JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Factors Affecting the Retention of Rennet in Cheese Curd

NIDHI BANSAL, PATRICK F. FOX, AND PAUL L. H. MCSWEENEY*

Department of Food and Nutritional Sciences, University College, Cork, Ireland

The coagulant retained in cheese curd is a major contributor to proteolysis during ripening. The objective of this study was to quantify the effects of several milk-related factors and parameters during cheese manufacture on the retention of coagulant in cheese curd. The amount of coagulant retained in curd was determined by its activity on a synthetic heptapeptide (Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu) using reversed-phase HPLC. The retention of chymosin in cheese curd increased significantly when the pH of milk was reduced at rennet addition below pH 6.1, the pH at whey drainage below pH 5.7, or the average casein micelle size in milk and when the ionic strength of milk was increased. The casein content of milk and the quantity of chymosin added to milk had no significant effect on the retention of chymosin in curd; the quantity of coagulant bound per gram of casein remained unchanged.

KEYWORDS: Residual coagulant; chymosin; proteolysis; cheese ripening

INTRODUCTION

Rennet is the general name for enzyme preparations used to coagulate milk in the production of rennet-coagulated cheese and rennet casein. The principal role of these enzymes is to coagulate milk, but they also contribute to proteolysis during ripening of most cheeses, particularly in low/medium-cooked varieties. Studies on cheese with a controlled microflora have shown that the residual coagulant is responsible for the level of proteolysis detected by gel electrophoresis and for most of the nitrogen soluble in water or at pH 4.6 (*1*–*3*). The residual rennet is partially responsible for the softening of cheese during ripening (*4*–*6*). Proteolysis by residual rennet also influences the flavor of cheese; some peptides produced by rennet are small enough to influence flavor directly. Also, peptides may be further hydrolyzed by microbial proteinases and peptidases to small peptides and amino acids, which contribute at least to the background flavor and, perhaps, to bitterness if the activity of such enzymes is excessive. Catabolism of amino acids by microbial enzymes and perhaps alterations via chemical mechanisms leads to a range of sapid compounds that are major contributors to characteristic cheese flavors.

Most of the rennet added to milk is lost in the whey. The amount of residual rennet in cheese varies with variety. Levels of residual rennet range from almost negligible in high-cooked varieties to about 15% of the original activity in Gouda cheese; about 50% rennet is retained in the cheese curd in high-moisture varieties such as Camembert cheese (*7*–*9*). Proportionate distribution of chymosin between the aqueous phases of whey and curds during cheesemaking would lead to about 5% of added chymosin activity being retained in the curd (*10*).

The level of coagulant retained in cheese curd plays an important role in the development of cheese texture and flavor; hence, it is very important to identify the factors that affect the quantity of rennet retained in cheese curd. Among the wide range of such factors, the most important are the parameters that vary during cheesemaking: the retention of chymosin increases with decreasing pH at rennet addition and the pH at which the whey is drained (*1*, *8*, *9*, *11*–*13*). The percentage of added rennet retained in cheese curd is also affected by the casein concentration in milk (*9*, *14*). It has been reported that the retention of rennet in cheese is linearly proportional to the amount of rennet added to milk (*1*, *15*). Also, the higher the cooking temperature during cheesemaking, the lower is the amount of rennet retained in cheese curd (*8*, *16*, *17*).

The quantity of rennet retained in cheese curd varies with the type of coagulant used; less fungal rennet, for example, *Rhizomucor meihei* proteinase, is retained in cheese curd than calf rennet (*9*, *11*). The pH of milk at rennet addition or the pH at which the whey is drained has no effect on the retention of *R. miehei* proteinase (*11*, *13*).

Considering the importance of residual rennet during the early stages of cheese ripening, much work has been done to develop methods for the determination of residual rennet in cheese curd. One of the earliest assays was to extract the residual rennet from cheese curd with a solvent and estimate the enzyme activity in the extract by measuring the milk clotting activity (*18*–*22*). Other methods include casein–agar diffusion (*11*, *23*, *24*), rocket immuno-electrophoresis (*25*), and enzyme-linked immunosorbent assay (*26*, *27*). For accurate and specific determination of active enzyme, a synthetic substrate has been used (*28*–*30*). Compared to other methods, this method is more sensitive, less

* Author to whom correspondence should be addressed (telephone +353-21-4902011; fax +353-21-4270001; e-mail p.mcsweeney@ucc.ie.

10.1021/jf071105p CCC: $37.00
Published on Web 10/04/2007

time-consuming, and less expensive and determines active enzyme rather than enzyme concentration. Most recently, a synthetic chromophoric heptapeptide (Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu) specific for aspartic proteinases has been used for the determination of residual enzyme activity in cheese curd (*31*).

The objectives of this study were to quantify the effects of the pH of milk at rennet addition and at whey drainage, the casein concentration in milk, the casein micelle size, the ionic strength of milk, and the quantity of chymosin added to milk on the retention of coagulant in cheese curd.

MATERIALS AND METHODS

Preparation of Coagulants. Two coagulants, chymosin (180 IMCU/mL, Maxiren-180, DSM Food Specialties, Delft, The Netherlands) and *Cryphonectria parasitica* proteinase (650 IMCU/mL, Suparen, DSM Food Specialties), were used. Unless otherwise stated, chymosin and *C. parasitica* proteinase preparations were added to milk at levels of 2 and 1.75 IMCU/mL, respectively, to give a coagulation time of ~50 min for commercial pasteurized skim milk. The effect of the pH of milk at rennet addition on the retention of both chymosin and *C. parasitica* proteinase in cheese curd was studied, but the other factors were studied only on the retention of chymosin.

Preparation of Casein Micelles. Casein micelles were prepared by ultracentifugation of raw milk at 100000*g* for 60 min at 20 °C, using an Optima LE-80K preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA), equipped with a Beckman-type 50.2 Ti rotor. Casein micelles differing in size were prepared from raw milk by centrifuging stepwise at forces ranging from 2600*g* to 100000*g* for 1 h each at 20 °C. The pellets of casein micelles obtained were freeze-dried.

Preparation of Cheese Curds. To study the effect of the pH of milk at rennet addition on the retention of rennet in cheese curd, samples of pasteurized skim milk (50 mL) were warmed to 30 °C and their pH values adjusted within the range of pH 6.7–5.3 and held overnight at 4 °C. Chymosin or *C. parasitica* proteinase was added to the pH-adjusted milk samples at 30 °C and the coagula were cut after 50 min. The curds–whey mixtures were centrifuged at 15000*g* for 10 min at 20 °C, and the whey was drained off. The curds were analyzed for moisture by drying to constant weight at 103 °C and for residual coagulant activity.

In a preliminary experiment the optimum cutting time for a control milk sample after the addition of chymosin was determined subjectively, and the corresponding G' (storage modulus) was measured by a controlled stress rheometer (Bohlin CS 50 Rheometer, Bohlin CVO, Bohlin Instruments, Cirencester, U.K.), according to the method of Hayes (*32*). The experimental milk samples with pH values in the range of 6.7–5.3 were then cut at that G' value. The curds were analyzed for moisture and for residual coagulant activity.

The effect of pH at whey drainage on the retention of chymosin in cheese curd was studied in curds prepared from 500 mL of pasteurized skim milk. The milk was warmed to 30 °C, chymosin solution was added to it, and the coagulum was cut after 50 min. A healing time of 10 min was allowed, with intermittent gentle stirring to prevent the curds from fusing. The pH of the curds–whey mixture was adjusted stepwise to values in the range of 6.6–4.9 by adding 1 N lactic acid. After each reduction in pH, the curds–whey mixture was stirred gently for 10 min to stabilize the pH and a sample of the curds (2–3 g) removed. The curds–whey mixtures were centrifuged at 15000*g* for 10 min at 20 °C, and the whey was drained off. The curd samples were analyzed for moisture and for residual chymosin activity.

To study the effect of the ionic strength of milk on the retention of rennet in cheese curd, samples (50 mL) of pasteurized skim milk were warmed to 30 °C and NaCl added at levels from 0.2 to 0.8 M. The pH of the milk samples containing NaCl was measured by a pH-meter, and the zeta-potential and casein micelle size were measured by a Zetamaster (Malvern Instruments Ltd., Malvern, Worchestershire, U.K.). Chymosin was added to the milk samples; preliminary experiments showed that the milk sample containing the highest quantity of NaCl formed a coagulum in 120 min and, therefore, all coagula were cut after 120 min. The curds–whey mixtures were centrifuged at 15000*g* for 10 min at 20 °C, and the whey was drained off. The curds were analyzed for moisture and for residual coagulant activity.

To study the effect of the casein concentration in milk on the retention of chymosin in cheese curd, the casein concentration (percent, w/v) in milk was increased to values from 2.24 (control milk) to 6.0% by dispersing different quantities of freeze-dried casein micelles (prepared as described under Preparation of Casein Micelles) in skim milk overnight, at 4 °C. To ensure complete dispersion of the casein micelles, the milk samples were stirred for a further 3 h at 40 °C. The casein content of some samples was reduced below 2.24% by diluting milk with the supernatant obtained by ultracentrifuging milk at 100000*g*. The supernatant was shown to be free of casein by urea–polyacrylamide gel electrophoresis (PAGE) (12.5% T, 4% C, pH 8.9) performed according to the method of Andrews (*33*), as modified by Shalabi and Fox (*34*). All milk samples were filtered through glass wool to remove any undispersed aggregates of casein micelles. The casein content of the milk samples was determined by subtracting the protein content of the whey (N × *6*.38) obtained by acid (1 M HCl) coagulation of milk at pH 4.6 from the total protein content of the milk, determined by the macro-Kjeldahl method (*35*). Chymosin was then added to the casein-adjusted milk samples at 30 °C, and the coagula were cut after 50 min. The curds–whey mixtures were centrifuged at 15000*g* for 10 min at 20 °C, and the whey was drained off. The curds were analyzed for moisture content and for residual coagulant activity. The quantity of curd produced from 50 mL of each milk sample was also measured.

To measure the effect of casein micelle size on the retention of chymosin in cheese curd, casein micelles of different sizes were dispersed as described above in synthetic milk ultrafiltrate (SMUF) (*36*) at a level of 2.4% protein. The size range of casein micelles in each sample was measured using a Zetamaster. The curds were then prepared and analyzed for moisture content and for residual coagulant activity.

To study the effect of the quantity of chymosin added to milk, chymosin was added at five different levels (2, 4, 6, 8, or 10 IMCU/mL of milk), and the curds were prepared and analyzed for moisture content and for residual coagulant activity.

Assay for the Residual Coagulant. Curd samples were analyzed for residual coagulant activity using the method of Hurley (*31*), except that the incubation time of citrate dispersions of curds with the heptapeptide substrate (Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu) was 24 h. After 24 h, reaction between the residual coagulant and the substrate was terminated by heating at 70 °C for 10 min, and the samples were centrifuged at 16000*g* for 10 min and filtered through Titan Syringe filters, RC 0.45 μm (Antech, Waterford, Ireland). The filtrate obtained was analyzed using reverse-phase (RP)-HPLC. The residual coagulant activity was expressed as units per hour per gram of dry matter, where 1 unit of enzyme activity is defined as the activity that releases 1 mmol of the product peptide [NO_2-Phe]-Arg-Leu from the heptapeptide substrate per hour at 37 °C and pH 3.2.

Statistical Analysis. Analysis of variance on the data was performed by one-way ANOVA using the statistical analysis software SPSS Version 11.0 for Windows XP (SPSS Inc., Chicago, IL). All results are the mean of triplicate analyses.

RESULTS AND DISCUSSION

Effect of pH at Whey Drainage on the Retention of Chymosin. In a separate study (Bansal et al., unpublished results), Cheddar-type cheeses were prepared and the residual chymosin activity was analyzed both in cheese and in whey samples. A careful mass balance proved that 100 ± 6% of chymosin added to milk can be recovered in a model system very similar to that used in this study. Chymosin solution was added to pasteurized skim milk, the coagulum was cut, and the pH of the curds–whey mixture was reduced to values in the range of 6.6–4.9. Curd samples were obtained at different pH values and analyzed for residual enzyme activity. The retention of chymosin did not change significantly ($P > 0.05$) in the pH range from 6.6 to 5.6, but increased significantly ($P < 0.05$)

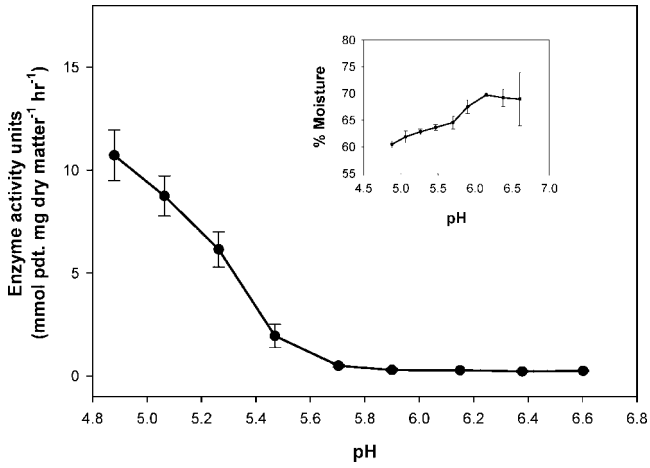

Figure 1. Enzyme activity (millimoles of product per milligram of dry matter per hour) in curds made using chymosin as coagulant as a function of the pH at whey drainage. (Inset) Percentage moisture in curds as a function of the pH at whey drainage. Values are means of replicates ($n = 3$); error bars indicate ± standard deviation.

when the pH was reduced to <5.6 (**Figure 1**). As expected, the moisture content of the curds decreased as the pH at whey drainage decreased.

The reason for the greater retention of chymosin at pH <5.6 may be that the casein micelles have a charge of about −20 mV at pH 6.6–6.8 and the isoelectric point (p*I*) of recombinant chymosin is about 4.75 (*37*). As the pH is reduced, the negative charge on both the micelles and the chymosin molecules decreases, as they approach their isoelectric point. Hence, the interaction of coagulant with casein micelles increases. The results of this study agree with those of Garnot et al. (*9*), who reported that during the manufacture of Camembert cheese, the enzyme concentration in whey decreased rapidly during the period of acidification by the starter.

Effect of the pH of Milk at Rennet Addition on the Retention of Rennet. To study the effect of pH of the milk on the retention of rennet, the pH of pasteurized skim milk was adjusted to values in the range of 6.7–5.3 and coagulated with chymosin or *C. parasitica* proteinase. In curds made with chymosin, the retention of residual enzyme did not change significantly ($P > 0.05$) in the pH range from 6.7 to 6.1, but increased significantly ($P < 0.05$) as the pH at rennet addition was reduced to <6.1 (**Figure 2a**). A similar trend was observed for curds made with *C. parasitica* proteinase, but a significant change ($P < 0.05$) in the retention of residual enzyme in curds was observed only below pH 5.8 (**Figure 2b**). In curds made with either chymosin or *C. parasitica* proteinase, the moisture content decreased when the pH of the milk was reduced (**Figure 2a**,**b**).

The results obtained in this study were similar to those reported in the literature; the retention of residual enzyme in cheese curds increased when the pH of milk at rennet addition was reduced (*8*, *11*, *13*). However, the pH below which any significant increase in the retention of coagulant is observed varies between studies. This could be due to many factors, such as differences in coagulants used, different methods for the quantification of residual coagulant, differences in systems in which measurements were done, or differences in the milk used for the study. In this study, the milk was kept overnight at 4 °C and rennet was added after a 15 min hold at 30 °C. It is possible that the milk may not have reached equilibrium fully after cooling. The effect of pH on the interaction of chymosin with caseins has also been studied in model systems. de Roos (*38*)

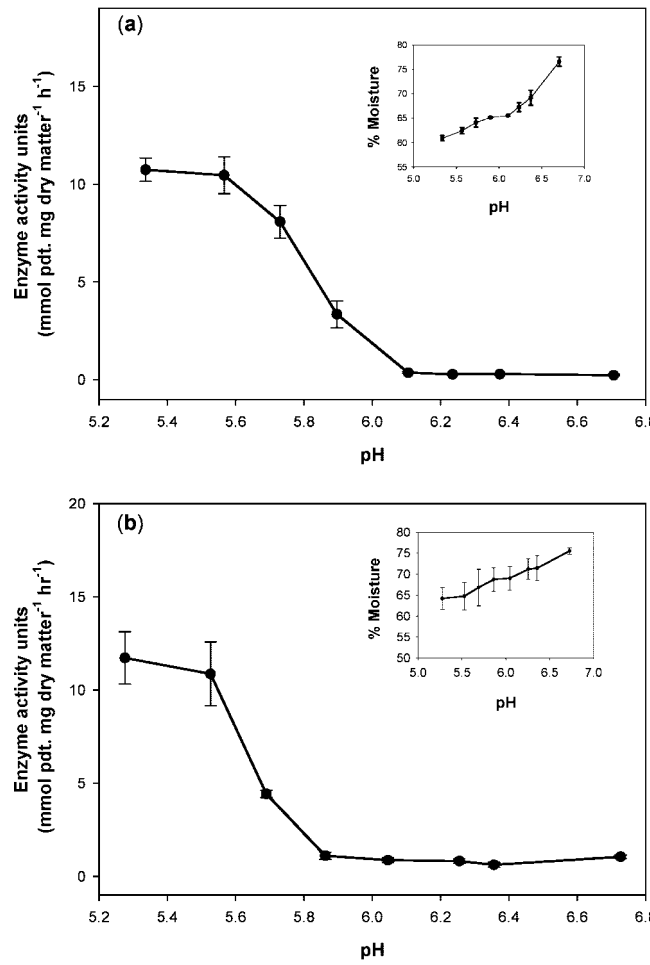

Figure 2. Enzyme activity (millimoles of product per milligram of dry matter per hour) in curds made using (**a**) chymosin or (**b**) *C. parasitica* proteinase as coagulant as a function of the pH of milk at rennet addition. (Inset) Percentage moisture in curds as a function of the pH of milk at rennet addition. Values are means of replicates ($n = 3$); error bars indicate ± standard deviation.

observed that the interaction of chymosin with *para*-κ-casein in an oil-in-water emulsion of soybean oil stabilized by α_s-, β-, or κ-casein increased when the pH of the emulsion was reduced. It was reported that when chymosin was added to a solution of κ-casein (dissolved in 0.05 M imidazole–HCl buffer; ionic strength of 0.04 M), the interaction of chymosin with *para*-κ-casein decreased as the pH increased (*10*). Larsson (*39*) found that the adsorption of chymosin (millimoles per mole of casein) on artificial casein micelles increased when the pH was reduced.

To study the effect of the firmness of the coagulum made from milks at different pH values, chymosin was added to pH-adjusted milk samples and the coagula were cut at the same G' value. The retention of enzyme in these curds was similar to that of the curds made from milk adjusted to different pH values and cut at the same time irrespective of their G' (**Figure 3**). The retention of chymosin in the curds taken from curds–whey mixtures adjusted to different pH values at whey drainage was lower than that in curds made from milk samples adjusted to the same pH values (**Figure 3**), presumably because the acid added to adjust the pH of curd–whey mixture may have needed time to diffuse throughout the curds, causing a difference in the pH.

Effect of Casein Concentration on the Retention of Chymosin. There was no significant difference in the moisture content of curds made from milk containing different concentra-

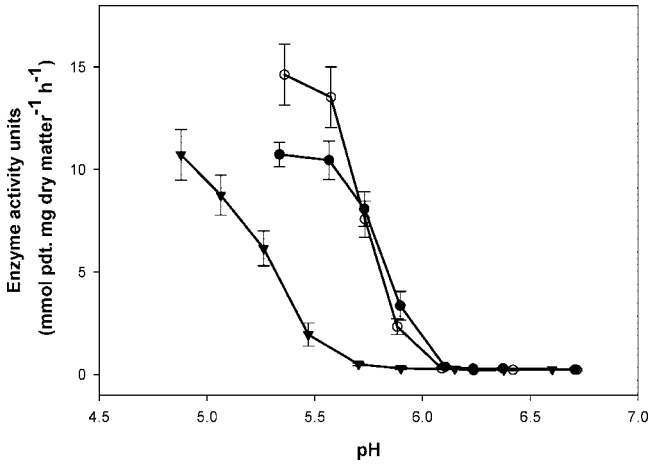

Figure 3. Enzyme activity (millimoles of product per milligram of dry matter per hour) in curds made using chymosin as coagulant as a function of the pH of milk at rennet addition and the coagulum cut at the same time (●) or at the same *G′* (○) or in curds from curd/whey mixtures with the pH adjusted to different values at whey drainage (▼). Values are means of replicates ($n = 3$); error bars indicate ± standard deviation.

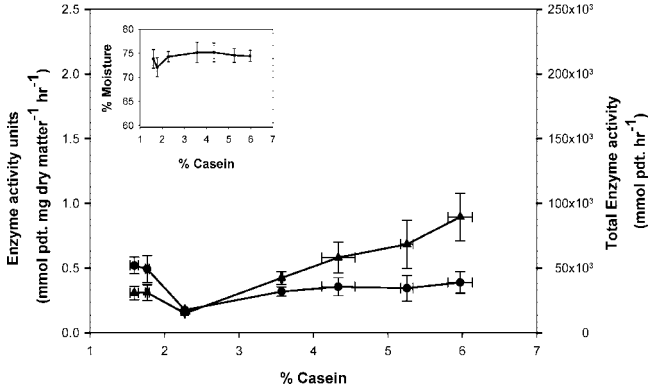

Figure 4. Enzyme activity per milligram of dry matter (millimoles of product per milligram of dry matter per hour, ●) and total enzyme activity (millimoles of product per hour, ▼) in curds made using chymosin as coagulant as a function of the percent casein in milk. (Inset) Percentage moisture in curds as a function of the percent casein in milk. Values are means of replicates ($n = 3$); error bars indicate ± standard deviation.

tions of casein (**Figure 4**), but the yield of curds (grams per milliliter of milk) increased when the casein content of milk was increased from 2.24 (control) to 6.0% (w/v) (results not shown). Increasing the casein content of milk had no significant ($P > 0.05$) effect on the retention of chymosin in curds (**Figure 4**), but the retention increased slightly when the casein content was reduced below that in the control. The reason for the increase in the retention of chymosin at low casein concentration may be that dilution of casein solution causes κ-casein to be less (self-) associated (*40–42*). Hence, in diluted milk, chymosin may associate more readily with *para*-κ-casein. The total retention of chymosin increased on either increasing or reducing the casein content, due to the increase in the total yield of curd.

Green (*14*) reported that the proportion of added rennet retained in curds made from unconcentrated milk was essentially the same as for curds made from milk concentrated to 1.7–4-fold by ultrafiltration. Garnot et al. (*9*) reported that the percentage of chymosin retained in curds made from ultrafiltered retentate was higher than that in curd made from unconcentrated milk. de Roos (*38*) observed that the percentage of adsorbed chymosin decreased on dilution of an emulsion of soybean oil

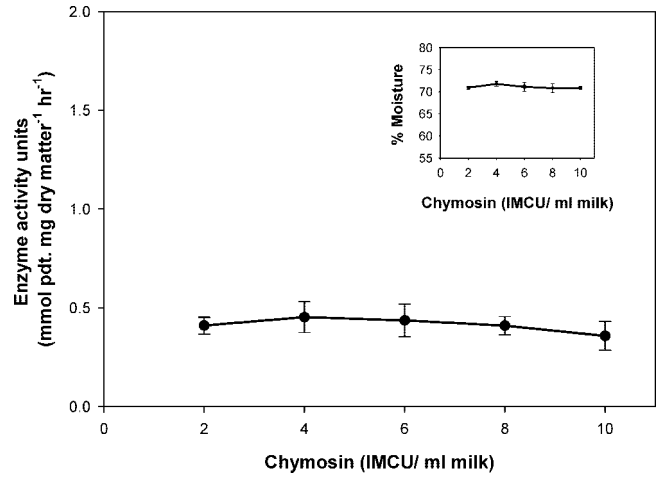

Figure 5. Enzyme activity (millimoles of product per milligram of dry matter per hour) in curds made using chymosin as coagulant as a function of the quantity of rennet added (IMCU/mL) to milk. (Inset) Percentage moisture in curds as a function of the quantity of rennet added (IMCU/mL) to milk. Values are means of replicates ($n = 3$); error bars indicate ± standard deviation.

stabilized by adsorbed κ-casein. However, the surface load (i.e., millimoles of chymosin bound per mole of *para*-κ-casein) increased as the degree of dilution increased. Dunnewind (*10*) observed that the binding of chymosin by κ-casein dissolved in imidazole–HCl buffer (pH 6.7, ionic strength of 40 mM) increased on dilution of a κ-casein solution with imidazole–HCl buffer at 30 °C.

Effect of Rennet Concentration on the Retention of Chymosin. There was no significant difference ($P > 0.05$) in the retention of chymosin in curds with increasing quantity of chymosin added to milk up to 5-fold above the normal level (**Figure 5**). Therefore, the percentage of added chymosin retained in cheese curd decreased with increasing quantity of chymosin added. Also, the moisture content of curds did not change significantly ($P > 0.05$; **Figure 5**). These results suggest that the casein micelles are saturated with respect to chymosin; that is, the amount of chymosin bound onto each casein micelle is almost constant. These results are supported by the fact that most of the coagulant added to milk during cheesemaking is lost in the whey. Some studies have reported, however, that the retention of rennet in cheese is linearly proportional to the amount of rennet added to milk (*1, 15*).

Effect of Average Casein Micelle Size on the Retention of Chymosin. To study the effect of average casein micelle size on the retention of chymosin in cheese curd, differently sized casein micelles were dispersed in SMUF at a constant protein level of 2.4%. These milk samples were then coagulated using chymosin, and residual chymosin activity in the curds was measured. The retention of chymosin increased significantly with decreasing average casein micelle size from 356 to ∼140 nm (**Figure 6**). The retention of chymosin in curds made from the control milk sample (containing casein micelles sedimented by centrifuging the milk at 100000*g* for 1 h) was not significantly different from that for the samples with casein micelles in the size range of ∼350–160 nm.

The amount of κ-casein in micellar casein is inversely related to micelle size (*43–46*). Also, there is an approximately linear relationship between the κ-casein content and the surface area of both natural and artificial casein micelles (*43, 47, 48*). The association of chymosin with casein micelles increases with increased κ-casein content in artificial micelles (*49*). Hence, the

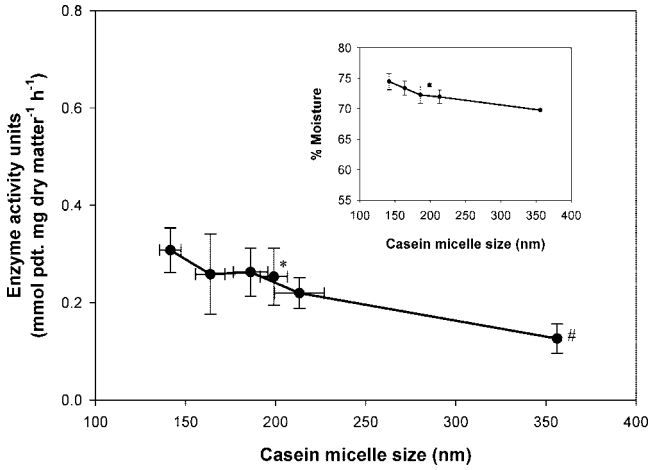

Figure 6. Enzyme activity (millimoles of product per milligram of dry matter per hour) in curds made using chymosin as coagulant as a function of the size of casein micelles (nanometers) in milk. (Inset) Percentage moisture in curds as a function of the casein micelle size (nanometers). Values are means of replicates ($n = 3$); vertical error bars indicate ± standard deviation for residual enzyme activity, and horizontal error bars indicate ± standard deviation for casein micelle size. The control milk (∗) consisted of casein micelles centrifuged at 100000*g* and redispersed in SMUF. (# indicates a milk sample prepared from a single lot of casein micelles with average size of 356 nm and analyzed in triplicate.)

Table 1. Zeta-Potential, Casein Micelle Size, and pH of Milk Samples to Which NaCl Was Added at Levels from 0.2 to 0.8 M

added NaCl (M)	zeta-potential[a] (mV)	pH of milk
0	−15.7 ± 2.1 a	6.67
0.2	−13.3 ± 2.5 b	6.55
0.4	−7.4 ± 2.3 c	6.51
0.6	−6.4 ± 3.1 d	6.46
0.7	−5.7 ± 3.4 d	6.45
0.8	−4.1 ± 2.2 e	6.44

[a] Numbers represent mean and standard deviation ($n = 6$); means within a column with different letters are significantly different (Tukey's HSD, $p < 0.05$).

retention of chymosin in cheese curds increases when the size of the casein micelles in milk is reduced. Casein micelle size had no statistically significant effect on the moisture content of the curds made from milk containing the differently sized casein micelles (**Figure 6**).

Effect of Ionic Strength on the Retention of Chymosin. The ionic strength of milk was increased above that of normal milk (∼0.08 M) by adding NaCl at levels from 0.2 to 0.8 M. The pH, casein micelle size, and zeta-potential of each milk sample were analyzed. Each milk sample was then renneted, and the curd samples were analyzed for residual enzyme activity. The pH of milk decreased from 6.67 to 6.44 when 0.8 M NaCl was added to milk (**Table 1**), probably due to the exchange of Na^+ for H^+ attached to the charged groups of the caseins (*50*). There was a very slight increase in the average casein micelle size with increasing ionic strength of milk, probably due to the increased hydration of casein micelles after the addition of NaCl to milk (*50–52*). The zeta-potential of the casein micelles decreased significantly from −15.7 to −4.1 mV with the addition of 0.8 M NaCl to milk (**Table 1**), partially due to neutralization of negative charge on the caseins by Na^+ (*50*). The moisture content of the curds increased with increasing ionic strength of milk (**Figure 7**). The retention of chymosin in curds ($P > 0.05$) increased significantly with increasing ionic strength of milk (**Figure 7**). This increase in the retention of chymosin

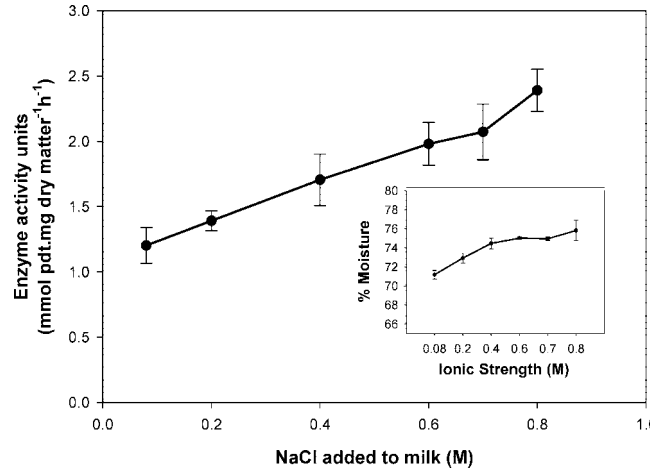

Figure 7. Enzyme activity (millimoles of product per milligram of dry matter per hour) in curds made using chymosin as coagulant as a function of NaCl (M) added to milk. (Inset) Percentage moisture in curds as a function of NaCl (M) added to milk. Values are means of replicates ($n = 3$); error bars indicate ± standard deviation.

in curds was probably due to the decrease in the zeta-potential of casein micelles with increasing ionic strength. At the ionic strength of control milk (∼0.08 M), both casein micelles and chymosin molecules are negatively charged and repel each other. As the zeta-potential of the casein micelles decreases, they become less negatively charged and the interactions between the casein micelles and chymosin molecules increase, increasing the retention of chymosin in curds. It has been reported that the binding of chymosin by κ-casein dissolved in imidazole–HCl buffer decreased with increasing ionic strength of the solution from 0.01 to 0.14 M (*10*).

As indicated by the results of this study, the effect of pH on the retention of chymosin became significant only when the milk pH was reduced below 6.1 (**Figure 2a**), and the average casein micelle size of milk had no effect on the retention of chymosin in curds (**Figure 4**). Therefore, the slight decrease in the pH of milk and the slight increase in the average casein micelle size of milk samples with increasing ionic strength should have no effect on the retention of chymosin.

Conclusions. This study showed that reducing the pH at whey drainage below pH 5.7 increased the retention of chymosin in cheese curd. The retention of chymosin and *C. parasitica* proteinase in cheese curd increased as the pH of milk at rennet addition was reduced below pH 6.1 and 5.8, respectively. The casein content of milk in the range from 2.24 to 6.0% (w/w) had no effect on the retention of chymosin in cheese curd, but reducing the casein concentration from 2.24 to 1.55% slightly increased the level of chymosin retained in the cheese curd. There was no change in the level of chymosin retained when the quantity of chymosin added to milk was increased from 2 to 10 IMCU/mL. Therefore, the quantity of coagulant bound per gram of casein remained unchanged. The residual chymosin activity increased significantly with decreasing average casein micelle size. The residual chymosin activity in cheese curds increased significantly when the ionic strength of the milk was increased above that of control milk (∼0.08 M) by adding NaCl. These results suggest that electrostatic forces play a role in the interaction of coagulants with casein micelles. Little is known about the forces that bind the coagulants to casein micelles, and these factors should be studied in more detail.

LITERATURE CITED

(1) Visser, F. M. W. Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 1. Description of cheese and aseptic cheesemaking techniques. *Neth. Milk Dairy J.* **1977**, *31*, 120–133.

(2) Visser, F. M. W.; de Groot-Mostert, A. E. A. Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 4. Protein breakdown: a gel electrophoretical study. *Neth. Milk Dairy J.* **1977**, *31*, 247–264.

(3) O'Keeffe, A. M.; Fox, P. F.; Daly, C. Proteolysis in Cheddar cheese: role of coalgulant and starter bacteria. *J. Dairy Res.* **1978**, *45*, 465–477.

(4) de Jong, L. Protein breakdown in soft cheese and its relation to consistency. 1. Proteolysis and consistency of 'Noorhollandse Meshanger' cheese. *Neth. Milk Dairy J.* **1976**, *30*, 242–253.

(5) Creamer, L. K.; Olson, N. F. Rheological evaluation of maturing Cheddar cheese. *J. Food Sci.* **1982**, *47*, 631–636.

(6) Creamer, L. K.; Zoerb, H. F.; Olson, N. F.; Richardson, T. Hydrophobicity of α_{s1}-I, α_{s1}-casein A and B and its implication in cheese structure. *J. Dairy Sci.* **1982**, *65*, 902–906.

(7) Ohmiya, K.; Sato, Y. Studies on the proteolytic action of dairy lactic acid bacteria. Part XII. Significant contribution of intercellular protease of lactic acid bacteria to the casein hydrolysis in cheese ripening. *Milchwissenschaft* **1972**, *27*, 417–421.

(8) Stadhouders, J.; Hup, G. Factors affecting bitter flavour in Gouda cheese. *Neth. Milk Dairy J.* **1975**, *29*, 335–353.

(9) Garnot, P.; Molle, D.; Piot, M. Influence of pH, type of enzyme and ultrafiltration on the retention of milk clotting enzymes in Camembert cheese. *J. Dairy Res.* **1987**, *54*, 315–320.

(10) Dunnewind, B.; de Roos, A. L.; Geurts, T. J. Association of chymosin with caseins in solution. *Neth. Milk Dairy J.* **1996**, *50*, 121–133.

(11) Holmes, D. G.; Duersch, J. W.; Ernstrom, C. A. Distribution of milk clotting enzymes between curd and whey and their survival during Cheddar cheese making. *J. Dairy Sci.* **1977**, *60*, 862–869.

(12) Vassal, L.; Gripon, J. C. Bitterness in Camembert cheese: role of rennet and *Penicillium caseicolum* and methods of control. *Lait* **1984**, *64*, 397–417.

(13) Creamer, L. K.; Lawrence, R. C.; Gilles, J. Effect of acidification of cheese milk on the resultant Cheddar cheese. *N. Z. J. Dairy Sci. Technol.* **1985**, *20*, 185–203.

(14) Green, M. L.; Glover, F. A.; Scurlock, E. M. W.; Marshall, R. J.; Hatfield, D. S. Effect of use of milk concentrated by ultrafiltration on the manufacture and ripening of Cheddar cheese. *J. Dairy Res.* **1981**, *48*, 333–341.

(15) Zoon, P.; Ansems, C.; Faber, E. J. Measurement procedure for concentration of active rennet in cheese. *Neth. Milk Dairy J.* **1994**, *48*, 141–150.

(16) Matheson, A. R. The immunological determination of chymosin activity in cheese. *N. Z. J. Dairy Sci. Technol.* **1981**, *15*, 33–41.

(17) Rampilli, M.; Raja, V.; Gatti, A. L. Valutazione dell'attività residua del caglio nel formaggio. *Sci. Tec. Latt. Cas.* **1998**, *49*, 29–41.

(18) Berridge, N. J. Some observations on the determination of the activity of rennet. *Analyst* **1952**, *77*, 57–62.

(19) Ernstrom, C. A. Heterogeneity of crystalline rennin. *J. Dairy Sci.* **1958**, *41*, 1663–1670.

(20) Douillard, D.; Ribadeau, Dumas, B. Determination using casein of the proteolytic activity of rennin, porcine pepsin and bovine pepsins. *Bull. Soc. Chim. Biol.* **1970**, *52*, 1429–1445.

(21) Dulley, J. R. The contribution of rennet and starter enzymes to proteolysis in cheese. *Aust. J. Dairy Technol* **1974**, *29*, 65–69.

(22) Stadhouders, J.; Hup, G.; van der Waal, C. B. Determination of calf rennet in cheese. *Neth. Milk Dairy J.* **1977**, *31*, 3–15.

(23) Cheeseman, G. C. Action of rennet and other proteolytic enzymes on casein in casein–agar gels. *J. Dairy Res.* **1963**, *30*, 17–24.

(24) Lawrence, R. C.; Sanderson, W. B. A micro-method for the quantitative estimation of rennets and other proteolytic enzymes. *J. Dairy Res.* **1969**, *36*, 21–29.

(25) Rothe, G. A. L.; Axelsen, N. H.; Jøhnk, P.; Foltman, B. Immunological, chromatographic, and milk-clotting activity measurements for quantification of milk-clotting enzymes in bovine rennets. *J. Dairy Res.* **1976**, *43*, 85–95.

(26) Boudjellab, N.; Rolet-Repecaud, O.; Collin, J. C. Detection of residual chymosin in cheese by an enzyme-linked immunosorbent assay. *J. Dairy Res.* **1994**, *61*, 101–109.

(27) Larsson, K. I.; Andrén, A.; Geurts, T. J.; de Roos, A. L. Enzyme-linked immunosorbent assay (ELISA) and milk clotting test (MCT) for determination of the affinity between chymosin and artificial casein micelles. *Neth. Milk Dairy J.* **1995**, *49*, 37–46.

(28) Hill, R. D. Synthetic peptide and ester substrates for rennin. *J. Dairy Res.* **1969**, *36*, 409–415.

(29) Polzhofer, K. P. Synthesis of a rennin-sensitive pentadecapeptide from bovine κ-casein. *Tetrahedron* **1972**, *28*, 855–865.

(30) Raymond, M. N.; Bricas, E.; Salesse, R.; Garnier, J.; Garnot, P.; Ribadeau-Dumas, B. A proteolytic unit for chymosin (rennin) activity based on a reference synthetic peptide. *J. Dairy Sci.* **1973**, *56*, 419–422.

(31) Hurley, M. J.; O'Driscoll, B. M.; Kelly, A. L.; McSweeney, P. L. H. Novel assay for the determination of residual coagulant activity in cheese. *Int. Dairy J.* **1999**, *9*, 555–558.

(32) Hayes, M. G.; Kelly, A. L. High pressure homogenisation of raw whole bovine milk (a) effects on fat globule size and other properties. *J. Dairy Res.* **2003**, *70*, 297–305.

(33) Andrews, A. T. Proteinases in normal bovine milk and their action on caseins. *J. Dairy Res.* **1983**, *50*, 45–55.

(34) Shalabi, S. I.; Fox, P. F. Electrophoretic analysis of cheese, comparison of methods. *Ir. J. Food Sci. Technol.* **1987**, *11*, 135–151.

(35) IDF. Determination of the nitrogen content (Kjeldahl method) and calculation of crude protein content, 20A; International Dairy Federation (IDF), Brussels, Belgium, **1986**.

(36) Jenness, R.; Koops, J. Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Neth. Milk Dairy J.* **1962**, *16*, 153–164.

(37) Bines, V. E.; Young, P.; Law, B. A. Comparison of Cheddar cheese made with a recombinant calf chymosin and with standard calf rennet. *J. Dairy Res.* **1989**, *56*, 657–664.

(38) de Roos, A. L.; Walstra, P.; Geurts, T. J. Association of chymosin with adsorbed caseins. In *Food Macromolecules and Colloids*; Dickinson, E., Lorient, D., Eds.; Royal Society of Chemistry: Cambridge, U.K., 1995; pp 50–57.

(39) Larsson, K. I.; Andrén, A.; Geurts, T. J.; de Roos, A. L.; Walstra, P. Association of chymosin with artificial casein micelles as influenced by micelle composition and pH. *Int. Dairy J.* **1997**, *7*, 43–46.

(40) Waugh, D. F. Casein interactions and micelle formation. *J. Phys. Chem.* **1961**, *65*, 1793–1797.

(41) Nakai, S.; Kason, C. M. A fluorescence study of the interactions between κ- and α_{s1}-casein and between lysozyme and ovalbumin. *Biochim. Biophys. Acta* **1974**, *351*, 21–27.

(42) Schmidt, D. G. Association of caseins and casein micelle structure. In *Developments in Dairy Chemistry–1. Proteins*; Fox, P. F., Ed.; Applied Science Publishers: London, U.K., 1982; pp 61–86.

(43) McGann, T. C. A.; Donnelly, W. J.; Kearney, R. D.; Buchheim, W. Composition and size distribution of bovine casein micelles. *Biochim. Biophys. Acta* **1980**, *630*, 261270..

(44) Dalgleish, D. G.; Brinkhuis, J.; Payens, T. A. J. The coagulation of differently sized casein micelles by rennet. *Eur. J. Biochem.* **1981**, *119*, 257–261.

(45) Davies, D. T.; Law, A. J. R. Variation in the protein composition of bovine casein micelles and serum casein in relation to micellar size and milk temperature. *J. Dairy Res.* **1983**, *50*, 67–75.

(46) Dalgleish, D. G.; Horne, D. S.; Law, A. J. R. Size-related differences in bovine casein micelles. *Biochim. Biophys. Acta* **1989**, *991*, 383–387.

(47) Sullivan, R. A.; Fitzpatrick, M. M.; Stanton, E. K. Distribution of κ-casein in skim milk. *Nature* **1959**, *183*, 616–617.

(48) Waugh, D. F.; Talbot, B. Equilibrium casein micelle systems. *Biochemistry* **1971**, *10*, 4153–4162.
(49) de Roos, A. L.; Geurts, T. J.; Walstra, P. The association of chymosin with artificial casein micelles. *Int. Dairy J.* **2000**, *10*, 225–232.
(50) Grufferty, M. B.; Fox, P. F. Effect of added NaCl on some physicochemical properties of milk. *Ir. J. Food Sci. Technol.* **1985**, *9*, 1–9.
(51) Donnelly, W. J.; Barry, J. G.; Buchheim, W. Changes in casein micelle in late lactation milk. *Food Science and Technology Research Report*, An Foras Taluntais: Dublin, Ireland, 1980; pp 14–15.
(52) O'Keeffe, A. M. Seasonal and lactational influences on moisture content of Cheddar cheese. *Ir. J. Food Sci. Technol.* **1984**, *8*, 27–37.

Received for review April 16, 2007. Revised manuscript received August 20, 2007. Accepted August 31, 2007.

JF071105P