Heat-Induced Interactions between Serum Albumin, Immunoglobulin, and κ-Casein Inhibit the Primary Phase of Renneting

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Skimmed bovine milk and casein micelles (in the presence and absence of various concentrations of individual whey proteins) were heated at 80 °C for 30 min. Cloned chymosin was added to the cooled preparations and the enzyme-catalyzed hydrolysis of κ-casein determined by measuring the formation of caseinomacropeptide. All of the whey proteins tested caused an inhibition of the enzymatic reaction. Heating micelles with individual whey proteins at temperatures as low as 60 °C also inhibited both the rate and the extent of the κ-casein hydrolysis. The results show that interactions between κ-casein and immunoglobulin G and serum albumin, in addition to the well-documented interaction with β-lactoglobulin, inhibit the primary phase of the renneting reaction.

Keywords: *Milk; casein micelles; renneting; heated; κ-casein; whey proteins*

INTRODUCTION

Milk is a colloidal system in which approximately 80% of the total protein is packaged into submicrometer sized particles called micelles. Although various models for the structure of these micelles have been proposed [see review by Holt (1992)], all agree that their integrity is crucially dependent upon the properties of one component, the κ-casein. Most of this protein is located on the surface of the micelle where the highly negatively charged C-terminal regions of the molecules extend outward into the aqueous phase to form a "hairy layer". Through a combination of steric and electrostatic repulsion, this layer prevents individual micelles from aggregating. Addition of the enzyme chymosin, traditionally extracted from the stomachs of calves (as rennet), but more recently produced in a cloned form in microorganisms, leads to hydrolysis of the κ-casein molecules at a specific phenylalanine–methionine peptide bond, resulting in the solubilization of the highly negatively charged, C-terminal caseinomacropeptide (CMP) fragment. This reduces the repulsion between individual micelles and leads to aggregation and curd formation. This is the first renneting step in cheese manufacture, the enzymatic stage of which is termed the primary phase and the aggregation stage, the secondary phase of the process.

The nonmicellar protein fraction consists principally of a number of highly folded, globular proteins, collectively called the whey protein fraction. Whereas the micellar caseins are extremely heat stable, these globular proteins are much less so. Heating causes a progressive loss of the native protein structures, leading to the formation of intermolecular disulfide-linked aggregates apparently via hydrophobically linked intermediate stages (Haque and Kinsella, 1988). Interactions between protein molecules of the same species result in the formation of homopolymers; interactions between proteins of different species give rise to heteropolymers. These heteropolymers may consist solely of whey protein molecules or, since both the κ- and α_{s2}-caseins contain cysteine residues, a combination of these caseins with whey proteins. The renneting properties of heated milk have been the subject of numerous investigations over recent years. It is now generally agreed that heating decreases both the rate and the extent of the enzymatic hydrolysis of the κ-casein, resulting in an increase in clotting time (Hindle and Wheelock, 1970; Wilson and Wheelock, 1972; Wheelock and Kirk, 1974; van Hooydonk *et al.*, 1987). This has been shown to be due to interactions between κ-casein and whey protein.

We have recently investigated the influence of the heating regime and pH on the primary phase of renneting of whole milk (Leaver *et al.*, 1995). Progressive denaturation of up to 10% of the total whey proteins resulted in increasing inhibition of the enzymatic reaction. Additional denaturation of whey protein had no further effect on the reaction. However, we were unable to determine if the inhibitory denaturation was due to reaction of the κ-casein with a single whey protein species. Most investigations of the inhibition of renneting have concentrated on interactions between κ-casein and the major whey protein, β-lactoglobulin (β-LG; Wheelock and Kirk, 1974; Reddy and Kinsella, 1990; Smits and van Brouwershaven, 1980). Potential interactions with the other globular whey proteins have largely been ignored. This concentration of effort appears to be due simply to the high concentration of β-LG in the whey fraction (3–4 g L^{-1}) since both the serum albumin (SA), present at about 0.3–0.4 g L^{-1}, and the immunoglobulin components, particularly immunoglobulin G (IgG), present at about 0.9 g L^{-1}, are more heat labile than β-LG as defined by their ability to form polymers (Law *et al.*, 1994). The remaining globular whey protein, α-lactalbumin (1.2 g L^{-1}), does not polymerize readily in the absence of other whey proteins

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even in the presence of casein micelles (Calvo *et al.*, 1993), presumably as a result of its lack of a free sulfydryl group. In the present work, the influence of the heat-induced denaturation of SA and IgG, as well as β-LG, on the chymosin-catalyzed hydrolysis of κ-casein at various protein concentrations and heating temperatures is reported.

MATERIALS AND METHODS

Bovine blood SA, β-LG, and IgG were purchased from Sigma Chemical Co., Poole, Dorset, U.K.

Milk, from the Hannah Institute's Friesian herd, was skimmed by centrifugation followed by filtration through glass fiber pads to remove any residual fat droplets. Casein micelles were separated from the serum phase by centrifuging skimmed milk at 40 000*g* for 2 h at 24 °C. Milk ultrafiltrate was prepared by filtration through a PM10 membrane using an Amicon ultrafiltration cell Model 420 (Amicon, Glos, U.K.) at a pressure of 380 kPa.

Casein micelles were resuspended in the original volume of milk ultrafiltrate by stirring overnight at 4 °C. Capillary electrophoresis showed that these micellar suspensions were free of whey proteins. The requisite weights of whey proteins were added in solid form to the micellar suspensions, which were stirred until complete dissolution of the added proteins occurred (15–20 min).

Heat Treatments. Aliquots (10 mL) of skimmed milk and micellar suspensions were heated in screw-cap tubes (25 mL) at 60–80 °C in a thermostatically controlled water bath. After heating, the tubes were immediately cooled on ice.

Chymosin Hydrolysis. Clonal chymosin (Maxiren; Gist-Brocades, Seclin, France) was diluted with distilled water and added at a concentration of 0.02 unit mL^{-1} to 10 mL of unheated or heated samples pre-equilibrated at 30 °C in a water bath. Samples were incubated at 30 °C, and aliquots (1 mL) were withdrawn from the reaction mixture at intervals. The enzymatic reaction was stopped by vigorously vortex mixing with 2 mL of a 6% TCA solution, resulting in a final TCA concentration of 4%.These samples were centrifuged at 20 000*g* for 20 min and the supernatant liquids containing the CMP retained.

Estimation of CMP. The CMP content of the TCA-treated supernatants was determined by HPLC at room temperature using an Apex WP C_{18} reversed-phase column (15 cm × 4.6 mm; Jones Chromatography Ltd., Hengoed, U.K.) as detailed by Lopez-Fandino *et al.* (1993). The CMP peaks were eluted using a 20–65% gradient of acetonitrile in 0.1% TFA over 11 min. Detection was at 214 nm. The initial rate of the reaction was estimated over the first 20 min.

Determination of Whey Protein Denaturation in Milk. The extent of denaturation of the various whey proteins was determined by gel permeation fast protein liquid chromatography (FPLC) of the acid filtrates from raw and heated milks (Law *et al.*, 1993).

RESULTS

Heating skimmed milk at 60, 70, and 80 °C caused a progressive increase in the extent of denaturation of the various whey proteins, as measured by the degree of aggregation (Figure 1). Heating at 80 °C for 30 min caused almost complete denaturation of the various whey proteins (Figure 1c) and, as a result of interactions between the whey proteins and κ-casein, reduced both the initial rate of the hydrolysis (V_i) and the maximum measured amount of CMP, by 19 and 10% respectively (Figure 2a). In contrast, heating micelles under the same conditions actually increased the rate and amount of CMP released (Figure 2b). These results are similar to those obtained by Reddy and Kinsella (1990), who used simulated milk salts buffer in place of ultrafiltrate as the resuspending medium for micelles.

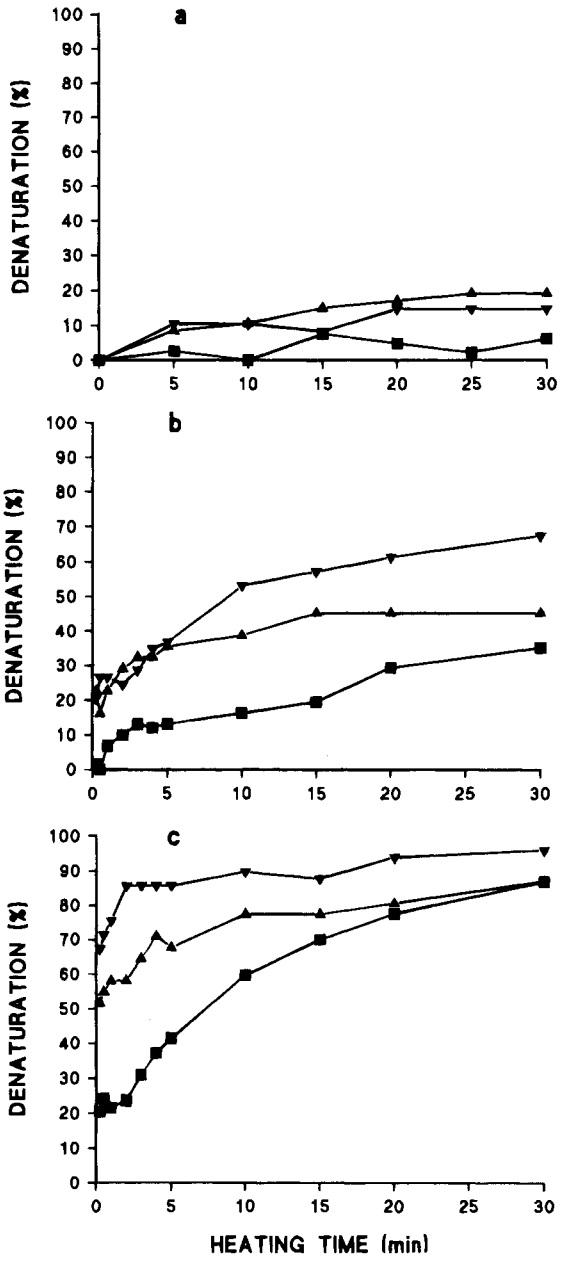

Figure 1. Denaturation of (■) β-LG, (▲) SA, and (▼) IgG in milk heated at (a) 60, (b) 70, and (c) 80 °C.

Heating micelles under these conditions in the presence of a physiological concentration of β-LG (3 mg mL^{-1}) decreased both the rate and the extent of CMP release (Figure 3a). V_i was reduced by 34% and the maximum level of CMP by 8%. Increasing the β-LG level to 9 mg mL^{-1} decreased these parameters by 54 and 32%, respectively. Changes in the values of these two parameters as a result of heating milk and micelles under a variety of conditions are summarized in Table 1.

In the presence of 0.4 mg mL^{-1} of SA (the physiological level), heating micelles at 80 °C had no measurable effect on V_i but decreased the maximum level of CMP by about 8% (Figure 3b). Increasing the SA concentration to 1.2 mg mL^{-1}, resulted in a 24% decrease in the initial rate and a 23% decrease in the maximum CMP level.

Due to the high cost of pure IgG, micelles were heated in the presence of the physiological concentration of this protein and also at one-third of this level to determine whether protein concentration influenced the extent of

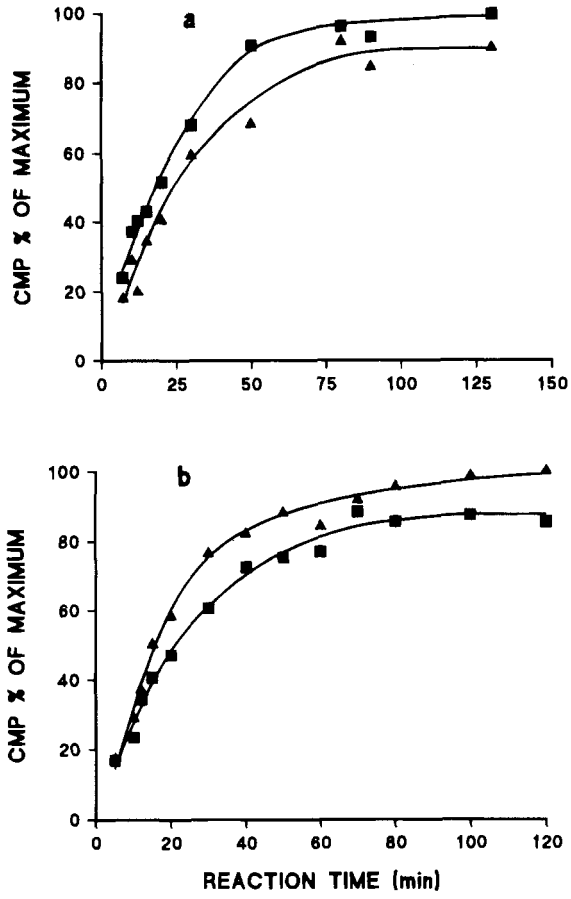

Figure 2. Rate of release of CMP from raw (■) and heated (▲) skimmed milk (a) and micelles (b).

inhibition. At the subphysiological level of 0.3 mg mL^{-1} V_i was reduced by 12% and the maximum level of CMP by 9%. Heating in the presence of the physiological level of 0.9 mg mL^{-1} resulted in an 18% reduction in V_i, but the decrease in the maximum level of CMP was the same as that measured at the lower protein level (Figure 3c).

The percentage denaturation of β-LG as a result of heating milk at 60, 70, and 80 °C for 30 min was 5, 35, and 88% respectively (Figure 1). The corresponding figures for the SA and IgG components were 19, 45, and 88% and 15, 68, and 95%, respectively. Heating micelles at 60, 70, or 80 °C in the presence of a single high concentration of the various whey proteins resulted in a reduction in both V_i and maximum CMP levels at all temperatures in the presence of each of the proteins (Figure 4; Table 1). In the presence of IgG and BSA, increasing the temperature had relatively little effect on the degree of inhibition. In the case of SA, increasing the temperature from 60 to 80 °C decreased V_i from 79 to 76% and the maximum level of CMP from 84 to 81% of that measured in unheated samples. With IgG, the decreases in these kinetic parameters were from 85 to 82% and from 92 to 89%, respectively.

Inhibition of the reaction as a result of heating in the presence of β-LG was slightly more temperature dependent, but even heating at 60 °C caused a severe inhibition of the reaction, with V_i and the maximum level of CMP decreasing to 47 and 75%.

DISCUSSION

Although in previous papers on the subject inhibitory effects on the rate of renneting arising from heat-induced interactions between the high molecular weight whey proteins and κ-casein have been ignored, the results presented in this paper clearly show that such interactions do occur between κ-casein and the high molecular weight SA and IgG fractions of milk in addition to the well-documented interactions with β-LG. The degree of inhibition of the chymosin-catalyzed hydrolysis of κ-casein resulting from interactions with physiological concentrations of IgG is, under the heating conditions reported here, sufficient to account for all of the inhibition of the reaction measured in heated skimmed milk. Which of these various whey protein/κ-casein interactions, if any, predominates in heated milk has still not yet been determined and must presumably await the isolation and characterization of

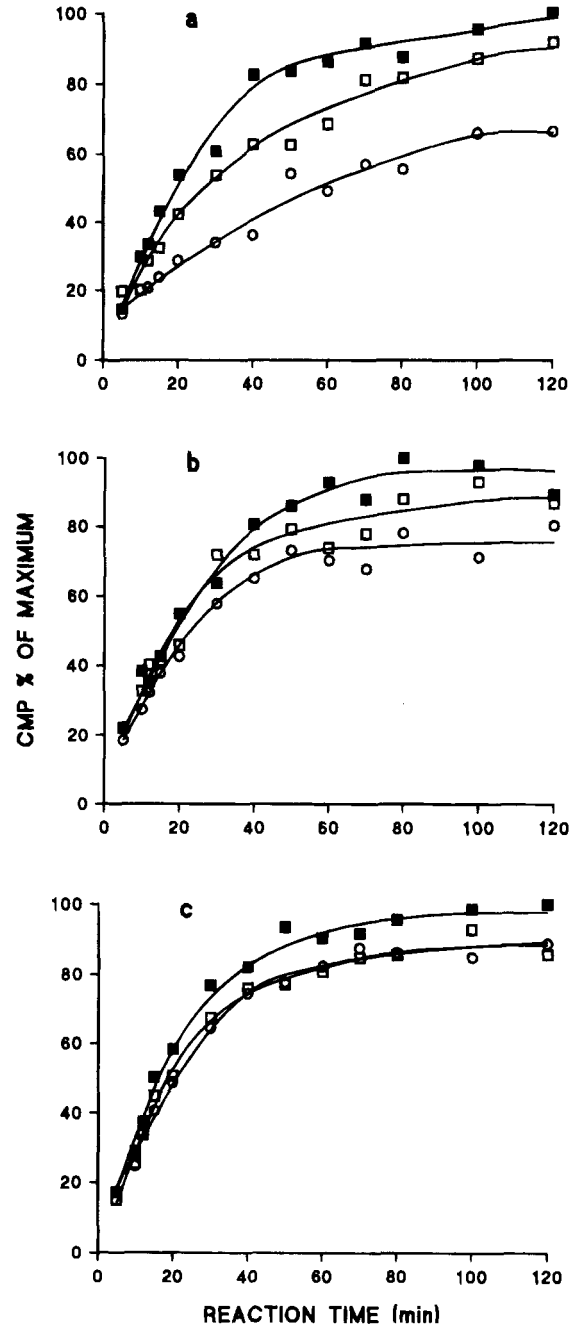

Figure 3. Rate of release of CMP from micelles heated for 30 min at 80 °C in the presence of different concentrations of whey proteins: (a) ■, no addition; □, + 3 mg mL^{-1} β-LG; ○, +9 mg mL^{-1} β-LG; (b) ■, no addition; □, + 0.4 mg mL^{-1} SA; ○, + 1.2 mg mL^{-1} SA; (c) ■, no addition; □, + 0.3 mg mL^{-1} IgG; ○, + 0.9 mg mL^{-1} IgG.

Table 1. Influence of Heating on the Initial Rate (V_i) of κ-Casein Hydrolysis and the Maximum Level of CMP Detected in Skimmed Milk and Micellar Suspensions[a]

system	added protein	level (mg mL^{-1})	conditions	V_i (%)	max CMP (%)
milk	none		heated	81	90
micelles	none		heated	134	112
micelles	β-LG	0	80 °C	100	100
		3		66	92
		9		36	68
micelles	SA	0	80 °C	100	100
		0.4		100	92
		1.2		76	77
micelles	IgG	0	80 °C	100	100
		0.3		88	91
		0.9		82	91
micelles	β-LG	9.0	60 °C	47	75
			70 °C	36	66
			80 °C	34	53
micelles	SA	1.2	60 °C	79	84
			70 °C	76	82
			80 °C	76	81
micelles	IgG	0.9	60 °C	85	92
			70 °C	82	89
			80 °C	82	89

[a] Unless otherwise indicated, all values are relative to unheated controls.

κ-casein/whey protein oligomers from heated milk. While the interaction of whey proteins with κ-casein and the subsequent effect which this has on the rate of the renneting reaction is of particular interest solely to cheese manufacture, chymosin is also acting as a probe of the surface properties of the micelle, and such interactions will be of importance in the manufacture of other dairy products in which a heating step is involved such as yogurts and sterilized milk.

Denaturation temperatures of the various whey proteins as measured by differential scanning calorimetry (DSC) have been reported to be 78, 72, and 64 °C for β-LG, Ig, and SA, respectively (deWit and Klarenbeek, 1984). Therefore, the extent of inhibition of κ-casein hydrolysis as a result of heating micelles at 60 °C in the presence of the various whey proteins is interesting, since this is below the denaturation temperature of any of these proteins. The gel permeation results indicate that some denaturation, as determined by the loss of native protein from the whey, occurs in milk heated at 60 °C. However, in the case of β-LG the extent of denaturation is only about 5%, and even with SA and IgG, this aggregated protein is less than 20% of the total. Denaturation of globular proteins is a complex, multistage process. Some steps are reversible, others are not. DSC profiles of β-LG show that in addition to the main denaturation peak, a reversible shoulder is observed at lower temperatures (Qi *et al.*, 1995). The actual positions of both the main denaturation peak and the shoulder are dependent on the protein concentration, pH, and heating rate. It has been suggested that the interaction between β-LG and κ-casein initially involves hydrophobic interactions between protein molecules followed by formation of more stable disulfide bridges (Haque and Kinsella, 1988). These workers measured decreases in the apparent hydrophobicity of κ-casein and β-lactoglobulin and mixtures of the two and also detected changes in the susceptibility of κ-casein to chymosin hydrolysis much earlier than any covalent interactions were detected as a result of heating soluble

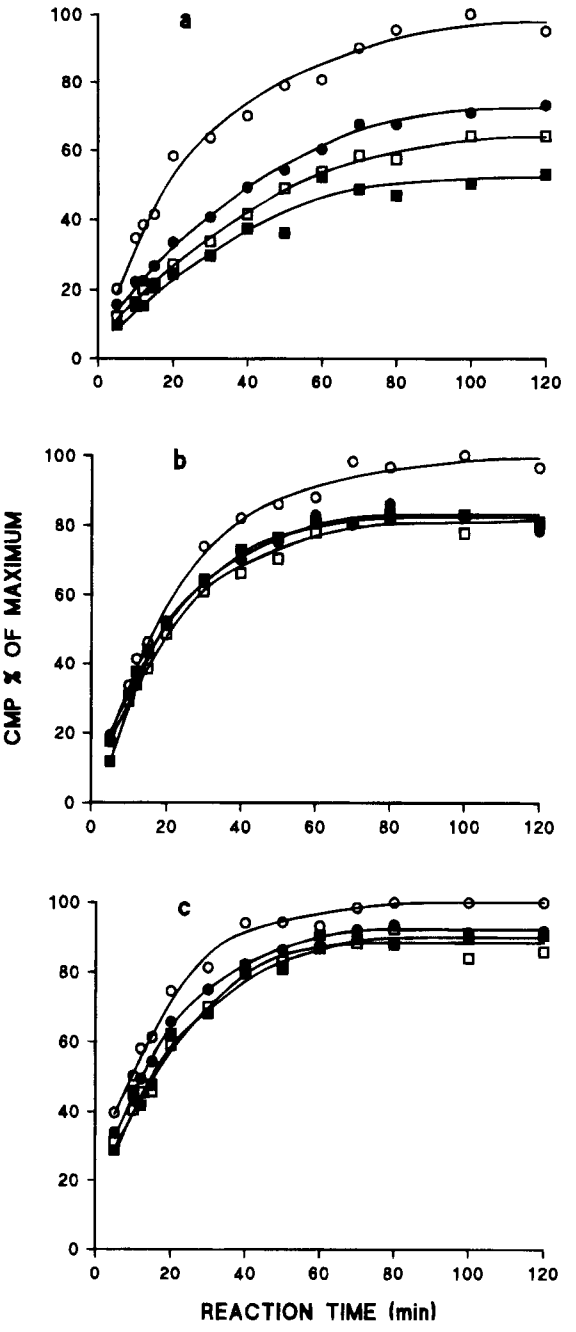

Figure 4. Rate of release of CMP from (○) unheated micelles and micelles heated at (●) 60, (□) 70 and (■) 80 °C in the presence of (a) 9 mg mL^{-1} β-LG, (b) 1.2 mg mL^{-1} SA, and (c) 0.9 mg mL^{-1} IgG.

proteins at 70 °C. Similar interactions may be occurring in the micellar/whey protein mixtures at 60 °C due to the formation of hydrophobic bonds resulting from conformational changes in the structure of the protein molecules. These interactions do not appear to be transient since they survive cooling and subsequent incubation at 30 °C. Whether they subsequently lead to the formation of intermolecular disulfide bridges has yet to be determined.

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