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Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size

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When skim milk at pH 6·55 was heated (75 to 100 °C for up to 60 min), the casein micelle size, as monitored by photon correlation spectroscopy, was found to increase during the initial stages of heating and tended to plateau on prolonged heating. At any particular temperature, the casein micelle size increased with longer holding times, and, at any particular holding time, the casein micelle size increased with increasing temperature. The maximum increase in casein micelle size was about 30–35 nm. The changes in casein micelle size were poorly correlated with the level of whey protein denaturation. However, the changes in casein micelle size were highly correlated with the levels of denatured whey proteins that were associated with the casein micelles. The rate of association of the denatured whey proteins with the casein micelles was considerably slower than the rate of denaturation of the whey proteins. Removal of the whey proteins from the skim milk resulted in only small changes in casein micelle size during heating. Re-addition of β-lactoglobulin to the whey-protein-depleted milk caused the casein micelle size to increase markedly on heat treatment. The changes in casein micelle size induced by the heat treatment of skim milk may be a consequence of the whey proteins associating with the casein micelles. However, these associated whey proteins would need to occlude a large amount of serum to account for the particle size changes. Separate experiments showed that the viscosity changes of heated milk and the estimated volume fraction changes were consistent with the particle size changes observed. Further studies are needed to determine whether the changes in size are due to the specific association of whey proteins with the micelles or whether a low level of aggregation of the casein micelles accompanies this association behaviour. Preliminary studies indicated lower levels of denatured whey proteins associated with the casein micelles and smaller changes in casein micelle size occurred as the pH of the milk was increased from pH 6·5 to pH 6·7.

Keywords: Milk, denatured whey proteins, casein micelles, α-lactalbumin, β-lactoglobulin.

Heating milk at temperatures above about 70 °C results in the thermal denaturation of the globular whey proteins in which the native conformation is disrupted and converted to a lower state of order. Denaturation of the whey proteins can result in the exposure of reactive amino acid side groups that are normally buried within the native conformation. Of particular importance is the increased reactivity of the free thiol groups of β-lactoglobulin, which can be involved in thiol-disulphide exchange reactions with other denatured whey proteins and with κ-casein at the casein micelle surface. However, denatured proteins are also more susceptible to aggregation via salt bridges and hydrophobic interactions.

There are numerous reports of studies examining the kinetics of the thermal denaturation of the major whey proteins when milk is heated under defined conditions (Hillier & Lyster, 1979; Dannenberg & Kessler, 1988; Kessler & Beyer, 1991; Bikker et al. 2000). These studies have been expanded to include heat treatments under typical processing conditions (Anema & McKenna, 1996; Oldfield et al. 1998*a*). Models that allow prediction of the degree of denaturation of the individual whey proteins when milk is heated at any temperature and time combination have been developed (Dannenberg & Kessler, 1988). An understanding of the denaturation reactions of the individual whey

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proteins provides information on the initial steps of a complex series of aggregation reactions that occur when milk is heated.

It has long been recognized that complexes are formed between the denatured whey proteins and the casein micelles when milk is heated, and the factors influencing these interactions have been intensively studied (Sawyer, 1969; Mulvihill & Donovan, 1987; Hill, 1989; Jang & Swaisgood, 1990). Zittle et al. (1962), using purified protein solutions, provided the first conclusive evidence that a heat-induced interaction between β-lg and κ-casein occurred. Under electrophoretic conditions, unheated solutions of κ-casein and β-lg formed discrete bands, whereas, on heating, a species of intermediate mobility was formed. The formation of a complex has been subsequently confirmed by numerous studies both in model systems and in heated milk (Sawyer, 1969; Smits & van Brouwershaven, 1980; Hill, 1989; Noh & Richardson, 1989; Jang & Swaisgood, 1990).

Subsequent investigations tended to focus on determining the types of bonding involved in complex formation and the stoichiometry of the complexes formed (Sawyer, 1969; Hill, 1989; Jang & Swaisgood, 1990; Corredig & Dalgleish, 1996*a*, 1999; Oldfield et al. 1998*b*). It is believed that the major interaction involves thiol-disulphide exchange reactions between the denatured β-lg and κ-casein at the micelle surface (Sawyer, 1969; Smits & van Brouwershaven, 1980; Jang & Swaisgood, 1990). Some reports suggest that ionic and/or hydrophobic interactions play a significant role in the aggregation of the denatured whey protein with the casein micelles, particularly in the early stages of the reaction (Mulvihill & Donovan, 1987; Hill, 1989).

When milk was heated slowly (e.g. laboratory waterbath conditions) about 80% of the denatured β-lg associated with the casein micelles (Smits & van Brouwershaven, 1980; Corredig & Dalgleish, 1996*a*). In contrast, when milk was heated rapidly (e.g. under processing conditions using direct heating systems) only about half the denatured β-lg and α-lactalbumin was found to be associated with the casein micelles. The rest of the denatured whey protein remained in the milk serum as disulphide-bonded and hydrophobically associated aggregates (Singh & Creamer, 1991; Oldfield et al. 1998*b*). Corredig & Dalgleish (1996*b*) showed that the rate and the degree of interaction of the whey proteins with the casein micelles were markedly dependent on the method of heat treatment. Higher levels of whey proteins associated with the casein micelles when skim milk was heated using indirect heating systems than when a direct steam injection system was used. Corredig & Dalgleish (1996*a*, 1999) suggested that, on heating milk, the α-la and β-lg initially aggregate in the serum phase at a ratio dependent on the initial individual whey protein concentrations. These complexes subsequently associate with the casein micelles on prolonged heating.

The interaction between casein micelles and denatured whey proteins should alter the physical properties of the micelles and the milk in which they are suspended. Jeurnink (1992) monitored the interaction of whey proteins with the micelles using turbidity techniques and showed that the rate of increase in turbidity paralleled the rate of denaturation of the whey proteins. Jeurnink & De Kruif (1993) reported increases in the viscosity of milk with heating and related this to the association of whey proteins with the casein micelles. A diffuse layer of denatured whey proteins around the casein micelles could be observed when skim milk was heated under mild conditions (Beaulieu et al. 1999).

The changes in both the viscosity and the turbidity of milk when the whey proteins interact with casein micelles suggest that the casein micelle size may be changing during the heat treatment; however, no systematic study on the changes in casein micelle size during mild heating of milk has been reported. In this study, the changes in casein micelle size during heating were monitored and relationships between the changes in casein micelle size and whey protein denaturation and their association with the micelles were examined.

Materials and Methods

Milk supply

Fresh milk samples were obtained from a local farm. The milk samples were skimmed at 40 °C and the resultant skim milk was used for the separation and purification of β-lg. Low-heat skim milk powder was obtained from Kiwi Cooperative Dairies, Pahiatua, New Zealand. This milk powder had a whey protein nitrogen index above 6 (Sanderson, 1970) and contained approximately 37% protein on a dry basis. Experimental skim milk samples were prepared by reconstituting low-heat skim milk powder to 10% (w/w) total solids in purified water (reverse osmosis followed by filtration through Milli-Q apparatus). The reconstituted skim milk samples were allowed to equilibrate at ambient temperature (about 20 °C) with gentle stirring for at least 4 h before further treatment. A small amount of sodium azide (0·01% w/v) was added to the milk as a preservative.

Preparation of β-lactoglobulin

Skim milk was warmed to 40 °C and the casein was precipitated by acidification to pH 4·6 using HCl. The resultant acid whey was concentrated by repeated freeze-thaw cycling, as described by Manderson et al. (1998). β-Lg was separated and purified from the concentrated acid whey using the low pH salt precipitation method that has been described by Mailliart & Ribadeau-Dumas (1988). The purified β-lg was freeze dried and stored at 5 °C. The purified β-lg contains approximately equal levels of the A and B variants of this protein, as determined by electrophoretic analysis.

Preparation of whey-protein-depleted (WPD) milk

WPD milk was prepared by subjecting milk to a combination of ultrafiltration and microfiltration techniques using

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the method described by Anema & Li (2000). A large volume (about 25 l) of reconstituted skim milk was ultrafiltered using a 10000 Dalton (H1P10-43) hollow fibre membrane cartridge and the associated pumping equipment (Amicon, Inc., Beverly, MA 01915, USA) until about 3 l of protein-free milk permeate was collected. A separate sample of milk (0·5 l) was microfiltered using a membrane cartridge with a 0·1 μm pore size (H1MP01-43). This membrane allows the passage of α-la and β-lg but retains the casein and most of the higher molecular weight whey proteins. The volume of microfiltrate was constantly monitored and an equivalent volume of the ultrafiltration permeate was added back to the milk. Using this method, a WPD milk containing only traces of the original α-la and β-lg was obtained.

Addition of β-lactoglobulin to WPD milk

The required quantity of β-lg was added directly to the WPD milk. The milk was allowed to stir for about 30 min before use.

Adjustment of pH and heat treatments

The majority of experiments were performed on milk at pH 6·55. However, some preliminary experiments on the effect of pH were conducted on milk samples at between pH 6·5 and pH 6·7. Experimental skim milk samples were adjusted to the required pH by the slow addition of 1 M-HCl or 1 M-NaOH to well-stirred solutions. The milk samples were allowed to equilibrate for 2 h before final pH reading and minor re-adjustment. The milk samples were preheated at 68 °C for 10 min before further use. Subsamples of milk (6 ml) were transferred to glass vials and heated, with continuous rocking, in a thermostatically controlled oil bath preset to temperatures in the range from 75 to 100 °C. After heat treatment, the milk samples were cooled to room temperature by immersion of the glass vials in cold running water.

Particle size analysis

Particle size measurements were made by photon correlation spectroscopy using a Malvern Zetasizer 4 instrument and the associated ZET5110 particle sizing cell (Malvern Instruments Ltd., Malvern, Worcs., UK). The temperature of the cell was maintained at 20±0·5 °C for the duration of the experiments. Measurements of the dynamics of the scattered light were collected at a scattering angle of 90° only. Average diffusion coefficients were determined by the method of cumulants and translated into average particle diameters using the Stokes–Einstein relationship for spheres. Skim milk samples were dispersed in Ca-imidazole buffer (20 mM-imidazole, 5 mM-$CaCl_2$, 30 mM-NaCl, pH 7·0) as has been described previously (Anema, 1997). Preliminary experiments showed that similar particle size changes were obtained when skim milk ultrafiltrate was used as the dispersant. Ca-imidazole buffer was used in preference owing to its ease of preparation and storage stability. The viscosity of the Ca-imidazole buffer was indistinguishable from water. The viscosity of the milk permeate at 20 °C was 1·24 Nsm^{-2}, which is identical to that determined by Pearce (1976).

Viscosity measurements

Viscosity was measured with a capillary viscometer (Cannon Instrument Company, State College, PA, 16804-0016 USA) using the method of Jeurnink & De Kruif (1993). The average flow times were ~154 s, ~180 s and ~270 s for water, milk permeate and skim milk respectively. The volume fraction of the unheated milk and heated milk was determined using the Einstein equation, as described by Jeurnink & De Kruif (1993).

Ultracentrifugation

Preliminary experiments were used to determine a suitable centrifugation speed. If a high centrifugation speed, and therefore a high ***g*** force, was used, higher levels of whey proteins were deposited with the pellet although there was little change in the serum casein content (results not shown). This suggests that, under high centrifugation force, some of the soluble whey protein aggregates were deposited even though these aggregates were not associated with the micelles. To reduce the possibility of centrifuging down soluble whey protein aggregates, the centrifugation speed chosen was the minimum required to effectively deposit the casein micelles/associated whey proteins as a firm pellet. Soluble whey proteins were therefore defined as those that did not sediment from the milk during ultracentrifugation at 30000 rpm (63000×***g*** average) for 1 h at 20 °C in a Beckman L8-80M ultracentrifuge and the associated Beckman Ti-80 rotor (Beckman Instruments Inc., Palo Alto, CA, USA). The clear supernatant was carefully removed from the pellets. The β-lg and α-la content of the supernatants was determined by gel electrophoresis and laser densitometry.

Gel electrophoresis and laser densitometry

The level of native whey protein in the unheated and heat-treated milk samples was determined using native polyacrylamide gel electrophoresis (native-PAGE), as described previously (Anema & McKenna, 1996). The casein and denatured whey proteins were removed from the milk by adjusting the pH to 4·6 and centrifuging out the precipitate using a bench centrifuge. The resultant supernatant was used for analysis by native-PAGE. The level of soluble whey proteins in the ultracentrifugal supernatants was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, as described previously (Anema & Klostermeyer, 1997).

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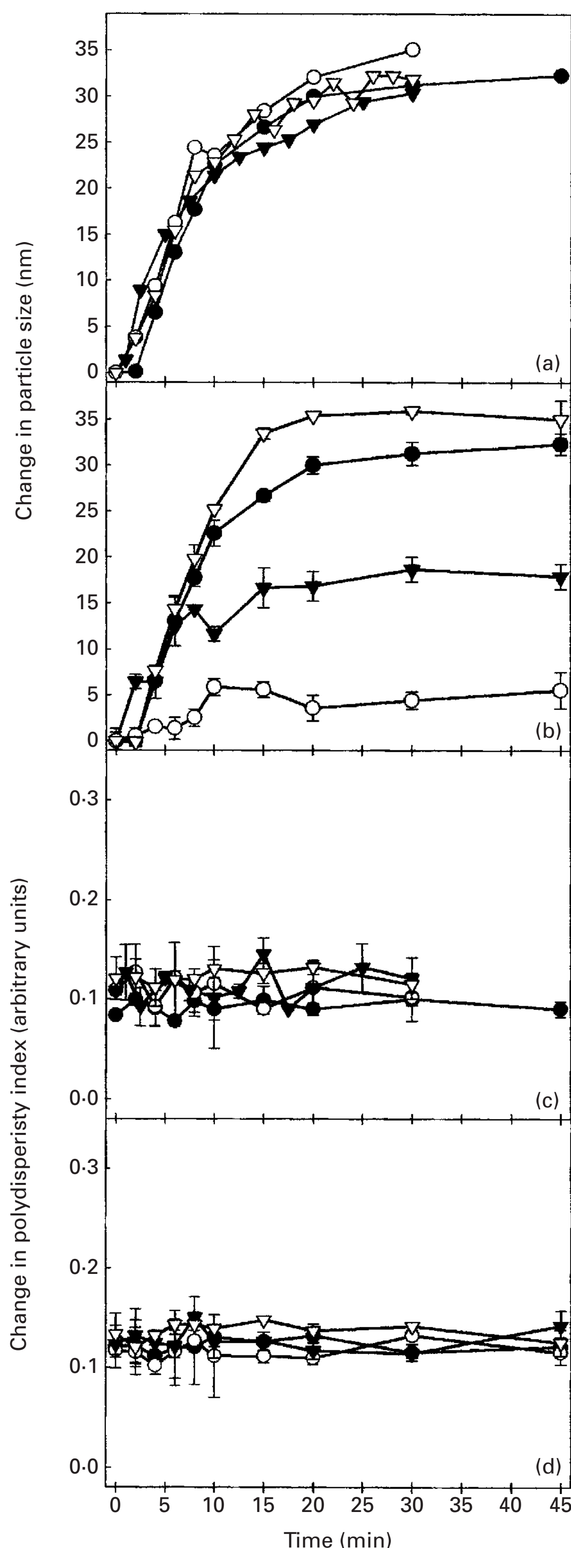

Fig. 1. a: Changes in casein micelle size for skim milk samples at pH 6·55 that were heated at 90 °C for various times. ●: Milk 1; ○: milk 2; ▼: milk 3; ▽: milk 4. b: Changes in casein micelle size for skim milk and whey-protein-depleted (WPD) milk with various levels of added β-lactoglobulin. All samples were at pH 6·55 and were heated at 90 °C. ●: skim milk; ○: WPD milk; ▼: WPD milk+0·15% β-lg; ▽: WPD milk+0·30% β-lg. Error bars represent the standard deviation of duplicate measurements.

Native-PAGE and SDS-PAGE gels were scanned using a Molecular Dynamics Model PD-SI computing densitometer (Molecular Dynamics Inc., Sunnyvale, CA, USA). The integrated intensities of the major casein bands were determined using the Imagequant software associated with the densitometer. The quantity of each protein in the ultracentrifugal supernatants was determined as a percentage of that in the original milk samples. The level of protein associated with the micelles was calculated as the difference between the levels of soluble protein in the unheated and heated milk samples.

All experiments reported have been repeated on several different milk samples. Although some variations existed between individual milks, the same trends and relationships as reported here have been found for all samples examined to date.

Results and Discussion

When milk is heated above about 70 °C, the whey proteins denature (Dannenberg & Kessler, 1988; Anema & McKenna, 1996; Oldfield et al. 1998*a*; Anema, 2000, 2001; Bikker et al. 2000). The denatured whey proteins can interact with other denatured whey proteins and with the casein micelles via thiol-disulphide exchange reactions, and hydrophobic and ionic interactions (Sawyer, 1969; Mulvihill & Donovan, 1987; Hill, 1989). The interaction of whey proteins with casein micelles in heated milk has been monitored indirectly using turbidity (Jeurnink, 1992) and viscosity (Jeurnink & De Kruif, 1993) techniques, or directly by centrifuging out the casein micelles and the associated whey proteins and measuring the whey protein content of either the pellet (Corredig & Dalgleish, 1996*a*, *b*, 1999) or the supernatant (Smits & van Brouwershaven, 1980; Singh & Creamer, 1991; Oldfield et al. 1998*b*, 2000).

The changes in the turbidity and viscosity of milk during heating suggest that the interaction of denatured whey proteins with the casein micelles may increase the size of the micelles. This change in casein micelle size should be measurable using sensitive sizing techniques such as photon correlation spectroscopy. Figure 1a shows the changes in casein micelle size when four separate skim milk samples at pH 6·55 were heated at 90 °C for up to 45 min. On heating, the casein micelles did increase in size. The size increased more rapidly during the early stages of heating and then tended to plateau after prolonged heating. Overall, the casein micelle size increased by about 30–35 nm on heating. The changes in casein micelle size were very similar for the four different skim milk samples, and were reproducible as long as the milk pH and heating conditions

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were carefully controlled. The polydispersity index (Fig. 1c) did not change during heating indicating that, within the limits of the instruments capability, the distribution was not broadening (or narrowing) on heating. Similarly, the scattering intensity of the particles did not increase as a consequence of heating indicating that there was not a marked increase in the mass of the particles as a consequence of the heating (results not shown).

In some milk samples, a small (~5 nm) decrease in casein micelle size at very short heating times preceded the gradual increase in size. This effect could be minimized (but not eliminated) by preheating the milk to 68 °C for a few minutes (results not shown). A similar decrease in micelle size has also been observed when measuring the turbidity (Jeurnink, 1992) and the viscosity (Jeurnink & De Kruif, 1993) of heated milk samples. It is unknown what causes this decrease in size. Jeurnink (1992) and Jeurnink & De Kruif (1993) suggested that this effect was due to the precipitation of calcium phosphate on to the casein micelles, which causes the micelles to shrink slightly. However, such precipitation would be expected to be reversible on subsequent cooling and storage unless the native colloidal calcium phosphate is solubilized in preference to the heat precipitated calcium phosphate.

To examine the effect of whey proteins on the changes in casein micelle size in heated milk, the majority of the whey proteins were removed from the skim milk samples using a combination of ultrafiltration and microfiltration techniques to produce a WPD milk. Heating the WPD milk (pH 6·55, 90 °C) caused small changes in casein micelle size, with an overall increase of only 5 nm after 45 min heating (Fig. 1c). As the WPD milk is not entirely free of whey proteins, the small increase in casein micelle size may be due to these residual whey proteins. Addition of β-lg to the WPD milk caused the casein micelle size to increase markedly on heating, with increases of about 18 and 35 nm when 0·15% and 0·3% β-lg, respectively, were added to the WPD milk. The addition of 0·3% β-lg to the WPD milk returned the β-lg concentration to a level similar to that observed in the original skim milk, and the size changes induced on heating were similar to those observed for this skim milk (Fig. 1b). As observed previously, no change in the polydispersity index was observed for all milk samples during heating (Fig. 1d).

These results clearly implicate the whey proteins, and β-lg in particular, as the major contributor to the changes to the casein micelle size during the heating of skim milk. The change in size on heating could be due to the uniform association of whey proteins with the casein micelle surface or, alternatively, through the partial aggregation of the whey protein coated casein micelles. It is unlikely that this size change is due to the formation of large whey protein aggregates as these would need to be either very large in size, or very numerous in order to cause the observed size increases. These types of aggregates are not seen in heated milk unless excess whey proteins are added (Beaulieu et al. 1999).

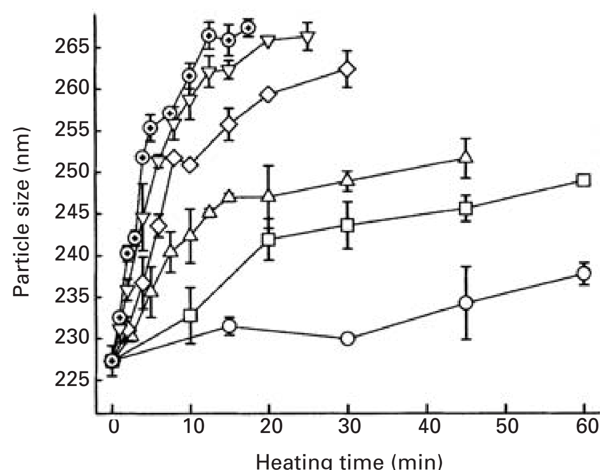

Fig. 2. Changes in casein micelle size for skim milk samples at pH 6·55 that were heated at 75–100 °C for various times. ○: 75 °C; □: 80 °C; △: 85 °C; ◇: 90 °C; ▽: 95 °C; ⊕: 100 °C. Error bars represent the standard deviation of duplicate measurements.

In more detailed experiments, reconstituted skim milk samples at pH 6·55 were heated at temperatures between 75 and 100 °C for times up to 60 min. The heated milk samples were analysed for casein micelle size, the level of whey protein denaturation and the level of whey proteins associated with the casein micelles. Figure 2 shows the changes in casein micelle size induced on heating the milk. The casein micelle size increased at all heating temperatures. At the lower temperatures (75–85 °C), the size increased relatively slowly throughout the heating time. At higher temperatures (90–100 °C), the size increased rapidly during the initial period of heating and tended to plateau on prolonged heating. As observed previously (Fig. 1a), the maximum increase in casein micelle size induced by the heat treatment was about 35 nm (Fig. 2).

The changes in the levels of denatured α-la, β-lg and the total denatured whey protein (defined as the denatured α-la and β-lg combined) in the unheated and heated milk samples were determined by native-PAGE (Fig. 3). For α-la, β-lg and the total denatured whey protein, the level of denatured protein increased on heating, and, as with the increase in micelle size, the degree of denaturation increased with both heating temperature and the duration of the heat treatment. At any defined heating condition, the levels of denaturation of α-la, β-lg and the total denatured whey protein were comparable to literature reports (Dannenberg & Kessler, 1988; Luf, 1988; Anema & McKenna, 1996; Oldfield et al. 1998*a*; Anema, 2000, 2001; Bikker et al. 2000).

The relationship between the degree of total whey protein denaturation and the change in the size of the casein micelles is shown in Fig. 4. There were only small changes in casein micelle size with protein denaturation levels up to about 80%, whereas the casein micelle size increased

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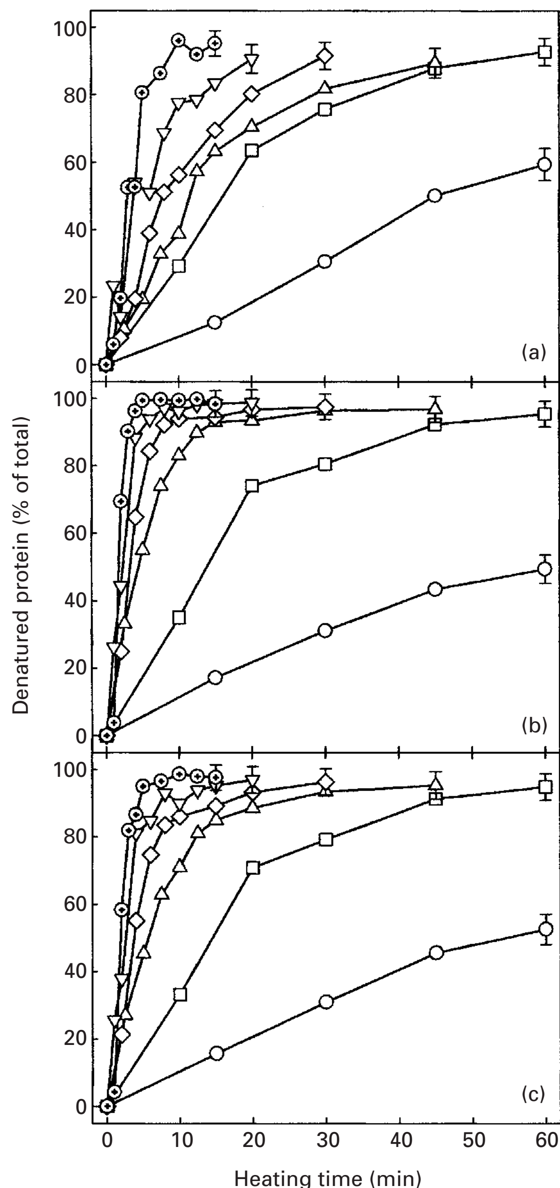

Fig. 3. Denaturation of whey proteins in skim milk samples at pH 6·55 that were heated at 75–100 °C for various times. (a): α-lactalbumin; (b): β-lactoglobulin; (c): total whey protein. ○: 75 °C; □: 80 °C; △: 85 °C; ◇: 90 °C; ▽: 95 °C; ⊕: 100 °C. Error bars, included on the last points only, represent the standard deviation of duplicate measurements.

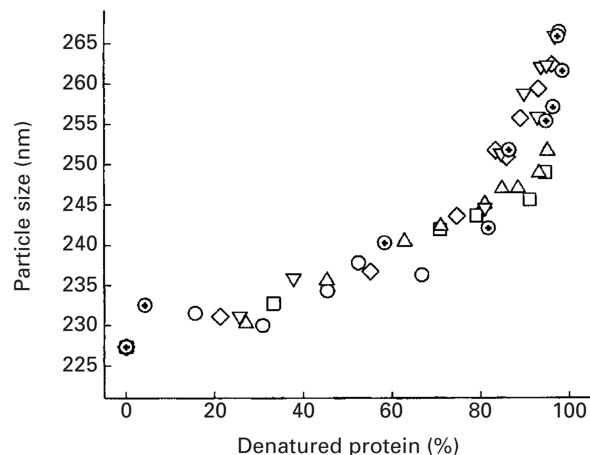

Fig. 4. Relationship between the level of total denatured whey protein and the particle size for skim milk samples at pH 6·55 that were heated at 75–100 °C for various times. ○: 75 °C; □: 80 °C; △: 85 °C; ◇: 90 °C; ▽: 95 °C; ⊕: 100 °C.

rapidly at denaturation levels between 80 and 100%. Although the general patterns for denaturation and the changes in casein micelle size were similar (compare Figs 2 and 3), it was evident that the rates of denaturation of the whey proteins were more rapid than the rates of change in the casein micelle size. These results indicate that the rates for the reactions involved in increasing the size of the casein micelles during heating are markedly slower than the rates for whey protein denaturation.

The association of the major whey proteins (α-la, β-lg and total whey protein) with the casein micelles was monitored by ultracentrifuging down the casein micelles and the associated whey proteins and analysing the supernatant for soluble whey protein content by SDS-PAGE (Fig. 5). As with the whey protein denaturation and the changes in the casein micelle size, the level of whey protein associated with the casein micelles increased with increasing temperature and with increasing holding time. At the lower temperatures (75–85 °C), the level of whey protein associated with the micelles increased relatively slowly throughout the heating time. At higher temperatures (90–100 °C), the level associated increased rapidly during the initial period of heating and tended to plateau on prolonged heating. It was evident that not all the denatured whey proteins were associating with the casein micelles. Under the conditions of this experiment, a maximum of about 70–80% of the denatured whey proteins associated with the micelles at any given heating temperature.

The relationship between the levels of denatured whey protein and the levels of whey protein associated with the micelles is shown in Fig. 6. For each whey protein fraction (α-la, β-lg and total whey protein), the association of denatured whey proteins with the micelles increased linearly with whey protein denaturation up to an association level of about 40%. Above this level, only small changes in denaturation level were observed yet considerably more whey protein continued to associate with the casein micelles (Fig. 6). In all cases, the level of denatured whey protein was markedly higher than the level that had associated with the casein micelles.

Figure 7a shows the relationship between the level of total whey protein associated with the casein micelles and the particle size, whereas Fig. 7b shows the relationship between the total whey protein associated with the casein micelles and the particle volume. In both cases there was a strong, almost linear relationship between the amount of whey protein associated with the casein micelles and the

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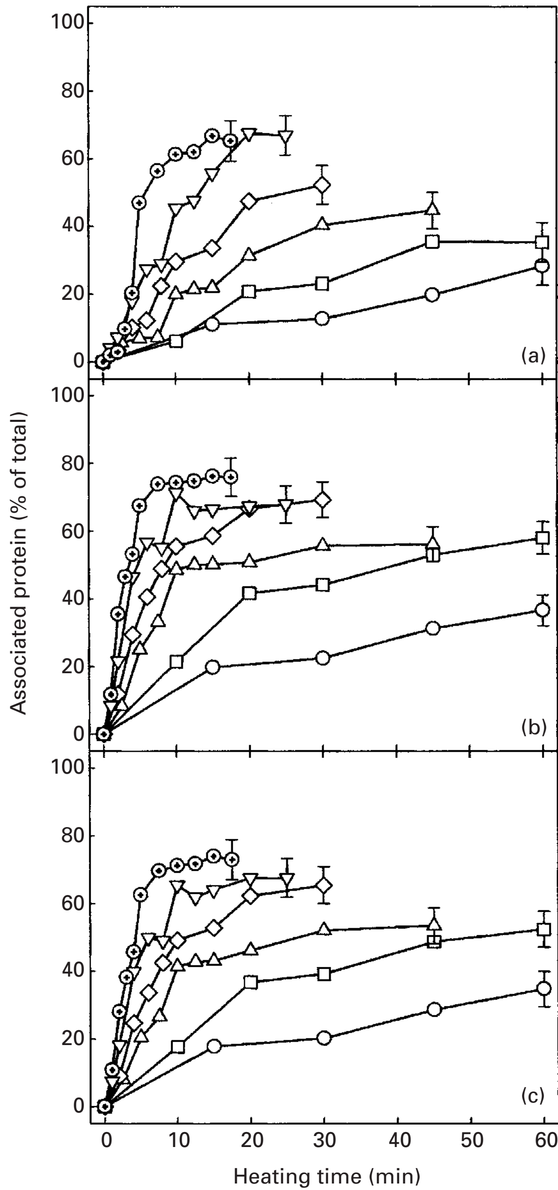

Fig. 5. Level of association of whey proteins with the casein micelles in skim milk samples at pH 6·55 that were heated at 75–100 °C for various times. (a): α-lactalbumin; (b): β-lactoglobulin; (c): total whey protein. ○: 75 °C; □: 80 °C; △: 85 °C; ◇: 90 °C; ▽: 95 °C; ⊕: 100 °C. Error bars, included on the last points only, represent the standard deviation of duplicate measurements.

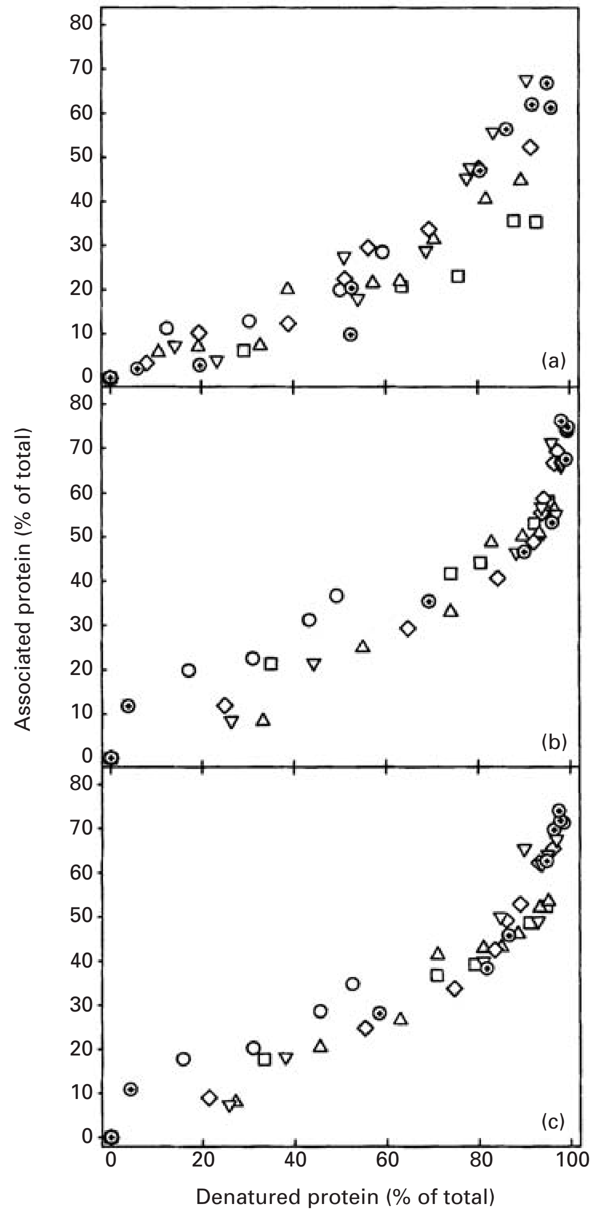

Fig. 6. Relationship between the level of denatured whey protein and the level of whey protein associated with the casein micelles for skim milk samples at pH 6·55 that were heated at 75–100 °C for various times. (a) α-lactalbumin; (b) β-lactoglobulin; (c) total whey protein. ○: 75 °C; □: 80 °C; △: 85 °C; ◇: 90 °C; ▽: 95 °C; ⊕: 100 °C.

casein micelle size/volume at all temperatures investigated. However, a slight deviation from linearity was observed at low levels of association, where smaller changes in size were observed relative to the level of whey protein that had associated with the micelles. This effect was probably due to the shrinkage of the casein micelles during the early stages of heating, as was described earlier. Although this effect could be minimized by preheating (68 °C/10 min), it could not be eliminated. If this effect is due to the shrinkage of the micelles, then the results in Fig. 7a suggest that the micelles may reduce in size by about 8 nm over and above the reduction induced by the preheating. A small reduction in the core micelle size will negate the increase in size as a consequence of the whey protein at the low levels of association and hence the size increase will not be as large as expected from the association level.

Figure 7b shows a line calculated by linear regression for the results above 15 % of associated whey proteins. The

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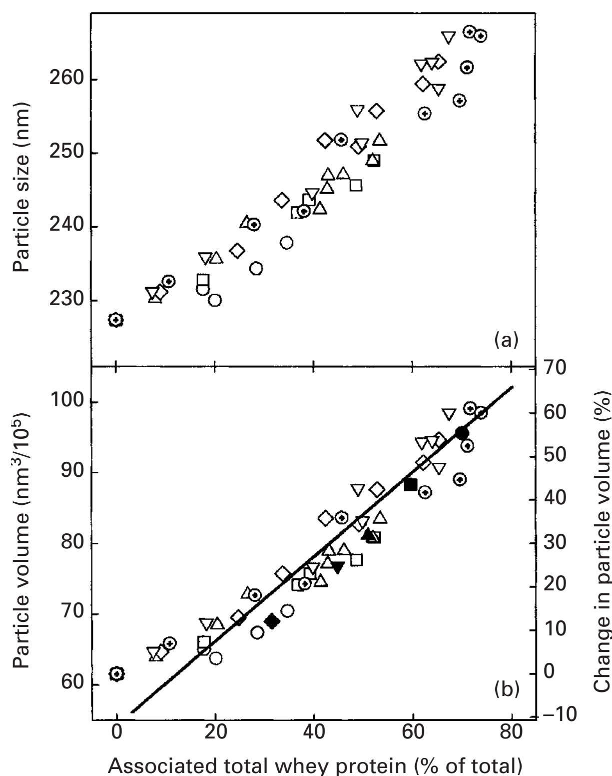

Fig. 7. Relationship between the level of associated total whey protein and (a) the particle size and (b) the particle volume for skim milk samples at pH 6·55 that were heated at 75–100 °C for various times. ○: 75 °C; □: 80 °C; △: 85 °C; ◇: 90 °C; ▽: 95 °C; ⊕: 100 °C. Figure (b) includes samples at various pH values that were heated at 90 °C for 30 min. ●: pH 6·5; ■: pH 6·55; ▲: pH 6·6; ▼: pH 6·65; ◆: pH 6·7.

residuals plot of this regression showed random scatter (results not shown) confirming that the relationship between the level of associated whey protein and the changes in particle volume were linear above this 15% association level. These results clearly indicate that the majority of the change in casein micelle size (Figs 1 and 2) are due to the association of the denatured whey proteins with the casein micelles. However, it is still not possible to determine whether the size changes observed are due exclusively to the association of denatured whey proteins with the casein micelle surface or due to the partial aggregation of casein micelles that occurs at the same time and is proportional to the levels of whey proteins that have associated with the casein micelles.

Oldfield et al. (1998*b*) suggested that the method of heating influenced the degree of association of denatured whey proteins with the casein micelles, and the composition of the associated protein. Under slow heating conditions (waterbath or indirect heating), the whey proteins denature over a longer time period and may exist as monomeric unfolded or small aggregated species for a significant time period. However, if the milk is heated rapidly (e.g. direct steam injection), all the whey proteins begin to denature in a short time period, allowing the whey proteins to form larger aggregated species. It was proposed that the smaller aggregates formed under the slow heating conditions may associate with the micelles more efficiently than the larger aggregates formed under rapid heating conditions. As a consequence, a greater level of whey proteins associates with the micelles under slow heating conditions than under rapid heating conditions (Oldfield et al. 1998*b*).

An alternative explanation for the differences in maximum association between the various studies may be related to the duration of the total heat treatment. In direct heating systems, the milk is rapidly heated to the required temperature, held for the desired time and then rapidly cooled. In contrast, under indirect heating conditions, the milk is heated relatively slowly to the desired temperature, held for the desired time and then cooled relatively slowly. Therefore, as the total heat load in indirectly heated milk is much higher than in directly heated milk, there is significantly more time available for the denatured whey proteins to associate with the micelles in the indirectly heated milk. This supposition is supported by the results in Fig. 6, which show that the rate of association of denatured whey protein with the casein micelles was markedly slower than the rate of whey protein denaturation. A substantial amount of the denatured α-la and β-lg associated with the micelles after more than 80% of the whey proteins had been denatured. This observation is in agreement with the results of Oldfield et al. (1998*b*) who, using a first order mathematical model to predict the level of association, showed that the rate constants for association of whey proteins with the micelles were lower than those for the thermal denaturation of the proteins.

In bovine casein micelles, it is generally believed that the κ-casein is located predominantly at the micelle surface as disulphide-linked polymeric species. The N-terminal *para*-κ-casein region is associated with the micelle core whereas the C-terminal glycomacropeptide region protrudes from the micelle surface as a flexible hair (Walstra, 1990). This arrangement accounts for the high stability of the casein micelles as the flexible hair provides both steric and electrostatic stabilization against aggregation. The cysteine residues (and therefore any disulphide bonds) are located on the *para*-κ-casein region of the κ-casein. For the denatured whey proteins to interact with the casein micelles via thiol-disulphide exchange reactions, they must first penetrate through the hairy surface of the casein micelle. The denatured whey proteins must then correctly orientate any free thiol groups with the disulphide bonds of κ-casein for the thiol-disulphide exchange reaction to be achieved. Considering the inherent nature of the reactions involved in the association process, it is not surprising that the rates of association are considerably slower that the rates of denaturation (Fig. 6).

The effect of pH on the association of whey proteins with the casein micelles was examined in preliminary experiments. Milk samples at between pH 6·5 and 6·7 were

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Table 1. Changes in the level of associated whey protein, particle size, volume, viscosity and the estimated volume fraction of casein micelles for skim milk samples at pH 6·5 to 6·7 that were heated at 90 °C for 30 min

pH	Associated whey protein† (%)	Particle size Change‡ (nm)	Particle volume Change§ (%)	Viscosity Change‖ (Nsm^{-2})	Volume fraction Change¶ (%)
6·50	70·10	31·5	55·3	0·23	53·8
6·55	59·70	25·5	43·6	0·20	44·7
6·60	51·00	19·3	31·9	0·17	32·1
6·65	44·80	15·3	24·8	0·13	22·6
6·70	31·50	7·8	12·1	0·08	11·1

† Determined by electrophoresis
‡ Determined by photon correlation spectroscopy
§ Calculated from particle size results using $4/3\pi r^3$, where r is the radius of the particles
‖ Determined using a capillary viscometer and calculated using the equation $\eta_1/\eta_2 = \rho_1/\rho_2 * t_1/t_2$; where η_1, ρ_1 and t_1 are the viscosity, density and flow times for the milk and η_2, ρ_2 and t_2 are the viscosity, density and flow times for the reference liquid (water)
¶ Calculated from viscosity using the Einstein equation: $\eta/\eta s = 1 + 2{\cdot}5\phi$ where η is the viscosity of the milk sample, ηs is the viscosity of milk serum and ϕ is the volume fraction of the particles

heated at 90 °C for 30 min. The samples were analysed for the levels of denatured whey protein (essentially 100% in all samples), the levels of whey protein associated with the casein micelles, the changes in particle size, and the changes in viscosity. The results are summarized in Table 1, along with calculations for volume change (based on the particle size analysis) and volume fraction change (based on the viscosity analysis). The associated whey protein/changes in particle volume results have been included in Fig. 7b (but not included in the regression analysis).

It is clearly evident that the association of whey proteins with the casein micelles is strongly dependent on milk pH. In this particular milk, the level of association decreased from about 70% at pH 6·5 to about 32% at pH 6·7. The change in particle size also decreased with increasing pH with the size increasing by about 32 nm when the milk was heated at pH 6·5, and only increasing by about 8 nm when the milk was at pH 6·7. These association levels and particle size results were entirely consistent with all other results of this study as the plot of calculated change in particle volume against association level essentially fell on the same line as all the previous results (Fig. 7b).

Smits & van Brouwershaven (1980) isolated casein micelles by centrifugation and resuspended them in a synthetic milk ultrafiltrate with added α-la and tritium labelled β-lg. The partition of β-lg was determined by measuring the radioactivity in serum and pellet phases. When these systems were heated at 90 °C a maximum of about 83% of the β-lg associated with the micelles at pH 5·8 and this decreased to about 76% at pH 6·3, 44% at pH 6·8 and 24% at pH 7·3. There was an increase in the level of β-lg in the interphase layer between pellet and supernatant, especially at pH 6·8 and pH 7·3. Corredig & Dalgleish (1996*a*) and Oldfield et al. (2000) also reported similar general trends on the effect of pH on the association behaviour. However, there was some variation in association levels at comparable pH. This may be related to the separation (centrifuging) techniques and/or the analysis techniques. For example, the present study used a centrifugal force of about 63000 ***g***, which was found to be sufficient to deposit the casein micelles. The study of Oldfield et al. (2000) used a substantially higher centrifugal force of 175000 ***g***; this may cause the deposition of some of the larger whey protein aggregates and this would increase the apparent level of association with the micelles.

If the particle size changes were due to the specific association of denatured whey proteins with the casein micelles then the results in Fig. 7 and Table 1 suggest that this association must substantially increase the volume fraction of the casein micelles. In this case, the associated whey proteins would need to be highly hydrated, or arranged at the micelle surface in such a way that a considerable quantity of serum is entrapped at the micelle surface. This would cause a measurable increase in the viscosity of the milk. Table 1 shows the measured change in viscosity for the heated milk samples at different pH. The viscosity was found to increase on heating and the increase was directly correlated the level of whey protein that had associated with the micelles, and therefore was also correlated with the particle size and the particle volume changes (results not shown). From the viscosity, it is possible to approximate the volume fraction using the Einstein equation (Jeurnink & De Kruif, 1993). The change in volume fraction is given in Table 1. Interestingly, the change in volume fraction, as approximated from the viscosity results, closely corresponded to the change in micelle volume, as calculated from the particle size results. This correspondence is consistent with the interpretation that the increase in particle size is due to the association of denatured whey with the casein micelles.

The question arises whether the association of whey proteins with the micelles could increase the casein micelle size by the level observed. This is difficult to determine without knowledge of the structure of the denatured whey proteins, the composition and structure of the casein micelle surface and the specific interactions involved in the association. The denatured whey proteins would probably be required to form linear polymers that attach to the

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micelle surface, extending the hairy layer and the hydration of the micelles. Griffin et al. (1993) indicated that the initial aggregation of β-lg in buffer systems did form linear rod-like particles but it is unknown whether these findings can be extrapolated to the aggregation in heated milk. If all available κ-casein is at the micelle surface then there are approximately two whey proteins available to interact with each κ-casein and this may not be sufficient to form polymers that can account for the size changes. However, if some of the κ-casein were unavailable for interaction, either because of their orientation at the surface or by being located in the micelle interior, then there would be more whey proteins available to interact with each κ-casein. In this case there may be sufficient whey protein to form linear polymers that could account for the observed size changes. Holt & Dalgleish (1986) indicated that as little as 10% of the total κ-casein was required to form the surface hairy layer.

It is still possible that an aggregation process could account for the change in particle size and also the increases in the viscosity and volume fraction. However, the aggregate structures would need to be of sufficient size and number to occlude the amount of water required to increase the viscosity and volume fraction by the level observed. Without further study it is not possible to conclude whether such voluminous aggregates could be formed in heated milk within the constraints of constant polydispersity and a limited (∼30 nm) increase in particle size.

Jeurnink & De Kruif (1993) measured the viscosity of milk as a function of heating time at pH 6·7; however, they did not measure the levels of whey protein that had associated with the micelles. The viscosity changes observed were very similar to those obtained in this study at a similar pH (where only about ∼30% of the whey proteins had associated with the micelles). Jeurnink & De Kruif (1993) concluded that the increase in viscosity was due primarily to an increase in volume fraction of the micelles, although some (non-permanent) changes in the weak attractions (or repulsions) between the micelles could account for some of the viscosity change. They state that they believe that there is no permanent clustering between the micelles in these heated samples i.e. the particle size and volume fraction did not increase due to permanent micellar aggregation. Similarly, Jeurnink (1992) concluded that the turbidity changes of heated milk at pH 6·7 were a consequence of the increase in volume fraction through the association of denatured whey proteins with the casein micelles, and not due a change in the interaction between casein micelles.

This study has demonstrated that the casein micelles in milk increase in size as a consequence of heat treatments. Both whey protein depletion experiments (Fig. 1b) and the relationship between the micelle size and the level of associated protein (Fig. 7) indicate that the association of denatured whey proteins with the casein micelle surface influences the micelle size changes. The rate of association of the whey proteins with the casein micelles is considerably slower that the rate of whey protein denaturation (Fig. 6). Further studies are required to determine with certainty whether the size changes are due explicitly to the association of denatured whey proteins with the casein micelle surface or due to the partial aggregation of casein micelles that occurs at the same time and is proportional to the levels of whey proteins that have associated with the casein micelles.

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