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pH-Dependent behaviour of soluble protein aggregates formed during heat-treatment of milk at pH 6·5 or 7·2

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The pH-dependent behaviour of soluble protein aggregates produced by the pre-heating of reconstituted skim milk at 90 °C for 10 min was studied, in order to understand the role of these aggregates in acid gelation of heated milk. The following milk samples were prepared: (1) control (unheated reconstituted milk, pH 6·5); (2) milk heat-treated at pH 6·5 (mHtd6·5) and (3) milk heat-treated at pH 7·2 (mHtd7·2). They were centrifuged and the supernatants (SPNT 1) pH-adjusted to yield a series of pH values ranging from 6·5 or 7·2 to 4·6 using HCl at 20 °C or GDL at 20 and 38 °C. pH-Adjusted SPNTs 1 were re-centrifuged. The resulting supernatants (SPNTs 2) were analysed by OD (at 600 and 280 nm) and SDS-PAGE in order to characterise proteins still soluble as a function of pH. Particle size in SPNTs 1 was analysed by Steric Exclusion Chromatography. The OD600 nm revealed that during acidification soluble casein in both control and heat-treated samples exhibits variations in its optical properties or size as previously shown with micellar casein. In heat-treated samples, soluble casein and heat-induced covalent soluble aggregates precipitate at the same pH value. A progressive acidification of the soluble phase did not separate them. Increasing the temperature of acidification from 20 to 38 °C resulted in an increase in the precipitation pH of the proteins. However choice of acidifier did not have a significant effect on OD profiles. The soluble covalent aggregates from mHtd7·2 were smaller, more numerous, and had a higher content of κ-casein than mHtd6·5. Both types of aggregates began to precipitate at the same pH value but precipitation occurred over a narrower pH-range for soluble aggregates prepared from mHtd7·2. This may explain the higher gelation pH of mHtd7·2 compared with mHtd6·5.

Keywords: Milk protein aggregates, pH, heat treatment, acidification.

Abbreviations:

SPNTs 1: supernatants from the centrifugation of milk
SPNTs 2: supernatants from the centrifugation of pH-adjusted SPNTs 1
MHtd6·5: milk heat-treated at pH 6·5
MHtd7·2: milk heat-treated at pH 7·2

Yoghurt manufacture requires a severe heat-treatment of milk that destroys pathogenic bacteria and also improves the texture of the final product. Indeed, acid gels made from heat-treated milk show lower syneresis and higher firmness than acid gels made with raw milk. It has also been shown that whey proteins such as α-lactalbumin, β-lactoglobulin and bovine serum albumin denature at temperatures above 70 °C and interact both with each other and with κ-casein to form protein aggregates (Smits & van Brouwershaven, 1980; Dannenberg & Kessler, 1988a; Dalgleish, 1990; Corredig & Dalgleish, 1999; Anema & Li, 2003a). These aggregates have been shown to be responsible for the improved rheological properties and increased gelation pH of acid milk gels prepared from heated milk compared with raw milk (Dannenberg & Kessler, 1988a, b; Lucey et al. 1997, 1998b, 1999; Vasbinder et al. 2001).

Heat-induced milk protein aggregates are located at the micellar surface (micellar aggregates) and in the aqueous phase (soluble aggregates). The changes in rheological properties of acid gels prepared from heat-treated milk

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were first attributed to micellar aggregates only (Lucey et al. 1998a, 1999). Yet, it has recently been shown that soluble aggregates can also contribute to these changes and may even be more efficient than micellar aggregates in increasing the firmness of acid gels (Vasbinder & de Kruif, 2003; Guyomarc'h et al. 2003a; Anema et al. 2004).

The adjustment of milk pH before heat treatment affects the relative proportions of the soluble and micellar aggregates formed. When the pH is raised above pH 6·7, a higher proportion of soluble aggregates and a lower proportion of micellar aggregates are induced (Singh & Fox, 1985, 1987; Singh & Creamer, 1991; Nieuwenhuijse et al. 1991; Anema & Klostermeyer, 1997; Vasbinder & de Kruif, 2003) and the resulting gel is firmer (Anema et al. 2004).

The aim of this study was to evaluate the pH-dependent solubility of heat-induced soluble protein aggregates, i.e. variations in their solubility during acidification, in order to get a further understanding of their role in acid gelation of heat-treated milk. A second objective involved the determination of differences in composition and pH-dependant behaviour of soluble protein aggregates formed in heat-treated milk at its natural pH or at pH 7·2. The effects of temperature during acidification of the aggregate suspension and of acidifying agent on the pH-dependent behaviour of soluble aggregates were also studied.

Materials and Methods

Chemicals

Hydrochloric acid, Tris-(hydroxymethyl) aminomethane (Tris), glucono-δ-lactone (GDL) and sodium hydroxide were purchased from VWR international Merck Eurolab SA (F-94126 Fontenay-sous-bois, France). β-Casein was supplied by CHARIS Ltd (Ayr, UK). Urea and citrate sodium were from Panreac Quimica SA (08110 Barcelona, Spain). Sodium Dodecyl Sulphate (SDS) was from Carlo Erba (20100 Milano, Italy) and glycerol and dithiothreitol (DTT) were from Sigma-Aldrich (St Louis, MO 63178, USA). Acrylamide was purchased from BIO-RAD (CA 94547, Hercules, USA) and Coomassie blue was from SERVA Electrophoresis GmbH (69115 Heidelberg, Germany). Sodium azide was from Fisher Scientific Labosi (F-78996 Elancourt, France).

Reconstitution of milk, adjustment of pH and heat-treatment

Skim milk was reconstituted from ultra low heat skim milk powder, prepared as described in Schuck et al. (1994), to a final solid content of 140 $g.kg^{-1}$ using distilled water. In this milk powder, the protein denaturation is very low (WPNI = 11) due to microbial purification by microfiltration prior to evaporation, during which the highest temperature value reached is 52–53 °C. The native quality of the proteins allowed rapid (stirred 1 h at ambient temperature) re-equilibration of this powder in water. For mHtd7·2, adjustment of pH was performed using 5 M-NaOH prior to heat-treatment. Neither mHtd6·5 nor the control sample were pH-adjusted. Milk samples (2 l), except control sample, were heated as described by Laligant et al. (2003). Milk temperature was maintained for 10 min at 90 °C. Including the time taken for temperature increase, holding and decrease, the equivalent time of the overall treatment at 90 °C was about 16 min for whey protein denaturation. After heat treatment, 0·2 g sodium azide/kg milk was added to milk.

Centrifugation of milk

The soluble and micellar phases were separated by centrifugation of about 200 ml of the milk at 19 200 ***g*** for 4 h at 20 °C in a Beckman J2-21 centrifuge (Beckman Coulter France SA, 95942 Roissy CDG, France) equipped with a JA-14 swinging rotor. The supernatant (SPNT) was collected by gently decanting from the tubes and was designated SPNT 1.

Size Exclusion Chromatography (SEC)

Particle size in SPNT 1 of control milk, mHtd6·5 and mHtd7·2 were compared using SEC. The separations were performed at ambient temperature by injection of 500 µl SPNT 1 diluted five times onto a Sephacryl S-500 Hi-Prep 16/90 column Amersham Biosciences UK Ltd. (Buckinghamshire, UK). The dilution and elution buffer used was 150 mM-NaCl, adjusted to pH 7. The diluted SPNT 1 samples were filtered through 1·2 µm filters before injection (Pall Gelman Laboratory, MI48109, Ann Arbour, USA). Flow rate of the elution buffer was 0·5 $ml.min^{-1}$. Absorbance of the eluate was monitored at 280 nm with a LKB 2141 detector (LKB Produkter AB, Broma, Sweden). The samples were injected manually. Flow rate of the mobile phase was regulated by a P-500 pump (Amersham Bioscience). The system was driven by a Liquid Chromatography Controller LCC-500 (Amersham Bioscience). Data were collected with a PE Nelson 900 series interface from GenTech Scientific Inc (NY 14009, Arcade, USA). The peaks were identified as described in Guyomarc'h et al. (2003b).

Acidification of the SPNT 1

SPNT 1 of all three milks was divided into 30 aliquots of 30 g in capped glass vials. These were acidified by addition of increasing quantities of GDL and holding at 20 or 38 °C (ten aliquots at each temperature) for 20 h. The remaining ten aliquots were cooled to 4 °C (to avoid local protein precipitation) prior to pH adjustment with 1 M-HCl and were held at 20 °C for 20 h. The final pH values, measured after the 20 ± 1 h incubation, ranged from the initial value (6·5 or 7·1) to 4·6. Circa 15 ml of all samples

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were re-centrifuged at 95 300 ***g*** for 12 min at 20 °C in a Beckman L8-55 centrifuge equipped with a fixed angle rotor 50 TI in order to separate the insoluble and soluble particles. The SPNTs were collected by gently decanting from the tubes and designated SPNTs 2.

Optical Density of SPNTs 2 at 280 nm ($OD_{280\ nm}$) and 600 nm ($OD_{600\ nm}$)

Optical Density of samples was measured at 280 nm and 600 nm in 1 cm path length cell using a KONTRON UVIKON 922 spectrophotometer (Buckinghamshire, UK). SPNTs 2 samples were subjected to dissociating and reducing conditions then total proteins present as a function of pH were measured by OD at 280 nm. Conditions of dissociation and reduction were as follows: SPNTs 2 were diluted 10-fold in a buffer containing 0·1 M-Tris, 8 M-urea, 13 g sodium citrate/kg, 10 g SDS/kg, 1 mM-DTT, pH 8. The SPNTs 2 were then incubated at 37 °C for 1 h followed by dilution 4 times in milli-Q water. This dissociation of particles prevented from light scattering within the sample. The blank reference was prepared as for samples using water rather than SPNT 2. A typical standard deviation was calculated on one unacidified SPNT 2, separated from mHtd6·5, by repeating dilution and measurement five times.

Monitoring of particles present in SPNTs 2 as a function of pH was performed by measurement of OD at 600 nm. The reference sample used was water. Standard deviation was calculated from 4 repetitions of the measurement. Unavoidable small time delays occurred in sample preparation before OD measurements were taken. To ensure that the $OD_{600\ nm}$ of samples remained constant over time, samples were assayed at regular intervals from the centrifugation time up to 5 h.

Electrophoresis SDS-polyacrylamide of the SPNTs 2 and laser densitometry

SDS PAGE was carried out according to Laemmli (1970) in a mini-protean II BIO-RAD system (Hercules, CA 94547, USA). HCl acidified SPNTs 2, at 20 °C, were diluted 1·5 time with water. They were then diluted twice with dissociating buffer and also with dissociating and reducing buffer. The dissociating buffer was composed of 0·125 M-Tris, 40 g SDS/kg, 200 ml glycerol/l, 0·4 ml bromophenol blue/l, pH 6·8. The reducing buffer had the same composition but with 0·6 M-dithiothreitol added. All samples were held for 1 h at 37 °C to ensure complete dissociation and reduction. An unheated reconstituted milk sample, containing 8·75 $g.kg^{-1}$ dry matter, was prepared in the same way with dissociating and reducing buffer and used as a standard. Samples (5 µl) were loaded on the gel. SDS-PAGE gels were prepared at a concentration of 14% and 4% acrylamide for the running gel and the stacking gel, respectively. The migration buffer consisted of 25 mM-Tris, 192 mM-glycine, 1 g SDS/l. The gels were run with a constant voltage of 200 V for approximately 45 min. The proteins were fixed in the gels with 7% (v/v) acetic acid, 40% (v/v) ethanol (ethanol–acetic acid) for 10 min and stained overnight (0·25 ml R250 Coomassie Blue/l ethanol–acetic acid). Following destaining, SDS-PAGE gels were scanned using an ImageScanner II (Amersham Biosciences) and the integrated intensities of the protein bands were determined using the ImageQuant TL software (Amersham Biosciences).

Protein bands were identified as described in Anema & Li (2000). For calculation of the protein quantities in SPNTs 2, the intensity of each protein band was divided by the intensity of the β-lg band of the unheated standard loaded on the same gel to normalize the effect of differences in staining between gels and therefore allow comparison.

Quantification of individual proteins involved in covalent aggregates formed through disulphide bridges was obtained by comparing intensities of protein bands in reduced and unreduced samples (unreduced aggregates being trapped in the stacking gel). To quantify the aggregation of κ-casein a method to correct for the presence of co-eluting β-casein was tested. The staining intensity of a sample of pure β-casein was measured under dissociating and dissociating-reducing conditions and shown to be identical. The extent of κ-casein association through disulphide bridges was therefore calculated as the intensity of reduced [κ-casein+β-casein] minus the intensity of unreduced [κ-casein+β-casein]. Only SPNTs 2 acidified with HCl at 20 °C were analysed by SDS-PAGE. For each SPNTs 2, three gels were prepared and analysed for reducing and non-reducing conditions. Standard deviations were calculated from these 3 repetitions.

A preliminary experiment was performed in order to measure the differences (weight, dry matter) of pellets from milk samples centrifuged at pH 6·5 or 7·2, since solvation at different pH may affect apparent protein concentration. After heat-treatment, mHtd6·5 and mHtd7·2 were centrifuged before and after pH adjustment. Correction factors of 1·009 (weight) or 1·06 (dry matter) were established in order to obtain equivalence of protein concentration between mHtd6·5 SPNT1 and mHtd7·2 SPNT1 samples.

Results and Discussion

Size Exclusion Chromatography

The peak of soluble protein aggregates from mHtd7·2 eluted at a higher retention time than that from mHtd6·5 (Fig. 1). This clearly indicated that the soluble protein aggregates were larger in mHtd6·5 than in mHtd7·2.

Optical Density of SPNTs 2: $OD_{280\ nm}$ and $OD_{600\ nm}$

The $OD_{280\ nm}$ of the SPNTs 2 following denaturation and reduction revealed the quantity of proteins still soluble as a function of pH (Fig. 2a). At initial pH values (6·5 or 7·1),

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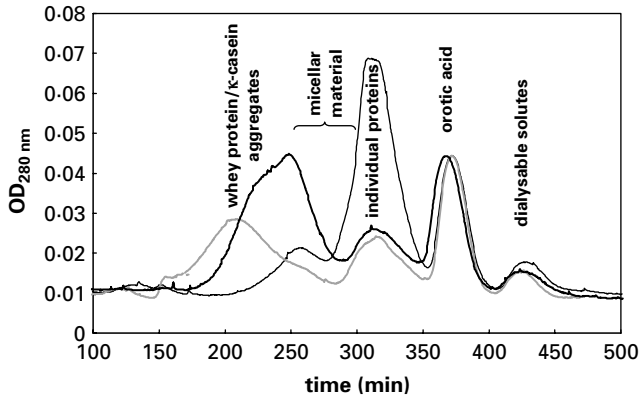

Fig. 1. Elution profiles of SPNT 1 from raw milk (—), mHt6·5 (–) and mHtd7·2 (—) obtained by SEC on a Sephacryl S-500 HR Hi-Prep 16/90 column in native conditions.

the protein concentration in SPNTs 2 was in the following order: mHtd7·2>control>mHtd6·5. From the initial pH to ~pH 5·5, the $OD_{280\ nm}$ remained fairly constant. On further decrease in pH, the $OD_{280\ nm}$ decreased and finally dropped in heated milk SPNTs 2 samples, whereas it decreased slowly and remained elevated at pH 4·6 in control milk SPNTs 2 samples due to the presence of native whey proteins. Protein precipitation occurred over a narrower pH range in soluble phase of mHtd7·2 (pH 5·4 to 5·1) than mHtd6·5 (pH 5·4 to 4·9).

$OD_{600\ nm}$ is related to the number, size and optical properties of particles. For SPNTs 2 prepared from each milk type, $OD_{600\ nm}$ decreased with pH (Fig. 2b), apart from an increase (pH 5·5–5·0), before a sharp decrease at the lowest pH values. The final sharp decrease indicated either dissociation or precipitation of all soluble particles. Fig. 2a indicates the main cause was precipitation. The three profiles followed similar trends; however there was a marked shift in position of the profiles that was clearly dependent on the treatment of milk. The $OD_{600\ nm}$ increase corresponded to the beginning of protein precipitation (Fig. 2a). It proved not to be due to a post-aggregation of particles after centrifugation as no changes in the $OD_{600\ nm}$ of samples were observed from the centrifugation time up to 5 h (data not shown). This increase was observed for the control SPNTs, showing that it was not specific to heat-induced aggregates but rather to soluble caseins. Guyomarc'h et al. (2003b) reported that the soluble casein, in the soluble phase from heat-treated milk prepared in a manner similar to this study, was in the form of particles. The soluble casein particles may have a similar water-binding capacity as micelles during acidification as described by Snoeren et al. (1984) and Van Hooydonk et al. (1986) who have shown the same profile of solvatation as a function of pH. The number, size or optical properties of particles explained the variations in $OD_{600\ nm}$.

Acidification temperature had a significant effect on the precipitation pHs of both particles and proteins which

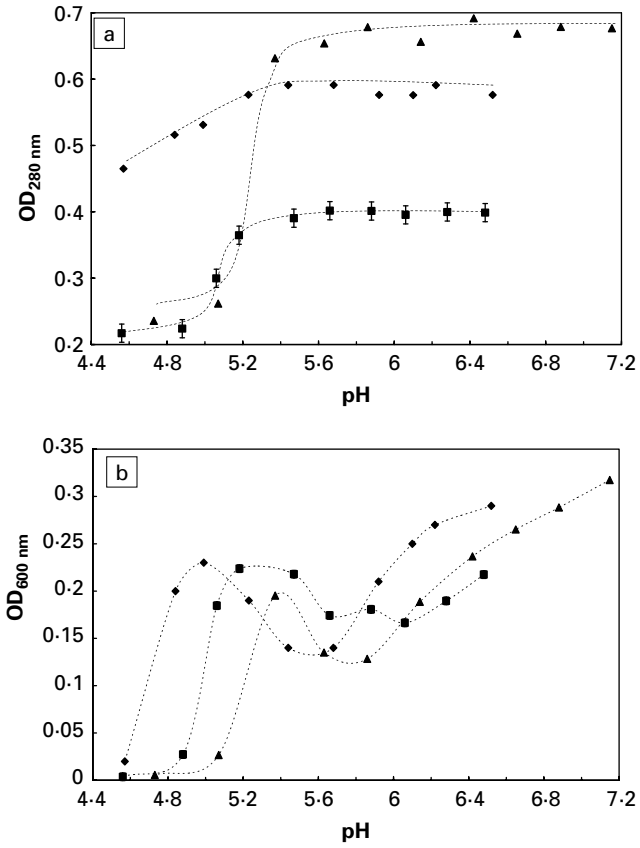

Fig. 2. Relation between the pH and the $OD_{280\ nm}$ (a) and $OD_{600\ nm}$ (b) of the SPNTs 2 isolated from raw milk (◆), mHtd6·5 (■), and mHtd7·2 (▲), acidified with HCl at 20 °C. Error bars represent the standard deviation of replicate measurements.

increased on raising temperature from 20 to 38 °C (results not shown). Acidification agent had no significant effect on the profiles of $OD_{600\ nm}$ or $OD_{280\ nm}$, at least when acidification was performed at 20 °C (results not shown).

Electrophoresis SDS-polyacrylamide of the SPNTs 2 and laser densitometry

Protein bands were more intense in reduced SPNTs 2 (Fig. 3b & d) than in unreduced SPNTs 2 from heat-treated milks (Fig. 3a & c) due to non-reduced aggregates prevented from migrating. As expected, in unheated samples, there were no differences between reduced and unreduced SPNTs 2 (not shown). For unreduced samples from mHtd6·5 (Fig. 3a), on decreasing pH, the intensity of the unpolymerised casein bands (β-casein mainly, a little αs-casein and very little κ-casein) faded, indicating their precipitation and subsequent sedimentation during centrifugation. Intensity of the whey protein bands also decreased with pH, albeit to a lesser extent, reflecting that part of the whey proteins were denatured but not polymerised. The intensity of protein bands was higher

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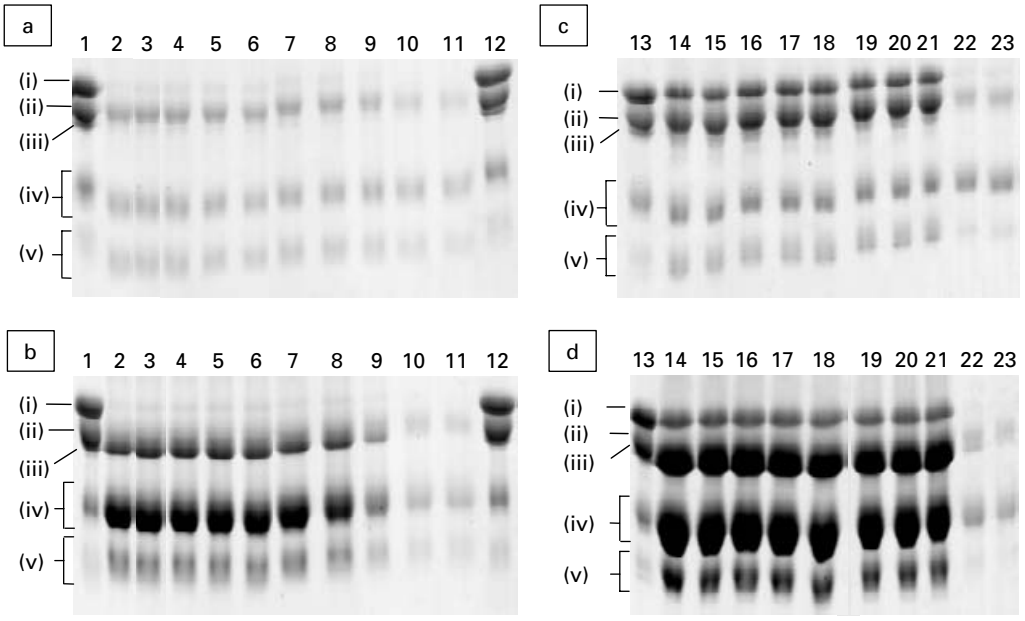

Fig. 3. SDS-PAGE patterns of SPNTs 2, isolated from (a, b) mHtd6·5 and (c, d) mHtd7·2, acidified with HCl at 20 °C, obtained in (a, c) dissociating conditions and in (b, d) dissociating and reducing conditions. Lanes 1, 12 and 13: standard milk after reduction; lanes 2–11: SPNTs 2 at pH 6·49, 6·28, 6·06, 5·88, 5·66, 5·47, 5·18, 5·06, 4·88 and 4·56; lanes 14–23: SPNTs 2 at pH 7·1, 6·88, 6·65, 6·42, 6·14, 5·86, 5·63, 5·37, 5·07, and 4·73. (i) αs-casein, (ii) β-casein, (iii) κ-casein, (iv) β-lactoglobulin and (v) α-lactalbumin.

in reduced samples (Fig. 3b) and decreased with pH indicating the precipitation of unpolymerised casein, unpolymerised denatured whey proteins and soluble heat-induced covalent protein aggregates. The same observations were made for gels containing SPNTs 2 from mHtd7·2. However the intensities of all protein bands were higher in these gels than in gels containing SPNTs 2 from mHtd6·5, in reduced conditions (Fig. 3d) and unreduced conditions (Fig. 3c & Table 1). The differences were not due to differences in hydration of caseins at pH 7·2 and pH 6·5 because increasing intensities at pH 6·5 by a factor 1·06 or 1·009 (see material and methods section) did not match the intensities at pH 7·2.

The higher concentration of unpolymerised β-lg and α-la in the soluble phase of mHtd7·2 than mHtd6·5 (Table 1) may be due to a higher concentration of denatured but non-covalently aggregated whey proteins. This agrees with the decrease in intensities of whey protein bands in heat-treated unreduced samples as pH decreased and Anema et al. (2004) reported that the rate of denaturation increases with the pH adjusted before heat treatment.

The intensities of the κ-casein bands in non-reduced samples were low, indicating little residual unpolymerised κ-casein, though the level was higher in non-reduced mHtd7·2 samples. The intensities of all casein bands in reduced samples were much higher in samples from mHtd7·2 than mHtd6·5 as reported in the literature (Singh & Fox, 1985, 1986; Anema & Klostermeyer, 1997; Anema & Li, 2000).

The covalent heat-induced protein aggregates, formed during heat-treatment of milk at pH 6·5 or 7·2, were composed of β-lg, α-la and κ-casein (Fig. 4). Small quantities of αs-casein were also detected. More covalent protein aggregates were present in the SPNT 2 isolated from mHtd7·2 than from mHtd6·5 as described in the literature (Smits & van Brouwershaven, 1980; Singh & Fox, 1985, 1986; Anema & Klostermeyer, 1997; Anema & Li, 2003a, b; Vasbinder & de Kruif, 2003; Anema et al. 2004). The onset of precipitation of covalent aggregates from mHtd6·5 and mHtd7·2 occurred at the same pH value of ~5·5, as already shown by $OD_{280\ nm}$. Nevertheless, precipitation of soluble aggregates from MHtd7·2 occurred over a narrower range of pH and aggregates formed by heat-treatment at pH 7·2 can also be more numerous (smaller in size, as shown by SEC, and involving more proteins) than those formed at pH 6·5. This may explain the higher gelation pH of milk heat-treated at pH 7·1 or 6·9 compared with milk heat-treated at pH 6·5 as previously

Table 1. Quantities of unpolymerised proteins at pH values higher than the onset of precipitation in unreduced SPNTs 2 from milk heated treated at pH 6·5 (mHtd6·5) and pH 7·2 (mHtd7·2). The quantities are expressed in intensities (arbitrary units) relative to the intensity of the β-lg band from standard milk sample in the same gel

Protein	mHtd6·5	mHtd7·2
αs-casein	$17^{a} \pm 4$	$243^{b} \pm 26$
β+κ-casein	$118^{a} \pm 11$	$452^{b} \pm 105$
β-lg	$101^{a} \pm 11$	$174^{b} \pm 21$
α-la	$63^{a} \pm 4$	$87^{b} \pm 13$

^{a,b}Means with different superscripts differ ($P<0·05$)

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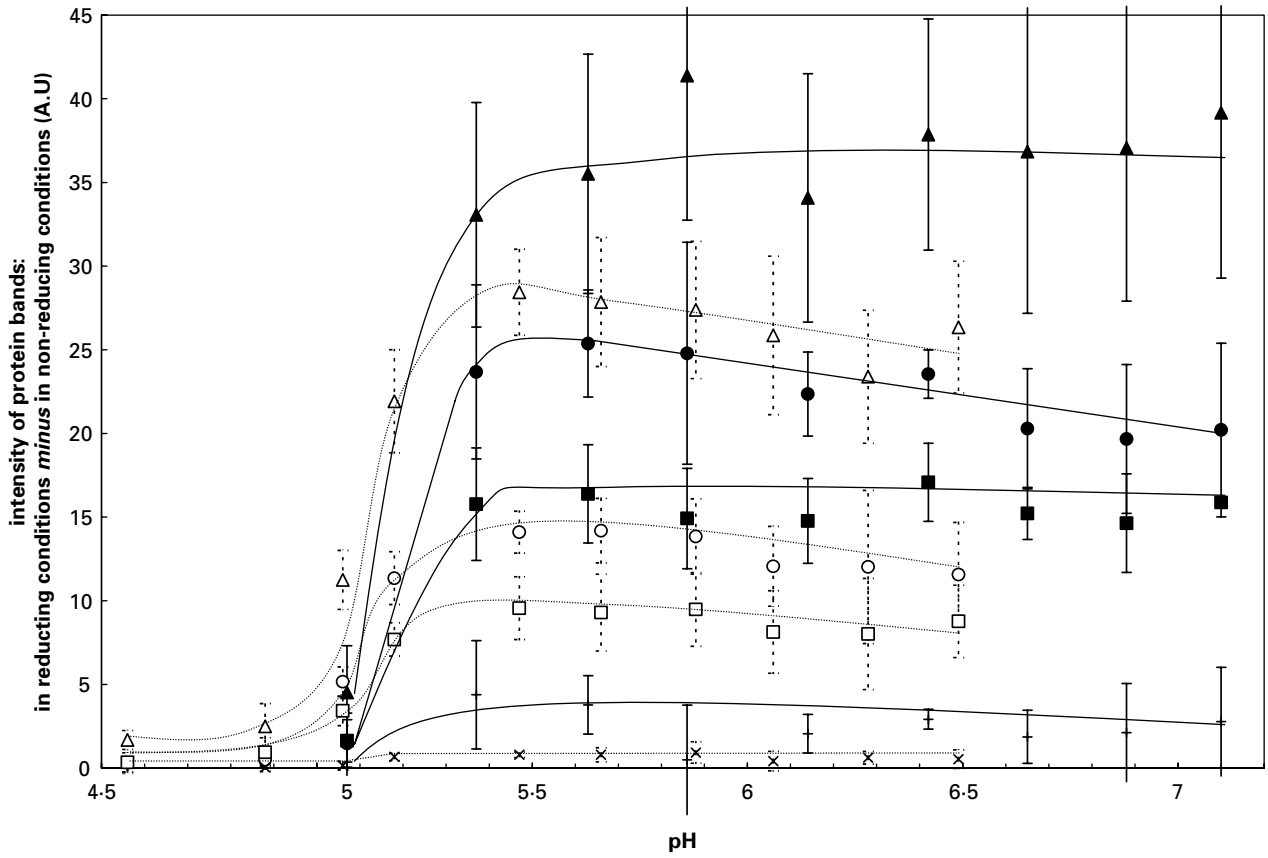

Fig. 4. Quantities of covalently aggregated proteins in SPNTs 2 as a function of pH. The quantity is expressed in intensity (arbitrary units) and calculated by the subtraction of the integrated intensities of each protein bands in the unreduced samples to the integrated intensities of the same protein bands in reduced samples. κ-casein (○, ●), β-lactoglobulin (△, ▲), α-lactalbumin (□, ■) and αs-casein (×, +). Empty symbols, x and dotted lines refer to SPNTs 2 from mHtd6·5 and plain symbols, + and continuous lines refer to SPNTs 2 from mHtd7·2. Error bars represent the standard deviation of replicate measurements.

observed (Vasbinder & de Kruif, 2003; Anema et al. 2004). However, the behaviour of the soluble phase during acidification is obviously different from that of the milk (dissociation of micellar casein, solubilization of calcium phosphate, increase in ionic strength, interactions between whey proteins and micelles).

Soluble aggregates (reduced – non-reduced) and unpolymerised caseins (non-reduced) began to precipitate at the same pH value (result not shown). Heat-induced protein aggregates were thought to have higher isoelectric pH than casein micelles (Lucey et al. 1998a; Vasbinder et al. 2001; Guyomarc'h et al. 2003a), and hence might also have a higher isoelectric pH than soluble casein particles. Hence, during acidification, it was expected that these protein aggregates would precipitate at higher pH value than soluble caseins. However, it was recently shown that the soluble heat-induced protein aggregates isolated from heated skim milk had an apparent isoelectric point of 4·7 (Jean et al. in press), which explains why unpolymerised casein and covalent aggregates co-precipitated.

The unpolymerised caseins in raw milk SPNTs 2 precipitate at the same pH as in heat-treated samples but over a larger pH range (result not shown). The precipitation of heat-induced aggregates may accelerate the precipitation of unpolymerised caseins probably due to some form of unknown association. A further explanation may be due to depletion of κ-casein in the soluble casein of heat-treated samples. κ-casein has probably a stabilising role in soluble aggregates, as it has in micelles. Despite large standard errors, the β-lg : α-la ratios were higher over all pH values tested in covalent aggregates from mHtd6·5 than mHtd7·2 (Fig. 5a). Soluble covalent aggregates from mHtd7·2 proved to be richer in κ-casein than those from mHtd6·5 (Fig. 5b). This result is consistent with the previous studies that have reported an increase in the dissociation of κ-casein from micelles when heat-treatment pH is increased above pH 6·5 (Singh & Fox, 1986; Anema & Klostermeyer, 1997; Anema & Li, 2000).

Aggregates from mHtd7·2 were richer in κ-casein, smaller and precipitated more rapidly than natural aggregates, which may indicate that aggregated κ-casein does not have the same stabilising role as in micelles. However it could be postulated that the proportion of κ-casein may be inversely proportional to the size of

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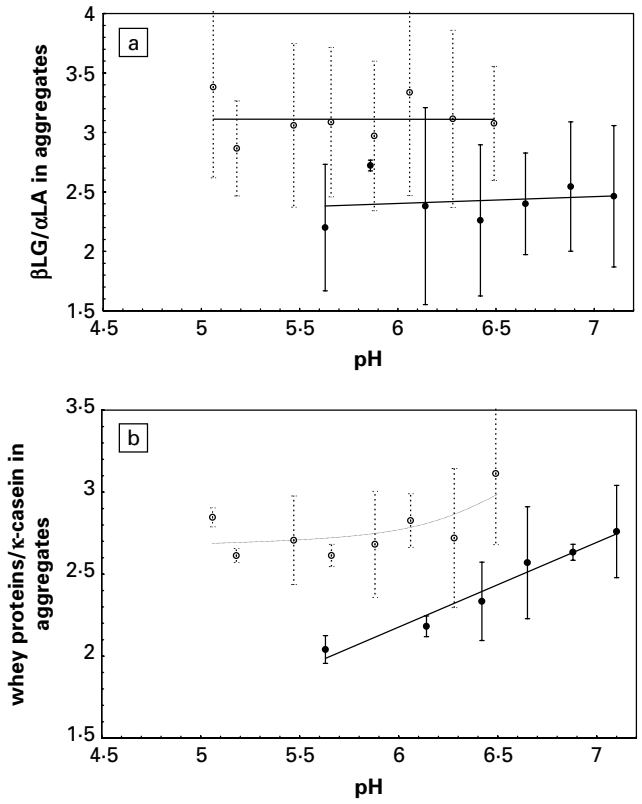

Fig. 5. Effect of pH of heat-treatment and of pH of SPNTs 2 on the protein composition of soluble covalent heat-induced protein aggregates. Ratios of (a) β-lactoglobulin : α-lactalbumin, (b) whey protein : κ-casein. Empty symbols or dotted lines and plain symbols or continuous lines refer to SPNTs from mHtd6·5 and mHtd7·2, respectively. Error bars represent the standard deviation of replicate measurements.

aggregates as previously reported for micelles (Donnelly et al. 1984; Dalgleish et al. 1989; Zbikowska, 1992).

The whey protein : κ-casein ratios were constant over all pH values before the onset of precipitation in soluble aggregates from mHtd6·5 but seemed to decrease with pH in aggregates from mHtd7·2 (Fig. 5b). At the same pH values, the protein concentration estimated from $OD_{280\ nm}$ was constant, therefore the decrease cannot be attributed to the presence of covalent aggregates of varying compositions that precipitate at higher pH values when containing lower κ-casein content. The quantity of covalently linked proteins increased in covalent heat-induced aggregates with decreasing pH – except for β-lg and α-la in aggregates from mHtd7·2 (Fig. 4). This increase may be explained by the formation of disulphide bridges during acidification as happens during acid gelation of heat-treated milk (Alting et al. 2000; Vasbinder et al. 2003). This suggests an incorporation of only κ-casein in aggregates from mHtd7·2 during acidification. It is known that more κ-casein dissociates from the micelle after heat-treatment at pH 7·2.

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