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Original article

Changes in the acid gelation of skim milk as affected by heat-treatment and alkaline pH conditions

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Abstract – In an attempt to improve the acid gelation properties of heated milk, or to reduce the heat load necessary to obtain significant acid gelation properties, skim milk adjusted at pH values ranging from 6.7 to 10.5 was heat-treated for 10 min at temperatures ranging from 25 to 95 °C then neutralised to pH 6.7. Protein composition of the serum and micellar phases of milk and separation of the protein particles present in the serum phase of milk indicated that formation of micelle-bound heat-induced whey protein/κ-casein aggregates was prevented from pH 7.5 upward, to the benefit of serum aggregates. Aggregates were formed in milk at pH 9.5 and 10.5 even at mild heating temperature (65–75 °C), while temperatures of 85 or 95 °C were necessary at lower pH values. Size of the aggregates decreased as pH of heat-treatment increased. Quality of the results was however reduced because of the extensive dissociation of caseins on alkaline treatment and of side mechanisms like Maillard reaction at high pH and temperature. However, some relationships could be found between the occurrence of aggregated whey protein and κ-casein and higher elastic modulus of the acid gels after heating at low temperature/high pH or high temperature/low alkaline pH values of skim milk.

whey protein / κ-casein / heat-treatment / pH / acid gel

摘要 – 热处理和碱性条件下对脱脂乳酸凝固作用的影响。调整脱脂乳的 pH 6.7~10.5，在 25~95 °C 鹾加热 10 min, 然后再中和到 pH 6.7，研究上述条件下加热牛奶酸凝固性的提高以及在减少热负荷的情况下牛奶的凝固特性。根据对牛乳清液相和酪蛋白微团相蛋白质组成的分析和牛乳清液相中蛋白质颗粒的分离，表明当 pH > 7.5 时，在酪蛋白微团相中热致的乳清蛋白 /κ- 酪蛋白聚合物的形成被抑制，但在清液相中则有利于乳清蛋白 /κ- 酪蛋白聚合物的形成。当牛乳在 pH 9.5 和 10.5 时，甚至在中温条件 (65–75 °C) 下，在乳中可以形成乳蛋白聚合物，而在 85 或 95 °C 鹾时，形成凝聚物 pH 值要较低一些，并且形成凝聚物的颗粒随着热处理条件下 pH 的增加而减少。由于在碱性条件下酪蛋白微团的解离以及在高 pH、高温下美拉德反应的发生，导致了最终产品品质降低。本研究探讨了脱脂乳在低温高 pH 或高温低pH条件下，乳清蛋白/κ-酪蛋白聚合物的形成和获得较高酸凝胶塑性模型之间的关系。

乳清蛋白 / κ- 酪蛋白 / 热处理 / pH / 酸凝胶

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Résumé – Modification de l'aptitude à la gélification acide du lait écrémé suite à un traitement thermique à pH alcalin. Dans l'objectif d'améliorer l'aptitude à la gélification acide du lait traité thermiquement ou de réduire la charge thermique nécessaire à l'obtention d'une aptitude significative, du lait écrémé a été ajusté à des pH variant de 6,7 à 10,5, traité thermiquement pendant 10 min à des températures variant de 25 à 95 °C puis neutralisé. La composition en protéines des phases sérique et micellaire du lait et la séparation des particules présentes dans le sérum indiquaient que la formation d'agrégats thermo-induits de protéines sériques et de caséine κ dans la fraction micellaire est inhibée à pH ≥ 7,5 au profit des agrégats sériques. Des agrégats étaient formés à pH 9,5 et 10,5 dès 65 °C, tandis que des températures de 85 ou 95 °C étaient nécessaires à pH moins élevé. La taille des agrégats était inversement proportionnelle au pH de traitement thermique. La qualité des résultats était cependant médiocre du fait de la dissociation importante des caséines à pH alcalin et de la dégradation des protéines à pH et températures élevés (e.g. réaction de Maillard). Cependant, une relation entre la présence d'agrégats et les qualités rhéologiques des gels acides formés a pu être identifiée à basse température/pH élevé et à haute température/pH modéré.

protéine sérique / caséine κ / traitement thermique / pH / gélification acide

1. INTRODUCTION

High heat-treatment of milk (85–95 °C for several minutes) is classically applied in the manufacture of yoghurt and other fermented milk, where the acid gel is expected to form early, to develop a firm texture and to show high whey retention capacity. This ability of the heated milk to form acid gels with good textural properties has been attributed to denaturation of the whey proteins and to their interaction with κ-casein through thiol-disulfide exchange and hydrophobic interaction to yield new particles, the whey protein/κ-casein aggregates, that are located both on the surface of the casein micelles (micelle-bound aggregates) and in the serum phase of milk (serum aggregates) [2–4, 9, 19, 43, 45, 46]. While whey proteins alone are able to form heat-induced complexes [12, 32], in milk various results have demonstrated the involvement of κ-casein in a large part, if not all, the aggregates formed [13, 19, 31] albeit the formation of κ-casein-free aggregates can not yet be excluded [46]. Recent studies have suggested that the serum aggregates provided the milk with improved acid gelation properties compared to those obtained with the micelle-bound aggregates [5, 6, 20, 40]. The proposed explanations for these effects were that the serum aggregates had a higher pH of gelation than that of the casein micelle (with or without attached denatured whey proteins), that stability of the casein micelles was reduced as a result of the involvement of κ-casein in the serum aggregates and that gelation was enhanced by the increased number of acid-precipitable particles and/or by the triggering effect of the formation of an intermediate gel when serum aggregates were present. It has also been shown that distribution of the heat-induced whey protein/κ-casein aggregates between the serum and the colloidal phases of milk depended on the pH of heat-treatment; namely, that formation of predominantly serum aggregates was favoured at alkaline pH values of heating [5, 6, 29, 40, 45]. All these studies used moderate alkaline pH values (pH ≤ 8.1); however, Monahan et al. [32] have shown that disulfide polymers of whey proteins could be formed in solutions of whey protein isolate (WPI) at pH 9 or 11 during mild or even in absence of heat-treatment, up to the formation of gels at sufficiently high pH or ionic strength [30, 32]. This method has been applied by Connolly [8] to aggregate and isolate proteins in mixtures of milk, whey and/or soy milk at temperatures below 70 °C. Combination of heat treatment and alkaline pH has also been applied to the manufacture of milk protein isolates (MPI), resulting in increased protein yield, increased rehydration properties and higher viscosity of the reconstituted powder in particular cases [7, 16, 17]. Grufferty and Mulvihill [16] have for instance obtained MPI from milk heated at pH 10 and 60 °C for 3 min whose protein yield competed with those obtained at neutral or mildly alkaline pH on heating at 90–95 °C for 15 min, and showed that these

results depended on the formation of disulfide aggregates of κ-casein and whey proteins. Conversely, Leaver and Law [23] have submitted skim milk at pH 10 to 12 for 2 h in order to bind the whey protein to caseins and yield co-precipitates; however, their analysis of the resulting sample by trypsine digestion and mass spectrometry did not evidence any significant formation of intermolecular disulfide bridges.

In some of these studies, recovery of the protein fraction was performed using acidification to pH 4.6 [16, 17]. The disulfide aggregates obtained in strong alkaline and mild heat-treatment conditions therefore seemed acid-precipitable, and consequently, seemed good candidates for the formation of acid yoghurt-type gels. The objective of this study was therefore to evaluate the interest of high pH conditions on heat-treatment of skim milk, either as a means to increase gel strength or as a means to reduce the heat load necessary to yield gel properties comparable to the reference gel texture obtained on acidifying milk pre-heated at pH 6.7 and at 95 °C for 10 min.

2. MATERIALS AND METHODS

2.1. Reconstituted skim milk

Milk was reconstituted as 100 $g \cdot L^{-1}$ ultra-low heat skim milk powder (Whey Protein Nitrogen Index = 9.5 [41]) and 0.2 $g \cdot L^{-1}$ sodium azide in stirred deionised water at 40 °C. The milk was stirred for at least one hour following complete dissolution, then left overnight at 5 °C to complete the process.

2.2. Milk ultrafiltration permeate

Milk ultrafiltrate (MUF) was prepared from fresh pasteurised milk on a 8-$kg \cdot mol^{-1}$ molecular weight cut-off TAMI membrane (Tami Industries, Nyons, France) and stored at 5 °C after addition of 0.5 $g \cdot L^{-1}$ sodium azide. pH of the MUF was 6.7.

2.3. Other materials

Other chemicals were from Sigma (St-Quentin-Fallavier, France), Panreac (Barcelone, Spain), Merck (Fontenay-sous-Bois, France), Prolabo (Fontenay-sous-Bois, France) or Carlo Erba (Val-de-Reuil, France) and were of analytical grade. Solvents were obtained from Carlo Erba and were of HPLC grade.

2.4. pH adjustement

Adjustment of the pH of heat-treatment was performed at least ~2 h prior to heating. Two hundred-mL samples of reconstituted skim milk were first equilibrated at 25 °C in a thermostated water-bath for 30 min, then pH was adjusted at pH 7.5, 8.5, 9.5 and 10.5 using NaOH 5 $mol \cdot L^{-1}$. The samples were left with agitation for 2h at room temperature, and during that time the pH was measured every ~30 min to ensure equilibration between measurements and further adjusted if necessary. Much care was taken so that the volume of NaOH added to the milk samples was minimum ($\leq$ 8 $mL \cdot L^{-1}$) to avoid significant dilution. Adjusted pH values were all within ± 0.05 pH units.

2.5. Heat-treatment

After alkalinisation, the 200-mL samples of milk were placed in 250-mL Schott bottles and heat-treated at 25, 65, 75, 85 or 95 °C for 10 min in a thermostated water-bath. The bottles were continuously hand-shaked throughout heat-treatment and cooling. The heating-up period was ~9 min at 65 °C, ~9.5 min at 75 and 85 °C, ~13 min at 95 °C and the milks were maintained at the targeted temperature for another 10 min. The milks were then cooled down to room temperature in agitated ice water, and the time to reach ~25 °C was ~3 min. The milk samples were then equilibrated at 25 °C in a thermostated water-bath for 1h. At this stage, a 30-mL sample of each milk was taken and ultracentrifuged (Sect. 2.8) prior to analysis of the supernatant (or serum phase) by size exclusion chromatography (SE-FPLC) as described in Section 2.9.

2.6. Neutralisation

The 200-mL samples of alkalinised, heat-treated skim milk equilibrated at 25 °C

were brought to pH 6.7 by addition of 5 mol·L^{-1} HCl under stirring and further correction every ~30 min until the pH was stable. The volume of HCl added to the milk samples was ≤ 6 mL·L^{-1}. At this stage, another 30-mL sample of each milk was taken and ultracentrifuged prior to analysis of the supernatant (or serum phase), the resuspended pellet (or micellar phase) and of the starting milk by reverse-phase high performance liquid chromatography (RP-HPLC) as described in Sections 2.8., 2.10 and 2.11. The milks were then stored at 4 °C until analysis.

2.7. Dynamic light scattering

Particle sizes in the alkalinised, heat-treated samples was measured prior to and after neutralisation to pH 6.7 within 2 d after heat-treatment using dynamic light scattering (DLS) at a set angle of 90° on a Zetasizer Malvern 3000 HS (Malvern Instruments, Orsay, France). The laser was a He-Ne laser, with 633 nm wavelength. The solution was brought to 25 °C in a thermostated water-bath, diluted in the appropriate milk ultrafiltration permeate (MUF) to meet the Zetasizer operating range, and allowed to stand at 25 °C for 20 min to ensure proper equilibrium of the diluted system prior to analysis. The solution was then transferred to 2-mL disposable cuvettes for measurement. The refractive index of the MUF was 1.3416 and the viscosity of the MUF was 0.99 mPa·s at 25 °C. The results given are the average of 10 readings, and each sample was analysed 2 or 3 times. Data was visualised using a CONTIN model, and the mode(s) of the weight-averaged particle size distribution were considered.

Ultrafiltration permeates of skim milk samples adjusted at pH 7.5, 8.5, 9.5 and 10.5 were obtained by ultracentrifugation of the milks to remove the casein micelles (see Sect. 2.8), and ultrafiltration at room temperature on a stirred cell system assembly (Pall Life Science, St Germain-en-Laye, France) equipped with a 10-kg·mol^{-1} cut-off membrane.The pH values of the collected permeates were respectively 7.5, 8.1, 8.5 and 10 instead of 7.5, 8.5, 9.5 and 10.5 and were adjusted to their target values using 5 mol·L^{-1} NaOH. These adjusted pH values were stable for at least 8 d. Low-shear viscosity measurement (Low-Shear 30 equipped with 11 and 12 mm coaxial cylinders, Contraves, Zurich, Switzerland) was performed at 25 °C to ensure that viscosity of the different MUF was unchanged with pH.

2.8. Separation of the serum and colloidal phases of skim milk

Separation of the serum and colloidal phases of heat-treated and control milk samples was performed on 15-mL aliquots of milk using ultracentrifugation on a Sorvall Discovery 90 SE centrifuge (Kendro Laboratory Products, Courtaboeuf, France) equipped with a 50.2 Ti rotor (Beckman Coulter, Fullerton, CA, USA). The samples were spun at 19 400 rpm (~33 000 average *g*) for 65 min at 20 °C. The supernatants were collected by simply pouring them into a container without draining or washing of the pellets. This fraction was designated as the "serum phase" or "serum". The total volume, as well as 12 mL of each serum, was weighted. From the data obtained with 12 mL of serum, the mass density of the serum was calculated, which allowed calculation of the total volume of the serums, Vs, to be used in calculation of the mass balance of targeted proteins (see Sect. 2.11). It was observed that the mass density of the serum phase showed a tendency to increase from 1.024 ± 0.005 to 1.033 ± 0.01 kg·L^{-1} as pH increased from 6.7 to 10.5, probably as a result of the dissociation of caseins (not shown). The pellets were resuspended in 12 mL of milk ultrafiltrate at room temperature for up to 48 h under constant agitation. This fraction was designated as the "colloidal" or "micellar phase" of the milk sample and indeed contains a large majority of the casein material of milk [19]. The pellets were resuspended in pH 6.7 milk permeate, no matter the initial pH of the milk sample, because these fractions were only used for the analysis of their protein composition under dissociating and denaturing conditions by reverse phase high-performance liquid chromatography (RP-HPLC).

2.9. Analysis of the milk serums by Size Exclusion Fast Protein Liquid Chromatography (SE-FPLC)

Size Exclusion FPLC analysis of the serum of heat-treated or control pH-adjusted milks was performed within 3 days after heat-treatment on a Sephacryl S-500 Hi-Prep 16/90 column (Amersham Biosciences, Orsay, France). The samples were filtered through 1.2 µm filters (Pall Life Science). The separation was performed at room temperature under isocratic conditions using 0.1 mol·L^{-1} Tris, 0.5 mol·L^{-1} NaCl and 10 mmol·L^{-1} NaN_3, pH 7, as the mobile phase. Loop size was 0.5 mL, flow-rate was 0.5 mL·min^{-1}, and absorbance was monitored at 280 nm. Eluate fractions corresponding to the SE-FPLC separation peaks were collected, dialysed against deionised water, concentrated by freeze-drying, resuspended in a volume of deionised water 200 times smaller than that of the initial collected peak eluate and immediately analysed by RP-HPLC.

2.10. Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

The protein composition of the initial milk samples, of its serum and colloidal phases separated by ultracentrifugation, and of the peak fractions collected during the separation of the serum by SE-FPLC were determined by RP-HPLC. The samples were diluted 5 times with denaturing buffer (7 mol·L^{-1} urea, 20 mmol·L^{-1} Bis-Tris Propane, pH 7.5, + 5 µL·mL^{-1} of fresh β-mercaptoethanol) then incubated for at least 1 h at room temperature. The column was an Apex wide-pore C18 column of 25 cm length, 0.46 cm inner diameter and 7 µm bead diameter (Jones Chromatography, Hengoed, UK). Buffer A was 0.106% v/v trifluoroacetic acid (TFA) in Milli-Q water (Waters, Molsheim, France). Buffer B was 0.1% v/v TFA in 80% v/v acetonitrile in Milli-Q water. The analysis was performed at 46 °C with a gradient of Buffer B increasing in steps from 43 to 100% v/v in 23 min. Loop size was 30 µL, flow-rate was 1 mL·min^{-1} and detection was at 214 nm.

2.11. Quantification of heat-induced protein transfers between the serum and the colloidal phases of milk

The proportions of κ-casein and whey proteins in both the micellar and the serum phases of milk were calculated by dividing the peak area of the protein (κ-casein or whey proteins) in each fraction (micellar or serum phase) by the average peak area of the same protein in the total reconstituted, unheated, pH 6.7 skim milk (6 samples). Because RP-HPLC analysis was directly performed on the serum (i.e., missing the volume of the pellet) and on the pellet resuspended in a consistent volume (12 mL) of milk ultrafiltrate slightly smaller than that of the serum actually removed, concentrations of the whey proteins and κ-casein were over-estimated and could not be directly balanced as fractions of the initial 15 mL of milk. After the volume of serum, Vs, was calculated (see Sect. 2.8), a correction factor of Vs/15 was therefore taken into account in the calculation of the proportions of κ-casein or whey proteins in the serum phase. Similarly, the volume of the resuspended pellet, Vc, was calculated as Vc = 12 + (15–Vs) mL and a correction factor of Vc/15 was applied to the RP-HPLC areas of the analysed proteins.

2.12. Viscoelastic properties of acid milk gels

Formation of the acid gels was monitored by measuring the elastic modulus (G') of the system under acidification at 38 °C using an AR1000 rheometer (TA Instruments, St Quentin-en-Yvelines, France) equipped with a coaxial geometry (DIN with a lateral gap of 1.95 mm and a bottom gap of 4 mm). After equilibration of a volume of 60 mL of milk at 38 °C, 11 g·L^{-1} of glucono-δ-lactone (GDL) was added to the milk under stirring. The milk was stirred for 1 min to allow dispersion of the GDL then poured into the cup of the rheometer. After installation of the geometry was completed, a thin Inlab 423 pH probe (Mettler-Toledo, Viroflay, France) was put in place so that the pH could be measured

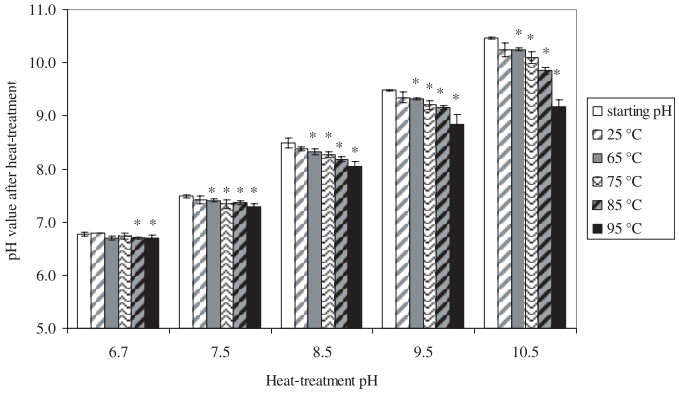

Figure 1. Average pH values of the milk samples adjusted at pH 6.7 to 10.5, prior to and after heat-treatment for 10 min at the indicated temperatures. The star symbol (*) indicates significant ($P_0 <$ 0.01) pH change on heating.

directly in the rheometer and recorded (pHM220 Meterlab, Radiometer Analytical SAS, Villeurbanne, France). The milk sample was covered with a thin layer of mineral oil to prevent evaporation during analysis. The applied deformation was 0.1%, frequency was 1 Hz, and gel formation was followed for at least 8 h. pH 4.9 (latest gelation points, see Results) was reached within 3 h.

2.13. Colour measurement

Colour measurement of the milk samples was performed after heat-treatment and neutralisation using chromametry (CR-300, Konica Minolta, Carrières-sur-Seine, France). Analyses yielded the coordinates for the tested samples in the Hunter L*a*b* colour space; L* is the luminance (increasing from dark/black to bright/white) and a* and b* are the coordinates that define the dominant hue of the sample and its saturation (purity and intensity of the hue) on a circular a*; b* spectrum plane. Axis (–a*; +a*) is from green to red and axis (–b*; +b*) is from blue to yellow.

2.14. Significance

The presented results were obtained from 2 (at 65 and 85 °C) or 3 (at 25, 75 and 95 °C) repetitions of complete sample preparation and analysis, except for SE-FPLC analysis where only 2 samples were obtained for each type of milk sample.

3. RESULTS

3.1. pH and external aspect of the milks

Figure 1 shows the average final values of the pH of the milk after heat-treatment at pH 6.7 to 10.5 and at 25 to 95 °C for 10 min.

The pH value of milk tended to decrease as a result of heat-treatment. This effect was increased as both the temperature and pH of heat-treatment were increased. It is well known that the heat-induced decrease of the pH of milk is essentially a consequence of the partial degradation of lactose due to the Maillard reaction, which is favoured in alkaline pH and high temperature conditions [47]. However, in the present study the pH differences that were applied between the pH-adjusted milk samples were rather well maintained during heating.

Figure 2 shows pictures of the samples obtained after alkalinisation to pH 7.5–10.5, heat-treatment at 25, 75 and 95 °C for 10 min prior to and following neutralisation to pH 6.7. The samples obtained at 65 and 85 °C showed intermediate behaviour (not shown).

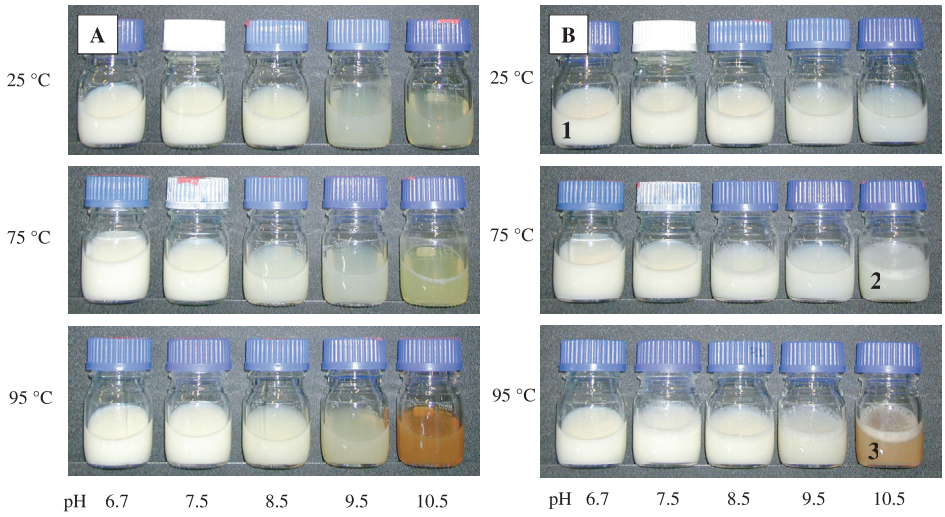

Figure 2. Pictures of typical skim milk samples as obtained after pH adjustment at pH 6.7 to 10.5 (A, top row), heat-treatment at 25 to 95 °C for 10 min (A) and neutralisation to pH 6.7 (B). The samples obtained at 65 and 85 °C showed intermediate aspects and were omitted. The numbers indicate typical samples of the 3 different aspects of milk samples described in the text.

Very significant changes of the external aspect of the milk occurred during alkalinisation, heat-treatment and neutralisation. Increasing pH to 10.5 induced a reduction of the whiteness of the milks, most likely as a result of the alkaline-induced dissociation of the casein micelles and loss of the associated light diffusion properties [1, 2, 23, 29, 36, 42, 44]. This suggestion was supported by the increased proportion, in % of the total weight in milk, of β and α_{s1}-caseins found in the serum phase rather than in the pellet phase when analysing these fractions by RP-HPLC (not shown). The effect of pH on whiteness of the milk was fully reversible up to pH 8.5 and only partially reversible at pH 9.5 and 10.5. Particle size measurement by dynamic light scattering (not shown) confirmed that the average particle size in milk alkalinised at pH 7.5 or 8.5 remained comparable to that found in unheated milk at pH 6.7 (~215 nm) throughout treatments. Conversely, two populations of particles were observed in milk at pH 9.5 or 10.5, heated or not. Diameter of the smaller particles was ~100 nm and that of the larger particles ranged from 300 nm to 1.5 µm, i.e. up to 7 times that of the initial average diameter of the casein micelles at pH 6.7. However, no sedimentation was visible. These particles were not identified. Dynamic light scattering is meant for the measurement of isolated, monomodal populations of particles in suspension; these results should therefore be taken with caution and are therefore not shown. However, these results indicated that, at least at pH 9.5 and 10.5, disruption and reformation of casein particles did not lead to the same protein and/or protein-mineral systems as the one initially present in the milk, despite the partial recovery of whiteness on neutralisation. The temperature of heat-treatment did not seem to have any visible effect on the recovery of whiteness or on changes in particle size on neutralisation. Direct observation of the milk samples (Fig. 2) also evidenced a large effect of the combination between high pH (≥ 9.5) and high temperature (≥ 85 °C) of heat-treatment on the development of

orange-brown color in the milk, most likely indicating advanced Maillard reaction [33, 47]. Reformation of casein particle on neutralisation partially attenuated the brown color of milk heated at high pH and temperature.

Colour measurement using chromametry L*a*b* (not shown) allowed the definition of 3 groups of samples with distinct characteristics. One first group was described with small colour coordinates, small absolutes meaning that the samples had no dominant hue; and the highest luminance (L*) values of all samples, meaning that whiteness was dominant. This group contained samples at pH 6.7 to 8.5, heated or not, i.e. white milk which did not show a significant colour change or transparency change on treatment. The second group was also described by the absence of dominant hue but showed lower L* values (i.e. decreased whiteness). This group contained samples at pH 9.5 to 10.5 heated at low temperatures, which showed partial or total disruption of the casein micelles but no browning. The last group was described by a yellowish hue and decreased whiteness (low L*). This group contained the samples submitted to a combination of high pH and high heating temperature. Typical samples of each group are indicated in Figure 2 as number 1, 2 and 3, respectively. These results showed that the visual observations made on the milk samples after heating were confirmed by objective measurements.

3.2. Heat-induced protein transfers between the serum and colloidal phases of milk

Because κ-casein and the whey proteins are the essential components of the heat-induced aggregates that form in both the serum and the micellar phases of milk, transfers of these two protein species between the two phases of milk were measured. Figure 3 shows the changes in the distribution of κ-casein and the whey proteins between the serum and micellar phases of skim milk after neutralisation, as a result of the temperature and pH of heat-treatment.

With these data, it is expected that the balance of the proportions of one protein between the two phases is 100%. Uniform integration errors induced a slight over-estimation of the whey protein area and under-estimation of the κ-casein area, as previously reported [29]. However, the 100% condition was met within ≤ 15% in most cases for the whey proteins and at low pH only for κ-casein. In the remaining cases, the overall balances in whey protein and/or κ-casein were significantly higher or lower than 100%, suggesting that changes occurred during alkalinisation and heat-treatment that affected the RP-HPLC response of the proteins. RP-HPLC areas for the whey protein in milk at pH ≥ 9.5 heated at 85 or 95 °C were larger than usual, yielding totals higher than 120% as compared to the unheated, pH 6.7, 100% reference. The general shape of the corresponding RP-HPLC profiles remained unchanged compared to that of the initial skim milk (same peaks, same retention times) but these profiles showed broad, smoothed, badly resolved peaks for all the milk proteins, especially in samples where a brown colour was observed. These changes of the RP-HPLC profiles were therefore attributed to Maillard reaction, where the binding of lactose molecules to the milk proteins might account for their increased area and modified profile during HPLC separation. The fact that the whey protein, involving the Maillard sensitive β-lactoglobulin [18, 24, 34, 35], were the only species to show an increase of their RP-HPLC area in the high pH, high temperature conditions supported this explanation.

Conversely, RP-HPLC areas for κ-casein showed a tendency to decrease with increasing pH and to a lesser extent, with increasing heating temperature. Even without heat treatment, submission of the milk to pH 9.5 or 10.5 for 3 h (2 h equilibration of the pH + 1 h equilibration at 25 °C prior to neutralisation) induced up to 21% decrease of the total κ-casein found at pH 6.7 (Fig. 3). The same tendency was observed for α_{s2} and β-caseins, but not for α_{s1}-casein whose area decreased markedly only at pH 10.5 and 95 °C (not shown). Alkaline hydrolysis of the proteins was suspected;

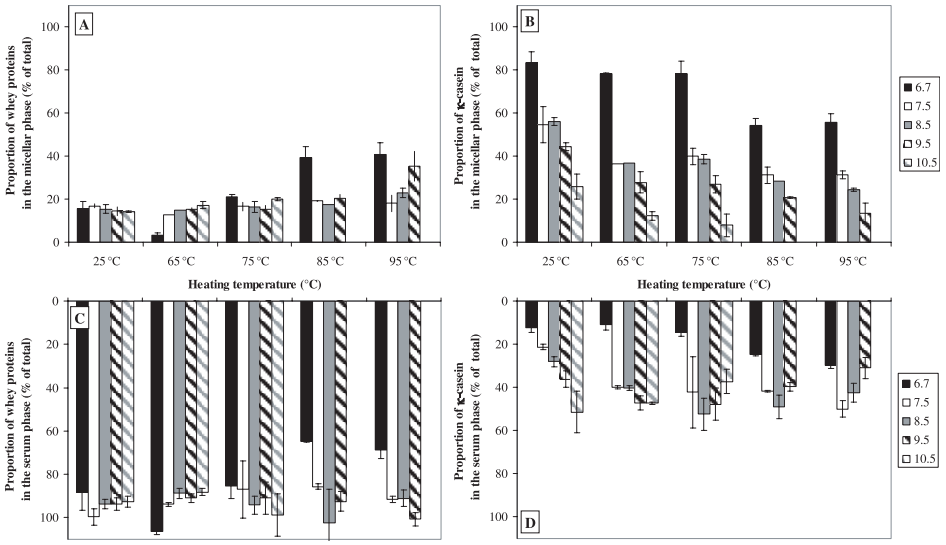

Figure 3. Distribution of the whey proteins (A, C) and of the κ-casein (B, D) between the ultracentrifugal pellet (A, B) and the supernatant (C, D) of skim milk adjusted at pH 6.7, 7.5, 8.5, 9.5 or 10.5, heated at 25, 65, 75, 85 or 95 °C for 10 min then neutralised to pH 6.7 prior to ultracentrifugation. The results are given in percent of the total considered protein (total RP-HPLC area) found in the initial unheated skim milk at pH 6.7. The results obtained at pH 10.5 and 85 or 95 °C were omitted because of obvious bias, as discussed in the result section.

however, Kjeldhal analysis of all the milk samples did not show any variation of the concentration in non-protein nitrogen (NPN) across pH nor temperature (not shown). This result thus ruled out alkaline hydrolysis as the main cause of the degradation of the milk proteins, unless this reaction was not extended enough to produce small peptides soluble in 12% trichloroacetic acid. Other possible causes are the formation of "unnatural" amino acids like lysino-alanine or lanthionine through the alkaline mediated elimination of the thiol of a cystein residue or of the organic phosphate moiety of a serine residue (both examples being particularly relevant to milk) and reaction of the dehydro-alanine protein with the ε-NH_2 group of lysine of another protein [11, 15]. However, if the cross-linked proteins are being hydrolysed to yield the release of lysino-alanine then it is expected that NPN increases in the milk; also, both αs1- and β-casein have been shown to produce lysinoalanine when heated at alkaline pH [28] where as α_{s1}-casein was barely affected in our conditions. Similarly, dephosphorylation under heating is common to α_{s1}- and β-casein [25], therefore none of the listed explanations satisfactorily explained the reduction of the concentration of, especially, κ-casein in alkalinised milk, heated or not.

Despite these drawbacks, some information on the transfers of the whey proteins and κ-caseins between the serum and micellar phases of milk could be provided by the results shown in Figure 3. Figures 3A and 3C showed that pH had no effect on the distribution of the whey proteins between the two phases at heating temperature ≤ 75 °C. In these conditions, ~90% of the total whey proteins were consistently found in the serum phase of milk. At 85 and 95 °C however, a significant transfer of about 20 to 30% of the total whey proteins from the

serum to the micellar phase could be observed at pH 6.7, but not at higher pH values. These results were in accordance with Ménard et al. [29], Vasbinder and De Kruif [45], Anema and Li [3, 4] or Anema et al. [6], where the heat-induced transfer of whey proteins to the micelle phase decreased from 70 to 80% at pH 6.5, ~40% at pH 6.6 and ~30% at pH 6.7–6.9 to ~10% at pH 7.1 then zero at higher pH values on heating milk at 90 °C for various times. However, to the authors' knowledge no data has been reported for pH values higher than 8.1. Figures 3B and 3D conversely showed that the distribution of κ-casein between the two phases of milk was strongly dependent on pH, as already suggested on observation of the clarification of milk as pH increased (Fig. 2). At 25 °C, ~85% of the total κ-casein of pH 6.7 milk was found in the micellar phase, but as pH increased the proportion of soluble κ-casein linearly increased up to about 60% of the total κ-casein (analysable by RP-HPLC) at pH 10.5. On heating, the displacement of κ- casein from the micelle phase was further increased. In milk at pH 6.7, no change in the distribution was observed until a temperature of 85 or 95 °C was used, where 10 to 20% of the total κ-casein were transferred from the micellar to the serum phase, yielding ~30% of the total κ-casein in the serum phase. These figures were in accordance with the data reported by Anema and Klostermeyer [1] and Anema and Li [2] where the proportion of non-sedimentable κ-casein increased from 15% in unheated milk to ~20% at 60 °C and 25–30% at 90 °C for various heating times. In milk at pH 7.5 or above, a heat-induced transfer of κ-casein from the micellar to the serum phase could be observed at temperatures as low as 65 °C but extent of the transfer was then rather constant across heating temperatures. At pH 7.5, the proportion of the total "RP-HPLC analysable" κ-casein in the micellar phase was 73% at 25 °C and 47% after heat-treatment at 65 °C; similarly, this proportion decreased from 33 to 17% at pH 10.5. About 15 to 25% of κ-casein therefore dissociated from the micellar phase as a result of heating at temperature ≥ 65 °C and pH ≥ 7.5. Of course, these calculations were made with the assumption that the alkaline degradation of κ-casein is proportional in the two phases of milk, therefore that the total "RP-HPLC analysable" κ-casein is distributed between the micellar and serum phases in the same proportions as before protein loss. Despite the poor quality of the results, they were in accordance with previous reports where a positive relationship was demonstrated between the pH of heat-treatment and the extent of the heat-induced dissociation of κ-casein [1, 2, 42], albeit the latter was also found to increase with temperature in milk at pH 6.9 or 7.1 [1, 2]. The latter studies found about 30% more κ-casein in the serum of milk heated at pH 7.1 and 90 °C than at pH 7.1 and 20 °C. Singh and Creamer [42] found 40 to 50% more κ-casein in the serum of concentrated milk at pH 6.85 after heat-treatment at 120 °C for a few min.

These results showed that, starting from a reference situation where κ-casein and the whey proteins are largely separated between the two phases of unheated, pH 6.7 skim milk, both alkaline and to a lesser extent heat-treatment favoured the contact of the two protein species. On alkalinisation, κ-casein was transferred from the micellar to the serum phase; this transfer was further enhanced by heating and yielded the co-location of the two protein species in the serum phase. At pH 6.7, heat-treatment induced the exchange of κ-casein and the whey protein, yielding a co-location of the two protein species in both the serum and micellar phases. In earlier reports, it has been proposed that the heat-induced co-location of κ-casein and the whey proteins suggested that thiol-disulfide exchanges and aggregate formation had occurred between the two protein species on heating [3, 4, 6, 29]. For pH values higher than 8, Monahan et al. [32] or Connolly [8] have shown that thiol-disulfide exchanges and aggregate formation could occur even at low heating temperature. However, in the present study disruption of the casein micelle at these pH and forced co-location of the caseins with the whey proteins as shown in Figure 3 did not provide enough indication of the formation of serum aggregates in these conditions.

3.3. Separation of the heat-induced serum protein aggregates and dissociated casein using SE-FPLC

The serum phase of the milk samples was analysed by SE-FPLC as a means to identify their components. Protein composition of the eluted peaks was analysed by RP-HPLC. The results are shown in Figure 4. For the sake of clarity, only those obtained at 25, 75 and 85 °C are shown. The profiles obtained at 65 °C were intermediate to those obtained at 25 and 75 °C. The profiles obtained at 95 °C were very similar to those obtained at 85 °C, and were not chosen because of the poor quality of the RP-HPLC results obtained at this pH.

Previous reports [19, 37] have shown that the protein fraction of the serum phase eluted between 130 min (exclusion peak) and 330 min. The material eluting at longer times is non-protein and was not considered in the present study.

The results showed that two different behaviours were obtained depending on the heating temperature of the milk. At 25, 65 and 75 °C, the protein material was separated in 3 poorly resolved peaks respectively eluting at ~250, 270 and 300 min (Figs. 4A and 4B). At 25 °C and pH 6.7–8.5, only the two peaks at 250 and 300 min were present. Analyses of the corresponding fractions by RP-HPLC showed that these peaks respectively contained small casein particles, with a high proportion of κ-casein, and native whey protein with some casein monomers. These results were in accordance with Guyomarc'h et al. [19] and Ménard et al. [29]. As pH increased, a third peak developed in the 270 min region, as also reported by Donato and Dalgleish [13] and Ménard et al. [29] in unheated milk. This peak contained a high proportion of α_s- and β-caseins and was identified as another type of small casein particles. This is also where sodium caseinate is eluted [37]. As pH increased from 6.7 to 10.5, the areas of the two peaks of small casein particles, i.e., eluting at 250 and 270 min, increased slightly up to pH 8.5 then dramatically up to pH 10.5, where reduction of the resolution induced fusion of the constitutive peaks (Fig. 4A). This result evidenced the alkaline-induced dissociation of the casein micelle. At pH 7.5 to 9.5, dissociation of the casein micelle in the form of κ-casein-rich and α_s/β-casein small particles increased as a function of temperature up to 75 °C. Barely any change was observed at pH 6.7 (little or no dissociation) and pH 10.5 (advanced disruption of the micelles even at 25 °C) across temperature. Figure 4B also showed that the area of the peak containing the native whey protein (at 300 min) seemed to decrease as pH increased as a result of heating at 65 °C (not shown) or 75 °C (Fig. 4B). Composition of the eluate fractions showed that higher proportions of κ-casein and whey proteins were then co-eluted with the α_s/β-casein fraction at 270 min. These proportions tended to increase with pH (not shown). Despite the low temperature, these results indicated that small quantities of κ-casein and whey protein were found together at retention times lower than that of protein monomers, therefore possibly forming small complexes. However, it was not possible to know whether the composition of the α_s/β-casein particle changed or whether new, heat-induced particles were formed in the serum on heating milk and co-eluted with them. On heating at 85 or 95 °C, the SE-FPLC profiles changed significantly (Fig. 4C). Area of the native protein peak (at 300 min) decreased as pH decreased, with dramatic heat-induced reduction at pH 6.7, 7.5 and 8.5, and little or no heat-induced change at pH 9.5 and 10.5. A new peak concomitantly appeared at short elution times, e.g. 215 min for heat-treatment at pH 6.7 and 85 °C, evidencing the formation of large particles. RP-HPLC analysis showed that this fraction was essentially composed of whey protein and κ-casein, which was interpreted as the formation of heat-induced whey protein/κ-casein aggregates [13, 19, 37, 40]. Elution time of this heat-induced peak increased with increasing pH, showing that smaller whey protein/κ-casein aggregates were formed at higher pH, in accordance with the reports by Donato and Dalgleish [13], Vasbinder and De Kruif [45], Renan et al. [39], Hoffmann et al. [21] or Ménard et al. [29]. At pH 9.5 and 10.5, poor resolution

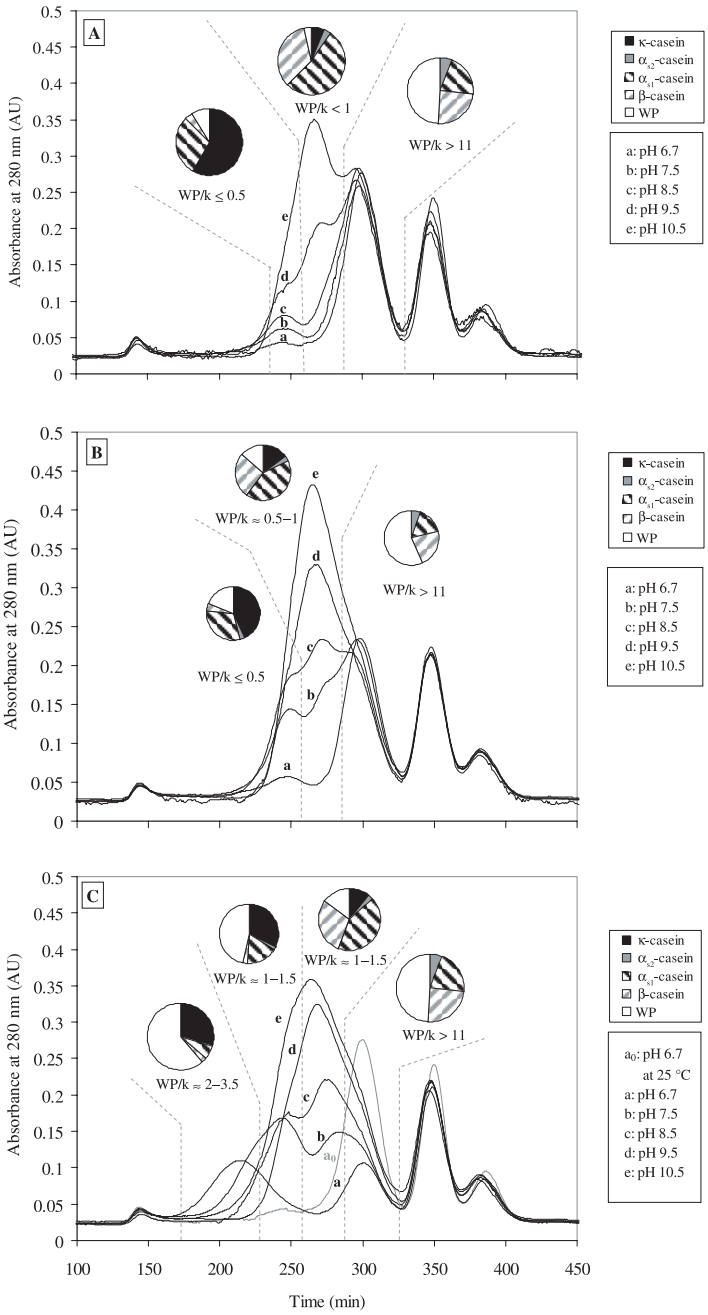

Figure 4. Typical SE-FPLC profiles of the ultracentrifugal supernatants of skim milk adjusted at pH 6.7, 7.5, 8.5, 9.5 or 10.5, heated at 25 °C (A), 75 °C (B) or 85 °C (C) for 10 min prior to ultracentrifugation. The inserted pie-charts show the typical protein composition, in % of the total RP-HPLC area, of the eluate fractions collected as shown by the dotted lines.

of the various types of small protein particles made interpretation difficult; however, elution of the merged peaks seemed to start from lower elution times at 85 and 95 °C than at lower heating temperatures. Composition of the corresponding fraction, at about 250 min, showed that whey proteins and κ-casein were present in high proportions (Fig. 4C), thus indicating that small whey protein/κ-casein aggregates were probably formed that co-eluted with the larger alkaline-dissociated casein material.

The results shown in Figure 4 therefore evidenced two different behaviours of the whey proteins in response to heat and alkaline treatment of milk. At low temperature (≤ 75 °C) and high pH values (≥ 9.5), some native whey proteins seemed to be lost and indications of the formation of small whey protein/κ-casein aggregates could be detected in the 250-min region. These changes slightly increased with increasing temperature up to 95 °C. At low pH values (6.7–8.5), high temperatures of heat-treatment (85–95 °C) were necessary for significant formation of serum aggregates; and the lower the pH, the larger the aggregates. This agreed with the results shown in Figure 3 for pH 6.7 milk, where significant heat-induced changes in the protein transfers between the serum and the micellar phases were only significant at 85 or 95 °C. However, determination of the denaturation rate of the whey protein using Kjeldahl analysis of the nitrogen fractions of milks at pH 6.7 and 10.5 (not shown) showed that denaturation was ~40% of the total native whey protein after heating at 75 °C at pH 6.7 and increased up to 74% at 95 °C, while heat-treatment at pH 10.5 yielded 7% denaturation at 25 °C, 55% at 65 °C and 62 to 72% at higher temperatures. This indicated that denaturation of the whey protein occurred at lower temperature when pH was strongly alkaline, in accordance with Monahan et al. [33], and supported the hypothesis that small disulfide complexes could be formed in these conditions. The fact that the SE-FPLC profiles of the serum of milk at pH 9.5 and 10.5 only slightly changed on heating at temperature 75 to 95 °C, where as aggregate formation at pH ≤ 8.5 was enhanced at 85 and 95 °C and increased as heat load increased might indicate different types of heat-induced protein complexes.

3.4. Properties of the acid gels

The wanted characteristics of acid milk gels like set yoghurt are gelation at high pH value, high elasticity (firmness) and low syneresis. The two first characteristics are well measured using low amplitude oscillation rheology throughout acidification of the milk. The gel point, or gelation pH, is defined as the pH value when G' > 1 Pa. Final G' is taken at pH 4.6, as about the pH value when the gel is cooled down and/or stirred in yoghurt manufacture. However, even 5 or more h after the gelation point, G' was still slightly increasing with time, i.e., the gels were not totally stable. This ability of acid milk gels to indefinitely rearrange well after gelation is well known [48]. Care should therefore be taken in interpreting the results, although it is likely that differences observed at pH 4.6 between two milk systems would be maintained on longer times. The rate of gelation is $|dG'/dpH|$, in $Pa \cdot min^{-1}$, i.e. the slope of the increase in G' from the gelation point onwards.

Figure 5 shows the increase in elastic modulus, G' (Pa), as a function of pH after addition of GDL to the neutralised milk samples. The results show that the acid gels formed with unheated (i.e. kept at 25 °C) milk samples only reached elastic modulus values, G', lower than 20 Pa at pH 4.6 and were therefore very soft gels. The inability of unheated milk to form elastic gel is widely reported in the literature [20, 26, 27]. However, unheated milk at pH 6.7 started acid gelation at higher pH values and reached higher G' values than all the other milks. This result could be explained by changes in the structure of the casein micelle on pH cycle (see Sect. 3.1), dilution of the milks by addition of NaOH and HCl or loss of the functionality of the proteins due to pH-induced changes as discussed in Section 3.2. When the milk had been heated at 65 or 75 °C at pH 9.5 or 10.5, higher gelation pH and final G' values were found compared to milk heated at pH 6.7–8.5. Final G' values of 160 Pa, i.e., about half that of skim milk at pH 6.7 heated at 85 or 95 °C, could for instance be reached by milk

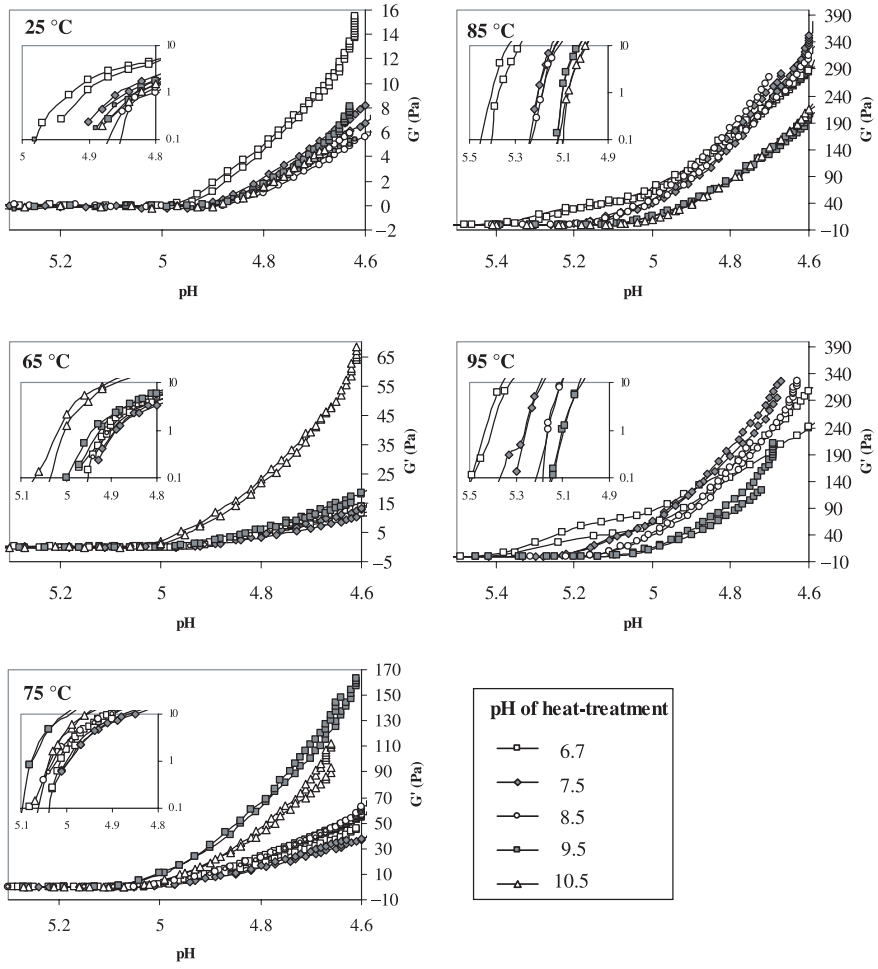

Figure 5. Typical acid gelation profiles of skim milk adjusted at pH 6.7, 7.5, 8.5, 9.5 or 10.5, heated at 25, 65, 75, 85 or 95 °C for 10 min, neutralised to pH 6.7 then acidified to pH 4.6 at 38 °C by addition of 11 g·L^{-1} GDL and measurement by low amplitude oscillation rheometry. The inserted figures (with G' in log scale) focus on the gel point (G' > 1 Pa) of the milk samples.

heated at pH 9.5 at as low a temperature as 75 °C. However, the positive effect of high pH of heat-treatment on gel formation seemed to be cancelled out when higher temperatures of heat-treatment were used. After heat-treatment at 85 or 95 °C, highest gelation pH values of ~5.4 were obtained with milk at pH 6.7, and decreased to pH ~5.1 as the pH of heat-treatment increased. However, the rate of gel formation seemed to be slightly lower at pH 6.7 than at pH 7.5 and 8.5. The final G' values obtained at the latter pH values were therefore slightly higher than those obtained at pH 6.7 ($P_0 >$ 0.05, not significant). Despite a low pH of gelation of 5.1, milk heated at pH 10.5 and 95 °C showed a higher gel formation rate than that of the other milks heated at 95 °C and

final G' values up to 340 Pa on acidification (not shown for reason of clarity). Since the ability of milk heated at pH 10.5 to form early and firm acid gels decreased with temperature from 65 to 85 °C, this result was rather surprising and remained unexplained.

It should be mentioned that despite standardised acidification conditions not all samples reached pH 4.6 after 8 h of analysis. For instance, the final pH of unheated skim milk at pH 6.7 or of skim milk heated at pH 10.5 and 75 °C tended towards 4.65 (Fig. 5). In general, it seemed that higher final pH values were observed when earlier and stronger gels were formed. This may be accounted for by differences in proton diffusion and/or performances of the pH probe depending on the gel properties.

As suggested by the results of Figure 4, formation of these acid milk gels depended on the heating temperature and pH. At low temperatures (65–75 °C), milk samples heated at pH 9.5 and 10.5 showed enhanced gelation properties while milk samples heated at lower pH values showed poor acid gelation properties. At high temperatures (≥ 85 °C), acid gelation properties of the milks heated at pH 9.5 or 10.5 decreased where as milk samples heated at pH 6.7–8.5 showed the highest gelation properties. To the authors' knowledge, no data is available on the acid gelation behaviour of skim milk heated at pH higher than 7.5. In the range 6.7–7.1, various studies conclusively reported a positive relationship between the pH of heat-treatment at 80 or 90 °C and an onset of gelation at higher pH values as well as higher gel strength of the acid gel [5, 40, 45]. The present study showed that the positive effect of alkaline pH values of heat-treatment on the increase of the final gel strength of acid gels could be partially and marginally extended up to pH 8.5. Some limitation of this increase therefore existed, as suspected by Rodriguez del Angel and Dalgleish [40] who observed a decrease of the final gel strength of acid gels after heating at pH 7.2. Another original result of this study was that significant ($P_0 < 0.05$) ability to acid gelation could be obtained by heat-treating milk at 75 °C, providing that pH was ≥ 9.5.

4. DISCUSSION

In this study, heat-treatment of skim milk at various pH values up to 10.5 was experimented as an attempt to reduce the heat load necessary to form whey protein/κ-casein aggregates and/or to enhance the acid gelation properties of heated skim milk by displacing these aggregates to the serum phase. The results indicated that the heat-induced transfer of the whey proteins from the serum to the micellar phase was inhibited at pH 7.5 or higher, while the transfer of the κ-casein from the micellar to the serum phase was increased on increasing pH, even at low heating temperature (Fig. 3). These results were in accordance with those obtained by Ménard et al. [29] and suggested that alkaline pH up to about 7.5–8.0 was enough to prevent formation of micelle-bound whey protein/κ-casein aggregates. The fact that little extra dissociation of κ-casein was induced by heat-treatment at pH ≥ 7.5 and temperature ≥ 65 °C where as no whey proteins were transferred to the colloidal phase in the meantime suggested that formation of the serum aggregates involved the alkaline dissociated κ-casein and that a limitation may exist in the quantity of serum aggregates possibly formed with κ-casein and whey proteins in the proportions found in milk. Conversely, the fact that some heat-induced dissociation of κ-casein did occur at pH ≥ 7.5 and temperature ≥ 65 °C in spite of the availability of extensively alkaline-dissociated κ-casein (Fig. 3B/D, 25 °C) also suggested that formation of the serum whey protein/κ-casein aggregates probably involved both the micelle-bound and dissociated κ-casein forms. This conclusion may be compared to that of Donato et al. (unpublished results), who observed that soluble κ-casein added to skim milk at pH 6.7 was not involved in the formation of aggregates on heating and concluded that a competitive route for the formation of serum aggregates was through the interaction of the denatured whey protein with κ-casein on the surface of the casein micelle.

Beyond indications of the formation and distribution of the heat-induced whey protein/κ-casein aggregates, as the scope of the present study, the mass balances showed in

Figure 3 also raised questions on the nature of the changes experienced by the milk system when submitted to high alkaline pH conditions. On heating milk at pH 10.5 and 65 °C for instance, the proportion of the total κ-casein that was found in the micellar phase did not exceed 20% after neutralisation. Yet, no visible precipitation of the reassociated "casein micelles" occurred in the milk samples (Fig. 2). The reduced opacity of the milks heated at pH 9.5 or 10.5 and neutralised, compared to that of control milk; the occurrence of light scattering particles smaller than casein micelles in these neutralised samples and the small size of the pellet obtained on centrifugation of the same samples suggested that most of the particles formed on neutralisation of milk samples previously heated at pH 9.5–10.5 remained in the supernatant, hence the low proportion of "micellar" κ-casein in these samples.

Similarly, visible precipitation of insoluble calcium phosphate could have been expected on heating milk at alkaline pH, e.g. on the walls of the Schott bottles. Both alkaline pH values [22, 44] and heat-treatment [38] induce the reduction of the concentrations in soluble calcium and phosphate ions. Whether the transfer of calcium and phosphate ions to the colloidal phase of milk is through the actual interaction with casein (growth of the calcium phosphate nanoclusters [22]) or through the formation of mineral crystals as in alkalinised whey [14] or both, is unclear. Vaia et al. [44] attributed the dissociation of the casein micelle on alkalinisation to the fact that depletion of the solvent phase in calcium ions probably increased the amount of free water and therefore solvent quality, hence solvatation of the calcium-sensitive casein molecules. Also, the occurrence of some large particles (μm range) in the alkalinised or neutralised milks as observed in the present study using light scattering may be accounted for by some mineral precipitation. However, Odagiri and Nickerson [36] reported that neutralisation of skim milk submitted to pH values up to 11 for 3 h did not induce changes in the calcium phosphate distribution in the milks, showing that changes are probably very slow. This may explain why no extensive mineral precipitation had been observed in the present study.

On heat-treatment of skim milk at low temperature (65–75 °C) and high pH values (9.5–10.5), the results obtained by SE-FPLC (Fig. 4) seemed to indicate the formation of serum whey protein/κ-casein aggregates that probably accounted for the significant increase in gelation pH and final gel strength obtained with these milks compared to that of the pH 6.7 control (Fig. 4). These results agreed with Monahan et al. [32] and Connolly [8] who used similar conditions to induce thiol/disulfide exchanges between globular proteins. However, present results showed that this combination of treatments did not produce acid gels whose gel strength could compete with those obtained from classically heated (85–95 °C, 5–10 min) skim milk at pH 6.7. On heat-treatment of skim milk at 85 or 95 °C, serum whey protein/κ-casein aggregates could be formed at all pH values (Fig. 4) and acid gels showed higher final gel strength values (Fig. 5). However, the pH of gelation tended to decrease as the pH of heat-treatment increased, and only milk heated at pH 7.5–8.5 showed final gel strength marginally higher than at pH 6.7. Limitation or inhibition of the increase in gelation pH and final strength of acid gels made with milk heated at alkaline pH, i.e., having high proportion of serum aggregates [5, 40, 45] might be accounted for by various reasons. First, changes in the properties of the casein micelles might have occurred. Reorganisation of the caseins on pH-cycling and changes in size distribution (see Sect. 3.1) might have affected the homogeneity of acid gels. Partial precipitation or other changes in the calcium phosphate equilibrium on heating at high pH and neutralisation might have also affected the ability of the casein micelle to acid gelation. Dialysis of skim milk against milk ultrafiltrate to reduce the total concentration in calcium and phosphate, followed by heat-treatment of the milk induces changes in the casein organisation (partial solubilisation, decreased zeta potential) and yields acid gels with variable pH of gelation and generally decreased final elasticity (Famelart, personnal communication). Also, the binding

of lactose on casein molecules on Maillard reaction could change their electrostatic properties and hence increase solubility of the casein micelles [10], thus possibly decreasing their ability to aggregation and gelation. Second, there may be a limitation for the size of the heat-induced whey protein/κ-casein aggregates below which their effect on texture formation is reduced, i.e., aggregates formed at pH ≥ 9.5 may be too small to act as fillers or to significantly affect growth and branching of the protein strands in the gel. Finally, it is very likely that degradation of the milk protein as observed in Figure 3, either by extensive Maillard reaction or by other reactions affecting caseins, affected gel formation through reduction of the actual concentration of proteins able to form an acid gel after heat-treatment at pH ≥ 9.5 and high temperature. These drawbacks have not been reported in any of the previous reports using high pH and heat-treatment of milk or whey [7, 16] or pH 12 for up to 2 h at room temperature [23]. Considering the improvement of the gelation properties of milk samples treated in milder conditions (high pH and low heating temperature or high heating temperature and moderate alkaline pH) and considering the results of Monahan et al. [32] for solutions of WPI in water, it could be interesting to perform high heat-treatment on delactosed skim milk or on a mixture of caseinate and whey protein in lactose-free medium, adjusted at high pH values to know whether such improvement does not occur at high pH/high temperature or whether it is counter-balanced by effects of the Maillard reaction. However, such processes would only have scientific interest since a possible reduction of the heat-load necessary to improve ability of heated milk to acid gelation would not, or barely, cover the cost of preliminary diafiltration or drying of the casein and WPI ingredients.

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