protein/casein micelle mixture that was heated together prior to gelation (20/80) and resulted in properties intermediate between those of systems C(80/20) and B(20/80). The syneresis results support this proposal and are directly linked to the microstructure of the gels. Gels formed with addition of unheated whey have a mainly casein network and show low syneresis, whereas those formed from denatured whey have a more particulate and heterogeneous structure and show the greatest syneresis. A second heating step reduced the syneresis due to the formation of a mixed casein/whey aggregate network.

This study has shown that gelation kinetics and properties of casein gels (microstructure, syneresis, gel strength) formed in the presence of whey are greatly influenced by heat treatment. For co-heat treated samples our results for acid gels are in good agreement with studies described in the literature on the gelation of skim milk. The influence of heat treatment on gelation depends on the gelation method used. The whey/casein complexes formed during heating give increased strength for acid gels, which contrasts with published results for rennet gels where rennet action is prevented, making the gels weaker.

This present paper highlights the importance of knowing the pre-treatment of milk and whey proteins if they are to be used together. Whey is often used to enrich the total milk protein level in products because it is cheaper than milk or casein and because it has interesting nutritional and functional properties. These results indicate that whey proteins offer an opportunity not only to control casein gelation but also to control subsequent properties such as syneresis. To achieve this, however, it is important to know the thermal history of the components in order to predict behaviour.

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