Influence of Milk-Clotting Enzyme Concentration on the $\alpha_{\text{s1}}$-Casein Hydrolysis During Soft Cheeses Ripening

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ABSTRACT

We studied the influence of the dose of milk-clotting enzyme on $\alpha_{\text{s1}}$-CN degradation, soluble nitrogen production, and sensory profile for an Argentinean soft cheese: Cremoso Argentino. Five different types of cheeses were produced: 1) control cheeses with normal technology, 2) cheeses with inactivated milk-clotting enzyme, 3) cheeses with inactivated milk-clotting enzyme, without starter (acidified with glucono delta lactone), 4) cheeses with a half dose of milk-clotting enzyme, and 5) cheeses with a double dose of milk-clotting enzyme. Proteolysis was assessed by isoelectric focusing electrophoresis of the insoluble fraction at pH 4.6, followed by densitometric quantification. Soluble nitrogen at pH 4.6, expressed as a percentage of total nitrogen and defined as ripening index was also performed. A sensorial panel evaluated the cheeses at the end of ripening. The hydrolysis level of $\alpha_{\text{s1}}$-CN depended on the milk-clotting enzyme dose used in cheese making. Cheeses without active coagulant did not show degradation at the end of ripening, while cheeses with half and whole doses showed proportional degradations to coagulant dose. Cheese with a double dose of coagulant did not show higher $\alpha_{\text{s1}}$-CN hydrolysis than normal cheese. No difference was found between cheeses with and without microbiological starter, indicating that the selected culture, composed of thermophilic strains, was unable to attack the whole casein. A high linear correlation was found between ripening index and the relation

$$\% \frac{\alpha_{\text{s1}}-I}{\alpha_{\text{s1}}-I + \alpha_{\text{s1}}},$$

Sensorial characteristics of cheeses agree with objective analysis. Cheeses without active coagulant were hard and crumbly, while cheeses with normal dose were soft and creamy.

(Key words: milk-clotting enzyme, $\alpha_{\text{s1}}$-CN, cheese ripening, proteolysis).

INTRODUCTION

Proteolysis is an event of great importance in most cheese varieties that is carried out by enzymes in the curd during ripening. Mainly residual milk-clotting enzyme and indigenous milk proteases are responsible for the primary breakdown of casein, while the complex peptidolytic system of microorganisms, both from starter and contamination, are responsible for secondary proteolysis (Fox and McSweeney, 1996).

Among the proteins forming the curd (i.e., para-$\kappa$, $\alpha_{\text{s1}}$, $\alpha_{\text{s2}}$, and $\beta$-CN), milk-clotting enzyme preferentially attacks $\alpha_{\text{s1}}$ at the primary site Phe23-Phe24 (Carles and Ribadeau Dumas, 1985). This transformation has been studied by incubation of $\alpha_{\text{s1}}$-CN with chymosin, the main proteolytic enzyme in rennet but has also been proved for other aspartic proteases such as pepsin from bovine and porcine origin (Fox, 1988). As a consequence of this cleavage, the peptides $\alpha_{\text{s1}}$-CN (f 1 to 23) soluble at pH 4.6 in water, and the peptide $\alpha_{\text{s1}}$-CN (f 24 to 199), also called $\alpha_{\text{s1}}$-I casein and insoluble in the same conditions, are originated (McSweeney et al., 1993). In soft cheeses, the main proteolytic agent is the residual milk-clotting enzyme, because the high moisture content and the absence of a cooking process enhance its retention in the curd and its activity on proteins.
(Noomen, 1978). In addition, environmental conditions discourage plasmin action (Fox et al., 1993), and the short ripening time limits further degradation of primary proteolysis products by peptidases from starter and nonstarter bacteria, which are released after cell death and lysis and are responsible for secondary proteolysis. These are clear advantages of the study of chymosin activity on a soft cheese model, considering that proteolysis products from the activity of chymosin have fewer breakdown products than do other cheese varieties.

The hydrolysis of $\alpha_{s1}$-CN in the bond Phe$_{23}$-Phe$_{24}$ is a most important transformation during the ripening of soft cheeses (Noomen, 1978; Hynes et al., 1999). The peptide $\alpha_{s1}$-I is more hydrophilic than the original $\alpha_{s1}$-CN, and the hydrolysis also helps to disrupt the bonds in the casein net. For these reasons, cheeses that have undergone the cleavage of $\alpha_{s1}$-CN have a greater ability to retain water and the creamy texture typical of these varieties at the end of ripening, such as Nordhollandse Meshanger (de Jong, 1975), Taleggio (Giangiacomo et al., 1993), Crescenza (Todesco et al., 1992), and Camembert (Vassal et al., 1986).

The aim of this work was to study the influence of the milk-clotting enzyme dose and the starter on the hydrolysis level of $\alpha_{s1}$-CN for Cremoso Argentino, a typical and widely consumed Argentinean soft cheese (production of Cremoso Argentino reached 231,202 tonne in 1998; CIL, 1999). The relation between the degradation of $\alpha_{s1}$-CN and the sensorial characteristics of the cheese with the ripening index (RI) was also investigated to provide a rapid test to assess the quality of the product.

**MATERIALS AND METHODS**

**Cheese Making**

The cheeses were made according to standard Cremoso Argentino technology (Hynes et al., 1999; Zalazar et al., 1999). A laboratory-scale cheese vat was used. Raw milk from a nearby farm was pasteurized at 63°C for 30 min, cooled to 30°C, and supplemented with 0.2 g L$^{-1}$ of CaCl$_2$ before cheese making. The starter was a lyophilized mixed culture of *Streptococcus termophilus* (CSL, Milano, Italy), 1.5 g of which was suspended in sterile reconstituted powdered skim milk, 40 min before milk was added. Porcine pepsin was used as the milk-clotting enzyme because it could be easily destroyed by high pH when required. Powered porcine pepsin (0.500 g; Diagramma, Santa Fe, Argentina) of 1:200,000 milk-clotting activity determined according to a modified Bergridge method (Huck and Zalazar, 1972) was chosen as a normal dose to clot milk in the usual coagulation time for Cremoso Argentino (approximately 10 min at 38°C). Pepsin was dissolved in 30 ml of acetic acid-sodium acetate buffer (pH 5.5) before addition.

When the coagulum strengthened to the typical consistency of this enzymatic curd, it was cut to gross cubes of approximately 2 cm$^3$. The mixture curd particles-whey was then gently stirred to allow light whey drainage, but without ulterior cutting of curd particles. This step was finished after 10 to 15 min, and then the curd was allowed to set in the bottom of the vat. Whey was pumped out and the curd was put in the mold. Before salting, the curd was placed in a hot chamber (40°C) until a pH 5.2 to 5.3 was reached. Salting was performed by immersion in saturated brine (26% wt/vol, pH 5.4) at 6°C for 1.5 h, in order to stop the starter activity. A 1.5-kg cheese was obtained from a 15-L vat.

The cheeses were vacuum packed in plastic bags and ripened at 6°C (±0.5°C) for 21 d.

Cheeses of the following types were made:

- **Experimental cheeses with inactivated milk-clotting enzyme (IMCE).** The instability of porcine pepsin at high pH was the basis of the cheese making procedure to obtain rennet-free cheeses. In a previous work (Meinardi et al., 1998), the conditions (pH and temperature) to inactivate porcine pepsin were established. The time of completion for the first step of milk coagulation at low temperature was also determined. Accounting for the results of the work, we established that hydrolysis of $\kappa$-CN was completed when milk was inoculated with porcine pepsin and incubated 80 min at 6°C, after which pH was increased to 7.8 to completely inactivate the milk-clotting enzyme in 45 min at 6°C. These results were applied to the design and construction of a laboratory vat for the manufacture of cheese that ripens without the influence of the milk-clotting enzyme. A 15-L cylindrical glass vessel was used. Heat exchange without stirring was achieved by placing a series of glass tube coils within the vessel. Cold or warm water was circulated in the tubes to maintain the vat temperature. Coils can be easily removed for vat cleaning.

To make rennet-free cheeses, we filled the vat with 15 L of whole, pasteurized and calcium-supplemented milk. The milk was cooled at 6°C, and then porcine pepsin was added. The milk was gently stirred and kept at 6°C for 80 min to allow completion of the first step of coagulation. After this incubation period, 20% (wt/ wt) NaOH was added under manual stirring until pH 7.8 was reached. The milk was further incubated at this pH for 45 min to inactivate the enzyme. Hydrochloric acid 10% (vol/vol) was subsequently added under manual stirring to adjust pH to 6.5. During neutralization with the acid, when the pH reached 7.0, the starter culture was added. The milk was then heated, without stirring, by circulating water at 38°C through the tube...
heating system. During the temperature increase, the second step of the coagulation process (nonenzymatic) began and gelation took place. Once the curd acquired the proper consistency, the heating tube system was removed, and the curd was cut. The curd was then processed through the standard steps of Cremoso cheese manufacture, including salting and ripening. Milk-clotting enzyme was therefore not implied in the proteolysis of these cheeses.

Experimental cheeses with inactivated milk-clotting enzyme and glucono delta lactone (IMCE-GDL). These were made by a procedure similar to IMCE cheeses, but the starter was replaced by a chemical-acidifying agent, glucono delta lactone (Giraffa et al., 1991). An antimicrobial agent was added to prevent the growth of microorganisms. These cheeses ripened free of starter and milk-clotting enzyme.

Control cheeses (C). They were made to obtain a standard Cremoso Argentino cheese. However, the milk underwent heating, cooling, and changes similar to IMCE cheeses. Pepsin was added after the high pH step, once it was re-adjusted to 6.4. This process supplied control cheeses with active milk-clotting enzyme and milk treatment similar to the experimental cheeses (Meinardi et al., 1998).

Cheeses with half dose of milk-clotting enzyme (HMCE). The procedure was the same as for control cheese, but using a half dose of milk-clotting enzyme, i.e., 0.250 g.

Cheeses with double dose of milk-clotting enzyme (DMCE). The same process was used as for control cheese, but with a double dose of coagulant, i.e., 1.000 g.

Five cheeses of each type were made, 25 cheeses in total, distributed randomly in five cheese-making days of five cheeses each.

Cheese Analysis and Proteolysis Monitoring

Moisture, protein content, pH, and NaCl concentration were determined by standard methods (IDF Standard 20:B 1993; IDF Standard 4:A 1962; IDF Standard 88:A 1988) on 21-d-old cheeses. Means of five determinations were compared by one-way ANOVA test. Molds and yeasts, total coliform bacteria, and total mesophilic microorganism counts were performed at 0 and 21 d, according to American Public Health Association standards (Frank et al., 1993).

Proteolysis was monitored by isoelectric focusing electrophoresis and by RI on 0- and 21-d-old cheeses.

Isoelectric focusing electrophoresis. A biophoresis horizontal electrophoresis cube with a power source model 3000Xi (Bio-Rad Laboratories, Richmond, CA) was used. The method of Krause and Belitz was employed (Krause and Belitz, 1985). The total monomer concentration (acylamide + bisacrylamide) in gel was 4.5%. The crosslinking monomer concentration (bisacrylamide) in relation to total monomer was 3%. The urea concentration was 8 M. 600 µl of anpholites were used for 10 ml of gel. The pH gradient ranged from 2.5 to 5.0. Polymerization was catalyzed by the addition of ammonium persulfate solution and TEMED. The casein samples were precipitated at pH 4.6 and purified by redissolution and washing, dissolved in sample buffer (20 mg of lyophilized casein/ml of sample), and seeded (15 µl) by using seeding papers on the gel. The run comprised a pre-focusing step, the seed, and, after two focusing steps, the gels were stained with Coomassie brilliant blue G250 using a method without decoloration (Blakesky and Boezi, 1977).

The bands of αs1-CN and αs1-I peptide were quantified by analyzing the gel slabs with a LKB Ultrascan XL densitometer (Bio-Rad Laboratories).

Taking into account the areas of αs1-CN and αs1-I peptide peaks, the relation % \(\frac{\alpha s1 - I}{\alpha s1 - I + \alpha s1}\) was calculated, where αs1 and αs1-I represent the peak surface for the casein and the peptide, respectively.

Nitrogen fractions. The RI was calculated as the relation between soluble nitrogen at pH 4.6 and total nitrogen. Soluble and total nitrogen content were determined by the Kjeldhal method (IDF Standard 20:B 1993).

Sensorial Analysis

The IMCE and C 21-d-old cheeses sensory characteristics were assessed by a panel of eight members trained in the sensory evaluation of soft cheeses. In the training step, a typical Cremoso Argentino cheese from the local market was used as reference. The general characteristics of this cheese were creamy aroma and taste, neutral white color, no holes mass, and a slight elastic, no-stick texture. Palate sensation of texture was also evaluated.

Samples identified by random numbers were presented in individual trays to the panelists. The evaluation of sensory attributes was performed in a conditioned room.

A five-point score scale was used for aroma, color, and taste intensity (1 = very low; 5 = normal), and a score scale of nine points was used for texture (1 = bad; 9 = very good). These scales were used by the panelists to score the control cheese used during training, with previous agreement about these scores (Amerine and Duran, 1965; Costell and Duran, 1982; Stone et al., 1974).

The analysis was performed in duplicate sessions. The second session began 20 min after the first ended.
Table 1. Gross composition of 21-d-old cheeses (means and standard deviations of five cheese makings). C = control cheeses, DMCE = cheeses with a double dose of milk-clotting enzyme, HMCE = cheeses with a half dose of milk-clotting enzyme, IMCE = experimental cheeses with inactivated milk-clotting enzyme, IMCE-GDL = experimental cheeses with inactivated milk-clotting enzyme and glucono delta lactone, S/M = salt-in-moisture.

<table>
<thead>
<tr>
<th>Cheese type</th>
<th>Moisture %</th>
<th>Protein %</th>
<th>pH</th>
<th>S/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMCE-GDL</td>
<td>54.96a ± 1.55</td>
<td>21.40a ± 3.21</td>
<td>5.10 ± 0.03</td>
<td>2.24 ± 0.25</td>
</tr>
<tr>
<td>IMCE</td>
<td>44.81b ± 0.40</td>
<td>29.19b ± 2.08</td>
<td>5.11 ± 0.04</td>
<td>2.39 ± 0.19</td>
</tr>
<tr>
<td>C</td>
<td>44.20b ± 1.29</td>
<td>30.10b ± 2.48</td>
<td>5.12 ± 0.04</td>
<td>2.33 ± 0.26</td>
</tr>
<tr>
<td>HMCE</td>
<td>46.24b ± 1.41</td>
<td>28.56b ± 2.39</td>
<td>5.10 ± 0.02</td>
<td>2.25 ± 0.21</td>
</tr>
<tr>
<td>DMCE</td>
<td>46.06b ± 0.38</td>
<td>28.19b ± 3.91</td>
<td>5.11 ± 0.02</td>
<td>2.23 ± 0.28</td>
</tr>
</tbody>
</table>

Means in the same column with different superscripts differ (P < 0.05).

The results were statistically processed by a one-way ANOVA test.

**RESULTS AND DISCUSSION**

The IMCE-GDL cheese had higher moisture (P < 0.05) content than the other cheeses (Table 1) because the curd was very gently cut and stirred as a consequence of its fragility and to prevent excessive fines production. Nevertheless, as results showed, this difference in moisture did not affect the proteolysis profile. Such behavior was not surprising, because IMCE-GDL were cheeses with neither active milk-clotting nor starter enzymes.

Protein content was somewhat lower for IMCE-GDL cheese as a consequence of their higher moisture content, but salt-in-moisture content was similar in all cases because IMCE-GDL cheeses were brined for a shorter time. Salt-in-moisture content and pH did not show significant variability between cheese makings. Protein and moisture content varied within the usual limits for the other types of experimental cheeses (Table 1).

The microbiological characteristics of all cheeses were very similar: total mesophilic count 10⁹ and 10⁷ cfu g⁻¹ for 0- and 21-d-old cheeses, respectively; total coliform bacteria 10² cfu g⁻¹ and mold and yeasts 10³ cfu g⁻¹ at the same stages of ripening.

Figure 1 shows the values of the relation %αs1-I − I for the different types of cheeses at 0 and 21 d of ripening, calculated by means of the areas of peaks for αs1-CN and αs1-I peptide in the densitogram. The hydrolysis level of αs1-CN for 0 d-old cheeses was very low and similar for the five types of experimental cheeses.

At the end of ripening, however, the hydrolysis of αs1-CN was dependent on the dose of milk-clotting enzyme used in cheese making.

In IMCE and IMCE-GDL 21-d-old-cheeses, such hydrolysis was not different from the hydrolysis level of 0-d-old cheeses. In HMCE, however, the hydrolysis at the end of ripening was significant, and it became very important, reaching more than 80%, in C and DMCE cheeses. The relation %αs1-I − I was nearly the same for C and DMCE cheeses, indicating that no hydrolysis increase is produced during a ripening period of 21 d by increasing of the milk-clotting enzyme dose at values higher than normal dose.

However, for cheeses of longer ripening periods (more than 90 d), a higher proteolysis of αs1-CN has been detected in products with a double dose of milk-clotting enzyme. Visser (1977) showed a linear relation between dose of milk-clotting enzyme and its retention in the curd. Nevertheless, this linearity was tested for a different interval of enzyme concentration than that used in the present study. Other studies have also confirmed that a double dose of coagulant does not mean a double
level of proteolysis, although an increase is observed at later stages of ripening (van der Berg and Exterkate, 1993).

The starter alone was not able to produce primary hydrolysis of αs1-CN, because no difference was noticeable between IMCE and IMCE-GDL cheeses. This result agrees with the fact that most streptococcus strains are not able to degrade whole proteins (Fox et al., 1993).

The RI presented in Figure 2, showed the same tendency of αs1-CN hydrolysis. The RI for 0-d-old cheeses was about 4 to 5%, and a one-way ANOVA test showed no significant difference between the five types of experimental cheeses. At the end of ripening, the RI was dependent of the milk-clotting enzyme dose, arriving at 15% in C and DMCE cheeses.

The values for RI and the relation \( \frac{\alpha s1 - I}{\alpha s1 - I + \alpha s1} \) were correlated by linear regression for the different doses of milk-clotting enzyme, and the relation between them was proved to be linear with a correlation index of 0.9848 (SD = 6.736; \( P = 0.00223 \)). This linearity indicates that both values are highly dependent. Effectively, while the increase of RI is due to the production of the \( \alpha s1 \) (Fl to 23) peptide and perhaps of its derivatives coming from the starter peptidases attack, also soluble at pH 4.6, the relation \( \frac{\alpha s1 - I}{\alpha s1 - I + \alpha s1} \) represents the increase in the concentration of the complementary peptide. Other cheese types with longer ripening times, and several active proteolytic agents allowing further degradation of \( \alpha s1 \) and \( \beta-CN \), probably do not show such a good adjustment.

The C cheese was found to have attributes similar to a typical Cremoso Argentino Cheese by the sensory panel.

On the other hand, IMCE cheese did not develop the characteristic aroma and color. The taste of this cheese was slightly creamy, acid, and bitter and was saltier than C cheese. Its texture was hard and crumbly, and the palate sensation was soft with a rough residual taste.

The results of the sensory evaluation are shown in Table 2. In all cases, the averages for C cheese were higher than IMCE cheese. Comparison tests showed that all attributes were significantly different for C and IMCE cheeses (\( P < 0.05 \)).

The differences between IMCE and C cheeses were higher for texture and taste than for aroma and color, confirming the absence of primary proteolysis detected by chemical analysis.

### CONCLUSIONS

The \( \alpha s1 \)-CN hydrolysis at the bond Phe23-Phe24 is the main proteolytic transformation during the ripening of Cremoso Argentino cheese. Hydrolysis was directly related to the milk-clotting enzyme dose used in cheese and reached more than 80% in C cheeses.

The RI correlates linearly with the \( \frac{\alpha s1 - I}{\alpha s1 - I + \alpha s1} \) relation, and, as a consequence, is a suitable index for monitoring \( \alpha s1 \)-CN hydrolysis in soft cheeses.

In the absence of a milk-clotting enzyme, the proteolytic system of the thermophilic starter alone is not able to attack whole casein.

The typical sensorial characteristics of Cremoso Argentino, especially texture, are dependent on the extensive hydrolysis of \( \alpha s1 \)-CN during ripening. The poor and crumbly texture and the slight creamy, acid and bitter taste of IMCE cheese proved this.

### Table 2. Sensorial attributes of the cheeses at the end of ripening (mean values of three trials). Aroma, taste and color: 1 to 5 scale. Texture: 1 to 10 scale. C = control cheeses, IMCE = experimental cheeses with inactivated milk-clotting enzyme.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>C</th>
<th>IMCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aroma</td>
<td>4.93\textsuperscript{a}</td>
<td>4.65\textsuperscript{b}</td>
</tr>
<tr>
<td>Color</td>
<td>4.99\textsuperscript{a}</td>
<td>4.79\textsuperscript{b}</td>
</tr>
<tr>
<td>Texture</td>
<td>9.32\textsuperscript{a}</td>
<td>7.11\textsuperscript{b}</td>
</tr>
<tr>
<td>Taste</td>
<td>4.84\textsuperscript{a}</td>
<td>3.98\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\( \textsuperscript{a,b} \) Means in the same row with different superscripts differ (\( P < 0.05 \)).
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