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Original article

Effect of heat treatment at alkaline pH on the rennet coagulation properties of skim milk

Olivia MÉNARD, Bénédicte CAMIER, Fanny GUYOMARC'H*

UMR 1253, Science et Technologie du Lait et de l'Œuf, Inra-Agrocampus Rennes, 65 rue de Saint-Brieuc, 35042 Rennes Cedex, France

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Abstract – Reconstituted skim milk was heated at 90 °C for 30 s at pH values ranging from 6.6 to 8.1, stored overnight at 5 °C then renneted at pH values ranging from 6.2 to 6.6. The heat-induced disimprovement of the rennet coagulation properties of the milk (longer rennet coagulation time and lower gel firmness) were partially reduced as heat-treatment pH increased, except at pH 6.6. These properties were related to the increased heat-induced dissociation of micellar κ-casein and subsequent formation of serum, rather than micelle-bound, heat-induced whey protein/κ-casein aggregates. It was postulated that such distribution of the denatured whey protein and κ-casein slightly reduced the detrimental effects of heating on the enzymatic coagulation, as well as helped earlier destabilisation of the micelles. These results are discussed with respect to the amplitude of the effect of the heat-treatment pH, as well as the side effects of alkalinisation and heat treatment on casein dissociation.

whey protein / heat treatment / rennet coagulation / pH

摘要 – 碱性 pH 条件下热处理对脱脂乳凝乳特性的影响。在 pH6.6~8.1 范围内将还原脱脂乳于 90 °C 加热 30s, 5 °C 储藏过夜，然后在 pH6.2~6.6 范围内添加凝乳酶凝乳。加热使得乳的凝固特性变差（凝乳时间延长，凝块硬度降低），但是随着加热过程中 pH（除了在 pH6.6 时）的升高，可部分改善这种现象。这种性质是由于加热使得 κ– 酪蛋白胶体解离后进入乳清相的原因，并不是由于乳清蛋白和酪蛋白胶体之间的结合，但加热可以引起 κ– 酪蛋白和乳清蛋白的聚集。可以假设变性的乳清蛋白和 κ– 酪蛋白的这种分布可轻微降低加热对酶凝乳的负面影响，同时有助于酪蛋白胶体在凝乳前的不稳定性。本文主要讨论热处理时 pH 对酪蛋白解离的影响程度以及碱化和热处理对酪蛋白解离的负面影响。

乳清蛋白 / 热处理 / 凝乳酶凝乳 / pH

Résumé – Effets d'un traitement thermique à pH alcalin sur l'aptitude du lait écrémé à la coagulation présure. Du lait écrémé reconstitué est traité thermiquement à 90 °C pendant 30 s à des pH variant de 6,6 à 8,1, stocké à 5 °C pendant une nuit puis emprésuré à des pH variant de 6,2 à 6,6. La perte d'aptitude à la coagulation présure des laits chauffés (temps de coagulation plus long et moindre fermeté) a été réduite pour des pH de traitement thermique croissants, sauf à pH 6,6. Ces propriétés ont été mises en relation avec un taux de dissociation accru de la caséine κ quand le pH de traitement thermique augmente, et donc une proportion accrue d'agrégats thermo-induits de protéines sériques dénaturées et de caséine κ dans la phase soluble du lait. Il est postulé que cette distribution en protéines réduit légèrement les effets rédhibitoires du traitement thermique sur la coagulation enzymatique, et accélère la déstabilisation des micelles de caséines. Ces résultats sont

* Corresponding author (通讯作者): fanny.guyomarch@rennes.inra.fr

discutés relativement à l'amplitude de l'effet du pH de traitement thermique ainsi qu'à la dissociation alcaline ou thermo-induite des autres caséines.

protéine sérique / traitement thermique / coagulation présure / pH

1. INTRODUCTION

Incorporation of the whey proteins into cheese has long been extensively investigated as a means of adding value to dairy products, for economic reasons as well as to increase nutritive or textural properties of cheeses. Ultrafiltration of cheese milk, addition of particulated whey proteins (by heating and homogenisation of whey) and/or heat treatment of the milk are the three methods generally used to meet these goals. Of the three methods, heating is the least satisfactory, as heated milk exhibits extended rennet coagulation time (RCT) and reduced gel firming rate, eventually leading to the formation of soft, grainy and humid curds [7, 19, 29, 31]. These heat-induced technological changes have been correlated to the denaturation of whey proteins and formation of both micelle-bound and soluble protein aggregates from the interaction between denatured whey proteins and κ-casein. The enzymatic phase of renneting is slowed due to steric hindrance of the Phe_{105}-Met_{106} bond of the κ-casein molecule by the attached whey proteins. Curd fusion is also inhibited as whey protein-coated micelles are better at retaining their integrity [10, 23, 31, 33].

Upon heating at pH > 6.7, heat-induced milk protein aggregates are reported to be smaller, more numerous [24] and with a higher proportion of disulphide bonds compared with hydrophobic interactions [12, 13]. Upon heating milk at pH > 6.9, the proportion of heat-induced whey protein/κ-casein aggregates found in the serum phase is increased, while casein micelles are depleted in κ-casein as a result of their interaction with denatured whey protein and dissociation [2–5, 26–28, 32]. The objective of this study was to investigate whether the rennet coagulation properties of skim milk heated at various pH values > 6.5 would be improved by the fact that the heat-induced whey protein/κ-casein aggregates would be present in the serum phase rather than on the micelle surface. The related depletion of the micelles in κ-casein could also help reduce flocculation time since less stabilising κ-casein is present from the start of the enzymatic reaction.

Information on the effect of alkaline heat-treatment of milk prior to renneting is scarce and results are contradictory due to the various experimental conditions used, especially with respect to post-heating treatments, e.g. pH changes or storage. Van Hooydonk et al. [31] reported poor renneting properties of milk heated at pH 7.5, while Leaver et al. [18] observed that the chymosin reaction rate decreased with increasing pH > 6.7, but not beyond 7.8. Conversely, Imafidon and Farkye [14, 15] found that milk heated at pH 7.5 then acidified or pH-cycled has a lower RCT than milk heated at pH 6.7, as later agreed by Singh and Waungana [29]. Cheeses obtained were not different with respect to composition, and Banks et al. [8] noticed that heat-induced defects of cheese curds were reduced when heating of the cheese milk was performed at pH 7.1 or 7.5. Vasbinder and de Kruif [32] reported better renneting properties of skim milk heated at pH 6.9 rather than 6.7.

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 [25]) and 0.5 $g \cdot L^{-1}$ 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 $kg \cdot mol^{-1}$ TAMI membrane (Tami Industries, Nyons, France) and stored at 5 °C after addition of 0.5 $g \cdot L^{-1}$ sodium azide.

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) and 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 adjustment

The adjustments of the pH of heat treatment or renneting were, respectively, performed ~2 h prior to heat treatment, and ~2 h prior to Formagraph analysis. The reconstituted skim milk samples were first equilibrated at room temperature for at least 1 h, then pH was adjusted using HCl and NaOH 5 $mol \cdot L^{-1}$ or 0.5 $mol \cdot L^{-1}$ at the following values: heat-treatment pH 6.6, 7.1, 7.6 and 8.1; renneting pH: 6.2, 6.3, 6.4, 6.5 and 6.6. Much care was taken so that the volume of HCl or NaOH added to the milk samples to reach the heat treatment or the renneting pH was minimum (≤ 3 $mL \cdot L^{-1}$) to avoid significant dilution.

Samples were left with agitation for a further ~30 min at room temperature to ensure equilibration prior to final pH adjustment. Adjusted pH values were all within ± 0.05 pH units.

2.5. Heat treatment

Six hundred mL of reconstituted skim milk were heat-treated at 90 °C for 30 s on a recirculating tubular heat-exchanger composed of an inox tubular coil (8 mm diameter, 7 m length) plunged in a thermostated water-bath and connected to a recirculating centrifuge pump (Micropump Inc., Vancouver, Canada). The flow rate was 135 $L \cdot h^{-1}$ and the flow was turbulent to ensure proper heat transfer (Re ~3200). The heating-up period was < 4 min. The milk was then cooled down to room temperature in agitated ice water. After 1 h at room temperature, samples were taken for separation of the micelle and serum phases, then the milk was stored overnight at 5 °C.

2.6. Rennet coagulation properties

Rennet coagulation properties of heat-treated or control pH-adjusted milks were evaluated on day 1 after heat treatment using a Formagraph rheometer (Foss Electric, Nanterre, France) through the measurement of the rennet coagulation time (RCT) and of gel firmness taken at 1 × RCT after coagulation (aR).

Ten-mL milk samples were equilibrated at 35 °C prior to inoculation with 30 µL of traditional rennet solution (Berthelot 530, Laboratoires Abia, Meursault, France) diluted to 10% w/w in deionised water. Formagraph measurement was monitored at 35 °C for 2 h.

2.7. 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 after heat treatment, cooling and equilibration of the milk at room temperature for 1 h. Separation was performed on 15-mL aliquots of milk using ultracentrifugation on a Sorvall Discovery 90 SE centrifuge (Kendro Laboratory Products, Courtabœuf, 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 pellets were resuspended in a volume of milk ultrafiltrate equivalent to the removed supernatant (~12.5 mL), at 5 °C for at least 48 h under constant agitation. This fraction was designated as the "colloidal" or "micellar phase" of the milk sample.

The materials found in the serum and micellar phases of heated and unheated skim milk separated by the above method have been extensively described elsewhere [11]. The low centrifugation speed used in this method prevented sedimentation of the protein materials present in the serum phase of the milk, at the expense of small quantities of residual micellar material that remained in the supernatant [11].

2.8. Quantification of heat-induced protein transfers by reverse-phase high performance liquid chromatography (RP-HPLC)

The protein composition of the initial milk samples and of their serum and colloidal phases separated by ultracentrifugation were determined by RP-HPLC as described in [16]. Briefly, 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 1 h at 37 °C. 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. Loop size was 30 µL, temperature was 46 °C, flow rate was 1 mL·min^{-1} and detection was at 214 nm.

The proportions of κ-casein and whey proteins in both the micellar and the serum phases of the 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 skim milk. This average peak area was the average of the peak areas of the total considered protein found in 16 reconstituted skim milk samples, at the various heat-treatment pH values, heated or not. The variation coefficients for the peak area of total κ-casein or total whey protein were < 10%, as expected, since pH change or heat treatment should not affect the overall protein composition of the milk. A correction factor of 12.5/15 was taken into account in the calculation of the proportions of κ-casein or whey proteins in the serum phase, as the removal of the centrifugal micellar pellet (~2.5 mL) from the milk samples (15 mL) mathematically increases the concentration of the serum species.

2.9. Analysis of the milk serums by size exclusion fast protein liquid chromatography (SE-FPLC)

Size exclusion FPLC analysis of the serums of heat-treated and control pH-adjusted milks was performed within 1 week after heat treatment on a Sephacryl S-500 Hi-Prep 16/90 column (Amersham Biosciences, Orsay, France). The samples were filtered through 5-µm filters (Pall Life Science, St-Germain-en-Laye, France). 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. Five mL eluate fractions were collected every 10 min, dialysed against deionised water, concentrated by freeze-drying and analysed by RP-HPLC as described above.

2.10. Micelle size measurement by dynamic light scattering (DLS)

The particle size in the resuspended pellet samples was measured within 1 week 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 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, the refractive index of the protein particles was 1.5 and the viscosity of the MUF was 0.99 mPa·s at 25 °C. The results

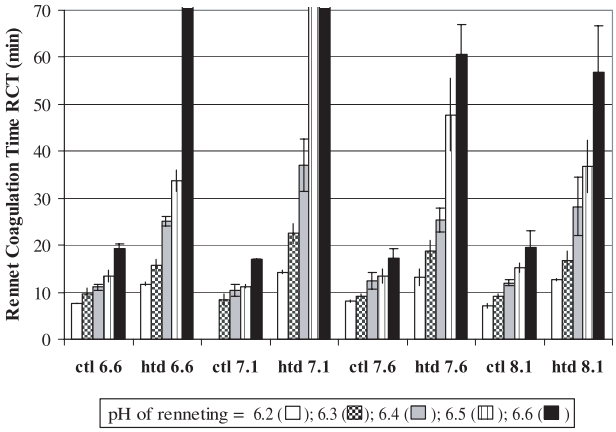

Figure 1. Rennet coagulation time (RCT) at 35 °C of reconstituted skim milk samples as a function of heat treatment (ctl = unheated control milk, htd = milk heated at 90 °C for 30 s), heat-treatment pH (6.6, 7.1, 7.6 or 8.1) and renneting pH (6.2, 6.3, 6.4, 6.5 or 6.6). The presented data were the average of 2 repeated experiments analysed twice each. Data is missing for control milk at pH 7.1. See Section 2.6 for experimental details.

given are the average of 10 readings, and each sample was analysed 2 or 3 times. The data was visualised using a Contin model, and the mode(s) of the weight-averaged particle size distribution were considered.

3. RESULTS

3.1. Rennet coagulation properties of milk heat-treated at alkaline pH

Figure 1 shows the average rennet coagulation time (RCT) values of the skim milk samples adjusted to pH values between 6.6 and 8.1, with or without heat treatment, stored overnight at the adjusted pH, then renneted at pH values ranging from 6.2 to 6.6. Variation of the renneting pH ensured that the optimum observation pH was visible, i.e. where gelation occurs and the effect of heating pH, if any, is not dominated by the effect of acidification on chymosin activity.

The effects of heating and of the renneting pH on the RCT were very significant ($P_0 < 0.001$). The heat treatment of milk induced considerable increase in the RCT, while acidification prior to renneting induced faster destabilisation of the milk system, as shown by reduction of the RCT. Both these effects are well documented, as stated in the introduction. Milk acidification prior to renneting is known to enhance chymosin activity, as well as the release of colloidal calcium into soluble form, decrease in repulsive electrostatic interaction between micelles, and subsequent destabilisation of micelles and increased gel firming [14, 20, 22, 32, 34].

Heating skim milk at pH values ranging from 6.6 to 8.1 also showed a significant effect on the RCT, albeit to a lower extent than either the heating pH or renneting pH factors ($P_0 < 0.01$). In heated samples, the RCT of the milk increased from pH 6.6 to 7.1, then decreased with increasing pH of heat treatment in the alkaline range 7.1 to 8.1 ($P_0 < 0.001$). The effect was strongest at renneting pH values of 6.4 to 6.6, where the positive effect of acidification on chymosin action did not totally screen the effect of heat-treatment pH.

Figure 2 shows the average gel firmness, aR, of the same skim milk samples adjusted to pH values between 6.6 and 8.1, with or without heat treatment, stored overnight at the adjusted pH, then renneted at pH values ranging from 6.2 to 6.6.

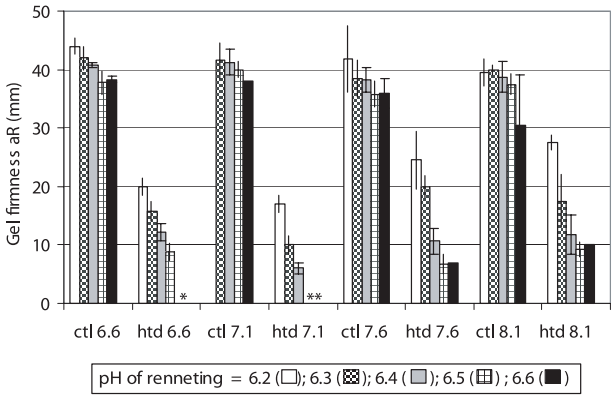

Figure 2. Gel firmness (aR) of reconstituted skim milk rennet gels obtained at 35 °C as a function of heat treatment (ctl = unheated control milk, htd = milk heated at 90 °C for 30 s), heat-treatment pH (6.6, 7.1, 7.6 or 8.1) and renneting pH (6.2, 6.3, 6.4, 6.5 or 6.6). The presented data were the average of 2 repeated experiments analysed twice each. The symbol (*) indicates 0 mm, i.e., no gel was formed. Data is missing for control milk at pH 7.1. See Section 2.6 for experimental details.

Again, the effects of both heat treatment and renneting pH were very significant ($P_0 < 0.001$). Heat treatment induced a reduction in the gel firming rate (not shown) and gel firmness, and acidification counteracted these effects due to a higher level of ionic calcium and decreased repulsive electrostatic interaction between micelles, as previously well reported in the literature. The effect of heat-treatment pH was not significant ($P_0 > 0.05$) when the aR response at all levels of the 3 factors (heat treatment, renneting pH and heat-treatment pH) are considered. It was, however, significant when heated samples were only considered ($P_0 < 0.01$). Gel firmness, aR, tended to increase upon increasing pH of heat treatment, except between pH 6.6 and 7.1. The positive effect of the heat-treatment pH on aR values was therefore strongest in the alkaline pH range 7.1 to 8.1 ($P_0 < 0.001$). This increase was more visible at renneting pH values of 6.5 or 6.6 where gels did not always form after heat treatment at pH 6.6 or 7.1 but reached significant aR values after heat treatment at pH 7.6 or 8.1.

These results showed a slight but significant recovery of the rennet coagulation properties of skim milk after heat treatment at alkaline pH values ranging from 7.1 to 8.1. The RCT responses obtained at pH 6.6 were often smaller than at higher pH, and aR values obtained at pH 6.6 were also often larger than those found at higher pH. These results distorted the otherwise rather linear and positive effect of alkaline pH of heat treatment on rennet coagulation properties of heated skim milk. These differences could be explained by the strong effect of acidification prior to rennet coagulation, as shown in Figures 1 and 2. Natural milk pH is usually 6.7 and adjusting the milk pH to even pH 6.6 introduces an acidification effect on skim milk rennet coagulation properties, as commonly exploited in the cheesemaking process [9, 21]. It is regrettable that the natural pH value of skim milk was not initially chosen as the start of the heating pH range explored in the present study.

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

Figure 3 shows the changes in the distribution of the κ-casein and of the whey proteins between the serum and the colloidal

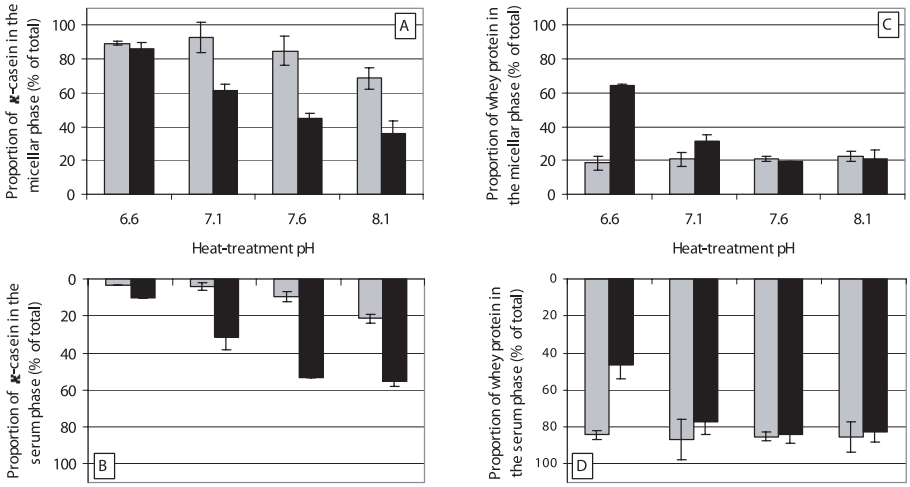

Figure 3. (Left) Proportions of κ-casein in the micellar phase (Fig. 3A) and in the serum phase (Fig. 3B) of reconstituted skim milk as a function of heat treatment (grey = unheated control; black = heated at 90 °C for 30 s) and heat-treatment pH. (Right) Proportions of whey proteins in the micellar phase (Fig. 3C) and in the serum phase (Fig. 3D) of reconstituted skim milk as a function of heat treatment (grey = unheated control; black = heated at 90 °C for 30 s) and heat-treatment pH. The results are given in percent of the total considered protein (total RP-HPLC area) found in the initial reconstituted skim milk. The presented data were the average of 2 repeated experiments analysed twice each. See Sections 2.7. and 2.8. for experimental details.

phases of reconstituted skim milk due to pH changes towards alkaline values or to heating at various heat-treatment pH values ranging from 6.6 to 8.1.

Figure 3 shows the pH-dependent protein exchanges between the colloidal and the serum phases during heating. The proportion of dissociated κ-casein significantly increased with increasing pH, reaching up to almost 60% of the total protein after heating at pH 7.6 and 8.1 (Figs. 3a and 3b). Conversely, little κ-casein was dissociated at pH 6.6 (~10%). The proportion of whey proteins found in the micellar phase significantly decreased with increasing pH, from ~60% of the total whey proteins at pH 6.6 to ~20% at pH 7.6 and 8.1 (Figs. 3c and 3d). No significant heat-induced change could be found at pH 7.6 and 8.1, which indicated that no (heat-denatured) whey proteins were bound to the micelles as a result of heat treatment at these alkaline pH values. The proportion of about 20% of the total whey protein found in the micellar pellet of unheated milk samples seemed too high to be accounted for only by the serum trapped within the casein micelles pellet upon ultracentrifugation. Another possible explanation may be based on the locally higher background noise of the RP-HPLC profiles in the whey protein region (not shown). Overall mass balance for the whey proteins consistently exceeded 100% by about 10%, which supports this explanation.

The results shown in Figure 3 therefore suggest that the heat-induced transfers of the two protein species were somehow related. More whey protein was attached to the micelles when less κ-casein was dissociated (at acidic pH values) or the whey protein remained in the serum phase when κ-casein extensively dissociated (at alkaline pH values).

Figures 3a and 3b also show a slight dissociation of the κ-casein from the micelle as the pH of the unheated control milk increased. About 10% and 20% of the total κ-casein was found in the serum phase of

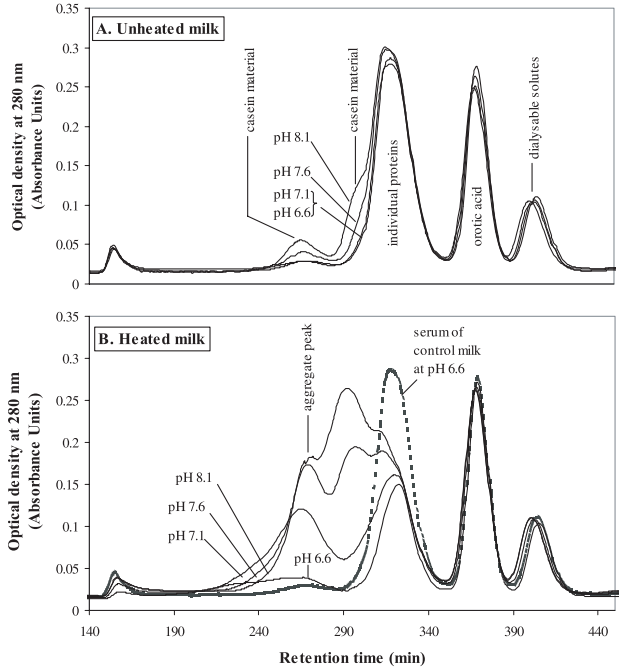

Figure 4. Typical elution profiles of the serum phase of control (Fig. 4a) and heated (90 °C for 30 s – Fig. 4b) skim milk samples at various heat-treatment pH values and analysed by size-exclusion fast protein liquid chromatography (SE-FPLC) on a Sephacryl S-500 HR Hi-Prep 16/90 column under isocratic conditions in 0.1 mol·L^{-1} Tris and 0.5 mol·L^{-1} NaCl. All samples were prepared and injected at least twice with satisfactory repeatability. See Sections 2.7 and 2.9 for experimental details.

the milk at pH 7.6 and 8.1, respectively, compared with less than 5% at pH 6.6 and 7.1 (Fig. 3b). Similar alkaline-induced dissociation rates were also calculated for β- and α_s-caseins, which indicated a non-specific phenomenon (not shown). The heat-induced dissociation rates were conversely much lower in the case of β- and α_s-caseins than for κ-casein (not shown). This indicated a significantly higher sensitivity of this casein to heat-induced dissociation from the micelles, in agreement with previous reports [1, 17, 30].

3.3. Concentration and size of the heat-induced serum protein aggregates

Concentration and size of the heat-induced serum whey protein/κ-casein aggregates were estimated by FPLC separation of the milk supernatants. Figure 4 shows typical elution profiles obtained for serums of the unheated skim milk samples at pH 6.6 to 8.1 (top) and serums of the same samples after heat treatment of the milk (bottom). Peak identification was performed after RP-HPLC analysis of the collected eluate fractions (and in comparison with earlier work [11]).

Figure 4a shows that the serums of unheated reconstituted skim milk contained individual proteins (mostly native whey proteins) as well as small non-protein components. The serums also contained two distinct protein materials eluting at ~270 and 300 min that were interpreted as two groups of submicelles or other specific casein materials (10^5 g·mol^{-1}-range) with consistent composition [11]. Figure 4a also shows

a significant increase in the two serum casein materials as the pH value of skim milk increased, in agreement with the alkaline-induced dissociation reported earlier (see Sect. 3.2.).

The heating of the milk induced a strong decrease in individual whey protein, as well as a large increase in the two peaks at 270 and 300 min (Fig. 4b). The latter results strongly suggest an increased casein dissociation upon heating, as reported earlier. The early eluting 270-min peak, however, became very rich in whey protein and κ-casein, and it is highly probable that a new co-eluting peak was generated during heating that corresponded to heat-induced aggregates composed of denatured whey proteins and κ-casein [11]. This aggregate peak eluted later, therefore the present aggregates were probably smaller than in previous reports [11, 16]. This was accounted for by the lower heat-treatment regime used in this study compared with previous studies which also used this FPLC method. The aggregate peak eluted at later elution times and its area tended to increase as heat-treatment pH increased (Fig. 4b). However, the concomitant increase and the slight shift toward earlier elution times of later eluting casein material showed less resolution at pH 8.1. These results indicate that a larger amount of smaller serum whey protein/κ-casein aggregates were formed upon heating milk at alkaline pH values.

3.4. Micelle size

The estimation of the amount and/or the size of micelle-bound whey protein/κ-casein aggregates was measured through micelle size measurement by dynamic light scattering (DLS) analysis of the centrifugal pellets of the milk samples at pH 6.6 to 8.1, with or without heating, resuspended in milk ultrafiltrate. Figure 5 shows the average values of micelle size as a function of heat-treatment pH and heating.

No significant differences were found between micelle sizes in control and heated milk samples, or across the pH values tested. There was a tendency for larger size dispersion as well as increased micelle diameter with increasing pH, possibly as a combined

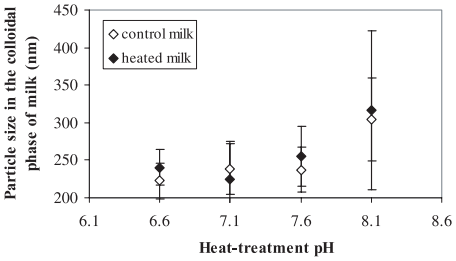

Figure 5. Average particle size as measured by dynamic light scattering (DLS) in the resuspended pellet fraction of control (open symbols) and heated (closed symbols) skim milk at various heat-treatment pH values. The presented data were the average of 2 repeated experiments analysed 3 to 5 times each. See Section 2.10 for experimental details.

result of increased dissociation of casein blocks (as seen in Fig. 4) and swelling of the casein micelle due to the increase in the electrostatic repulsion between negatively-charged casein molecules. Earlier studies have reported in detail the effect of heat-treatment pH values ranging from 6.5 to 6.7 and heating regime [3, 4]. In those studies, it was clearly demonstrated that increasing heating time or temperature, as well as decreasing the heat-treatment pH led to an increase in average micelle size. These results correlated well with the amount of denatured whey protein found to be associated with the casein micelles as a function of heating time, heating temperature and heat-treatment pH [3, 6]. The effect of the heat-treatment pH was, however, negligible and the effect of the heat-treatment itself seemed barely significant, at as low a heating regime as 90 °C for 30 s [3, 4], as used in the present study.

4. DISCUSSION

The results obtained in this study show that more κ-casein dissociated from the micelles and less denatured whey proteins were bound to the micelles as heat-treatment pH increased from 6.6 to 8.1. As a consequence, the two protein species were in the same phase at the end of the heating

process. This heat-induced distribution of the two protein species was accounted for by their interaction through hydrophobic bonding and thiol/disulfide interchanges to form heat-induced serum whey protein/κ-casein aggregates, in agreement with previous reports [3–6, 26, 32]. The analysis of the serum phase of reconstituted skim milk heated at pH values ranging from 6.6 to 8.1 suggested that smaller but more numerous heat-induced aggregates were present in the serum phase as heat-treatment pH increased, in agreement with previous studies [12, 24, 32]. The analysis of the micellar phase of the same milk samples did not, however, indicate that less whey protein/κ-casein aggregates could be bound to the casein micelles as heat-treatment pH increased. Earlier studies using the same method, however, reported a clear correlation between a lower heat-treatment pH and a higher proportion of micelle-bound whey protein/κ-casein aggregates [3–5]. The results of the present study were accounted for by the mild heating regime. They could also be explained due to the effect of increasing pH values in the alkaline range on the swelling of the micelle and on the proportion of whey protein transferred to the micellar phase upon heating (i.e. very little or none, see Fig. 3).

The increased proportion of heat-induced aggregates in the serum phase of milk, as pH increases, appears to be related to the increased dissociation rate of the casein molecules, and especially, of κ-casein, in alkaline pH and/or high temperature conditions [1]. The interaction between κ-casein and denatured whey proteins, and formation of aggregates upon heating, further enhance the dissociation rate of κ-casein, but the question of whether this interaction precedes, or follows, the dissociation of κ-casein is still debated [2, 4]. The smaller size of the heat-induced (serum) aggregates of whey proteins and κ-casein, as pH increases, may also be related to the higher dissociation rate of κ-casein ([24], Fig. 3), leading to a higher proportion of κ-casein in the aggregates [11]. The poor resolution of the heat-induced aggregates present in the serum samples by SE-FPLC (Fig. 4) did not, unfortunately, allow satisfactory quantitative analysis of their composition.

Furthermore, original relationships were drawn between this pH-dependent distribution of the heat-induced whey protein/κ-casein aggregates across the serum and colloidal phases, and the rennet coagulation properties of the heated skim milk. Good correlations ($R^2 > 85\%$) were found between the average values of both RCT and aR (taken for a renneting pH of 6.5) and the heat-induced protein exchanges between the serum and the micelle phases, at pH values > 6.7. The RCT decreased and gel firmness, aR, increased as heat-induced κ-casein dissociation increased and whey protein association decreased, as postulated. These R^2 values were, however, probably affected by the distribution of the data for the dissociation of κ-casein and the association of the whey protein, where the values found at pH 7.6 and 8.1 were very close and both quite different from those found at pH 7.1 (Fig. 3). Further research with a better controlled and wider spread of dissociation/association proportion values would be needed to confirm these relationships.

In an earlier study, Imafidon and Farkye [14] heated milk at pH 7.5 and at 75 °C for 16 s, followed by acidification to pH 6.4 prior to renneting. The RCT of the alkaline heat-treated milk was 6.07 min, versus 21.91 min for the control sample (not heat-treated, not acidified). The RCT was even shorter when the alkaline heat-treated milk sample was pH-cycled (i.e. stored overnight at pH 5.5 then adjusted to pH 6.4) prior to renneting. Singh and Waungana [29] confirmed that alkaline heat-treatment followed by pH-cycling gave the highest rennet coagulation properties for heated milk. These results indicated a positive effect of alkaline heat-treatment on rennet coagulation properties of heated milk; however, this effect was often also dependent on other experimental conditions such as storage conditions or pH changes that made it difficult to study independently. The present study used very standardised storage conditions with respect to temperature and duration, and kept the samples at their heat-treatment pH up to the last couple of hours before renneting in order to limit possible effects of pH changes other than that under study.

The present results show slightly improved rennet coagulation properties of heated skim milk, when the heat treatment is performed at alkaline pH up to 8.1. These results could be of interest to partially recover the renneting properties of a milk, heated to improve cheese yield through the recovery of the heat-denatured whey proteins. Further research, however, needs to be undertaken to ensure that the heat-induced whey protein/κ-casein aggregates are or can be retained in the curd upon cutting and drainage. It is suggested that cycling heat-treatment pH values could help further aggregate the serum whey protein/κ-casein aggregates up to sizes that would ensure proper trapping in the rennet curd. Optimisation of the alkaline heat-treatment method would also be needed, e.g. by controlled neutralisation of the milk after heat treatment, to ensure that minimum casein loss is suffered from alkalinisation of the milk and further technological operations up to moulding.

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