Detection of $\alpha_s$-l casein in mozzarella Fiordilatte: A possible tool to reveal the use of stored curd in cheesemaking

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ABSTRACT

The assessment of the origin and quality of raw materials is pivotal for the protection and valorization of typical dairy products. Italian high moisture mozzarella cheese, also called "Fiordilatte", is manufactured by stretching in hot water the curd, which can be produced inside the dairy (fresh curd) or purchased as semi-finished product from specialized companies (pre-made stored curd). The employment of stored curd allows cheese manufacturers to obtain economic profit thanks to the reduction of the production costs. The use of the semi-finished product has not to be mentioned in the label according to the actual EU regulation, and this has given rise to competition between local milk farmers and curd-producing companies causing misinformation among the consumers, and unfair competition to the disadvantage of the traditional dairies. In the present research, a proteomic approach was used to investigate "Fiordilatte" produced with or without the employment of stored curd. The results obtained allowed us to identify a molecular marker which is present in very small amounts in cheese made from fresh curd and at high levels in cheese made from stored curd. The marker is $\alpha_s$-l casein (fragment 24–199), the main product of primary proteolysis formed by the action of chymosin on $\alpha_s$ casein. It can be easily detected by electrophoresis in the presence of urea (urea-PAGE) and is measured by image analysis. Even though the kinetic of formation of this proteolytic product has been well known for several decades, in mozzarella it has a peculiar behavior compared to other cheeses. As a matter of fact, it originates during the first part of cheesemaking, and has a very slow increase during storage of the cheese, due to the denaturation of the clotting enzyme by the hot water stretching process. The results obtained demonstrated that the quantification of $\alpha_s$-l fragment should be recommended to guarantee the production of "Fiordilatte" obtained from fresh milk under normal conditions, and could be the basis for the development of a method able to fully protect the traditional cheesemaking procedure.

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1. Introduction

Mozzarella cheese is the most commonly produced cheese worldwide, due to its characteristics that allow consumption both as fresh table cheese (high moisture type, made both from buffalo and cow milk) and as an ingredient for very popular food specialties such as pizza (low moisture type, made from cow milk). Most of the scientific knowledge available on this cheese regards low moisture mozzarella, which is very different with respect to the table type. In fact, it is shaped in parallelepiped blocks weighing a few kilograms and has a maximum moisture content of 52%; furthermore, it needs a short ripening period to fully develop the required textural and functional properties, and can be preserved for more than 1 month (Gunasekaran & Ak, 2003; Joshi, Muthukumarappan, & Dave, 2004; Kindstedt, 1993; Kindstedt & Fox, 1993; Rowney, Roupas, Hickey, & Everett, 2004; Rudan, Barbano, Yun, & Kindstedt, 1999). Instead, table mozzarella cheese has a spherical shape, a weight of 10–300 g and 60–70% moisture content. In Italy it is also called “Fiordilatte” when manufactured from cow milk, it is packaged into plastic bags or vessels with water as preserving liquid and has a very short shelf-life, ranging from a few days to two weeks (De Candia et al., 2008; Del Nobile, Gammariello, Di Giulio, & Conte, 2010; Faccia, Mastromatteo, Conte, & Del Nobile, 2012; Faccia, Trani, & Di Luccia, 2009; Minervini et al., 2012). The functional and organoleptic properties of low moisture mozzarella are deeply influenced by proteolysis: softening and changes in other functional properties during storage are

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attributed to casein hydrolysis (Creamer, 1976; Farkye, 1995; Farkye, Kiely, Allhouse, & Kindstedt, 1991; Tunick et al., 1993). In general, primary proteolysis in this cheese is considered to progress quite slowly, due to partial thermal inactivation of residual rennet during the stretching process (Mc Goldrick & Fox, 1999). However, the rate can also be influenced by: the type and amount of rennet used in cheesemaking (Farkye et al., 1991; Kindstedt, Yun, Barbano, & Laroze, 1995; Yun, Kiely, Kinstedt, & Barbano, 1993); the extent of its retention in the curd (Dave, McMahon, Oberg, & Broadbent, 2003); the curd cooking temperature (Tunic et al., 1993; Yun, Kiely, Barbano, & Kinstedt, 1993); the time and temperature of stretching (Renda, Barbano, Yun, Kindstedt, & Mulvaney, 1997); and finally by pH and calcium content of cheese (Feneey, Guinee, & Fox, 2002). As regards the role of microorganism, Yun, Barbano, Kiely, and Kinstedt (1995) reported that secondary - but not primary - proteolysis is significantly influenced by starter populations even when it reaches very high levels in cheese; as for the contaminant microflora, Baruzzi, Lagonigro, Quintieri, Morea, and Caputo (2012) and Morales, Fernandez-Garcia, and Nunez (2003), reported that a certain role can be played in cheese by environmental non lactic acid-bacteria only if they reach very high cellular densities (more than 108). As can be played in cheese by environmental non lactic acid-bacteria Fernandez-Garc

2.2. Two dimensional gel electrophoresis (2DGE) of curd

For 2DGE, an immobilized pH gradient (IPG) was used as the first dimension and an 8%–18% SDS-PAGE orthogonal pore gradient as the second dimension (O’Farrell, 1975). Sixty micromgrams of curd total protein were dissolved in a rehydration buffer (8 M urea, 1 M thiourea, 50 mM DTT, 2% CHAPS, 2%IPG buffer pH 3–10) and loaded onto a 13 cm IPG Dry Strip pH 3–10 (Amersham Bioscience, Uppsala, Sweden). The first dimension was provided using the Multiphor II electrophoresis unit (Amersham Bioscience) in 4 steps: 500 V/h, 1000 V/h, a gradient to 8000 V over 110 min, and 8000 V for 3 h. After the IPG electrophoresis, the strips were equilibrated for 25 min in a solution containing 1.5 M Tris–HCl, pH 6.8, 6 M urea, 30% glycerol (pH 8.3), 2% SDS, 64 mM dithiothreitol, 135 mM iodoacetamide, and a trace of bromophenol blue. The equilibrated IPG Dry Strips were loaded on top of the 8%–18% SDS-PAGE gradient gel (14 × 16 cm), and electrophoresis was carried out overnight at a constant voltage of 100 V and at 15 °C. The 2-dimensional maps were stained with Brilliant Blue Coomassie G250, destained with double distilled water, and analyzed using the Image Master Platinum software (Amersham Bioscience).

2.3. Mass spectrometry identification

Protein spots from 2 DGE were manually excised and an in situ digestion was applied. They were destained, reduced, and alkylated by carboxymethylation and then digested in situ by trypsin (Sigma Aldrich, St. Louis, MO, USA) overnight at 37 °C, according to the procedure of Shevchenko, Wilm, Vorm, and Mann (1996). The peptides resulting from the digestion step were extracted 3 times with 40 µL of AcN, water, and formic acid solution (50:45:5, vol/vol/vol) and then concentrated by vacuum centrifugation for MS analysis. Finally, the peptide mixtures were dissolved in aqueous 50% (vol/vol) AcN containing 1% (vol/vol) formic acid. Duplicate aliquots of peptide mixture solution were mixed (1:1) with matrix solution (2–cyano-4-hydroxy-cinnamic acid in 50% v/v acetonitrile containing 0.5% v/v trifluoroacetic acid). This mixture (0.3 µl) was applied in duplicate (two different spots or bands) on a sample slide tray and allowed to dry in air. Mass spectra were acquired in reflection mode, using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences). Calibration of the time-to-mass scale was performed using two external standard peptides (ile7AngIII, M+H 897.531, monoisotopic, and hACTH 18–39, M+H 2465.191, monoisotopic). MALDI-ToF analysis was carried out in triplicate and also using the internal standard peptide hACTH 18–39.

2.4. Polyacrylamide gel electrophoresis in the presence of urea (urea-PAGE) of curd and cheese

The samples were dissolved in 9 M urea and loaded onto a discontinuous gel polyacrylamide in the presence of urea (16 × 18 cm). Electrophoresis was conducted as reported by Andrews (1983) and the gels were stained with Silver Blu (Candido et al., 2004) and destained in water. The image of the destained gel was scanned and subjected to densitometry analysis using software Quantity One (Biorad, Hercules, CA, USA). The optical densities obtained, were expressed in relative quantity (RQ)
compared to the total area of the identified bands in the electrophoretic pattern of each sample.

2.5. Kinetic study on αs1-1 casein in fresh curd and cheese produced at laboratory level

Pasteurized whole bovine milk was purchased from a local shop to prepare fresh curd at the laboratory (five replicates were carried out by using 2 L of five different milk brands). Processing involved direct acidification of the milk to pH 5.75 with citric acid and coagulation by addition of single (0.18 mL/L) or triple (0.54 mL/L) dose of rennet (1:10,000 strenght, chymosin/pepsin ratio 75:25, Sacco srl, Cadorago, Italy). Each of the curds obtained were split into two parts that were kept at different conditions: in whey at 37 °C for 6 h and out of whey at 10 °C for 50 h (Fig. 1). The αs1-I-CN formation was monitored at 1, 2, 4, 6 h of storage in whey and at 20, 26, 44 and 50 h of storage out of whey; after collection, the samples were immediately dissolved in urea 9 M and stored at 4 °C until the series was completed for analysis. From the curd stored in whey, samples of “Fiordilatte” were made by stretching under controlled conditions (1:1 water/curd ratio, temperature of the paste 60 °C) aliquots of curd taken at 1 and 6 h. The cheeses obtained were stored in pot water at 4 °C and analyzed at 1, 2 and 5 days by urea-PAGE as above indicated.

2.6. Determination of αs1-1-casein in industrial cheese

Samples of “Fiordilatte” were produced at a local dairy from the same batch of fresh milk (24 h from milking) by two different technologies: direct acidification with citric acid and acidification with natural whey starter culture as described by Faccia, Gambacorta, Quinto, and Di Luccia (2010). After production the cheeses were cooled, packaged in plastic vessels filled with chilled pot water, and transported at the laboratory where they were stored at 10 °C for 15 days. Sampling was made at 0, 7, 10 and 15 days of storage. Besides these samples produced under controlled conditions, 81 samples of freshly produced “Fiordilatte” were randomly collected from the storage warehouse of 27 dairies after verification of the lot number/production date and storage temperature (4 ± 2 °C). Out of 81 samples, 48 were declared by the producers as manufactured from fresh curd and 33 with added preserved curd. All samples were subjected to one-dimensional electrophoresis analysis, as previously reported.

2.7. Statistical analysis

All data showed were means obtained from at least three analytical determinations. Descriptive statistics, regression analysis and t Student test were performed by SPSS v.19 (IBM, New York, USA).

3. Results and discussion

3.1. Proteomic investigation

In Fig. 2 the 2DGE maps of fresh and preserved curds are shown; for identification, all the gel spots were cut and digested with...
tripsin. By the peptide mass fingerprints (PMF) obtained by MALDI-TOF mass spectrometry and by the position of the spots in terms of molecular weight (MW) and isoelectric point (pI) identifications were provided. As shown in Table 1, PMFs of spot 1 and spot 3 were recognized as s1-I-casein (CN) and s1-I-CN, respectively. The actual spectrum of whole s1-CN (inset of Fig. 3) shows two higher signal at 1267 and 1760 m/z than s1-I-CN, whose spectrum shows the absence of the signal at 1760 m/z corresponding to the peptide 8—22, and of the signal at 1384 corresponding to the peptide 23—34. By comparing the maps, the main difference regards spot 3, which is characterized by MW of about 20 kDa and pl of 4.10: it is very intense in the preserved curd sample, whereas it is slightly detectable in the fresh one.

Fragment of s1-I-CN is the main proteolytic product obtained by rennet digestion of this casein (Mc Sweeney, Olson, Fox, Healy, & Healy, 1993; Mulvihill & Fox, 1979). In cheesemaking, the production of this large peptide is closely associated with chymosin: its formation takes place already at the earliest stages of production, but proceeds during the whole ripening period at a rate that depends on temperature, water activity, amount of rennet resituated in the paste, pH, NaCl concentration, etc. The results of the 2DGE analysis are fully explainable by the different ages of the curds analyzed, and were expected: the higher the age of the curd, the higher the presence of products of proteolysis. Several samples of fresh and preserved curd were then analyzed by alkaline urea-PAGE, the most widely used electrophoretic technique to investigate primary proteolysis in cheese and able to reveal several large casein fragments. As shown in Fig. 4 the fragment was confirmed to be always detectable at much higher intensities in the stored curd samples than in the fresh ones; the intensity among the samples was different, probably depending on the age and manufacturing/storage conditions. Through the electrophoretic mobility and PMF obtained by MALDI-TOF mass spectrometry, the identity of the band was confirmed, and the casein fragment was called “ALMI”", according to the Italian Patent n. 0001397624, for application in this work. In order to ascertain in cheese the relationships between the ALMI fragment and the amounts of stored curd used, we also analyzed samples of “Fiordilatte” made by mixing increasing amounts (from 0 to 80%) of stored curd with freshly prepared curd. The results shown in Fig. 5 indicated that the intensity of the band was almost linearly related to the amount of stored curd used, and by the image analysis it was ascertained that the value of the optical density was about 4 RQ in the fresh curd control cheese (Fig. 6).

3.2. Kinetic study of s1-I casein on laboratory-manufactured samples

For a possible utilization of the ALMI fragment as a sort of “molecular marker” of quality, the kinetic of its formation in curd and cheese has to be well understood. Since it is a very complex argument, adequate deepening was carried out both by studying the literature and by a specifically addressed experimentation. The literature reports that s1-I-CN increases very slowly in low-moisture mozzarella, and even though it can be detected at significant amounts after several weeks of ripening, it is only slightly present in fresh cheese (Costabel, Pauletti, & Hynes, 2007; Sheehan, O’Sullivan, & Guinee, 2004). As for “Fiordilatte”, as already said, information about proteolysis is scarce, but it seems that the s1-I fragment is even less abundant than in the low moisture type, and is barely detectable in few days-aged cheeses (Baruzzi et al., 2012; Di Matteo, Chiovitti, & Adddeo, 1982). Following these considerations, we postulated the hypothesis that the presence of the fragment could be suitable to detect the use of stored curd if analysis is performed in freshly produced cheeses. If it is found at a high level of intensity already in the first days after production, the use of semi-finished products should be revealed. For the sake of precision, little controversial information does exist: Feehney et al. (2002) found the presence of this casein fragment already at day 1 after production under certain conditions. In our opinion, this finding does not conflict with our hypothesis since it should derive from the different conditions of cheesemaking with respect to high moisture mozzarella (Guinee, Feehney, Auty, & Fox, 2002). In Table 2 the main technological differences between the two cheeses are summarized: as can be seen, the processing cycle of the low moisture type, involves higher processing temperature, longer holding times before the stretching of the curd and slower cooling of the finished product. The specifically addressed experimentation aimed to verify if such technological differences can cause a different kinetic of formation of the “ALMI” fragment in “Fiordilatte”, and if a sort of “tolerance level” for its presence in the cheese obtained from fresh curd could be fixed. To do so, the kinetic was

![Fig. 2. Two-dimensional electrophoresis analysis of two curd samples: freshly produced (A) and stored (B). Identification of the bands is reported in Table 1.](image-url)
investigated both in curd and cheese, which were produced in the laboratory and stored under normal and “forced” conditions (single or triple dose of rennet, short or long keeping of the curd at optimum conditions for proteolysis). Figs. 7 and 8 show the kinetic of formation of the fragment (expressed as RQ of the electrophoretic band) in fresh curd maintained under whey at \( +37 \) °C for 6 h and at \(+10\) °C for 50 h, respectively. The RQ values in the curd maintained in whey were negligible but, as expected, they increased when a triple dose of rennet was used, reaching level 7 after 6 h: this value exceeded level 4 previously found for fresh curd “Fiordilatte” (Fig. 6). This could appear a weak point to propose the ALMI fragment as molecular marker of quality, but such a rennet dose is not used in “Fiordilatte” cheesemaking, since it is not compatible with the attainment of a soft cheese. The kinetic study at \(+10\) °C confirmed the slow formation of the ALMI marker, whose RQ value remained under level 4 after 50 h storage. The urea-PAGE pattern of

<table>
<thead>
<tr>
<th>Trypsin Theoretical peptide mass</th>
<th>Measured peptide mass of ( \alpha s_1 ) (ALMI)</th>
<th>Measured peptide mass of ( \alpha s_1-I ) (ALMI)</th>
<th>Position</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2321.0813</td>
<td>/</td>
<td>/</td>
<td>59-79</td>
<td>QMEAESISSSEEIVPNSVEQK</td>
</tr>
<tr>
<td>2316.1369</td>
<td>2316.187</td>
<td>2316.121</td>
<td>133-151</td>
<td>EPMIGVQELAYFYPELFR</td>
</tr>
<tr>
<td>1767.7589</td>
<td>/</td>
<td>/</td>
<td>43-58</td>
<td>DIGSESTEDQAMEDIK</td>
</tr>
<tr>
<td>1759.9449</td>
<td>1760.056</td>
<td>/</td>
<td>8-22</td>
<td>HQGLPQEVLNENLLR</td>
</tr>
<tr>
<td>1580.8278</td>
<td>/</td>
<td>/</td>
<td>106-119</td>
<td>VPQLEIVPNSAEER</td>
</tr>
<tr>
<td>1384.7299</td>
<td>1384.886</td>
<td>/</td>
<td>23-34</td>
<td>FFVAPPPEVFEGK</td>
</tr>
<tr>
<td>1267.7045</td>
<td>1267.873</td>
<td>1267.673</td>
<td>91-100</td>
<td>YLGYLEQLLR</td>
</tr>
<tr>
<td>910.4741</td>
<td>/</td>
<td>/</td>
<td>125-132</td>
<td>EGIHAQQQK</td>
</tr>
</tbody>
</table>

Fig. 3. MALDI-ToF profiles of tryptic digest relative to spots 1 and 3, identified as \( \alpha s_1-I-CN \) and \( \alpha s_1-CN \). The MALDI spectrum of \( \alpha s_1-CN \) is displayed into the inset of \( \alpha s_1-I-CN \) spectrum. The table reports the comparison among theoretical mass of peptides from tryptic cut of \( \alpha s_1-CN \) sequence and those observed from in situ digestion of the spots.
the cheeses confirmed negligible detection of the ALMI fragment both after stretching (Fig. 9) and until the 5 days storage at +10 °C (Fig. 10). As can be seen, the rate of formation during cheese storage, dramatically dropped with respect to curd, due to chymosin denaturation, and the intensity of the band remains under an RQ value of 4 up to 5 days. On the whole, considering that these manufacturing conditions exceed those normally adopted in traditional cheesemaking, the RQ level 4 (found under our electrophoretic conditions) could be fixed as the tolerance limit for “Fiordilatte” obtained from freshly prepared curd.

3.3. Determination of αs1-I-casein in industrial cheese

The evolution of the ALMI fragment was then investigated in industrial “Fiordilatte” stored at commercial temperature
conditions (+10 °C). The urea-PAGE pattern shown in Fig. 11 allows a comparison between two different cheesemaking technologies (direct acidification and fermentation with natural whey starter culture). The results confirm that the fragment is only slightly detectable until 5 days of storage and remains under the level of 4 RQ independently from the type of acidification used. After 5 days the band becomes more intense, exceeding the value 4, although differences between the two production technologies are only detectable at day 15. We concluded that the production technology has no significant effect on primary hydrolysis of αs1 casein, and our method can be applied to both types of "Fiordilatte" if analyzed within a few days from manufacture. The suitability of the method was finally tested on 81 freshly produced “Fiordilatte" samples taken from different dairies, as reported in the materials and methods section. On the whole, 95% of the samples from fresh curd fell within the RQ range 4.18–6.19, and the mean RQ value of ALMI fragment for fresh curd cheese was 5.3 (higher than that found in the laboratory experimentation) and that for those containing preserved curd was 16.6. The Boxplot reported in Fig. 12 demonstrates that the cheeses declared to be made with or without the employment of preserved curd were well discriminated: the difference between the two groups is significant by Student t test, at

![Fig. 9. Urea-PAGE pattern of samples of Fior di latte obtained from curd stored in whey at +37 °C for 6 h. Lanes 1–4 and 5–8 are two series of cheeses obtained from curd at 12, 46 h; lane 9 is a fresh curd; lane 10 is a frozen curd (12 months of storage). Identification of the bands is the same as indicated in Table 1.](image)

![Fig. 10. Kinetic of formation of the ALMI fragment during storage at +10 °C of Fiordilatte obtained by stretching of the curd after 1 and 6 h holding in whey.](image)

![Fig. 11. Urea-PAGE of samples of industrial Fior di latte manufactured by direct acidification with citric acid or acidification with natural whey starter culture, and stored at +10 °C. Lanes 1, 3, 5, 7: direct acidification samples at 0, 5, 7, 15 days; lanes 2, 4, 6, 8: starter culture samples at 0, 5, 7, 15 days. Identification of the bands is the same as indicated in Table 1.](image)

![Fig. 12. Boxplot of the results obtained by analysis of 81 samples of industrial Fior di latte, freshly produced.](image)
p < 0.001. Nevertheless, in a small part of Boxplot the two groups appear to overlap, giving rise to a sort of “grey zone” in which the discrimination does not seem to be certain. However, we found that Shapiro–Wilk test of normality and skewness showed an abnormal distribution of data in fresh products. More precisely the RQ of ALMI fragment had a positive skewness and, consequently, the median was lower than the mean. The sampling method could account for this statistical behavior and, in particular, the absence of information about milk used at the dairies. Eventually some slight proteolysis in milk during long storage before cheesemaking should overlap to that caused by rennet: more investigation on this subject is actually in progress.

4. Conclusions

The results obtained indicate that the use of stored curd in “Fior-dilatte” cheesemaking can be revealed by quantification of fragment-24–199 of αs1-CN. Its formation in this cheese takes place in two phases, at different rates: it is rapid in the curd (first phase, until stretching), since chymosin is fully active, whereas it is slow in cheese (second phase, from stretching until consumption), since chymosin is more or less denatured. If we consider that high moisture mozzarella is stored at low temperature and has a short shelf-life, the intensity of the peptide in the finished product mainly depends on the first phase, and time and temperature conditions at which the curd is stored become critical points. Under normal conditions, the formation of the casein fragment is negligible, since the curd is promptly stretched; in the case of stored curd, the formation becomes as more intense as storage time is prolonged. A level of about 4 RQ band intensity could be the “tolerance level” that must not be exceeded in “Fior-dilatte” obtained from fresh curd. However, being densitometry a semi-quantitative method, the tolerance level should be always verified by using a standard sample. Even if the evaluation of the ALMI fragment seems already useful in obtaining an indication about the use of semi-finished products in cheesemaking, further investigation is needed to evaluate the feasibility of the method and, in particular, to better establish the RQ tolerance level for cheese from fresh curd. At the moment, the method is useful to certify the attainment of “Fior-dilatte” under controlled technological conditions of production and storage, as is often required by the official protocol of production of EU typical products (DOP, IGP, GTS).

References