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Influence of cheese making technologies on plasmin and coagulant associated proteolysis

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ABSTRACT

The aim of this work was to study the influence of different cheese-making technologies applied for three varieties of Argentinean cheeses on the action of coagulant enzyme, plasmin-plasminogen system and proteolysis. For this, Cremoso (soft cheese), Pategrás (semi-hard cheese) and Reggianito (hard cooked cheese) cheeses were analyzed for composition, nitrogen fractions, enzymes activities and elecrophoresis throughout ripening. As for coagulant, no reactivation of the enzyme was registered during ripening. Changes were mainly related to cooking temperature, as decreasing cooking temperature increased coagulant activity, being superior in Cremoso, followed by Pategrás and then by Reggianito cheeses. In regards of plamin/plasminogen system, it was observed greater activity of inactive plasminogen in Reggianito and Cremoso cheeses; while in Pategrás cheese the level was very low probably because plasminogen activation was enhanced during cheese making by the elimination of plasminogen activators inhibitors by curd washing. Indeed, the highest plasmin activity was found in Pategrás cheese, which indicates that curd washing combined with soft thermal treatment of the curd favored plasminogen activation. However, the environment defined by Reggianito cheese matrix was more suitable for maintaining the stability of plasmin activity along ripening. Results were consistent with proteolysis registered in electrophoresis and nitrogen fractions.

1. Introduction

Proteolysis is a complex primary event that occurs in most cheese varieties, especially relevant in internal bacterially ripened cheeses such as Cheddar, Swiss, and Gouda (McSweeney, 2011). It is of great interest to characterize the extent and pattern of proteolysis in cheeses, as it represents an index of maturity and influences the final quality of the product. Even if cheese proteolysis has been extensively studied, comparative research on cheese making technologies and their influence on different proteolytic agents and profiles are still lacking.

Breakdown of intact caseins – especially αs1 and β caseins - is mostly caused by non-microbial proteases in cheese, i.e. coagulant and plasmin (Chitpinityol & Crabbe, 1998; Grufferty & Fox, 1988). It has been correlated with texture development and physical properties of the cheese, although discussion on the actual extension of its influence is ongoing (Mistry, 2012). Subsequent changes on casein-derived peptides, especially N-terminal peptides, are mainly attributed to the microbiota and makes significant contribution to flavor. Free amino acids and small peptides can impart umami and bitter taste notes, but they are much more relevant as flavor precursors via amino acid catabolism, a transformation also caused by lactic microbiota, that may lead to 50% of cheese flavor compounds (Upadhyay, McSweeney, Magboul, & Fox 2004; Yvon, 2006).

Cheese making technology, especially regarding to milk pretreatment, curd washing, curd cooking and pH curve, affects mainly primary proteolysis and can be related *a priori* with changes in plasmin and coagulant activities (Bansal, Fox, & McSweeney, 2007; Grufferty & Fox, 1988; Hynes, Delacroix-Buchet, Meinardi, & Zalazar, 1999), while secondary proteolysis and flavor formation are mainly mediated by lactic acid bacteria in cheese (Sousa, Ardo, & McSweeney, 2001). The differentiate effect of cheese making operations on plasmin activity is due to the fact that plasmin, its precursor, inhibitors and activators have diverse heat resistance and solubility (Grufferty & Fox, 1988; Sommers & Kelly, 2002). Therefore, the hydrolysis of β and α_{s2}-caseins, preferential substrates of

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plasmin, has been reported to depend on heat treatment and curd washing (Rampilli & Raja, 1998; Sommers & Kelly, 2002).

As for coagulant, its retention and activity in cheese, and the consequent hydrolysis of α_{s1} casein, depends on technological factors such as pH at draining, cooking temperature or cheese moisture (Gaiaschi et al., 2000; Jacob, Jaros, & Rohm, 2010).

The association between cheese making technology, coagulant and plasmin activity and the proteolysis pattern during ripening has been studied only for few cheese varieties, not including Argentinean cheeses (Bansal et al., 2007; Nega & Moatsou, 2012). In the present work, we studied the most produced and consumed cheeses in Argentina, which in turn represent soft (Cremoso), semi hard (Pategrás) and hard cooked (Reggianito) cheese families. They are all cow's milk cheeses enzymatically-coagulated and produced with starters of thermophilic lactic acid bacteria. Argentina is one of the main world cheese producers, with 563.943 tons per year, most of it consumed in the domestic market (12.44 Kg per capita per year), and 54,088 tons of cheese exports in 2012 (Minagri, 2012).

The aim of this work was to assess and compare the effect of cheese making process on plasmin and coagulant activities and proteolysis patterns of three types of Argentinean cheeses, representative of soft, semi hard, and hard-cooked cheese varieties.

2. Material and methods

2.1. Cheeses

We studied three cheese varieties widespread in Argentina: Cremoso, Pategrás and Reggianito. The most important aspects of the cheese making technologies of these traditional cheeses are shown in Table 1. For this, three replicates were obtained from a nearby dairy plant. Replicates came from different cheese making batches and different days. Young cheeses were transported to our laboratory the same day they came out of brine. All the cheeses were ripened in our ripening room at 80% relative humidity with the following conditions of temperature and time of storage: 7 °C and 22 days for Cremoso cheeses; 12 °C and 50 days for Pategrás cheeses, 12 °C and 180 days for Reggianito cheeses.

Cheeses were sampled according to the International Dairy Federation standard method (IDF, 1995) for the analytical determinations at different ripening times (Table 2).

2.2. Analyses

2.2.1. Gross composition

Gross composition was assessed by standard methods: moisture was determined by oven drying (IDF, 1982), fat content by Gerber-Van Gulik method (IDF, 1997), and pH according to APHA (Bradley et al., 1992).

2.2.2. Proteolysis

Proteolysis was described by assessing nitrogen content (SN) in fractions of the cheese extract soluble at pH 4.6, in trichloroacetic acid (TCA, 12 mL/100 mL) and in phosphotungstic acid (PTA, 2.5 g/100 mL) according to Gripon, Desmazeaud, Le Bars, and Bergère (1975). The nitrogen content was determined by the macro-Kjeldahl method (IDF, 1993) and the values were expressed as percentage of total nitrogen.

2.2.3. Electrophoresis

The insoluble residue at pH 4.6 was analyzed by Urea-PAGE in a Mini-Protean II cube (BioRad Laboratories, California, USA) by the Andrews (1983) method, with a concentration of acrylamide of 7.5 g/100 mL (Hynes et al., 1999). Proteins were stained by Coomasie Blue G-250.

2.2.4. Plasmin and plasminogen activities

Plasmin and plasminogen activities were determined according to Richardson and Pearce (1981). A non-fluorescent substrate, N-succinyl-L-alanyl-L-phenylalanil-L-lysyl-7-amido-4-methyl coumarin (Sigma Chemical Co., St. Louis, MO, USA) was cleaved by plasmin to yield a fluorescent product, 7-amino-4-methyl coumarin (AMC). The activity derived from plasminogen, was measured by difference after its activation with urokinase (EC 3.4.21.73, Sigma Chemical Co., St. Louis, MO, USA). In order to compare the different cheeses, the activities were normalized to the dry matter content of cheeses. The plasmin and plasminogen activities in cheese samples were expressed in AMC units (nmoles AMC released per min) g^{-1} cheese (dry matter).

2.2.5. Residual coagulant activity

Residual coagulant activity was determined as described by Hurley, O'Driscoll, Kelly, and McSweeney (1999) with some modifications. Briefly, 250 mg of finely grated cheese samples were dispersed in 5 mL of trisodium citrate buffer pH 7.0 and incubated at 37 °C for 60 min. Then, the samples were centrifuged at 2000 × g for 5 min and the aqueous layer was separated and used for the reaction with an heptapeptide substrate: Pro-Thr-Glu-Phe-[NO2-Phe]-Arg-Leu (Bachem California, Inc., Torrance, USA). For that, 30 μL of the synthetic heptapeptide 1 mg mL^{-1} was added to 200 μL of formate buffer 0.1 mol/L pH 3.2, and finally 70 μL of cheese sample dispersion were added. The assay mixture was incubated at 37 °C/2 h, and then the enzymatic reaction was stopped by heat treatment at 70 °C/10 min. A volume of 60 μL filtered through 0.45 μm membranes (Millex, Millipore, São Paulo, Brazil) was injected into a chromatograph performance liquid chromatography (HPLC) equipment (Series 200, Perkin Elmer, Norwalk, CT, USA) for the quantification of the tripeptide produced by the coagulant activity. Separation was achieved on a 220 × 4.6 mm Aquapore OD-300 C18, 5 mm–300 A° analytical column (Perkin Elmer) under a gradient between two solvents: A-0.1% trifluoroacetic acid (TFA) in HPLC-grade water and B- 0.1% TFA in acetonitrile. The flow rate was 1 mL min^{-1}, the column temperature was 25 °C, and the UV detection was performed at 270 nm. The results were expressed as the quantity of the tripeptide (nmol) released by the activity of coagulant per hour per g of dried cheese.

Table 1
Important technological aspects of cheese making for the three varieties studied in the present work (Zalazar, Meinardi, & Hynes, 1999).

Cheese variety	Technological description
Cremoso	Bovine milk, pasteurization step, enzymatic coagulation (chymosin) at 37 °C, starters of *Streptococcus thermophilus,* no cooking performed. Brining pH of 5.30–5.20, reached in the molds after cheese making, no pressing. Ripening time: 20–30 days at 5 °C/7 °C.
Pategrás	Bovine milk, pasteurization step, enzymatic coagulation (chymosin) at 37 °C, starters of *Streptococcus thermophilus*, semi-cooking step up to 45 °C (1 °C/min); curd washing step, a pH of 6.30–6.50 is reached at the end of cheese making and acidification to 5.3–5.2 during pressing. Ripening time: 40–50 days at 12/15 °C.
Reggianito	Bovine milk, pasteurization step, enzymatic coagulation (chymosin) at 33 °C, starters of Lactobacillus helveticus combined with organic acids or acidogens, cooking step at 52 °C vs 54–56 °C, a pH of 6.3 is registered after cooking, and the drop of pH to 5.2 is produced after pressing and airing. Ripening time: 180 days at 12/15 °C.

Table 2
Sampling days for Cremoso, Pategrás and Reggianito cheeses.

Cheese type	Sampling days
Cremoso	0, 9, 22
Pategrás	0, 12, 38, 50
Reggianito	0, 30, 80, 130, 150, 180

All analyses were performed in duplicate.

2.2.6. Statistical analysis

One way ANOVA was applied on composition, proteolysis and enzyme activity data, as well as linear regression was applied on nitrogen fractions as a function of ripening time.

3. Results and discussion

3.1. Cheese composition

Gross composition of the cheeses met the standards established by the Argentinean regulation, Código Alimentario Argentino (ANMAT, 2009) (Table 3). Cremoso cheeses had the lowest protein to fat ratio, due to high moisture and fat retention in the curd, followed by Pategrás and Reggianito cheeses. As for pH, the lowest values were found in Cremoso cheeses, which is related to the pH curve for this cheese and the retention of high moisture and lactose. The pH in Pategrás and Reggianito cheeses remained somewhat higher than Cremoso, which is attributed to a lower initial content of lactose at the end of cheese making. Besides, as we discuss below, Pategrás and Reggianito cheeses underwent a more intense proteolysis, which also contributes to increasing pH.

3.2. Enzyme activities

The highest levels of plasmin activity were found in Pategrás cheeses on the manufacturing day (Fig. 1). At the beginning of the storage, Cremoso and Reggianito cheeses had similar plasmin activities, but then the levels decreased in Pategrás and Cremoso, reaching the lowest values in the latter of all cheeses ($p < 0.05$).

This inhibition of plasmin activity during ripening is probably due to the environment conditions of Cremoso cheese, especially pH (Watkinson et al., 2001).

Plasminogen activity did not vary significantly over time of storage for all cheeses: no activation of the precursor was found (Fig. 2). Despite a diminution in the activity of Cremoso cheeses was noticed at the beginning of ripening, it was not significative ($p > 0.05$). The lowest values for plasminogen were recorded in Pategrás cheeses, which agrees with the highest values found in this cheese type for plasmin, and suggests that plasminogen activation may have proceeded during milk processing/cheese manufacture, i.e., before ripening. Activation of plasminogen during Pategrás cheese making is probably due to curd washing. Indeed, other authors attributed high hydrolysis of β-caseins in Gouda cheeses to the enhanced activation of plasminogen caused by curd washing, as plasminogen activators inhibitors are found in the whey (Fox, Law, McSweeney, & Wallace, 1993; Grufferty & Fox, 1988). In this sense, other studies suggest that the inclusion of an increased amount of whey proteins in cheese (e.g. cheese made with micro filtrated milk) increase the inhibition of plasmin activity, which could be partially explained by increased plasma-derived inhibitors (Benfeldt, 2006).

Plasmin activity was intermediate for Reggianito cheeses (Fig. 1). Previous research reports that increasing curd cooking temperature increases plasmin activity due to thermal inactivation of inhibitors of plasminogen activators (Delacroix-Buchet & Fournier, 1992; Sommers & Kelly, 2002). Our results suggest that curd washing combined with soft thermal treatment of the curd in Pategrás cheese were more effective than Reggianito curd cooking to increase plasmin activity in the cheeses. However, in Reggianito cheeses the activity of the enzyme remained without changes during ripening ($p > 0.05$). Pategrás and Cremoso cheeses, showed a significant decrease on plasmin activity during ripening, retaining 70% of the initial activity at the end of this period. These results indicate that Reggianito cheese matrix favor enzyme stability, compared to Cremoso and Pategrás. When talking about the enzyme activity during cheese ripening, variable results were reported. Richardson and Pearce (1981) found that plasmin activity in Swiss cheeses tended to decline, with 70% of the initial activity after 42 days of ripening, while in Cheddar cheeses it was constant during three months. Bastian, Lo, and David (1997) did not found significant decrease of plasmin in Swiss cheeses during storage. Rampilli and Raja (1998), and Sommers and Kelly (2002), reported increases in plasmin activity during ripening of different type of cheeses, cooked at different temperatures.

Coagulant activity remained without significant changes during ripening in all the cheeses studied (Fig. 3). The highest values were found for Cremoso, followed by Pategrás and then by Reggianito cheeses. Decrease of the activity correlate with increasing cooking temperature for the three cheese types included in this study. Besides, lower pH in Cremoso cheeses is more favorable to chymosin (Chitpinityol & Crabbe, 1998). Delacroix-Buchet and Fournier (1992) demonstrated that an increase of cooking temperature from 52 °C to 56 °C in Gruyère cheese making, diminished α−s1 hydrolysis. Sheehan, Oliveira, Kelly, and McSweeney (2007) found similar results in semi-hard cheeses cooked at temperatures of 47, 50 or 53 °C. More recently, Nega and Moatsou (2012) found that high cooking at 50–52 °C affected negatively the residual chymosin activity in Gruyere type cheeses. However, comparison of cheese making technologies also include other variables such as pH in the final activity of the enzyme, but main effect can be attributed to thermal treatment which in turn influences on moisture and coagulant retention and on enzyme inactivation (Bansal, Fox, & McSweeney, 2009).

3.3. Nitrogen fractions and electrophoresis

Nitrogen content in all soluble fractions were highest in Reggianito, followed by Pategrás and then Cremoso cheeses (Table 4). This trend is due to longer ripening times for Reggianito and Pategrás cheese varieties, as proteolysis has been shown to

Table 3
Composition of Cremoso, Pategrás and Reggianito cheeses at the end of ripening. (Mean and standard deviation of three replicates are reported.)

Cheese type	Proteins (g/100 g)	Moisture (g/100 g)	Fat (g/100 g)	Protein/Fat ratio	pH
Cremoso	$20.64^{a} \pm 1.31$	$48.80^{a} \pm 0.90$	$33.65^{a} \pm 0.38$	$0.61^{a} \pm 0.04$	$5.33^{a} \pm 0.09$
Pategrás	$28.07^{bc} \pm 0.72$	$38.20^{b} \pm 0.932$	$33.94^{a} \pm 0.38$	$0.83^{b} \pm 0.01$	$5.55^{b} \pm 0.06$
Reggianito	$31.71^{c} \pm 0.63$	$33.50^{c} \pm 1.026$	$28.1^{b} \pm 1.22$	$1.13^{c} \pm 0.03$	$5.43^{ab} \pm 0.05$

[a,b] Values in the same column with different superscripts differ significantly ($p \leq 0.05$).

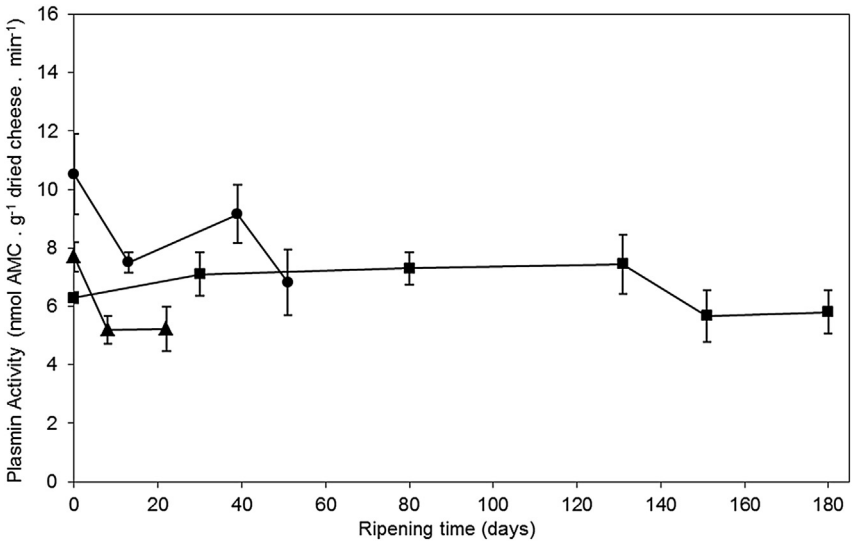

Fig. 1. Plasmin activity (nmol AMC g^{-1} of dried cheese. min^{-1}) of Cremoso (▲), Pategrás (●) and Reggianito (■) cheeses during ripening time.

increase during ripening for a number of cheese types (Diezhandino, Fernández, González, McSweeney, & Fresno, 2015; Hynes, Bergamini, Suárez, & Zalazar, 2003; McSweeney & Fox, 1993). In this research we confirmed that the proportion of SN pH 4.6/NT, TCA/NT, PTA/NT increased during ripening; lineal relation of the nitrogen content with time was significant in all cases ($p \leq 0.05$). For SN 4.6 the values of R^2 for Cremoso, Pategrás and Reggianito were similar among them and denoted a high correlation with time (0.95, 0.94, 0.93; respectively). The rate at which soluble nitrogen at pH 4.6 is produced in cheese was higher for Reggianito cheese, with a slope of 7.03% of N/day followed by 5.4 and 5.1% of N/day for Pategrás and Cremoso, respectively. TCA and PTA indexes did not show as high correlation coefficients as SN 4.6 for Cremoso cheese. For TCA, Cremoso cheese R^2 was 0.86, and for PTA, the R2 was 0.63. The same indexes showed much better correlation indexes with ripening time for Pategrás and Reggianito (TCA: 0.9 and 0.92; PTA: 0.87 and 0.94, respectively). Primary proteolysis, described by SN 4.6, was well correlated with time for the three cheese varieties studied, but secondary proteolysis was not as deep in Cremoso cheeses as in Pategrás and Reggianito. Especially in Reggianito cheese, long ripening time and highly proteolytic lactic cultures of *Lactobacillus helveticus* sp determine extensive proteolysis (Candioti et al., 2002). Consequently, TCA and PTA fractions were more performant to describe proteolysis in Reggianito and Pategrás.

Electrophoretic patterns varied according to cheese type (Fig. 4). Plasmin-mediated proteolysis of β-casein to γ-caseins was observed in Pategrás and Reggianito cheeses, but not in Cremoso cheeses. The absence of this breakdown may be due to low plasmin activity: Cremoso cheeses showed the lowest values for the enzyme, and even if initial records were similar between Reggianito and Cremoso cheeses, soon during ripening plasmin activity decreased only in Cremoso. In addition, adverse environmental conditions and short ripening time may affect the production of γ caseins in Cremoso cheese; as well as high moisture retention in this variety. In this sense, the proteolytic action of plasmin "*in situ*" may be influenced by relatively high amounts β lactoglobulin. This main whey protein which concentration increases with moisture retention in cheese, has been previously reported as a plasmin inhibitor (Bastian, Hansen, & Brown, 1993; Rollema & Poll, 1986). In turn, long-ripened Reggianito eventually showed γ caseins formation. These fractions were also evident in Pategrás cheese where

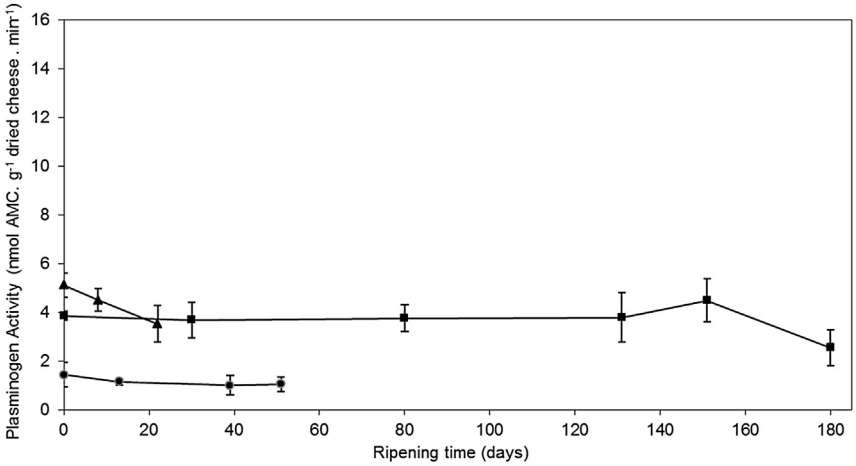

Fig. 2. Plasminogen activity (nmol AMC g−1 of dried cheese. min−1) Cremoso (▲), Pategrás (●) and Reggianito (■) cheeses during ripening time.

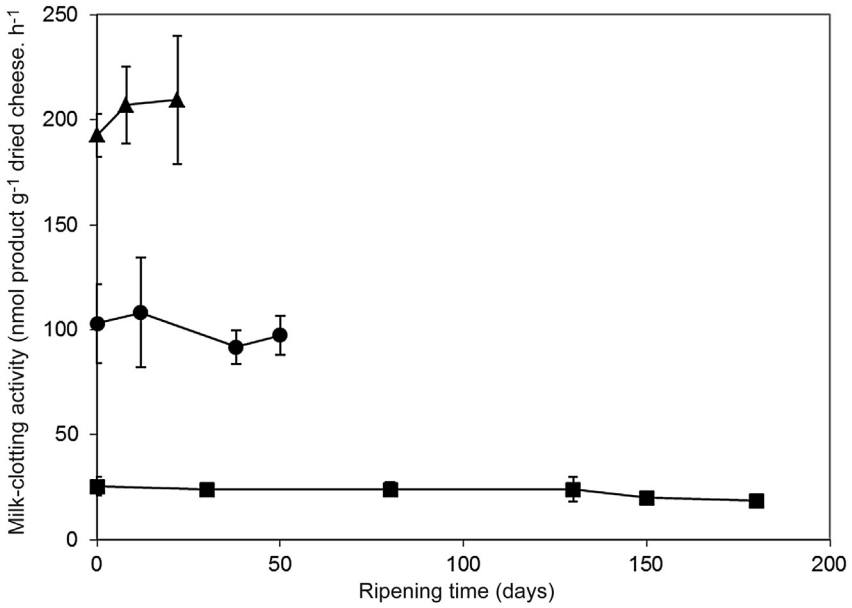

Fig. 3. Residual chymosin activities (nmol product. g−1 of dried cheese. h−1) Cremoso (▲), Pategrás (●) and Reggianito (■) cheeses during ripening time.

Table 4
Soluble nitrogen content (SN) at pH 4.6, in trichloroacetic acid (TCA) 12% and in phosphotungstic acid (PTA) 2.5% during ripening for Cremoso, Pategrás and Reggianito cheeses. (Mean and standard deviation of three replicates are reported.)

Type of cheese	Time of ripening (days)	SN pH 4.6/NT (g/100 g)	SN TCA/NT (g/100 g)	SN PTA/NT (g/100 g)
Cremoso	0	5.24 ± 0.39	2.61 ± 0.02	0.57 ± 0.15
	9	6.31 ± 0.22	3.05 ± 0.05	1.01 ± 0.13
	22	8.97 ± 0.60	4.54 ± 0.05	1.29 ± 0.22
	30	10.89 ± 0.49	5.51 ± 0.52	1.90 ± 0.16
Pategrás	0	6.27 ± 0.58	2.37 ± 0.16	0.81 ± 0.10
	12	7.98 ± 0.62	4.14 ± 0.05	0.96 ± 0.01
	38	13.28 ± 0.74	6.66 ± 0.18	2.24 ± 0.25
	50	15.55 ± 0.93	8.35 ± 0.76	3.39 ± 0.01
Reggianito	0	6.68 ± 0.95	3.95 ± 0.78	1.76 ± 0.06
	30	12.21 ± 1.18	9.82 ± 1.44	5.32 ± 1.19
	117	19.73 ± 3.04	15.97 ± 2.57	9.87 ± 1.85
	150	21.42 ± 3.04	17.53 ± 3.64	11.51 ± 1.13
	180	21.84 ± 1.26	19.88 ± 0.75	12.60 ± 0.07

plasmin had the highest activity. As can be seen, ripening time may be an important factor to detect the expression of the enzyme activity on cheese caseins. Results are in concordance with other authors that applied electrophoretic analyses to describe proteolysis of Argentinean cheeses, but according to our knowledge there are not studies of plasmin activities in Cremoso and Pategrás cheeses. Milesi, Candioti, and Hynes (2007) and Milesi, McSweeney & Hynes (2008) found little or no degradation of β-casein in Cremoso cheeses; Ceruti, Zorrilla, and Sihufe (2012) observed the formation of γ caseins in Reggianito cheeses.

Coagulant activity was also consistent with results of proteolysis, especially as described by eletrophoresis analysis. Hydrolysis of

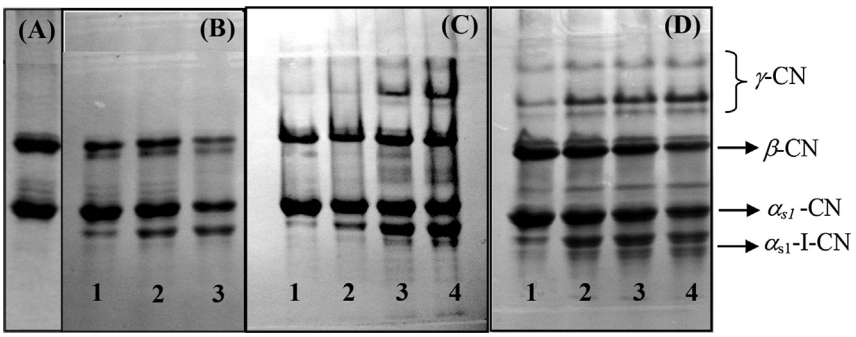

Fig. 4. Urea-PAGE electrophoretograms of cheeses. One replicate is shown as an example. (A) Standard Caseinate (B) Lanes 1 to 3: Cremoso cheeses at days 0, 11, and 22. (B) Lanes 1 to 4: Pategrás cheeses at days 0, 12, 38, 50. (C) Lanes 1 to 4: Reggianito cheeses at days 0, 30, 80, 180.

αs1 casein and production of chymosin-derived αs1-I was evidenced in all samples, but it was earlier and more pronounced in Cremoso cheeses. Indeed, this transformation has been previously reported as the main biochemical change during ripening of Cremoso Argentino (Hynes, Delacroix-Buchet, & Zalazar, 2001; Hynes et al., 1999). In Pategrás and Reggianito, the intensity of the band corresponding to αs1-I was very weak at the beginning of ripening, and became stronger later (at 11 and 38 days respectively). Increasing of αs1-I is probable due to accumulation during ripening, as no increase of coagulant activity was found. Unlike other authors, in the present work we did not found a reactivation of chymosin during cheese ripening. Hayes, Oliveira, McSweeney, and Kelly (2002) had difficulties to correlate proteolysis pattern and enzyme activity in Cheddar and Swiss with modified cooking temperature: they found similar chymosin activity but electrophoresis showed lower α-s1 hydrolysis in Swiss curds. The authors attributed the results to reactivation of chymosin during the citrate-extraction from cheese during the assay; strongly supporting the hypothesis that chymosin inactivation during curd cooking is reversible. Hynes, Aparo, and Candioti (2004) found that chymosin reactivated during ripening of Reggianito cheese scalded at 45, 52 and 60 °C, especially for samples cooked at 52 °C, a trend that confirms the work carried out by Hayes et al. (2002). However, it is not known yet the proportion of the denaturation in the cheese and the factors involved in the extent of its reversion (Hayes et al., 2002; Hynes et al., 2004).

4. Conclusions

Results in our study show that chymosin activity is mainly related to thermal treatment of the curd, while for plasmin, multiple factors are involved. As for chymosin, decreasing cooking temperature increased coagulant activity, being superior in Cremoso, followed by Pategrás and then by Reggianito cheeses. No reactivation of the enzyme was registered during ripening. With respect to plasmin activity, curd washing step had a stronger influence on it that increasing cooking temperature for Pategrás and Reggianito cheeses, respectively. As for plasmin stability during time, we did not find changes from plasminogen to plasmin during ripening of our cheeses, suggesting that such changes took place during milk processing/cheese manufacture, especially for Pategrás cheese. The enzyme activity remained constant in Reggianito, and decreased 30% in Pategrás and Cremoso during the first part of ripening, showing that Reggianito cheese matrix favored enzyme stability.

The present work is a contribution to increase knowledge about the influence of cheese making technology on no-microbial enzymes plasmin and coagulant associated proteolysis. This could be applied in cheese making technology with the potential view of accelerating ripening. In fact, we are carrying out this type of studies in our Institute.

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