Impaired Rennetability of Heated Milk; Study of Enzymatic Hydrolysis and Gelation Kinetics

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ABSTRACT

Casein micelles in milk are stable colloidal particles with a stabilizing hairy brush of κ -casein. During cheese production rennet cleaves κ -casein into casein macropeptide and para- κ -casein, thereby destabilizing the casein micelle and resulting in aggregation and gel formation of the micelles. Heat treatment of milk causes impaired clotting properties, which makes heated milk unsuitable for cheese production. In this paper we compared five different techniques, often described in the literature, for their suitability to quantify the enzymatic hydrolysis of κ -casein. It was found that the technique is crucial for the yield of casein macropeptide and this yield then affects the calculated enzymatic inhibition caused by heat treatment, ranging from 5 to 30%. The technique, which we found to be the most reliable, demonstrates that heat-induced calcium phosphate precipitation does not affect the enzymatic cleavage, while whey protein denaturation causes a very slight reduction of enzyme activity. By using diffusing wave spectroscopy, a very sensitive technique to monitor gelation processes, we demonstrated that heat-induced calcium phosphate precipitation does not affect the clotting. Whey protein denaturation does not affect the start of flocculation but has a clear effect on the clotting process. This work adds to a better understanding of the processes causing the impaired clotting properties of heated milk.

(Key words: renneting, heated milk, enzymatic hydrolysis, gelation kinetics)

Abbreviation key: **CMP** = casein macropeptide, **DWS** = diffusing wave spectroscopy, **HAC** = acetic acid, **NaAc** = sodium acetate, **RCT** = rennet clotting time, **RP** = reverse phase.

INTRODUCTION

A casein micelle is an association colloid with a stabilizing hairy brush of calcium-insensitive κ -casein on its surface. Chymosin, the major enzyme in rennet, is an endopeptidase, which in milk cleaves very specifically to the Phe105-Met106 bond of κ -casein. The subsequently formed para- κ -casein is insoluble, while the released casein macropeptide (**CMP**) is soluble. Para- κ -casein lacks the colloid-protective property of κ -casein; therefore, extensive cleavage of the κ -caseins will result in destabilization and aggregation of the micelles into a coagulum (Walstra and Jenness, 1984; Dalgleish, 1990b). Manufacturing of cheese is preferably carried out with unheated or low-pasteurized milk. Under these conditions the action of the chymosin is well investigated. The activity of the enzyme is dependent on pH, temperature, and calcium concentration. An overview of the effects of these parameters on enzymatic cleavage, clotting, and syneresis is provided by Walstra and Jenness (1984).

Chymosin acts only on the casein micelles; native whey proteins are not included in the curd. Upon heat treatment whey proteins interact with the micelles through thiol-group-disulphide bridge exchange reactions (Jang and Swaisgood, 1990) and are transferred to the curd. However, heat treatment of the milk leads to off flavors in cheese and causes impaired clotting properties resulting in a weaker curd, therefore, making it less suitable for cheese manufacturing (Wauguna et al., 1996; Singh and Wauguna, 2001). In the literature, various explanations are given for this phenomenon, which either alone or in combination would cause the impaired clotting, such as 1) incomplete enzymatic hydrolysis, 2) reduced concentration of serum calcium due to precipitation of calcium phosphate, and 3) stabilization of casein micelles due to the coating with charged denatured whey proteins.

However, conflicting results in the literature hamper complete understanding of impaired rennetability caused by heat treatment. Depending on the isolation method applied to determine the degree of hydrolysis,

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inhibition varies from negligible to a significant inhibiting factor (Hindle and Wheelock, 1970; Wilson and Wheelock, 1972; Wheelock and Kirk, 1974; Walstra and Jenness, 1984; Marshall, 1986; Hooydonk et al., 1987; Singh et al., 1988; Reddy and Kinsella, 1990). No evidence can be found in the literature for a direct relation between heat-induced (70 to 90°C), impaired rennetability and precipitated calcium (van Hooydonk, 1986; Singh et al., 1988; Schreiber, 2001; Singh and Waunguna, 2001) or denatured whey proteins (Waunguna et al., 1996; Raynal and Remeuf, 1998; Steffl et al., 1999; Singh and Waunguna, 2001).

In the present paper, we compared five different techniques to monitor enzymatic action of rennet in heat-treated milk, i.e., precipitation by acetic acid and by TCA (2, 8, and 12%), followed by CMP analysis and monitoring of para- κ -casein with reverse phase (RP)-HPLC. This allowed us to select the most suitable technique and to investigate the inhibition by calcium phosphate precipitation and whey protein denaturation. In addition, diffusing wave spectroscopy (DWS) is used as a technique to study the rennet-induced flocculation and gelation behavior of heated milk. The DWS analyzes fluctuations in light scattered back from the sample using correlation techniques to determine a characteristic relaxation time for the mobility of colloidal particles in the system (Maret, 1997). It is therefore a very suitable technique to investigate the influence of process parameters on the gelation point (Vasbinder et al., 2001). After gelation the network still exhibits a thermally driven motion, counteracted by the viscoelasticity of the gel network. As a result the scattered light intensity still fluctuates but now contains information on the viscoelasticity of the gel (Mason et al., 1997; Gisler and Weitz, 1999). Combining DWS with two model-systems, i.e., whey-protein-free milk and whey-protein-free milk with added whey proteins, allows us to investigate the effect of calcium phosphate precipitation on the clotting properties and the additional contribution of whey protein denaturation.

MATERIALS AND METHODS

Skim Milk

Skim milk heated for 10 min at 72°C was obtained from the pilot plant at NIZO Food Research (Ede, The Netherlands). Sodium azide (0.02%) was added to prevent bacterial growth, and the milk was stored at 4°C.

Whey Protein Isolate Solution

Whey protein isolate (Bipro; Davisco Food International Inc., Eden Prairie, MN) solution was prepared at a concentration of 9% (wt/wt). The solution was stirred

for 2 h at room temperature and subsequently filtered through a 0.45 μ m filter; 0.02% (wt/wt) NaN₃ was added.

Whey-Protein-Free Milk

Reconstituted whey-protein-free milk was prepared by dissolving 9.23 g of milk powder prepared by microfiltration and ultrafiltration (NIZO Food Research) in 90.77 g of distilled water. The milk was stirred at 45°C for 1 h. To prevent bacterial growth 0.02% NaN₃ was added, and the milk was kept overnight at 4°C before use. The initial pH of the milk was 6.67 ($\pm$ 0.01). Before further use, 19.45 g of whey-protein-free milk was mixed with either 1 g of distilled water or whey protein isolate solution.

Heat Treatment of Milk

The cold milk was adjusted to room temperature for 2 h. Glass tubes (8 ml, diameter 1 cm) were filled with 5 ml of milk and heated for 10 min in a waterbath at the required temperature ranging from 70 to 90°C and cooled with tap water to room temperature.

Renneting of the Milk for CMP and Para- κ -Casein Analysis

The heat-treated milk was incubated for 75 min at 30°C. An aliquot was withdrawn (blank) before addition of rennet. Then 500-times-diluted rennet (CSK 10.800, The Netherlands, 150 international milk-clotting units/ml), was added to a final concentration of 0.005% rennet in the milk. After 4 h of incubation, the enzymatic reaction was stopped by addition of 10 μ l/ml of pepstatin solution (5 mg of pepstatin (Sigma) per milliliter DMSO). These milk samples were used for CMP and para- κ -casein isolation.

CMP Isolation

Acetic acid precipitation. Renneted milk (0.4 g) was mixed with 0.8 g of distilled water (40°C) and 40 μ l of acetic acid (HAc) (10%) in an Eppendorf tube (2 ml). After mixing (vortex) and 10 min waiting, 40 μ l of sodium acetate (NaAc; 1 M) and 0.72 g of distilled water were added, and the solution was mixed again. After 1 h standing the solution was centrifuged for 5 min at 3000 $\times$ g. The CMP in the supernatant was determined by RP-HPLC.

TCA precipitation. The renneted milk was mixed in an Eppendorf tube with a 24% (wt/wt) TCA solution to a final concentration of 2, 8, and 12% TCA (wt/wt), stirred thoroughly and incubated for 30 min at 20°C. The sample was then centrifuged for 5 min at 5000 $\times$ g, and the

supernatant was mixed with buffer (0.1 M bis-Tris pH 7, 8 M urea, 45 mM citrate) to a final pH of 3.0. The supernatant:buffer ratios were 1:1 for 2% TCA and 1:2 for 8 and 12% TCA. The mixture was filtered through a 0.22 μ m filter before injection on the RP-HPLC column.

Para- κ -Casein Isolation

Renneted milk was mixed 1:1 with buffer (0.1 M bis-Tris pH 7, 8 M urea, 45 mM citrate) containing mercaptoethanol (0.3%, vol/vol). After 1 h of incubation at 20°C, 0.5 g of this solution was added to 1.5 g of buffer (6 M urea, adjusted to pH 3 with trifluoroacetic acid). The sample was mixed thoroughly, filtered through a 0.22 μ m filter, and analyzed by RP-HPLC.

RP-HPLC Analysis of CMP

A description of the RP-HPLC method used is given by Minkiewicz et al. (1996). A gradient was applied of solvent A (acetonitrile:water:trifluoroacetic acid 100:900:1, vol/vol/vol) and solvent B (same components 900:100:0.8, vol/vol/vol). The gradient was started with 15% of solvent B and increased from 15 to 28% over 13 min, 28 to 32% over 22 min, 32 to 70% over 3 min, and finally kept constant for 5 min before returning to the initial conditions. Analytical separations were carried out at 30°C, a flow rate of 0.8 ml/min, peak detection at 220 nm, and an injection volume of 100 μ l (Hi-Pore reversed phase C18 column).

RP-HPLC Analysis of Para- κ -Casein

Apart from a different gradient applied and an injection volume of 50 μ l the same protocol as presented in HPLC analysis of CMP was used for para- κ -casein. The gradient was started with 26% of solvent B and increased from 26 to 40% over 50 min, 40 to 70% over 2 min, and finally kept constant for 5 min before returning to the initial conditions.

DWS Measurement of Renneted Milk

The heat-treated milk was incubated for 75 min at 30°C. Then 500-times-diluted rennet was added to a final concentration of 0.005% in the milk. The rennet-induced clotting of the milk was monitored by DWS. Light from a 5 mW He-Ne laser (632.8 nm) was passed through a multimode fiber into the milk. The backscattered light was monitored by a single-mode fiber located at 3.0 mm from the input fiber. The scattered light was detected with a photo multiplier tube (ALV SO-SIPD and fed to a PC-interfaced autocorrelator (Flex 5000, correlator.com). The correlator calculates the autocorrelation

Figure 1. The $\tau_{1/2}$ traces of fresh skim milk unheated ($\circ$) and heated at 70 ($\blacksquare$), 75 ($\blacktriangledown$), 80 ($+$), 85 ($\bullet$), and 90°C (∇) for 10 min subjected to rennet treatment at 30°C.

functions in real time. The time at which the autocorrelation curve has decayed to 50% of its maximum plateau level is defined as $\tau_{1/2}$ (see Figure 2 in Vasbinder et al., 2001) and the value is determined by linear interpolation. The renneting was monitored in time at intervals of 2 min. All data were normalized by the $\tau_{1/2}$ value (average of five measurements of 1 min) of the same sample before rennet was added. This eliminates variations between the samples and fibers. The gelation point is defined in the plot of $\tau_{1/2}$ against renneting time as the time at which $\tau_{1/2}$ is increasing. The error in determining the gelation point is at most 5 min.

RESULTS AND DISCUSSION

Rennet-Induced Gelation of Heated Skim Milk Studied by DWS

The rennet-induced clotting of casein micelles in preheated skim milk monitored by DWS, as a function of time, is shown in Figure 1. The parameter $\tau_{1/2}$ reflects the mobility of the particles in solution, i.e., the casein micelles in milk, and is therefore a sensitive parameter for monitoring the clotting of the micelles and the beginning of the subsequent gelation (Maret, 1997; Mason et al., 1997; Gisler and Weitz, 1999; Vasbinder et al., 2001). All curves show a gradual and reproducible decrease in $\tau_{1/2}$ towards a minimum at 200 min. This minimum is followed by an increase in $\tau_{1/2}$, but lower values are reached with increasing temperature of heat treatment (70 to 90°C). Unheated milk and milk heated at 70°C show a rather similar behavior, a steep increase in $\tau_{1/2}$

during renneting. The $\tau_{1/2}$ curves of milk heated at 85 and 90°C both show hardly any increase in time.

The decrease of $\tau_{1/2}$ to a minimum in the first 200 min of incubation means that the mobility of the micelles increases during renneting due to a decrease in size, which is caused by enzymatic cleavage of the κ -caseins in the hairy brush (Walstra and van Vliet, 1986; de Kruif and Zhulina, 1996; Mellema et al., 1999). The fact that this minimum is observed for all the milks studied demonstrates that proteolytic cleavage takes place both in unheated and heated milk. All studies published on rennet clotting times (RCT) of heat-treated milk report that increasing the temperature of heat treatment results in an increase in RCT. The RCT is defined as the time at which flocculation is observed visually. For heated milks, it is increased by a factor 10 to 15 relative to unheated milk (Singh et al., 1988; Dalgleish, 1990b; Allogio et al., 2000). The DWS, which is a very sensitive technique for measuring flocculation, adds complementary information to these observations. It demonstrates that the onset of clotting of milk is not affected by the heat treatment but only the kinetics of flocculation. This means that the micelles aggregate independent of the heat treatment applied, but the casein micelles are no longer able to form a gel. This latter observation corresponds with the increased RCT and gelation times and the decreased G' values as reported in the literature (van Hooydonk et al., 1986; Singh et al., 1988; Dalgleish, 1990b; Wauguna, 1996; Raynal and Remeuf, 1998; Steffl et al., 1999; Allogio et al., 2000), which is ascribed to a decrease in enzymatic activity, precipitation of calcium, and denaturation of whey proteins. In this paper we systematically investigate the effect of heat treatment on these three parameters by comparing fresh skim milk, whey-protein-free milk, and whey-protein-free milk with added whey proteins.

Enzymatic Hydrolysis in Heated Skim Milk

To study the enzymatic activity in heated milk, various methods are possible, i.e., CMP isolation by precipitating the other milk proteins with Hac/NaAc precipitation or with 2, 8, and 12% TCA or determination of para- κ -casein. In this section we will compare the four different CMP isolation techniques with respect to quantity and composition of CMP present in the supernatant. All five techniques were used to determine the enzymatic activity in milk heated at 90°C for 10 min. The technique, which was validated to be the most reliable, was used to study the enzymatic hydrolysis in heated milk.

Comparison of Four CMP Isolation Methods in Unheated Milk. Figure 2 shows four RP-HPLC chromatograms of CMP present in unheated milk treated with rennet and isolated by precipitation with Hac/NaAc and

Figure 2. Reverse-phase-HPLC chromatograms of casein macropeptide (CMP) released by rennet treatment of unheated fresh skim milk. Isolation of CMP occurred by precipitation of the proteins in milk with acetic acid/sodium acetate (A), 2% TCA (B), 8% TCA (C) and 12% TCA (D). The peaks corresponding with nonglycosylated CMP A and B and the glycosylated CMP are indicated.

with 2, 8, and 12% TCA. The figure reveals that the isolation technique used has a large effect on the total area of the peaks present in the chromatogram. The shape and position of the RP-HPLC pattern of supernatants obtained by Hac/NaAc and 2% TCA precipitation are almost identical. However, with increasing TCA concentration a clear decrease in area is observed. The nonglycosylated CMP B peak disappears completely after precipitation with 8 and 12% TCA. Nonglycosylated CMP A precipitated partly in 8% and almost completely in 12% TCA. Table 1 shows the total area of CMP, the area of glycosylated and nonglycosylated CMP, and nonglycosylated CMP as a percentage of total CMP. With increasing TCA concentration, the area of total CMP diminishes by 60 and 80%, for 8 and 12% TCA, respectively, compared with 2% TCA. The same trend is observed for the nonglycosylated CMP, but the decrease is even stronger, i.e., about 90 and 100% for 8 and 12% TCA, respectively. The area of glycosylated CMP shows a clear decrease for 8 and 12% TCA compared with 2%

Table 1. Casein macropeptide (CMP) content in renneted unheated milk (4 h, 0.005% at 30°C) isolated by precipitation with acetic acid/sodium acetate (HAc/NaAc), 2, 8, and 12% TCA.¹

CMP isolation method	Total CMP (V*s)	Glycosylated CMP (V*s)	Nonglycosylated CMP (V*s)	% Nonglycosylated CMP of total CMP
HAc/NaAc	35 ± 1 ^a	16 ± 2 ^a	20 ± 1 ^a	55 ± 3 ^a
2% TCA	48 ± 4 ^b	25 ± 2 ^b	23 ± 3 ^a	48 ± 3 ^a
8% TCA	16 ± 3 ^c	13 ± 3 ^a	3 ± 1 ^b	21 ± 1 ^b
12% TCA	11 ± 1 ^c	11 ± 1 ^a	0 ± 0 ^b	2 ± 1 ^c

¹The CMP is presented as total CMP, glycosylated CMP and nonglycosylated CMP. The nonglycosylated CMP is composed of the peaks of CMP A and B as indicated in Figure 2. In the last column the percentage of nonglycosylated CMP obtained is shown. The results are an average of four independent measurements. The standard error of the mean is indicated in the table. The superscript letters a, b, and c are based on an analysis of the variance among the methods, showing if a difference in yield and percentage between the four methods is significant.

TCA. Isolation by acetic acid precipitation yields less total CMP, glycosylated and nonglycosylated CMP than with 2% TCA. Although the absolute yield is clearly less than by isolation with 2% CMP, the relative amounts are very similar. Nonglycosylated CMP represents about 50% of the total in the case of HAc/NaAc and 2% TCA but only 21 and 2% for 8 and 12% TCA, respectively. This is in agreement with the results of Vreeman et al. (1986), who showed that 90% of the nonglycosylated CMP precipitates at 8% TCA and 100% precipitates at 12% TCA. They also observed that at very high TCA concentrations (12%) even glycosylated CMP becomes sensitive to precipitation. We can conclude that the technique used for CMP isolation is crucial for the yield of CMP observed in the supernatant. For determination of the absolute amounts of CMP present in milk, isolation with 2% TCA seems favorable and for relative measurements also isolation by acetic acid seems a reliable technique. Isolation with 8 and 12% TCA yields only a fraction of total CMP and nonglycosylated CMP is precipitated selectively.

Comparison of Five Methods to Study Enzymatic Hydrolysis in Heated Milk. The effect of heat treatment of milk (10 min at 90°C) on the enzymatic activity of rennet monitored by the four different CMP isolation methods and determination of para- κ -casein is depicted in Figure 3. The amount of CMP and para- κ -casein released was determined after 4 h of incubation, which is the time at which a visible gel was formed in unheated milk and milk heated at 70°C. At this point 85% of CMP was released in unheated milk. For all fractionation methods used, the amount of CMP released is expressed as the percentage of the area observed for the unheated milk. At 90°C the percentage of CMP released is 95, 92, 67, 78, and 88% in the case of precipitation by HAc/NaAc and 2, 8, and 12% TCA and determination of para- κ -casein, respectively. The standard deviations are indicated in the figure. Precipitation by 2% TCA clearly shows the lowest standard deviation.

These results reveal that analysis of the same samples with different isolation techniques can cause almost 30% difference in determined degree of hydrolysis. This may lead to completely different conclusions. The results obtained with HAc/NaAc and 2% TCA show only a limited inhibition of the enzymatic activity induced by heat treatment, while the results with 8% TCA suggest a significant inhibition of the enzymatic activity. Comparing these findings with reports in the literature on enzymatic cleavage of κ -casein shows that in several studies fractionation was carried out with 2% and 12% TCA and analyzed by determining the total nitrogen content (Hindle and Wheelock, 1970; Wilson and Wheelock, 1972; Wheelock and Kirk, 1974). In 2% TCA, both carbo-

Figure 3. Relative enzymatic hydrolysis (%) in fresh skim milk heated for 10 min at 90°C. Comparison of five different methods to determine hydrolysis, i.e., precipitation of the proteins in milk by acetic acid/sodium acetate (HAc/NaAc) and 2, 8, and 12% TCA, and monitoring of para- κ -casein. The degree of hydrolysis is represented as percentage of hydrolysis determined in unheated milk with the same isolation method. In the graph the standard deviation of the different methods is indicated.

Figure 4. Enzymatic hydrolysis of fresh skim milk (white bars) and whey-protein-free milk (hatched bars) subjected to heat treatment from 70 to 90°C represented by percentage of CMP released, which was isolated by precipitation with 2% TCA. The standard deviation is indicated in the graph.

hydrate-containing and carbohydrate-free macropolypeptides are soluble; however, their quantification by determination of total nitrogen is difficult due to large amounts of nitrogenous material, like native whey proteins, which is also soluble in the milk (Chaplin and Green, 1980). Therefore, the comparison of unheated and heated milks by this procedure is hampered as denatured whey proteins are precipitated in 2% TCA. This explains the large decrease in soluble nitrogen found in 2% TCA for heated milk compared with unheated. Combining 2% TCA precipitation with RP-HPLC, which separates the whey proteins from CMP, yields a very reliable technique. Experiments performed by precipitation with 8% TCA show variations in CMP release ranging from 10 to 30% (van Hooydonk et al., 1987; Reddy and Kinsella, 1990). In contrast, Marshall (1986) showed that determination of the enzymatic activity by monitoring the release of para- κ -casein in milk heated for 10 min at 80°C caused only 4% reduction of enzymatic hydrolysis compared to unheated milk. Apart from this paper most papers show a clear decrease in enzymatic cleavage, which is also the general opinion held in the literature (Walstra and Jenness, 1984; Dalgleish, 1990a). Here, we conclude that enzymatic activity or, better, the amount of CMP split off, is only slightly influenced by heating milk at 90°C (10 min). The variable results in the literature reflect insufficient awareness of the interference of the analytical methods with the interpretation of experimental data (Table 1; Vreeman et al., 1986).

Enzymatic Hydrolysis as a Function of Temperature of Heat Treatment. In Figure 4 the enzymatic

hydrolysis, which is reached after 4 h of renneting of fresh skim milk and whey-protein-free milk, is shown as a function of the temperature of heat treatment. The CMP isolation was performed by precipitation with 2% TCA as this results in a maximum yield of CMP, both glycosylated and nonglycosylated, and the results show a very low standard deviation. Heat treatment at 70°C causes a slight increase in enzymatic hydrolysis compared with unheated milk, which slowly levels off at higher temperatures of heat treatment to 92% of unheated milk. No effect of heat treatment was observed for whey-protein-free milk: at all temperatures of heat treatment the release was similar to the unheated whey-protein-free milk.

Comparing the results of fresh skim milk and whey-protein-free milk shows two differences, i.e., the maximum in CMP release at 70°C for fresh skim milk and the slight reduction in CMP release at 85 and 90°C. The absence of whey proteins in whey-protein-free milk allows us to study the heat-induced calcium phosphate precipitation. The results demonstrate that heat treatment has no effect on enzymatic hydrolysis; therefore, we can exclude heat-induced calcium phosphate precipitation as a factor influencing the enzymatic hydrolysis. The maximum in CMP release observed in milk heated at 70°C has not previously been reported in the literature. A restructuring of the casein micelle due to mild heat treatments could increase the accessibility of the κ -caseins. According to Farrell et al. (1998) heat treatment of a purified κ -casein solution causes increased κ -casein- κ -casein interactions. Although whey-protein-free milk powder is obtained by microfiltration at rather low temperatures, the spray-drying process might cause these interactions. This could prevent restructuring of the casein micelle during heat treatment at 70°C and therefore explain the absence of a maximum in CMP release. To study this phenomenon, whey-protein-free milk is probably not a good model system. Although more research is required to understand the mechanism causing this maximum, it can be concluded that it does not affect the rate of clotting (Figure 1). As heat-induced calcium phosphate precipitation has no effect on enzymatic hydrolysis, it seems very likely that the small reduction in enzymatic hydrolysis at higher temperatures of heat treatment is caused by the denaturation of whey proteins, which is the general opinion in the literature (Walstra and Jenness, 1984; Dalgleish, 1990a). However, the reduction is very limited, i.e., 6% less than in unheated milk, while heat treatment at 70°C shows an increase of 9%. Comparing unheated milk and milk heated for 10 min at 80°C (60% of denaturation) shows an enzymatic activity, which is very similar for both milks, while the flocculation rate decreased by a factor of 2 (Figure 1). These observations demonstrate

Figure 5. The $\tau_{1/2}$ traces of whey-protein-free milk unheated ($\circ$) and heated for 10 min at 70 ($\blacksquare$), 75 ($\blacktriangledown$), 80 ($+$), 85 ($\bullet$), and 90°C (∇) subjected to rennet treatment at 30°C.

Figure 6. The $\tau_{1/2}$ traces of whey-protein-free milk with added whey proteins unheated ($\circ$) and heated for 10 min at 70 ($\blacksquare$), 75 ($\blacktriangledown$), 80 ($+$), 85 ($\bullet$), and 90°C (∇) subjected to rennet treatment at 30°C.

clearly that slight changes in enzymatic hydrolysis cannot explain the observed effects on the rate of clotting.

Rennet-Induced Gelation of Heated Milk

Calcium phosphate precipitation. Figure 5 illustrates the effect of heat treatment on whey-protein-free milk as monitored by DWS. A steady decrease of $\tau_{1/2}$ towards a minimum was observed, which is situated around 150 min, followed by a sharp increase. The milks behaved identically under all the heat treatments applied. The start of flocculation of whey-protein-free milk occurred 50 min earlier than in the case of fresh skim milk. Apparently, spray drying and reconstitution of the milk affect the stability of the casein micelles. Heat treatment of whey-protein-free milk did not affect the gelation properties. The major difference between skim milk and whey-protein-free milk is the absence of whey proteins; therefore, comparison of the two milk types should reveal the effect of heat-induced calcium precipitation, which is usually masked by the effect of whey protein denaturation. Raynal and Remeuf (1998) observed a 10 to 15% loss of diffusible calcium in skim milk upon heat treatment (10 min, 75 to 90°C). However, heat treatment did not affect the clotting of whey-protein-free milk, which indicates that 10 to 15% loss of calcium does not affect this process. At temperatures above 100°C effects of heat treatment on renneting properties of whey-protein-free milk were found (Schreiber, 2001). A clear decrease in the gel strength was observed. The higher the temperatures applied (100 to 140°C), the weaker the final rennet gels obtained (60 to 90% weaker than unheated milk).

A decrease in soluble calcium of 30 and 50% was caused by a heat treatment for 10 min at 100 and 120°C, respectively (Schreiber, 2001). This indicates that precipitation of a significant amount of calcium can affect the gel formation. By interpolating these results to the temperature regime of 70 to 90°C, it seems likely that a reduction in gel strength would occur. However, this has not yet been investigated. The role of calcium with respect to rennet-induced gelation appears to be rather complicated. At temperatures of heat treatment of 70 to 90°C, it has no effect on the clotting of the casein micelles, but it seems likely to influence the gel strength. However, one main conclusion can be drawn: at heat treatments below 100°C precipitation of calcium is not responsible for the impaired clotting of heated skim milk.

Whey protein denaturation. Figure 6 shows the effect of whey protein denaturation caused by heat treatment on the rennet-induced gelation of whey-protein-free milk with added whey proteins as measured with DWS. All curves show a slight decrease in $\tau_{1/2}$ towards a minimum around 150 min, followed by an increase, which is dependent on the heat treatment applied. Unheated milk and milk heated at 70°C show an almost identical behavior; heat treatment at 75 and 80°C caused a less steep increase of the DWS trace, but still a steady increase was observed. Milk heated at 85 and 90°C showed a weaker increase of $\tau_{1/2}$, which leveled off at 600 min. No gel formation was observed in these milks. Comparing Figures 5 and 6 shows that addition of whey proteins causes changes in the flocculation behavior of the casein micelles. The minimum is not affected by the heat treatment applied both in either the presence or

the absence of whey proteins. This reveals that casein micelles start to interact and to form flocs independent of the presence of whey proteins. In the presence of whey proteins a clear effect of heat treatment was observed on clotting behavior, while no effect at all was found in whey-protein-free milk. Therefore, we can conclude that the impaired clotting of casein micelles in heated milk is entirely attributable to coating of the casein micelles with denatured whey proteins. This confirms one of the hypotheses often proposed in the literature (Dalglish, 1990b; Wauguna et al., 1996; Raynal and Remeuf, 1998; Steffl et al., 1999; Singh and Wauguna, 2001).

CONCLUSIONS

We can conclude that the impaired clotting of casein micelles in heated milk can be entirely attributed to whey protein denaturation. However, this denaturation does not affect the start of flocculation but inhibits the process of clotting. No effect of calcium precipitation was found on either the enzymatic activity of rennet or the clotting of the casein micelles. The slight inhibition of enzymatic activity above 80°C in skim milk does not appear to contribute much further to the already very poor clotting properties of heated milk.

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