ORIGINAL RESEARCH

Influence of residual milk-clotting enzyme and proteolysis on melting properties of soft cheese

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In this work, we assessed the influence of coagulant residual activity and primary proteolysis on Cremoso Argentino cheese melting properties. For that purpose, we made Cremoso soft cheeses using different amounts of coagulant, and also obtained samples in which milk-clotting enzyme was inactivated. Primary proteolysis correlated with residual activity of coagulant in early stages of cheese ripening; however, it was similar in all cheeses after 30 days. The hydrolysis of caseins did not significantly affect the melting ability of the cheeses, expressed as the area increase after heating samples under standardized conditions. Samples with similar proximate composition showed some changes in meltability; those seemed related to pH evolution during ripening.

Keywords Coagulant enzyme, Melting properties, Proteolysis, Soft cheeses.

INTRODUCTION

Cremoso Argentino is a soft cheese variety manufactured with full fat pasteurized milk and a thermophilic starter culture (Código Alimentario Argentino 2007). Unlike most other soft cheeses, Cremoso is not surface ripened by moulds or bacteria. Cheesemaking is also relatively simple as it does not comprise cooking or washing of the curd and it is not pressed. As a consequence, the product has a characteristic taste, slightly acid and buttery, and a creamy texture; it shows very low consistency and can flow or spread easily upon heating. Cremoso Argentino is the most consumed cheese in Argentina, generally as an inexpensive substitute for Mozzarella (Centro de la Industria Lechera 2007).

Melting ability (or meltability) is a useful property to describe the behaviour of cheeses during heating. It can be defined as the ability of cheese to flow and spread at high temperatures. In a research study (Lefevere *et al*. 2000), cheese was described as a sponge-like structure in which the protein forms a network around the fat globules. Upon heating, the cheese fat melts, resulting in weakening of the cheese, which cannot support its own weight. According to this concept, all factors affecting either the fat content of the cheese, its distribution, moisture content, or the extent and strength of the protein network, would influence cheese meltability. For instance: the pH of cheese has been reported to impact on meltability, by modifying the capacity of proteins to bind water and interact (Mercanti *et al*. 2004). Similarly, fat content and time of ripening correlate well with melting properties of Cheddar and Mozzarella (Muthukumarappan *et al*. 1999a,b; Lefevere *et al*. 2000; Kuo *et al*. 2001).

Proteolysis, which is linked to ripening time as it normally advances during this period, was also studied as a potential factor influencing meltability, with diverse results. Some authors proposed that proteolysis diminished the strength of the protein matrix by degrading casein molecules into shorter peptides, improving cheese meltability (Lefevere *et al*. 2000). On the contrary, other work reported that this property was not significantly modified by proteolysis. The study also showed that meltability of Mozzarella cheese increased with age, but did not exhibit significant changes when coagulant concentration was modified (Kindstedt *et al*. 1995).

Proteolysis is a relevant biochemical transformation during the ripening of most cheeses, and it has been extensively studied and reviewed (Sousa *et al*. 2001; Upadhyay *et al*. 2004; Mcmahon *et al*. 2005). In soft cheeses, the main proteolytic agent is the residual milk-clotting enzyme, because the high moisture content and the lack of a cooking step enhance its retention in the curd and its activity in the food environment (de Jong 1976; de Jong 1977; Giangiacomo *et al*. 1993). Milk-clotting enzyme preferentially attacks α_{s1} casein at the primary site Phe_{23}-Phe_{24}, releasing the peptide α_{s1}-CN (f1-23), which is soluble in water and at pH 4.6, and α_{s1}-CN (f24-199)—formerly known as α_{s1}-I CN—which is insoluble in the same conditions (Carles and Ribadeau-Dumas 1985; Mcsweeney

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et al. 1993). This reaction has been reported as the main biochemical event in soft cheese ripening (Hynes *et al*. 2001).

In addition, although it is generally accepted that α_{s1} casein hydrolysis has a significant role in the development of appropriate texture in soft cheeses (Visser 1993; Hynes *et al*. 1999; Guinee 2003; Upadhyay *et al*. 2004), so far this event has not been related either with the residual activity of milk-clotting enzyme or with melting properties.

The aim of the present work was to study the influence of the residual activity of milk-clotting enzyme and proteolysis extent on the melting properties of Cremoso Argentino cheese. For that purpose, we performed cheesemaking experiments using different amounts of coagulant, and studied meltability, residual milk-clotting activity and proteolysis in the resultant cheeses.

MATERIALS AND METHODS

Cheesemaking

Bulk raw milk was obtained from a nearby dairy plant, taken into the laboratory under refrigerated conditions and batch pasteurized at 65°C for 30 min. After cooling to 37°C, $CaCl_2$ was added up to a final concentration of 0.2% (w/v). Cheeses were made according to the standard process for Cremoso Argentino cheese, adapted to laboratory conditions, on two different scales:

Miniature cheeses

Three types of mini-cheeses were prepared, using different amounts of coagulant: (i) control cheeses (C_{mini}), in which 0.5 ml/L was added into milk; (ii) cheeses with a half dose of coagulant ($1/2C_{mini}$, 0.25 ml/L); and (iii) cheeses with a double dose of coagulant ($2C_{mini}$, 1.0 ml/L of milk). In all cases, adult bovine coagulant was used (230 IMCU, Naturen, Chr. Hansen, Quilmes, Argentina). Miniature cheeses were prepared in large-neck 2-L containers, using miniature stainless steel tools, according to a protocol previously described (Milesi *et al*. 2007). They were ripened at 4°C for 30 days.

Laboratory-scale cheeses

Cremoso cheeses in which the milk-clotting enzyme was inactivated (IC_{lab}) were made in a specially designed 15-L vat (Meinardi *et al*. 1998). The milk-clotting enzyme was porcine pepsin (3450 IMCU, Diagramma S.A. Santa Fe, Argentina) which was added to milk and inactivated after the first step of milk coagulation, by pH cycling. The pH cycle adopted was 6.4–7.8–6.4 (Meinardi *et al*. 1998; Hynes *et al*. 2001). Control cheeses (C_{lab}) were obtained in the same vat by performing the pH cycle before the addition of porcine pepsin.

Replicate cheesemakings were carried out in different days. All cheeses were vacuum packed in plastic bags and ripened at 4°C for 90 days.

Cheese analysis

Cheeses were analysed for dry matter and protein content according to the International Dairy Federation (IDF Standard 4:A 1962 and IDF Standard 20:B 1993, respectively). Fat content was determined by the Gerber method, and pH was assessed with a Horiba pH meter (Horiba, Kyoto, Japan).

Protein hydrolysis in the cheese samples was assessed by nitrogen fractionation and urea polyacrylamide gel electrophoresis (urea-PAGE). Crude citrate extracts of the cheeses were precipitated at pH 4.6. The soluble fraction was analysed for nitrogen content by the macro-Kjeldahl method, and the result expressed as a percentage of the total nitrogen content of the cheese (%SN-pH 4.6) (Gripon *et al*. 1975; Hynes *et al*. 2001). The insoluble fraction was purified and analysed by urea-PAGE in a Mini-Protean II cube (Bio-Rad Laboratories, CA, USA). Acrylamide concentration was 7.5%, and proteins were stained with Coomassie Blue G-250 (Andrews 1983).

The residual activity of milk-clotting enzyme in the cheeses was quantified as described by Rampilli *et al*. (1998). An aliquot of cheese extract was added to a standard milk substrate and incubated under controlled conditions. Proteins were then precipitated with trichloroacetic acid and the supernatant, containing the casein macro-peptides (CMP) released from κ-CN, was analysed by liquid chromatography. The amount of CMPs was proportional to the residual activity of coagulant in the cheese, and the two parameters were related by a calibration curve obtained using known amounts of milk-clotting enzyme. Reverse phase liquid chromatography conditions were as described by Zoon and Faber (1994), Rampilli *et al*. (1998) and Hynes *et al*. (2004). The HPLC equipment consisted of a quaternary pump, an on-line degasser and UV/VIS detector, all Series 200, purchased from PerkinElmer (PerkinElmer, Norwalk, CT, USA). It was provided with an analytical column 250 mm × 4.6 mm Aquapore OD-300, C18, 5 nm-300 A°. An interface module connected to a computer was used to obtain the chromatographic data with the software TURBOCHROM (PerkinElmer, Norwalk, CT, USA).

Cheese melting properties were determined by the Schreiber test, modified by Muthukumarappan *et al.* (1999a). Cheese disks (3 mm thick, 42 mm diameter) were obtained by cutting cheese blocks with a standardized device. The disks were placed on glass plates and heated in an oven with natural-convection at 130°C for 15 min. Then the plates were taken out of the oven and left until they were at room temperature. The surface covered by the

Table 1 Gross composition, pH, soluble nitrogen at pH 4.6 (%SN-pH 4.6) and residual activity of coagulant enzyme in miniature cheeses prepared with different rennet amounts after 30 days of ripening. $1/2C_{mini}$: cheeses with half of the dose of coagulant; C_{mini}: control cheeses; and $2C_{mini}$: cheeses with double dose of coagulant. Means and standard deviations of three replicate cheeses are presented

Cheese type	*Moisture (%)*	*Fat (%)*	*pH*	*%SN-pH 4.6*	*Residual coagulant activity (μL cheese kg^{-1})*
$1/2C_{mini}$	54.88 ± 1.02	21.5 ± 0.95	5.08 ± 0.03	13.30 ± 1.63	240 ± 18[a]
C_{mini}	56.19 ± 0.98	22.7 ± 1.15	5.27 ± 0.05	14.27 ± 1.39	490 ± 31[b]
$2C_{mini}$	56.20 ± 1.37	22.6 ± 1.33	5.08 ± 0.01	14.85 ± 1.58	717 ± 40[c]

[a,b,c]: Values in the same column with different superscripts showed significant differences ($P < 0.001$)

melted cheese was calculated by scanning the plates in a regular scanner (Snapscan Touch, AGFA, Petalin Jaya, Malaysia) connected to a computer provided with specially—designed software (Área, Departamento de Física, Facultad de Ingeniería Química, UNL). The software is based on the difference of colour between the melted cheese and the surrounding area: cheese surface is calculated by adding all the pixels whose colour is similar to the cheese's, after selecting a small area as colour reference. Surface can be expressed in cm^2 after calibration with a paper sheet of known area.

Two replicated samples were taken from each cheese to assess melting ability, and the area was measured thrice.

Statistical analysis

Soluble nitrogen values and meltability measurements were analysed by one-way ANOVA. Principal components analysis (PCA) was applied to the studied variables after standardization (correlation matrix), to explore the origin of data variation and detect subjacent structures among samples. Linear partial least squares (PLS) regression was also used, to analyse the relationship between melting ability (Y-block) and chemical parameters (X-block) (Hough *et al*. 1996; Lawlor *et al*. 2001; Poveda *et al*. 2004). Statistics were performed with Unscrambler ® (CAMO Inc., Oslo, Norway).

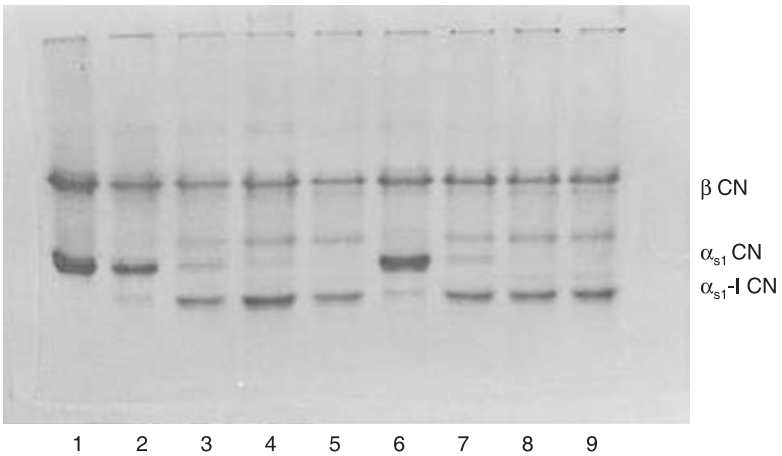

Figure 1 Electrophoresis of insoluble nitrogen at pH 4.6, for mini-cheeses made with half ($1/2C_{mini}$), normal (C_{mini}), and double dose ($2C_{mini}$) of milk-clotting enzyme. Lane 1: standard (isoelectric caseinate), lanes 2 and 6: curd of C_{mini} cheese before moulding, lanes 3–5 and 7–9: $1/2C_{mini}$, C_{mini} and $2C_{mini}$ 30-day-old cheeses, respectively. Cheeses from two replicate cheesemakings are shown.

RESULTS AND DISCUSSION

Miniature cheeses

Gross composition of the cheeses, as well as pH values, % SN-pH 4.6 and residual activity of coagulant, is shown in Table 1. Gross composition of the different types of mini-cheeses was not significantly different ($P > 0.05$); it was similar to the typical proximate composition of standard Cremoso cheeses (Milesi *et al*. 2007).

Not surprisingly, the residual activity of milk-clotting enzyme differed significantly from one type of cheese to another ($P < 0.01$). Increasing the coagulant dose by a factor of two actually doubled the residual activity of coagulant in Cremoso cheese, which differs from previous observations in other cheese varieties (Visser and de Groot Mostert 1977; de Jong 1978; van der Berg and Exterkate 1993). However, in these previous studies the residual activity of milk-clotting enzyme was not measured, but inferred from the production of α_{s1}CN(f24-199), which in our case too, was very similar in $1/2C_{mini}$, C_{mini}, and $2C_{mini}$ cheeses at the end of the ripening (Fig. 1). It has been reported that α_{s1}CN(f24-199) can undergo further degradation by rennet (Visser 1993). That would explain the similar final amount of α_{s1}CN(f24-199) in all cheeses. However, SN-pH 4.6 did not significantly differ either, indicating that primary proteolysis was equivalent in the three types of miniature cheeses. Only a numerical, nonsignificant difference was detected for $1/2C_{mini}$ cheeses. Both α_{s1}CN(f24–199) production detected by urea-PAGE and nitrogen content in pH 4.6 soluble fraction are indexes of primary proteolysis in cheese (O'Keefe *et al*. 1978).

Taking into account these results, it was reasonable to propose that: (i) coagulant retention and

Table 2 Gross composition of laboratory scale cheeses. C_{lab}: control cheeses, CI_{lab}: coagulant inactivated cheeses. Means and standard deviations of three replicate cheeses are presented; proximate composition of both types of cheese did not differ ($P > 0.05$)

Cheese	*Moisture (%)*	*Fat matter (%)*	*Protein content (%)*
C_{lab}	47.17 ± 1.29	24.40 ± 1.60	21.69 ± 1.97
IC_{lab}	48.66 ± 1.51	23.90 ± 0.99	20.99 ± 2.04

residual activity in the curd were proportional to the added dose of the enzyme, and (ii) 50% of the usual rennet dose was enough to cause extensive hydrolysis of the intact caseins after 30 days of ripening, especially the breakdown of α_{s1} casein to give α_{s1}CN(f24-199) and its complementary peptide, α_{s1}CN(f1-23) (Noomen 1978; Noël and Lefler 1991; Hynes *et al*. 2001).

As for melting ability, it did not significantly change from one type of cheese to another, which was not surprising: although they did differ in residual activity of milk-clotting enzyme, proteolysis level was very similar, as well as gross composition (Figure 2).

Multivariate analysis confirmed the trends already discussed. PCA showed that the dimensionality of the system could be reduced from eight variables (moisture, fat matter, protein content, initial pH, final pH, SN-pH 4.6, residual activity of rennet, and area of melted cheese) to one PC which represented 99.76% of the variation. The only variable that significantly influenced PC1 was residual activity of rennet, and sample grouping along the PC1 axis was only due to this activity. PLS results were not satisfactory as melting ability did not correlate well with the rest of the studied variables (most of them did not vary), and residual activity of rennet did not show a significant influence (results not shown).

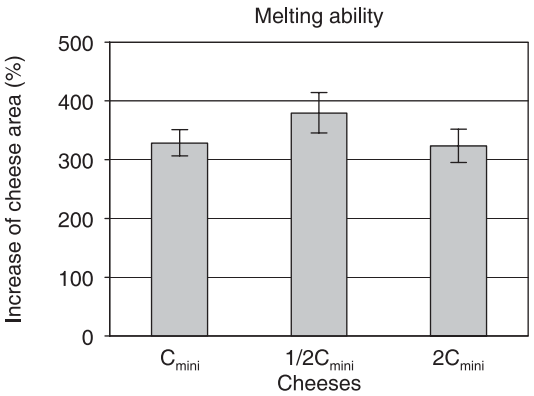

Figure 2 Melting ability of miniature cheeses prepared with half (1/2C_{mini}), normal (C_{mini}), and double dose (2C_{mini}) of milk-clotting enzyme, expressed as the increase of cheese area after heating in an oven in standardized conditions (%). Means and standard deviations are shown.

Laboratory-scale cheeses

Taking into account the results obtained using the mini-cheese model, a cheesemaking experiment on a laboratory scale was performed, aimed at obtaining cheeses with different proteolysis levels. Ripening period was extended up to 90 days in order to monitor proteolysis and melting ability for a longer time.

Gross composition of control and experimental cheeses did not differ (Table 2). As a consequence of the inactivation of the enzyme, rennet residual activity in IC_{lab} cheeses was very low, while C_{lab} cheeses showed an activity at least 10 times higher, which remained constant during all ripening (Table 3). Variation in rennet activity during ripening for each type of cheese was considered not relevant, taking into account the detection and quantification limits of the method (Aparo *et al*. 2004). Consequently, these results suggest that primary proteolysis was significantly different between IC_{lab} and C_{lab} cheeses, especially at the beginning of the ripening period. The amount of SN-pH 4.6 differed in samples younger than 30 days (Table 3), and peptide α_{s1}CN(f24-199) was detected in IC_{lab} only after 15 days of ripening (Figure 3). However, values tended to level up at the end of ripening period. This result suggested that a very low residual activity of coagulant enzyme was enough to produce the hydrolysis of α_{s1} casein and the consequent appearance of α_{s1}CN(f24-199), although more slowly than in control cheeses. Another explanation could be a poor correlation between the rennet activity quantified by HPLC and the actual expression of the enzyme displayed as α_{s1} casein degradation in cheese. Finally, the breakdown of α_{s1} casein could be occasioned by other proteases present in the curd, such as cathepsin D (Hurley *et al*. 2000a; 2000b; Larsen *et al*. 2000).

Despite the difference in proteolysis extent verified during the first month of ripening, melting ability of IC_{lab} and C_{lab} cheeses did not differ ($P > 0.05$). Both cheeses showed good melting properties even at the beginning of the ripening, when α_{s1}CN(f24-199) was very low in C_{lab} and not detected in IC_{lab} cheese, and %SN-pH 4.6 was lower than 6.0 in both cheeses. After 60 days of ripening, we observed a highly significant change between the meltability of IC_{lab} and C_{lab} cheeses:

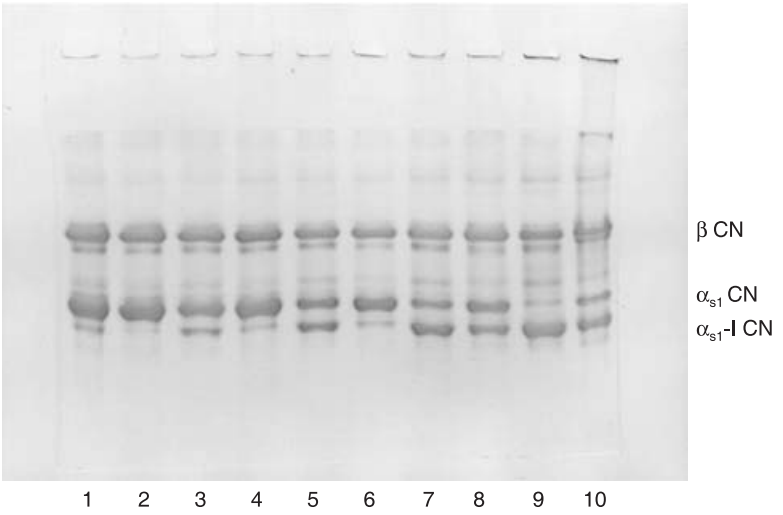

Figure 3 Electrophoresis of insoluble nitrogen at pH 4.6 for laboratory-scale cheeses. Lanes 1, 3, 5, 7, and 9: control cheese (C_{lab}) cheese at 1, 7, 14, 30, and 60 days of ripening, respectively. Lanes 2, 4, 6, 8, and 10: cheese with inactivated coagulant (IC_{lab}) at 1, 7, 14, 30, and 60 days of ripening, respectively. Electrophoretic patterns of samples from other cheesemaking days were similar.

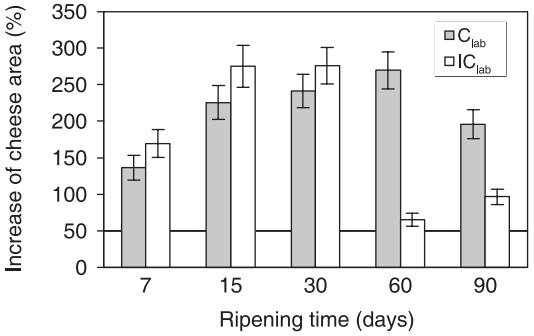

Figure 4 Melting ability of control cheese (C_{lab}) and cheese with inactivated coagulant (IC_{lab}) obtained at laboratory scale, expressed as the increase of cheese area after heating in an oven in standardized conditions (%). Means and standard deviations are shown.

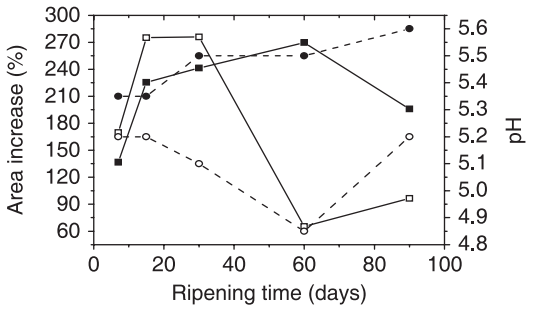

Figure 5 Melting ability (solid lines) and pH (dash lines) evolution during ripening of control cheese (■,●) and cheese with inactivated coagulant (□,○) obtained at laboratory scale. Points in the curves are the average of three measurements; variation was always lower than 20%.

the increasing of the area of IC_{lab} after heating was much lower than that of C_{lab} cheese (Figure 4). However, this difference could not be attributed to casein hydrolysis, as the studied indexes of proteolysis were similar for both cheeses at this time of ripening (Table 3, Figure 3).

PCA showed that the main source of variation among cheese samples was, as expected, residual activity of rennet and SN-pH 4.6, but no correlation among proteolysis and meltability was detected (results not shown).

The results suggested that meltability of Cremoso cheeses depended on factors other than proteolysis, such as changes in pH or in colloidal calcium content, which are concurrent events during ripening. Recent research has reported that proteolysis takes place simultaneously with solubilization of colloidal Ca included in the paracasein matrix, leading to the consequent loss of cross-linking material and weakening of the cheese body (Lucey *et al*. 2005; O'Mahony *et al*. 2005).

In our work, the pH of IC_{lab} and C_{lab} cheeses evolved very differently. Even if they started the ripening process with relatively close pH values (5.17 and 5.35, respectively), after 60 days the difference of pH between the two types of cheese was as high as 0.65 U (Figure 5). Starter activity was probably not the cause of such pH differences,

Table 3 Soluble nitrogen at pH 4.6 (%SN-pH 4.6) and residual activity of coagulant enzyme in laboratory-scale cheeses. Means and standard deviations of three replicate cheeses are shown. C_{lab}: control cheeses, IC_{lab}: coagulant inactivated cheeses

Ripening time (days)	*%SN-pH 4.6*		*Residual coagulant activity (μL cheese kg^{-1})*	
	C_{lab}	IC_{lab}	C_{lab}	IC_{lab}
7	6.2 ± 0.7^{a},	3.4 ± 0.2^{b},	360 ± 22^{c},	58 ± 14^{d}
15	12.1 ± 1.6^{a},	5.9 ± 0.3^{b}	338 ± 36^{c},	25 ± 9^{d}
30	14.9 ± 0.8^{a},	6.5 ± 0.5^{b}	279 ± 19^{c},	22 ± 9^{d}
60	20.56 ± 1.9	20.34 ± 2.3	356 ± 10^{c},	69 ± 12^{d},
90	22.7 ± 1.5	20.9 ± 0.9	347 ± 27^{c},	25 ± 15^{d},

^{a,b} and ^{c,d}: Values with different superscripts showed significant differences ($P < 0.001$)

as the starter composition was the same and the inoculation method was not changed from IC_{lab} to C_{lab} cheeses. The unexpected result is probably a consequence of the pH cycling for milk-clotting enzyme inactivation, performed during cheese-making. The method used to inactivate coagulant in IC_{lab} cheeses enzyme employed the incubation of milk with milk-clotting enzyme at 4°C by 45 min for the accomplishment of the enzymatic step of milk coagulation; followed by 60 min at pH 7.80 at the same temperature, to inactivate the enzyme; and then adjusting the pH value back to 6.40, before heating renneted milk to obtain coagulation (Meinardi *et al*. 1998; Hynes *et al*. 2001). C_{lab} cheeses suffered similar temperature and pH changes, but in a shorter period of time and before the addition of rennet. Acid development and pH have been shown to play a key role in the extent of calcium solubilization in cheese (Lee *et al*. 2005). Ionic calcium increase has also been reported for milk maintained at refrigeration temperatures (Raynal and Remeuf 2000).

In addition, lower pH in IC_{lab} cheeses may have promoted coagulant activity (Noomen 1978), explaining the high proteolysis levels found after 30 days in these cheeses, despite the low milk-clotting enzyme amount. Likewise, an increase in the impact of plasmin on proteolysis of less acidic C_{lab} cheeses could have been expected (Grufferty and Fox 1988); however, hydrolysis of its preferential substrates, i.e. β and α_{s2} caseins, was not increased in control cheeses (Figure 3).

Even when the changes of pH were not intended and, in fact, were probably a collateral result of our experimental treatment, it is important to observe that the highest differences between meltability values were found when the pH of IC_{lab} and C_{lab} cheeses differed; pH and meltability showed similar trends during ripening for both type of cheeses (Figure 5).

CONCLUSIONS

Proteolysis in soft cheeses with low levels of residual milk-clotting enzyme was slowed down, but not diminished in comparison with control cheeses. Hydrolysis of the caseins did not significantly affect soft cheese meltability. For the studied samples, which presented similar proximate composition, the changes in melting ability seemed to be related to pH evolution during ripening. New studies including physical analyses of the cheeses are currently in course in our laboratory.

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