Factors Affecting the Rennet Clotting Properties of Ewe's Milk

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The effect of temperature (65−85 °C), heating time (0−35 min), pH (6.2−6.8) and $CaCl_2$ concentration (0−10 mM) on rennet clotting properties of ewe's milk was studied using a Formagraph apparatus. Heating at 85 °C for 35 min caused slight changes in the rennet clotting time, although β-lactoglobulin was completely denatured. The rennet clotting time decreased as the concentration of $CaCl_2$ increased. Rennet clotting time decreased and curd firming rate increased as pH decreased. Under the conditions of this study, Ca^{2+} concentration was the most significant factor affecting rennet clotting properties of ewe milk.

Keywords: *Ewe milk; rennet clotting*

INTRODUCTION

The cheese-making properties of milk are affected by a number of factors, including temperature and time of heating, pH of milk, and presence of divalent cations; thus to standardize cheese quality, these factors have to be controlled.

The effect of heating on the rennet clotting (RC) properties of cow's milk is to render the milk less susceptible to coagulation. As the intensity of heating increases, the clotting time is progressively lengthened, and the curd consistency decreases (McMahon and Brown, 1982; Ustunol and Brown, 1985; Pagnacco and Caroli, 1987; Singh et al., 1988). Although changes in salt equilibrium are involved (Kannan and Jenness, 1961; Amram et al., 1982) the factor responsible for the increased rennet clotting time (RCT) of heated milk is the complex between κ-casein and β-lactoglobulin (Marziali and Ng-Kwai-Hang, 1986; Pellegrini et al., 1994) or α-lactalbumin (Shalabi and Wheelock, 1976).

Acidification of milk samples before heating reduces the adverse effect of severe heat treatment on RCT (Tsugo and Yamauchi, 1959; Shalabi and Fox, 1982). Van Hooydonk et al. (1986) suggested that both the accessibility of the Phe-Met bond of κ-casein at the surface of the micelle and the interaction between chymosin and para-κ-casein are affected by pH.

Addition of Ca^{2+} decreases the RCT of cow milk (Kannan and Jenness, 1961; Marshall et al., 1982; Gouda, 1987; Lucey and Fox, 1993); the reduction of the potential RCT by about 40% has been attributed to the addition of Ca^{2+} to negatively charged phosphoserin residues or carboxylic acid groups of α- and β-casein (Lucey and Fox, 1993).

Most of these investigations on factors affecting the clotting properties have been conducted on cow's milk; however, given the differences in milk composition among species, the results obtained cannot be extrapolated to other ruminant species. Some authors have worked with ewe's milk. Pellegrini et al. (1994) reported that RCT rises while the gel firming rate becomes slower with advancing lactation.

The production of ewe's milk constitutes an alternative to cow's milk of increasing importance. Ewe's milk is mainly used in the cheese industry for the manufacture of ewe's milk cheeses as well as cheeses from mixtures with other milk species. Since the renneting behavior is a major factor in cheese making, information dealing with rennet clotting properties of ewe's milk, compared to that of cow's milk, would allow the improvement of cheese-making processes from this type of milk.

MATERIALS AND METHODS

Samples. Raw milk from cow and ewe herds in the Central Region of Spain were used. The Ca^{2+} in the milk was increased by adding 5 M $CaCl_2$ solution at room temperature until a final concentration of 0.2−10 mM on milk, these concentrations increase the [Ca^{2+}] of the milk between 2 and 7.85 mM (Singh et al., 1988). Milk samples were adjusted to pH values in the range 6.2−6.8 with 1 M HCl or 1 M NaOH immediately prior to heating. Samples were stirred vigorously during addition of acid to avoid localized coagulation.

Heat Treatments. Portions (15 mL) of milk were heated at 65, 70, 75, 80, and 85 °C in a water bath for 5−35 min in tightly sealed Pyrex glass tubes (16 × 162 mm). Heated samples were immediately cooled and kept at 30 °C for 1 h before analysis.

All experiments were replicated four or nine times using milk samples collected on different days.

Renneting Properties. Rennet solution was obtained by diluting 300 mg of rennet powder containing 85% of chymosin and 15% of bovine pepsin (Hansen, Copenhagen, Denmark, rennet strength 1:100.000) to 100 mL with 0.2 M sodium citrate buffer at pH 5.2.

The renneting properties were determined by use of the Formagraph apparatus (McMahon and Brown, 1982); 10 mL of milk were allowed to equilibrate to 30 °C for 30 min, then 200 μL of rennet solution was added to each sample, and the RC properties were determined. This apparatus draws a "firmness versus time" diagram as RC occurs. The RCT is the time from addition of enzyme until the point at which the two lines diverge. The time in minutes, from RCT until the two lines are 20 mm apart, represents the rate of curd firming (K_{20}). The curd at this time is considered to be firm enough for cutting. The a_{30} value is the width of the graph 30 min after enzyme is added.

Protein Analysis. Undenatured milk serum proteins were obtained by acidification of milk to pH 4.6 by addition of 2 M HCl followed by centrifugation; soluble whey proteins were determined by HPLC (Resmini et al., 1989). The denaturation for each protein in a heated sample was expressed as the percentage of the area of the corresponding peak in the acid filtrate from its raw milk.

Composition of Milk. The total solids were determined after drying to constant weight at 115 °C. The proteins were determined by N content × 6.38 as measured by the Kjeldahl technique. Fat was determined by the Gerber method and ash after burning at 550 °C for 16 h.

Statistical Analysis. Means, standard deviation (SD) among batches, and variance were calculated by a BMDPpv2 program (Brussels, Belgium, 1983) with a CDC Ciber 180/855

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Table 1. Composition of Four Samples of Cow's Milk and 10 Samples of Ewe's Milk

species	sample batch	milk composition, %			
		fat	ash	total solids	proteins
cow	1	3.64	0.63	11.42	3.41
	2	3.72	0.62	11.58	3.26
	3	3.71	0.66	11.67	3.42
	4	3.74	0.67	11.52	3.47
	mean	3.70	0.65	11.55	3.39
	SD[a]	0.043	0.023	0.105	0.091
ewe	1	8.14	0.93	19.53	5.51
	2	7.38	0.84	18.50	5.52
	3	8.44	0.86	18.29	5.85
	4	7.95	0.85	18.39	5.39
	5	7.90	0.80	18.60	5.40
	6	8.01	0.84	18.92	5.41
	7	7.40	0.76	17.26	5.04
	8	8.52	0.79	17.20	4.99
	9	7.39	0.96	19.79	6.11
	10	7.50	0.83	18.63	5.35
	mean	7.86	0.85	18.51	5.46
	SD	0.431	0.061	0.830	0.334

[a] SD = standard deviation among batches.

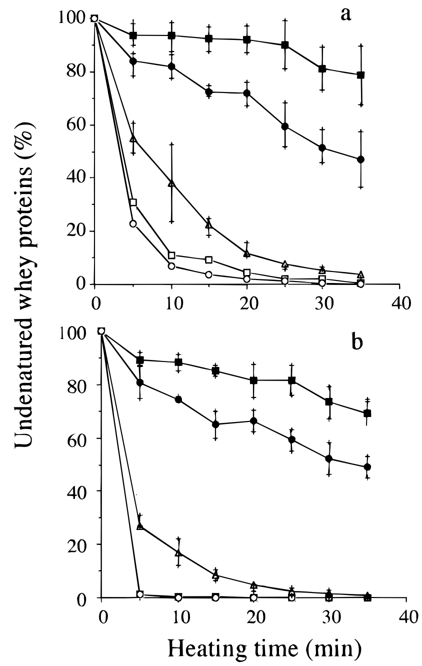

Figure 1. Percentage of undenatured α-lactalbumin (a) and β-lactoglobulin (b) of ewe's milk heated at 65 °C (■), 70 °C (●), 75 °C (△), 80 °C (□), and 85 °C (○) for 5−35 min.

computer. A one-way analysis of variance was calculated to test the influence of the time, temperature, and $CaCl_2$ concentration on rennet clotting properties of ewe's milk

RESULTS AND DISCUSSION

Effect of Heat Treatments. Since the milk composition could influence the RCT properties, the composition of four cow's milks and nine ewe's milks were analyzed (Table 1). The contents of ash, fat, and proteins in ewe's milk were higher than those of cow's milk. These results are in agreement with the results of Storry et al. (1983) and Anifantakis (1985).

To slow the coagulation rate of ewe's milk and achieve an accurate measurement on the Formagraph chart, a lower concentration of rennet was used (rennet strength 1:100.000) in all experiments.

To study the influence of the temperature and time on RC properties of ewe's milk, four samples were heated to temperatures of 65−85 °C for up to 35 min; Table 2 shows the variance analysis of the influence of heat and temperature treatments on RC properties of ewe's milk. RCT of ewe's milk was not significantly ($P < 0.05$) affected by the heating time and temperature. The K_{20} remained practically unaltered when ewe's milk was heated at 65 °C and increased significantly ($P < 0.01$ or $P < 0.001$) in samples heated at higher temperatures but were not affected by the time. The a_{30} value was not affected by the heat treatments used ($P < 0.05$).

Because complex formation between denatured whey proteins and κ-casein can affect cow's milk RCT (Swayer, 1969; Marziali and Ng-Kwai-Hang, 1986; Pagnacco and Caroli, 1987), whey protein denaturation during heat treatment of ewe's milk was studied (Figure 1). α-Lactalbumin was less denatured than was β-lactoglobulin. More than 20% of proteins were denatured for

Table 2. Rennet Clotting (RC) Properties of Ewe's Milk Submitted to Different Heat Treatments

heating time, min	RC properties	heating temperature,[e] °C				
		65	70	75	80	85
5	RCT (min)	$4.6^{a,A}$ (0.25)	$4.5^{a,A}$ (0.57)	$5.6^{a,A}$ (1.03)	$5.2^{a,A}$ (1.32)	$5.3^{a,A}$ (1.31)
10		$4.3^{a,A}$ (0.50)	$4.6^{a,A}$ (0.47)	$5.3^{a,A}$ (0.64)	$4.5^{a,A}$ (1.22)	$5.0^{a,A}$ (1.11)
15		$4.3^{a,A}$ (0.28)	$4.9^{a,A}$ (0.48)	$5.3^{a,A}$ (0.64)	$4.5^{a,A}$ (0.91)	$4.5^{a,A}$ (1.58)
20		$4.4^{a,A}$ (0.25)	$5.1^{a,A}$ (0.48)	$5.4^{a,A}$ (0.92)	$4.5^{a,A}$ (0.95)	$4.2^{a,A}$ (0.95)
25		$4.5^{a,A}$ (0.40)	$5.3^{a,A}$ (0.64)	$5.3^{a,A}$ (0.95)	$4.8^{a,A}$ (0.95)	$4.5^{a,A}$ (1.08)
30		$4.3^{a,A}$ (0.64)	$5.3^{a,A}$ (0.47)	$5.3^{a,A}$ (0.13)	$4.0^{a,A}$ (0.74)	$5.3^{a,A}$ (1.00)
35		$4.2^{a,A}$ (0.41)	$5.5^{a,A}$ (0.29)	$4.6^{a,A}$ (0.78)	$4.1^{a,A}$ (0.75)	$4.3^{a,A}$ (0.86)
5	K_{20} (min)	$2.5^{a,A}$ (0.70)	$2.8^{a,A}$ (0.64)	$4.3^{b,A}$ (1.50)	$3.7^{b,A}$ (1.09)	$5.3^{b,A}$ (2.13)
10		$2.5^{a,A}$ (0.71)	$3.1^{a,A}$ (1.14)	$5.5^{b,A}$ (1.14)	$4.9^{b,A}$ (0.48)	$6.8^{b,A}$ (1.37)
15		$2.8^{a,A}$ (1.10)	$3.1^{a,A}$ (1.31)	$5.3^{b,A}$ (0.75)	$4.2^{a,A}$ (0.96)	$5.9^{b,A}$ (1.46)
20		$2.5^{a,A}$ (0.57)	$3.5^{b,A}$ (0.57)	$5.3^{c,A}$ (0.64)	$4.7^{b,c,A}$ (1.14)	$7.5^{d,A}$ (1.51)
25		$2.7^{a,A}$ (0.75)	$4.1^{a,c,A}$ (0.85)	$5.1^{b,c,d,A}$ (0.94)	$5.0^{a,d,A}$ (1.03)	$6.6^{b,A}$ (1.65)
30		$2.8^{a,A}$ (0.86)	$4.0^{a,c,A}$ (0.91)	$5.3^{b,c,d,A}$ (0.47)	$4.7^{a,c,b}$ (0.57)	$6.8^{b,A}$ (1.27)
35		$2.3^{a,A}$ (0.96)	$4.1^{a,c,A}$ (0.86)	$4.6^{b,c,B}$ (2.02)	$4.1^{b,c}$ (1.23)	$6.3^{b,c,A}$ (1.50)
5	a_{30} (mm)	$30.7^{a,A}$ (2.98)	$38.8^{a,A}$ (5.31)	$36.8^{a,A}$ (2.50)	$38.5^{a,A}$ (1.91)	$33.0^{a,A}$ (1.50)
10		$34.2^{a,A}$ (5.34)	$38.5^{a,A}$ (2.38)	$38.5^{a,A}$ (3.60)	$38.8^{a,A}$ (1.89)	$35.0^{a,A}$ (2.51)
15		$33.0^{a,A}$ (5.90)	$38.5^{a,A}$ (5.19)	$38.5^{a,A}$ (1.20)	$39.8^{a,A}$ (0.50)	$32.2^{a,A}$ (1.70)
20		$32.5^{a,A}$ (5.18)	$44.0^{a,A}$ (4.97)	$44.0^{a,A}$ (2.30)	$39.7^{a,A}$ (2.16)	$32.3^{a,A}$ (2.73)
25		$36.6^{a,A}$ (8,42)	$37.0^{a,A}$ (4.70)	$37.0^{a,A}$ (3.10)	$36.5^{a,A}$ (2.08)	$32.3^{a,A}$ (2.33)
30		$33.7^{a,A}$ (3.36)	$35.3^{a,A}$ (5.80)	$35.3^{a,A}$ (3.00)	$35.5^{a,A}$ (8.34)	$38.3^{a,A}$ (4.69)
35		$35.2^{a,A}$ (7.50)	$34.9^{a,A}$ (1.97)	$35.0^{a,A}$ (2.21)	$38.2^{a,A}$ (4.43)	$32.8^{a,A}$ (2.21)

[a−d] Means in the same row without a common superscript differ ($P < 0.05$). [A,B] Means in the same column without a common superscript differ ($P < 0.05$) [e] Means and standard deviation were calculated from four different samples. [f] RCT = rennet clotting time, K_{20} = time in minutes from RCT until the two lines are 20 mm apart and represents the rate of curd firming and a_{30} is the width of the graph 30 min after enzyme is added.

Table 3. pH of Ewe's Milk Containing Various Amounts of $CaCl_2$ and Submitted to Heat Treatments

	pH of milk samples		
$CaCl_2$ concn, mM	after $CaCl_2$ addition	after heating at[a] 70 °C	after heating at 80 °C
0	6.69[b]	6.54	6.51
0.2	6.66	6.53	6.50
0.5	6.65	6.53	6.48
1	6.63	6.51	6.46
2	6.61	6.49	6.46
4	6.57	6.48	6.45
6	6.52	6.45	coagulated
8	6.50	6.43	coagulated
10	6.45	6.41	coagulated

[a] Before heat treatments samples were adjusted to pH 6.7, changes in pH after heat treatment at 70 or 80 °C for 30 min are also shown in the table. [b] Mean of four different milk samples.

samples heated at 70 °C for 20 min. More than 90% of α-lactalbumin and all of the β-lactoglobulin were denatured in samples heated at 80 °C for 15 min. These results indicated that whey protein denaturation should not have appreciably influenced the RCT of ewe's milk.

The differences in the RCTs of cow's and ewe's milk could be due to the differences on milk composition and micelle composition and conformation. As can be observed in Table 1 the protein and ash concentrations are higher in ewe's than in cow's milk. O'Connor and Fox (1977) have reported a higher concentration of calcium and phosphorus in ewe's than in cow's milk. Anifantakis (1985) has not found great differences in β-lactoglobulin concentration between the two studied species; differences between composition and size distribution of micelle have been reported (Storry et al., 1983; Anifantakis, 1985).

The differences in the salt composition and the changes in the salt equilibrium during heating could be very important in RCT of ewe's milk, since salt composition has a great influence on the aggregation of the micelles. On the other hand, since the composition and conformation of the micelles are very important in the enzymatic attack of the chymosin and the coagulation of the destabilized micelles, the differences on the components and the composition of the casein micelles of the two species could influence both the enzymatic action of the chymosin and the approach of the micelles sufficiently close to coagulate.

The differences on milk composition of ewe's and cow's milk could also influence other rennet clotting properties. Some differences in the casein concentration of the two species have been found by different authors (Storry et al., 1983; Anifantakis, 1985), and Pagnnaco and Caroli (1987) and Marziali and Ng-Kwai-Hang (1986), studying the effect of genetic polymorphism on coagulation properties of milk, found that the amount and composition of κ-casein were the most significant factors affecting curd firmness.

Effect of Addition of $CaCl_2$. Calcium chloride (0–10 mM) was added to raw milk prior to heating at 70 or 80 °C for 30 min. The pH of the milk decreased with $CaCl_2$ addition (Table 3). The pH was readjusted to the initial pH before heat treatment; after heat treatments the changes in the pH values were determined. Addition of $CaCl_2$ above 4 mmol/L caused coagulation of ewe's milk during heating at 80 °C for 30 min; similar results were obtained when goat's milk was submitted to the same treatment (Montilla et al., 1995).

The effect of $CaCl_2$ concentration on the RC properties of ewe's milk is shown in Table 4. Addition of $CaCl_2$ decreased significantly ($P < 0.05$) the RCT of raw and heated samples; these results agree with those found by Storry et al. (1983) and Trevala et al. (1985) on cow's milk and by Montilla et al. (1995) on goat's milk.

Both k_{20} and a_{30} values decreased significantly ($P < 0.05$) for raw milk when the $CaCl_2$ concentration increased; however, the influence of $CaCl_2$ was not notice-

Table 4. Analysis of Variance of Four Ewe's Milk Samples Containing Different $CaCl_2$ Concentrations

$CaCl_2$ concn, mM	RC properties[b]	raw milk	milk heated at 70 °C for 30 min	milk heated at 80 °C for 30 min
0	RCT (min)	5.5^{a,A} (0.71)	6.1^{a,A} (2.13)	4.5^{a,A} (2.13)
0.2		5.2^{a,A,C} (0.35)	5.5^{a,A,C} (0.71)	3.5^{b,B,C} (0.71)
0.5		5.0^{a,A,C} (0.00)	5.3^{a,b,A,D} (0.50)	4.3^{a,b,A,C,D} (0.50)
1		5.0^{a,A,C} (0.00)	5.3^{a,A,C,E} (0.50)	4.7^{a,A} (0.50)
2		4.5^{a,A,C,D} (0.00)	4.8^{a,b,B,C,F} (0.50)	3.6^{a,b,B,D} (0.50)
4		4.3^{a,B,C,D,E} (0.35)	4.2^{a,B,D,E} (0.25)	2.9^{b,B} (0.25)
6		3.5^{a,B,D} (0.00)	3.6^{a,B} (0.47)	coagulated
8		3.5^{a,B,D} (0.00)	3.5^{a,B,F} (0.40)	coagulated
10		2.8^{a,B,E} (1.06)	3.4^{a,B,F} (0.25)	coagulated
0	K_{20} (min)	1.5^{a,A} (0.71)	1.7^{a,A} (0.29)	1.8^{a,A} (0.29)
0.2		1.3^{a,A,C} (0.35)	1.7^{a,A} (0.25)	1.1^{a,A} (0.25)
0.5		1.3^{a,A,C} (0.35)	1.5^{a,A} (0.00)	4.3^{a,A} (0.25)
1		1.0^{a,B,C} (0.00)	1.5^{a,A} (0.00)	4.7^{a,A} (0.58)
2		1.0^{a,B,C} (0.00)	1.5^{a,A} (0.25)	3.6^{a,A} (0.71)
4		1.0^{a,B,C} (0.00)	1.3^{a,A} (0.29)	2.9^{a,A} (1.41)
6		1.0^{a,B,C} (0.00)	1.2^{a,A} (0.29)	coagulated
8		1.0^{a,B,C} (0.00)	1.3^{a,A} (0.29)	coagulated
10		1.0^{a,B,C} (0.00)	1.5^{a,A} (0.00)	coagulated
0	a_{30} (mm)	51.5^{a,A} (3.53)	57.3^{b,A} (0.57)	57.7^{a,A} (2.21)
0.2		53.0^{a,A} (2.83)	58.6^{b,A} (1.52)	52.7^{a,A} (2.50)
0.5		50.0^{a,A} (1.41)	53.5^{a,A} (4.11)	54.5^{a,A} (1.29)
1		58.0^{a,A,C} (4.24)	56.0^{a,A} (4.54)	51.0^{a,A} (4.50)
2		50.5^{a,A} (3.53)	56.3^{a,A} (4.86)	54.0^{a,A} (2.94)
4		52.0^{a,A} (2.82)	52.3^{a,A} (5.31)	42.0^{a,B} (7.97)
6		48.5^{a,A,D} (2.12)	52.3^{a,A} (3.69)	coagulated
8		48.0^{a,A,D,E} (8.48)	52.5^{a,A} (2.08)	coagulated
10		39.5^{a,B,D,E} (3.53)	51.3^{b,A} (1.52)	coagulated

[a] Means in the same row without a common superscript differ ($P < 0.05$). [A–F] Means in the same column without a common superscript differ ($P < 0.05$). [b] RCT is the rennet clotting time, K_{20} is the time when the curd firmness is adequate for cutting the cheese curd; a_{30} is the width of the graph 30 min after enzyme is added.

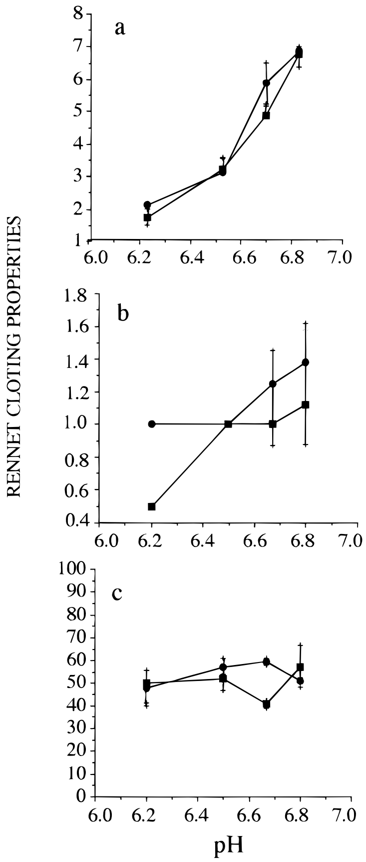

Figure 2. Influence of pH on (a) rennet clotting time, (b) K_{20}, and (c) a_{30} of raw milk (■) and milk heated at 70 °C for 5 min (●).

able in heated samples. This could be due to the influence of the changes in the [Ca^{2+}] during heating of the micelles aggregation.

Effect of pH. Samples of ewe's milk were adjusted to pH 6.2−6.8. At pH lower than 6.5, ewe's milk coagulated when heated to 70 °C for 30 min. However, heating at 70 °C for 5 min did not cause coagulation in the pH range studied.

Figure 2 shows the RC properties of ewe's milk under different pH conditions. Similar behavior was observed for RCT of raw and heated milk samples (70 °C for 5 min). The marked decrease in RCT with decreasing pH found was in agreement with that previously reported for cow's milk (Tsugo and Yamauchi, 1959; Shalabi and Wheelock, 1982). The K_{20} of all samples deceased with pH. No influence of pH was found on the a_{30} value.

The RC properties of ewe's milk were not affected by heat treatments but were influenced by $CaCl_2$ concentration and pH. Because acidification increased Ca^{2+}, RC properties of ewe's milk are probably influenced largely by Ca^{2+} concentration.

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