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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.