Effect of pH on the Thermal Denaturation of Whey Proteins in Milk

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The effect of pH on thermal denaturation of four main whey protein fractions in skim milk was examined by gel permeation FPLC. On heating skim milk at 80 °C for 0.5−20.0 min over the pH range 5.2−8.8, the extent of denaturation, based on loss of solubility at pH 4.6, increased with heating time and was usually in the order immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin > α-lactalbumin. Rates of denaturation of the immunoglobulins and the serum albumin/lactoferrin fraction were highest at the lower end of this pH range, whereas those of β-lactoglobulin and α-lactalbumin increased over most of the pH range. The effects of pH, addition of Ca, and reduction of disulfide bonds on the rates of the unfolding and aggregation stages of denaturation are discussed.

Keywords: *Whey protein; denaturation; aggregation; pH*

INTRODUCTION

Milk for liquid consumption or for processing requires an initial heat treatment to control the growth of microorganisms. Additional heating may be necessary at different stages in the manufacture of various products such as evaporated milks, coprecipitate, and milk powders. Heat treatment, however, may cause problems such as the formation of deposits on heat exchangers (Burton, 1968; Fryer et al., 1995), milk instability or gelling during the production of concentrated milks (Singh and Creamer, 1992), and age-thickening of concentrated milks (Harwalkar, 1992). Some of these difficulties are related to denaturation of the whey proteins on heating and are affected by the pH at which heat treatment is carried out.

Extensive studies have shown that denaturation of the whey proteins involves two main stages in which, on heating above 60 °C, the proteins lose their globular conformation, and then undergo aggregation (Rüegg et al., 1977; De Wit, 1981; Dannenberg and Kessler, 1988). On mild heating the changes in conformation may be reversible but on more severe heating the unfolded whey proteins become associated with other whey proteins or with the casein micelles (Mottar et al., 1989). The association may initially be through hydrophobic interaction, and later by formation of disulfide bonds with other whey proteins or κ-casein (Smits and Van Brouwershaven, 1980; Haque and Kinsella, 1988). Various studies have shown that the processes of unfolding and aggregation are quite distinct and occur to different extents depending on the pH of heating, protein concentration, and ionic strength (Smits and Van Brouwershaven, 1980; De Wit, 1981; Dannenberg and Kessler, 1988).

Several workers have studied the effect of pH of heating on the conformational changes in isolated whey protein fractions using direct scanning calorimetry (Rüegg et al., 1977; Bernal and Jelen, 1984, 1985; Lindström et al., 1994). Other workers have examined the effect of pH on the degree of aggregation of the whey proteins by measuring their change in solubility at pH 4.6 (Lyster, 1970) or the extent of their incorporation into rennet curd (Singh et al., 1988).

Because of their association with the casein micelles, the denatured whey proteins can be recovered from heat-treated milk on a commercial scale together with the casein by acid precipitation, as coprecipitate. Also, in the manufacture of cheese, the denatured whey proteins remain associated with the micelles during rennet coagulation and are retained in the curd, giving substantial increases in yield (Law et al., 1994). There is little information, however, on how the rates of thermal denaturation of the individual whey proteins vary with the pH at which the milk is heated. In a previous study (Law et al., 1994), we described changes in the rates of denaturation of β-lactoglobulin and α-lactalbumin in milk with pH for a single short heating time (90 °C for 30 s). In the present work, we have extended the scope of the study and have examined the effects of pH with a range of heating times up to 20 min at 80 °C on rates of denaturation of four main whey protein fractions in milk. We have also studied the effects of changes in pH and temperature prior to thermal treatment, as these are manipulations commonly carried out during processing and are known to affect the serum−micellar equilibrium.

When milk is acidified, colloidal calcium phosphate is removed from the casein micelles, and levels of serum Ca increase. On the other hand, at alkaline pH, the intramolecular disulfide bonds that help to maintain the secondary structure of the whey proteins are more easily broken. To try and understand the separate effects of pH on the unfolding and aggregation stages, we have studied changes in the rates of denaturation of the individual whey proteins in milk on adding $CaCl_2$ to increase the serum concentration of Ca, and on adding 2-mercaptoethanol (ME) to disrupt intramolecular disulfide bonds in the whey proteins and promote disulfide interchange reactions.

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10.1021/jf981302b CCC: $19.00
Published on Web 02/26/2000

MATERIALS AND METHODS

Milk Samples. Milk samples were collected from the bulk milk of Friesian cows in the Institute herd. The milks were skimmed by centrifugation at 1000*g* for 30 min at 4 °C, and equilibrated at 20 °C for about 1 h before adjusting the pH.

pH-Adjusted Milks. A range of 18 milks between pH 5.2 and 8.8, at 20 °C, was prepared by the addition of 1.0 M HCl or NaOH, with rapid stirring. The samples were stirred for an additional 15 min before heat treatment.

Heat Treatment. Skim milk samples (10 mL) were placed in thin-walled glass test tubes 18 mm in diameter and 150 mm in length. The tubes were placed in a waterbath at 80 °C and allowed 1 min to warm to temperature. Samples (1.0 mL) were removed at intervals of 0.5, 1.0, 3.0, 5.0, 10.0, and 20.0 min, and rapidly cooled in ice.

Determination of Levels of Denaturation of Whey Proteins. Skim milk samples (1.0 mL) from raw and heat-treated milks were diluted with 1.0 mL of water and acidified to pH 4.6 to precipitate casein and denatured whey proteins by the addition of 1.0 mL of a 1:1 mixture of 0.83 M acetic acid and 0.2 M sodium acetate. The solutions were stirred for 20 min and centrifuged at 1000*g* for 5 min in a Mistral 6L centrifuge (Fisher Scientific, Crawley, Sussex, U.K.). Each supernatant was passed through a 0.2 μm filter. Gel permeation FPLC (fast protein liquid chromatography) of acid filtrates from raw and heated milks was carried out on a Superdex 75 HR 10/30 column (Pharmacia Biotech, St. Albans, U.K.), and levels of denatured whey proteins were calculated as described previously (Law et al., 1993).

The effects of pH-cycling (reducing or increasing the pH and then restoring to that of the original milk), cold storage, and addition of $CaCl_2$ or 2-mercaptoethanol (ME) on levels of heat denaturation of the whey proteins were also examined as described below.

pH-Cycled Milks. Milk was cooled to 4 °C and acidified to pH 5.2 by the addition of 1.0 M HCl. The milk was maintained at this pH for 45 min and then warmed to 20 °C and readjusted to pH 6.7 by the addition of 1.0 M NaOH. The milk was kept at pH 6.7 for 1 h before heat treatment. A second sample of milk was adjusted to pH 9.5 at 20 °C by the addition of 1 M NaOH. The milk was maintained at this pH for 10 min and then readjusted to pH 6.7 by the addition of 1.0 M HCl. The milk was kept at this pH for 15 min before heat treatment.

Cold Storage. A sample of skim milk was cooled to 4 °C and stored for 22 h. The skim milk was reequilibrated at 20 °C for 2 h before heat treatment.

Addition of $CaCl_2$. To examine the effect of Ca^{2+} on levels of denaturation of the whey proteins, $CaCl_2$ was added at a concentration of 30 mM to give a serum concentration similar to the total Ca concentration in skim milk. The pH was adjusted to that of the original skim milk by the addition of a small amount of 1.0 M NaOH, and the milk was stirred for 30 min before heat treatment.

Addition of ME. ME was added to selected skim milk samples at concentrations of 5 and 10 mM (a large excess in relation to the total concentration of sulfhydryl groups in the milk proteins), and the milk was stirred for 1 h before heat treatment. ME was also added at a concentration of 10 mM to some samples after heat treatment, with stirring for 1 h before the levels of denaturation of the whey proteins were determined .

Determination of Level of Serum Casein. To examine the effect of ME on casein micelles, levels of serum casein in raw milk before and after adding ME were determined. Micellar casein in skim milk, and in skim milk treated with ME at a concentration of 70 mM for 1 h, was sedimented at 20 °C by centrifugation for 2 h at 70 000*g* in a 6 × 38 mL swing-out rotor in an MSE Superspeed 65 ultracentrifuge (MSE Scientific Instruments, Crawley, U.K.). The levels of serum casein, before and after addition of ME, were determined by Kjeldahl analysis for total and non-casein N in the skim milks and supernatants as described previously (Davies and Law, 1983).

RESULTS

Effect of pH of Heating. Milk samples were heated at 80 °C for 0.5−20 min, and the extent of denaturation of four whey protein fractions, on the basis of loss of solubility at pH 4.6, was determined by gel permeation FPLC. The reproducibility of the method was satisfactory, and values for the standard deviations in determination of the levels of percentage denaturation of the whey proteins were as follows: total whey proteins, 0.9; immunoglobulins, 1.7; serum albumin/lactoferrin, 1.5; β-lactoglobulin, 0.7; α-lactalbumin, 2.1. Between pH 5.2 and 5.6, extensive coagulation of the milk occurred as it was warmed to 80 °C. As the pH was increased, coagulation occurred less readily, and at pH 5.8, 6.0, and 6.1 coagulation times were less than 3, 5, and 20 min, respectively. Above pH 6.1, there was no obvious coagulation of the milk with heating times up to 20 min.

The rates of denaturation of the four whey protein fractions increased with heating time, and varied considerably with pH (Figure 1). Over most of the pH range, for equivalent heating times, the susceptibility of the whey proteins to denaturation was in the order immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin> α-lactalbumin. For most heating times, the rates of denaturation of the immunoglobulins were highest at about pH 6.0, decreasing to a minimum about pH 7.4, increasing up to about pH 7.8, and then decreasing slightly at higher pH. The rate of denaturation of the serum albumin/lactoferrin fraction, however, tended to be highest at low pH, decreasing to a minimum about pH 7.4, increasing up to about pH 7.8, and then decreasing slightly at higher pH. The rate of denaturation of β-lactoglobulin increased rapidly between pH 5.2 and 6.1, decreased to about pH 6.8, and then increased rapidly up to pH 8.8. The minimum in the rate of denaturation of β-lactoglobulin near the natural pH of milk became less pronounced with increasing heating time. The rate of denaturation of α-lactalbumin decreased between pH 5.2 and 6.0, increased slightly close to pH 6.2, and then increased fairly rapidly up to pH 8.8.

Denaturation of the immunoglobulins and the serum albumin/lactoferrin fraction did not follow simple kinetics. It was possible, however, to determine the orders of reaction and rate constants for denaturation of β-lactoglobulin and α-lactalbumin at each pH by examining the closeness of fit of the denaturation data to two expressions derived from the general rate equation as described previously (Lyster, 1970; Hillier and Lyster, 1979; Dannenberg and Kessler, 1988). For a reaction order, $n \neq 1$, $(C_t/C_0)^{1-n} = 1 + (n-1)kt$, where C_0 is the initial protein concentration, C_t is the native protein concentration at time t, and k is the rate constant for denaturation. In the special case of a first-order reaction, $\ln(C_t/C_0) = -kt$.

Plots of $(C_0/C_t)^{n-1}$ against holding time showed that the apparent reaction order (n) for denaturation of β-lactoglobulin at pH 5.2 was 1.5 (Figure 2a; Table 1). Between pH 5.8 and 6.2, the value increased to about 1.8, but between pH 6.3 and 7.0, the value for the reaction order decreased to about 1.6. Above pH 7.0 the reaction order increased gradually up to 2.3.

Plots of log (C_t/C_0) against holding time (Figure 2b; Table 1) indicated that the apparent reaction order for denaturation of α-lactalbumin between pH 5.2 and 7.8 was close to 1.0. Plots of $(C_0/C_t)^{n-1}$ against holding time

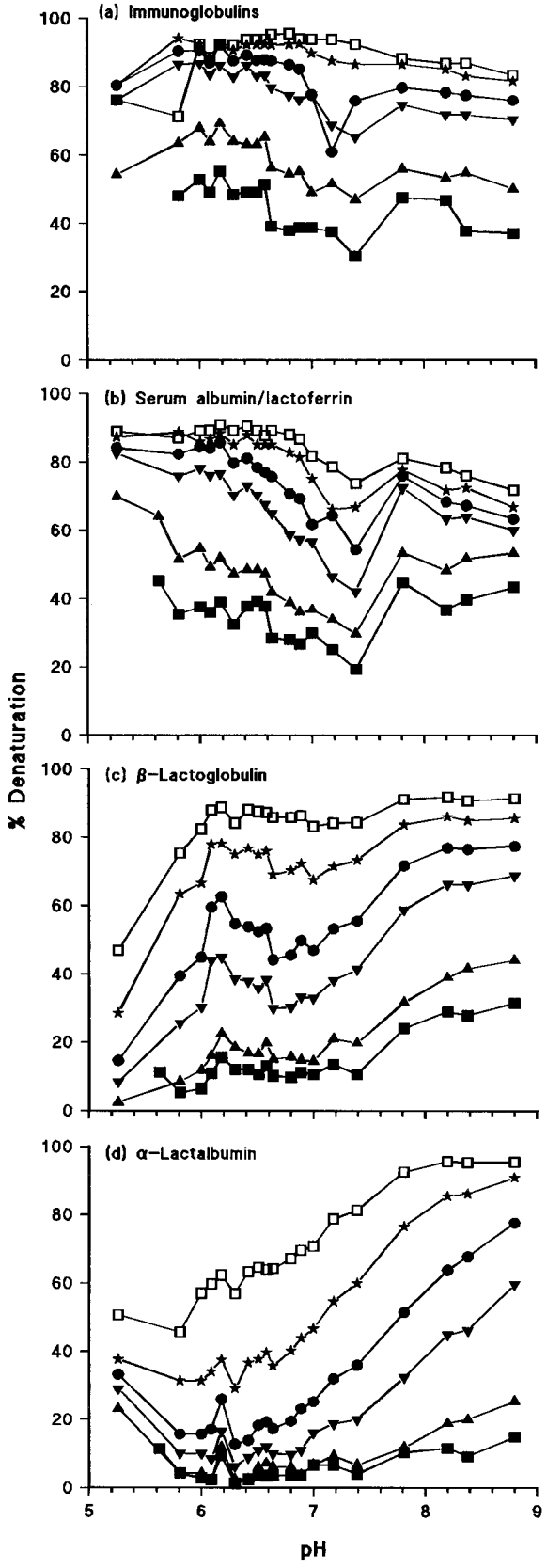

Figure 1. Effect of pH on rates of denaturation in skim milk of (a) immunoglobulins, (b) serum albumin/lactoferrin, (c) β-lactoglobulin, and (d) α-lactalbumin. Holding times (min) at 80 °C were (■) 0.5, (▲) 1.0, (▼) 3.0, (●) 5.0, (★) 10.0, and (□) 20.0.

(Figure 2c; Table 1) showed that above pH 7.8 the order of reaction increased rapidly to 1.6.

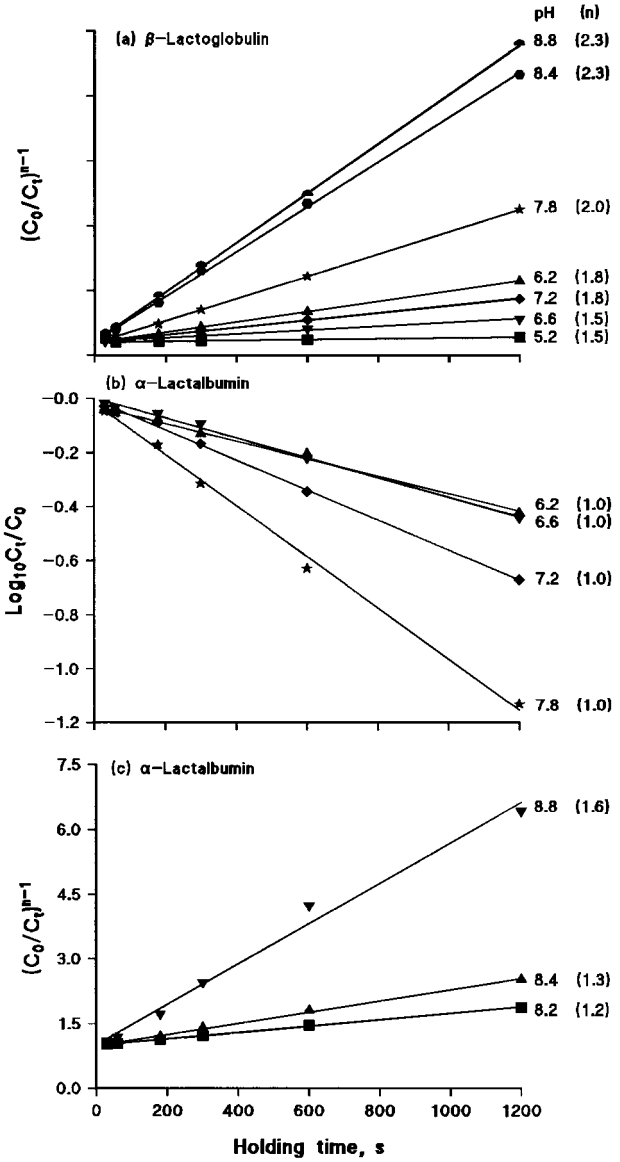

Figure 2. Kinetics plots for denaturation of whey proteins on heating skim milk (80 °C) between pH 5.2 and 8.8: (a) β-lactoglobulin represented with reaction orders between 1.5 and 2.3, (b) α-lactalbumin below pH 7.8 represented with a reaction order of 1.0, and (c) α-lactalbumin above pH 7.8 represented with reaction orders between 1.2 and 1.6.

The rate constants for denaturation of β-lactoglobulin and α-lactalbumin were calculated from the slopes of the respective lines (Figure 2; Table 1) as described by Dannenberg and Kessler (1988). The plots of the rate constant for β-lactoglobulin (Figure 3) show that there was an increase between pH 5.2 and 6.2, followed by a decrease to about pH 7.0, and thereafter a rapid increase to pH 8.8. The rate constant for denaturation of α-lactalbumin, however, increased from pH 5.2 to 8.8, the increase being more rapid above about pH 7.5.

Composition of Undenatured Whey Protein. The composition of undenatured whey protein, and of denatured whey protein that precipitated together with casein at pH 4.6, varied with the pH of heating (Figure 4). Compared with undenatured whey protein in skim milk heated at pH 6.7, that from milk heated (80 °C for 5 min) at pH 5.2 contained more β-lactoglobulin and less α-lactalbumin, whereas that from milk heated at pH 6.2 contained less β-lactoglobulin and more α-lactalbumin. Above pH 6.7, the relative proportions of β-lactoglobulin

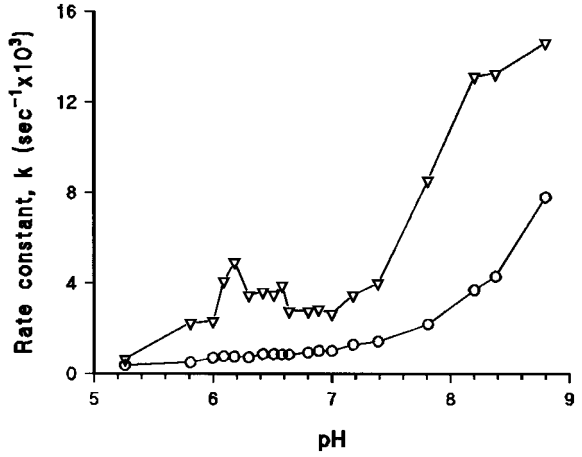

Figure 3. Changes in the rate constants for denaturation of (▽) β-lactoglobulin and (○) α-lactalbumin with pH. The rate constants were determined from kinetics plots as shown in Figure 2.

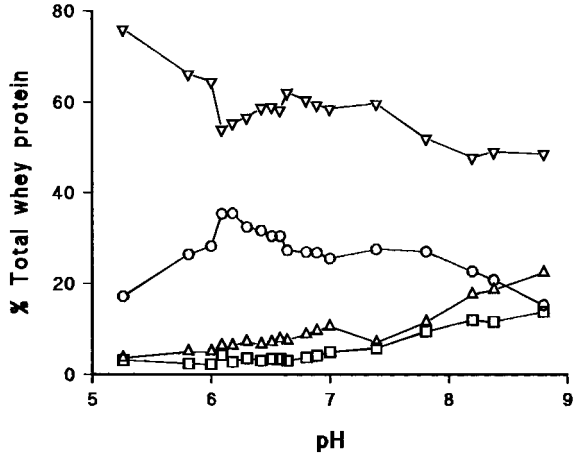

Figure 4. Effect of pH of heating (80 °C for 5 min) on the percentage composition of undenatured whey proteins in skim milk: (□) immunoglobulins, (△) serum albumin/lactoferrin, (▽) β-lactoglobulin, and (○) α-lactalbumin.

Table 1. Apparent Reaction Orders (*n*) and Rate Constants (*k*) for Denaturation of β-Lactoglobulin and α-Lactalbumin between pH 5.2 and 8.8

	β-lactoglobulin		α-lactalbumin	
pH	*n*	k ($s^{-1} \times 10^3$)	*n*	k ($s^{-1} \times 10^3$)
5.2	1.5	0.64	1.0	0.37
5.8	1.8	2.25	1.2	0.50
6.0	1.5	2.32	1.0	0.70
6.1	1.7	4.10	1.0	0.77
6.2	1.8	4.94	1.0	0.75
6.3	1.6	3.48	1.0	0.72
6.4	1.6	3.63	1.0	0.86
6.5	1.6	3.50	1.0	0.86
6.6	1.5	2.78	1.0	0.85
6.8	1.5	2.78	1.0	0.93
6.9	1.5	2.86	1.0	1.01
7.0	1.6	2.65	1.0	1.01
7.2	1.8	3.48	1.0	1.28
7.4	1.9	4.01	1.0	1.42
7.8	2.0	8.55	1.0	2.18
8.2	2.2	13.15	1.2	3.70
8.4	2.3	13.26	1.3	4.30
8.8	2.3	14.63	1.6	7.80

and α-lactalbumin in undenatured whey protein decreased gradually whereas those of the immunoglobulins and the serum albumin/lactoferrin fraction increased.

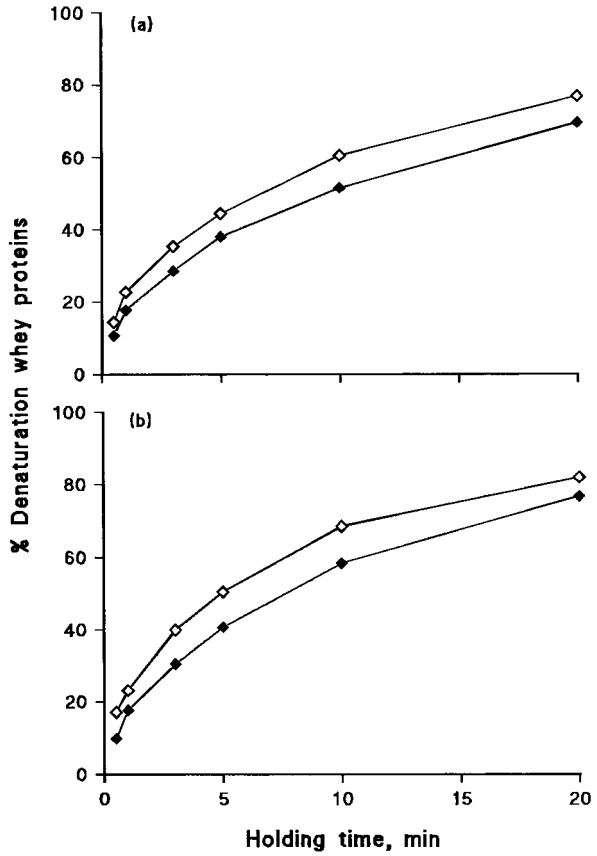

Figure 5. Rates of denaturation (80 °C) of total whey protein in (a) (◇) skim milk, (◆) skim milk adjusted to pH 5.2 at 4 °C for 45 min and readjusted to pH 6.7 at 20 °C for 1 h, and (b) (◇) skim milk, (◆) skim milk adjusted to pH 9.5 at 20 °C for 10 min and readjusted to pH 6.7 at 20 °C for 15 min.

pH-Cycled Milk. When milk was acidified to 5.2 and readjusted to pH 6.7 before heating at 80 °C, the level of denaturation of total whey proteins (Figure 5a) was significantly reduced ($P < 0.001$). The level of denaturation of immunoglobulins also decreased slightly whereas that of the serum albumin/lactoferrin fraction increased slightly (results not shown). There were more pronounced reductions in the levels of denaturation of β-lactoglobulin and α-lactalbumin, and the orders of reaction and rate constants for denaturation were calculated as described above. Following pH adjustment, the orders of reaction for denaturation of β-lactoglobulin and α-lactalbumin were 1.5 and 1.0, respectively, as found for the original milk. However, after pH adjustment of milk, the rate constant for denaturation of β-lactoglobulin decreased from 2.03×10^{-3} to 1.43×10^{-3} s^{-1}, and that for α-lactalbumin decreased from 0.65×10^{-3} to 0.42×10^{-3} s^{-1}.

When milk was adjusted to pH 9.5 and readjusted to pH 6.7 before heating at 80 °C, there was a significant reduction ($P < 0.001$) in the rate of denaturation of total whey protein similar to that obtained after acidification and readjustment to pH 6.7 (Figure 5b).

Effect of Cooling and Rewarming. On storing milk at 4 °C for 22 h, and reequilibrating at 20 °C for 2 h before heat treatment, there was no significant change in the rate of denaturation of total whey protein (Figure 6).

Addition of $CaCl_2$. On adding $CaCl_2$ (30 mM) and heating at 80 °C and pH 6.7, the milk coagulated in less than 2 min. In the presence of Ca^{2+}, up until the point

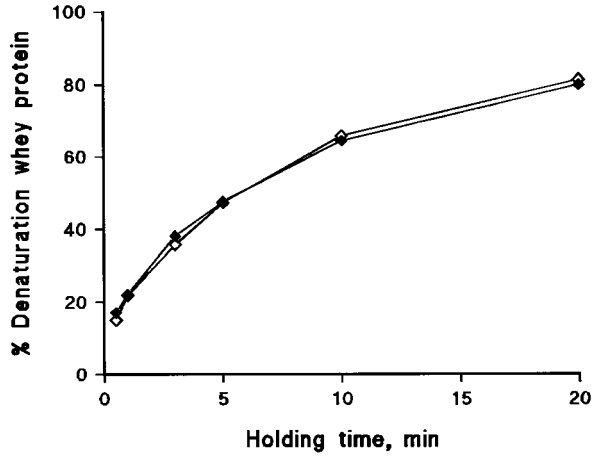

Figure 6. Rates of denaturation (80 °C) of total whey protein in (◇) skim milk and (◆) skim milk cooled to 4 °C for 22 h and reequilibrated at 20 °C for 2 h before heating.

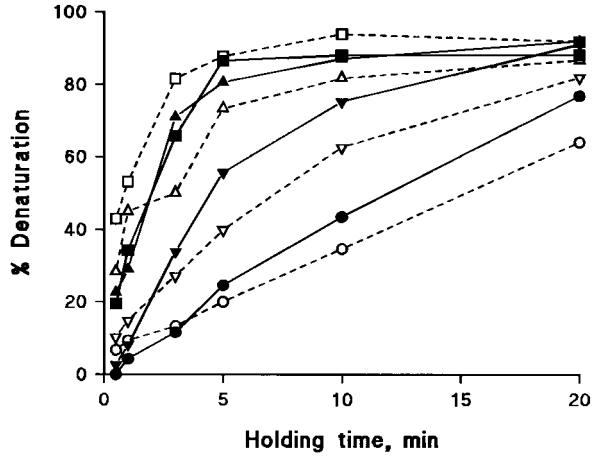

Figure 7. Effect of addition of $CaCl_2$ on rates of denaturation of individual whey proteins in skim milk heated at 80 °C: (□) immunoglobulins, (△) serum albumin/lactoferrin, (▽) β-lactoglobulin, and (○) α-lactalbumin. Skim milk with $CaCl_2$ (30 mM): (■) immunoglobulins, (▲) serum albumin/lactoferrin, (▼) β-lactoglobulin, and (●) α-lactalbumin.

at which coagulation of the milk occurred, the rate of denaturation of each of the whey proteins was slightly lower than in the original skim milk (Figure 7), but thereafter the rates of denaturation of all of the whey proteins, except the immunoglobulins, were significantly greater ($P < 0.001$) than in skim milk. The orders of reaction for denaturation of β-lactoglobulin and α-lactalbumin were 1.5 and 1.0, respectively, as found for the original milk. However, on addition of $CaCl_2$, the rate constant for denaturation of β-lactoglobulin increased from 2.22×10^{-3} to 3.97×10^{-3} s^{-1}, and that for α-lactalbumin increased from 0.81×10^{-3} to 1.24×10^{-3} s^{-1}.

Addition of ME before Heat Treatment. The addition of ME (5 mM) to milk 1 h before heat treatment gave significant increases ($P < 0.001$) in the rates of denaturation of all the whey proteins, particularly of α-lactalbumin (Figure 8). Doubling the concentration of ME from 5 to 10 mM gave further slight increases in the rates of denaturation of the whey proteins. Following addition of ME, the order of reaction for denaturation of β-lactoglobulin was 1.5, as found in the original milk. However, with ME at concentrations of 5 and 10 mM, the rate constant for denaturation of β-lactoglobulin increased from 2.00×10^{-3} for the control to 8.28

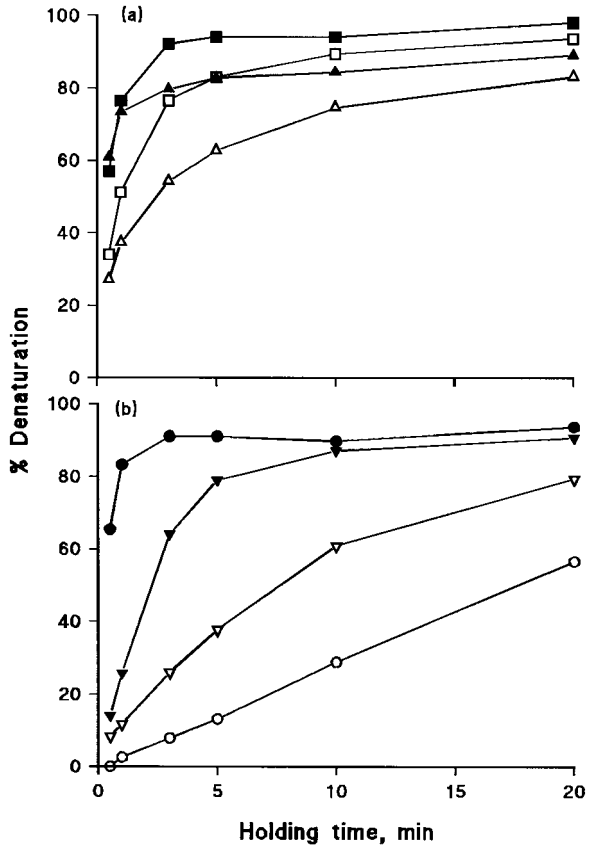

Figure 8. Effect of addition of ME to skim milk before heating at 80 °C, on denaturation of whey proteins: (a) (□) immunoglobulins and (△) serum albumin/lactoferrin. With ME (5 mM): (■) immunoglobulins and (▲) serum albumin/lactoferrin. (b) (▽) β-lactoglobulin and (○) α-lactalbumin. With ME (5 mM): (▼) β-lactoglobulin and (●) α-lactalbumin.

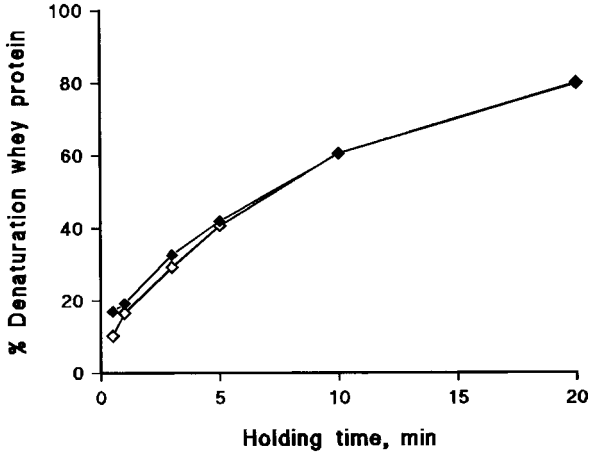

Figure 9. Effect of addition of ME to skim milk after heating at 80 °C, on denaturation of whey proteins: (◇) whey proteins and (◆) whey proteins with 10 mM ME.

$\times 10^{-3}$ and 9.34×10^{-3} s^{-1}, respectively. In the presence of ME, denaturation of α-lactalbumin occurred too rapidly at 80 °C to permit accurate determination of the reaction order or rate constant.

Addition of ME after Heat Treatment. ME (10 mM) was added to milk samples that had been heated at 80 °C and cooled to 20 °C, and stirred for 1 h. Results show that the addition of ME after heating had no significant effect on the extent of denaturation of total whey protein (Figure 9) or individual whey proteins, as measured by the present method.

DISCUSSION

Over most of the pH range 5.2−8.8, the susceptibility of the whey proteins to denaturation, determined on the basis of loss of solubility at pH 4.6, was in the order immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin > α-lactalbumin. However, the rates of denaturation of the immunoglobulins and the serum albumin/lactoferrin fraction tended to decrease with increasing pH, whereas those for β-lactoglobulin and α-lactalbumin increased markedly with pH.

Grufferty and Mulvihill (1987) also found that when milk was heated at 80 °C for 15 min the degree of denaturation of total whey protein, determined by the amount of total protein precipitated at pH 4.6, increased as the pH of heating was increased between 6.5 and 7.5. However, in contrast to the present work, these workers found that the level of denaturation decreased as the pH was further increased to 8.0. Lyster (1970) found that, between pH 6.2 and 6.9, the rates of denaturation of β-lactoglobulin and α-lactalbumin, as measured by changes in solubility at pH 4.6, were independent of pH of heating. Above pH 6.9, as in the present work, the rates of denaturation of both proteins increased. Lyster (1970) also found that, in contrast to the present study, the rates of denaturation of β-lactoglobulin and α-lactalbumin both increased below pH 6.2.

The changes in rates of denaturation of β-lactoglobulin and α-lactalbumin with pH found here were similar to those in a previous study (Law et al., 1994) in which milk was heated (90 °C for 0.5 min) between pH 6.2 and 9.1. The present work, however, gives additional information about changes over the pH range with increased heating time. There was some indication, for example, that the minima obtained for the rates of denaturation of β-lactoglobulin and α-lactalbumin near pH 6.7 and 6.4, respectively, for short heating times, were less pronounced at longer heating times. We are not aware of any comparable studies of changes in the rates of denaturation of the immunoglobulins and serum albumin/lactoferrin fractions with pH, but the results here clearly show differences in their behavior from β-lactoglobulin and α-lactalbumin.

Because the rates of denaturation of the individual whey proteins varied in different ways with pH, the composition of the undenatured whey protein, and of denatured whey protein that precipitated at pH 4.6, was affected by the pH at which milk was heated. Similarly, the relative amounts of denatured whey proteins incorporated into rennet curd would be affected by the pH of the thermal treatment. For a given heating time at high pH, compared with coprecipitate or rennet curd from milk heated at pH 6.7, that from milk treated at high pH would contain lower relative amounts of immunoglobulins and serum albumin/lactoferrin fractions, and higher proportions of β-lactoglobulin and α-lactalbumin. The pH of heating also affects the functional properties of the whey protein isolate or coprecipitate. Grufferty and Mulvihill (1987) found, for example, that the protein which precipitated from milk heated above pH 7.5 had better solubility properties than that from milk heated at its natural pH of 6.5.

In the present study, lowering or increasing the pH of milk, followed by reequilibration at pH 6.7, reduced the rates of thermal denaturation of the whey proteins. On acidifying milk to pH 5.0 at 4 °C for short periods, most of the colloidal calcium phosphate is solubilized and the caseins begin to dissociate from the micelles. On adjusting the pH of skim milk to 9.5, however, the concentration of calcium phosphate in the serum decreases, whereas the level of serum casein increases. It is not clear to what extent these changes are reversed when the milk is readjusted to pH 6.7. Lucey et al. (1996) have shown that, following acidification and readjustment of the pH, there is an increase in Ca^{2+} activity in the milk and little reformation of colloidal calcium phosphate. Using electron microscopy, the above workers showed that after acidification and reneutralization there was increased clustering of casein particles, which they attributed mainly to a reduction in electrostatic repulsion during the acidification period. The increased clustering may be partly responsible for the reduction in rates of aggregation of the whey proteins found in the present work, particularly of β-lactoglobulin and α-lactalbumin, which are known to become attached to the micellar surface (Mottar et al., 1989). The increase in ionic strength of the serum on adding NaOH and HCl to adjust the pH of the milk may also help to promote aggregation of the denatured whey proteins.

On cooling milk to 4 °C, and reequilibrating at 20 °C for 2 h, the rates of subsequent thermal denaturation of the whey proteins were unchanged. Cooling milk to 4 °C for 22 h leads to extensive dissociation of β-casein from the micelles, and rewarming to 20 °C gives a decrease in the level of serum β-casein (Davies and Law, 1983). It has not been established, however, to what extent the micelles regain their original structure. The results of the present work indicate that cooling and rewarming milk before heat treatment had little effect on the subsequent interaction of denatured whey proteins with the micelles.

The addition of ME to milk before heat treatment gave substantial increases in the rates of denaturation of all the whey proteins, particularly of α-lactalbumin. The levels of serum casein in skim milk before, and 2 h after, addition of ME were 7.9 and 9.1% of the total casein, respectively. It is unlikely that the increase in rates of denaturation of the whey proteins in the presence of ME was due to changes in the serum−micellar equilibrium, unless the small increase in serum casein was due to selective dissociation of κ-casein from the micelles. Extensive studies have shown that during the unfolding stage of heat denaturation of β-lactoglobulin and other whey proteins, intramolecular disulfide bonds are broken, and −SH groups are free to react with disulfide bonds in other whey proteins or in κ-casein. Addition of ME would similarly disrupt intramolecular disulfide bonds and promote unfolding of the whey proteins, thereby increasing the overall levels of denaturation. The results of direct scanning calorimetry have shown that α-lactalbumin unfolds on heating but also readily renatures. The marked effect of ME in increasing thermal denaturation of α-lactalbumin may be due to prevention of reformation of intramolecular disulfide bonds.

The addition of ME after heat treatment had no effect on the rates of denaturation of the whey proteins as measured by the present method. These results are consistent with previous findings that, although aggregation of whey proteins on heating includes the formation of disulfide bonds, other forces are also involved in maintaining the complexes. In a study of the heat-induced interaction of β-lactoglobulin and κ-casein in micelles, Smits and Van Brouwershaven (1980) showed

that ionic strength, concentration of Ca^{2+}, and pH also affected the degree of association. Haque and Kinsella (1988) showed that hydrophobic interactions were important in maintaining the complex especially in the initial stages, and that covalent disulfide bonds were formed later.

In this study, addition of $CaCl_2$ to skim milk before heat treatment gave a marked increase in the rates of denaturation of all the whey proteins, except for the immunoglobulins. Few studies have been carried out on the effect of increasing Ca^{2+} concentration on rates of denaturation of whey proteins in milk itself. Work on whey and whey protein isolates has shown that increasing the concentration of Ca^{2+} affects both the unfolding and aggregation stages of β-lactoglobulin and α-lactalbumin. Bernal and Jelen (1984) showed that the binding of small amounts of Ca^{2+} by α-lactalbumin actually increased its heat stability by promoting renaturation on cooling. However, Li et al. (1994) found from ^1H NMR studies that Ca^{2+} stabilized the unfolded formation of β-lactoglobulin and, therefore, promoted its denaturation. On studying the effect on aggregation in rennet whey heated at 90 °C for 10 min (pH 6.5), Donovan and Mulvihill (1987) found that the addition of $CaCl_2$ (10 mM) gave a marked increase in the amount of sedimentable N. Similar increases in the extent of aggregation have been found for whey protein isolate, α-lactalbumin (Patocka and Jelen, 1991) and β-lactoglobulin (Xiong et al., 1993; Sherwin and Foegeding, 1977). Donovan and Mulvihill (1987) established that aggregation occurred more readily below pH 6.0 and was less sensitive to the addition of $CaCl_2$. They concluded that Ca^{2+} promoted aggregation by binding to the whey proteins and reducing their net negative charge.

Results of the present study indicate that the rates of denaturation of the whey proteins may depend on the rates of both the unfolding and aggregation stages of the individual whey proteins, and these vary in different ways with pH. The isoelectric points of most of the whey proteins are in the lower region of the pH range studied (Eigel et al., 1984). Over the greater part of the pH range 5.2−8.8, therefore, the net negative charge on most of the whey proteins increases with pH. As the pH and net negative charge increase, the intramolecular electrostatic repulsion promotes unfolding of the molecules, allowing aggregation to occur. Using radiolabeled whey proteins, Noh and Richardson (1989) showed that, on heating, some of the β-lactoglobulin and α-lactalbumin become associated with κ-casein in the micelles. In previous work with heating conditions similar to the present study, it was found that at pH 6.7 the denatured whey proteins were almost even distributed between the serum and the casein micelles (Law, 1996). Above pH 7.0, the ionization and activity of thiol groups increase, and similarly promote unfolding of the globular conformations of the whey proteins. In the present work, addition of ME which reduces intramolecular disulfide bonds and promotes unfolding of the whey proteins gave a marked increase in rates of thermal denaturation of all the whey proteins, particularly of α-lactalbumin.

β-Lactoglobulin, and to a lesser extent α-lactalbumin, also showed an increase in their rates of denaturation on heating milk at about pH 6.2. On acidifying milk to this pH, there is an appreciable increase in the concentration of serum Ca^{2+} due to solubilization of colloidal calcium phosphate, and this may promote aggregation. In this study, addition of $CaCl_2$ before heating gave an increase in the rate of aggregation of each of the whey proteins except the immunoglobulins. Previous studies have shown that Ca^{2+} promotes aggregation by binding to the whey proteins and effectively reducing their net negative charge (Donovan and Mulvihill, 1987). Although the concentration of serum Ca^{2+} continues to increase as the pH is reduced below 6.0, the effect of this increase would be diminished as the net negative charge on the proteins decreases appreciably.

Detailed studies have shown that changes in the conformation of β-lactoglobulin on heating involve a Tanford-like transition with a midpoint about pH 6.3 at 70 °C (Dupont, 1965). This transition is accompanied by dimer−monomer dissociation, loss of charge on a His residue, and greater accessibility of the free −SH group. The increase found, in the present study, in the rate of denaturation of β-lactoglobulin at pH 6.2 and 80 °C may result in part from the greater accessibility of the free −SH group and increased intermolecular disulfide linkage.

The results of this study show that the changes in the rates of thermal denaturation of the whey proteins with pH are complex and depend on the rates of the unfolding and aggregation stages. The rates of denaturation of the immunoglobulins and the serum albumin/lactoferrin fraction tended to decrease with pH whereas the rates of denaturation of β-lactoglobulin and α-lactalbumin were highest at high pH. These differences in the behavior of the individual whey proteins may have practical benefit and, by selection of suitable heating conditions, provide a means of preparing coprecipitates or rennet curd with specific proportions of denatured whey proteins and different functional properties.

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Received for review November 30, 1998. Revised manuscript received June 23, 1999. Accepted December 30, 1999. This research was funded by the Scottish Office Agriculture, Environment and Fisheries Department (SOAEFD).

JF981302B