Characterization of Silter Cheeses Produced in Valley and Alpine Pastures by a Proteomic Approach

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Abstract

Silter is an Italian hard cheese manufactured with milk produced by cows fed at different altitude, valley or alpine pasture. The chemical, rheological and sensory properties of cheeses can be affected by the modification in milk composition due to the breed which at different altitude causes the modification of protein content, κ-CN glycosylation, plasmin activity, and coagulation properties. The influence of milk plasmin activity on dairy production was investigated in seven Silter cheeses, four produced in the valley and three from alpine mountain, through alkaline urea-polyacrylamide gel electrophoresis; two-dimensional gel electrophoresis coupled to mass spectrometry and image analysis. Results demonstrated that Silter cheese obtained from cows reared in alpine pasture is characterized by a more evident proteolysis, determining high levels of β-CN and αl-CN fragments. Therefore, the most relevant fragmentation was attributed to a more intense activity of plasmin and to a different dosage of rennet to make up for the reduced coagulation properties of alpine milk.

Keywords: Silter cheese; Proteomic study; Plasmin action; Valley milk; Alpine milk

Introduction

Silter is an Italian hard or semi-hard cheese produced in summer in the Valcamonica area from milk of cows fed with valley or alpine pastures. Silter cheese has an ancient origin, very likely from a word of Celtic origin that means Casera in Italian, corresponding to a ripening room [1]. Silter cheese received EU recognition of protected designation of origin (PDO) with Reg. 2081/92. Silter PDO refers to a half-fat hard cheese made with raw milk from cows fed mainly on fodder from the production area. At least 80% of the animals must belong to typical alpine breeds-Grigio Alpina, Pezzata Rossa and Bruna-the latter of which must represent at least 60% of the lactating cows present on each individual farm [2].

The chemical, rheological and sensory properties of cheeses can be affected by the modification in milk composition due to the breeding at different altitudes, as already demonstrated by several authors [3-5], causing the modification of protein content, κ-CN glycosylation, plasmin activity, and coagulation properties.

Bugaud et al. [6] and Buchin et al. [4] found an increase of plasmin and plasminogen in milk of cows grazing at high-alpine pastures with respect to those kept down in the valley, especially under unfavorable feeding and climatic conditions. Differences in texture between the cheeses produced in the two areas were also observed and ascribed to the primary proteolysis, partly due to different amounts of plasmin and plasminogen present in the milk [4]. On the contrary, Coulon et al. [7] and Leiber et al. [8] found that neither alpine feed nor altitude-related hypoxia caused the increase of plasmin or plasminogen in milk, while feeding alpine hay even significantly reduced plasminogen. However, Leiber et al. [8] found that hypoxia is related to reduced milk yield and protein content because of a low metabolic energy from impaired energy availability from the alpine pasture that, consequently, reduces protein synthesis. Energy deficit is also accompanied by low plasma glucose levels [9] that reduce the capacity for protein glycosylation, and then, the degree of glycosylation of κ-CN [8]. The negative influence on rennet coagulation properties of alpine milk was attributed to several factors such as reduced protein content, casein proportions, κ-CN content and degree of glycosylation [8]. Casein proportion can be modified by plasmin, an endogenous protease of milk acting on all caseins (CNs), but especially on β-CN [4,10,11], producing three γ-CN, polypeptides known as γ1 (βfragment 29-209), γ2 (βfragment 106-209) and γ3 (βfragment 108-209). If plasmin proteolysis happens prior to coagulation, the breakdown products are lost with the whey [10,12]. As a consequence, plasmin activity adversely affects rennet coagulation properties [13], cheese-making properties and cheese yield. Thus, an up-regulated plasmin system during alpine grazing, enhancing proteolysis during the ripening of cheeses, could also affect cheese taste either worsening it or contributing to specific flavours [4]. Therefore influence of alpine pastures on the plasmin activity in milk is still debated, but generally accepted.

Exogenous enzymes from calf rennet carry out another important proteolytic activity occurring in cheeses. The major components are chymosin (88-94% milk clotting activity, MCA, EC 3.4.23.4) and bovine pepsin (EC 3.4.23.1; 6-12% MCA). The main target of chymosin is the cleavage of the bound Phe105-Met106 of the κ-CN, which promote milk coagulation and curd formation. In solution,
chymosin also acts on both αs1-CN and β-CN [14], but in cheese, it appears to hydrolyze mainly αs1-CN [15-17]. The residual chymosin activity in the curd has a considerable impact on the proteolysis of cheese and consequently on its quality.

Therefore, our work aimed to evaluate the differences that occur in Silter cheeses from milk produced by cows reared in Alpine and valley farms. Our study was focused to the identification of the casein fragments, resulting from endogenous and exogenous enzymes activities, mainly chymosin and plasmin, by a proteomic approach. Moreover, we also evaluated the presence of molecular marker to trace cheeses from milk of cow bred at low or high altitude.

Materials and Methods

Chemicals

All reagents, for the sample preparation and electrophoresis analysis, were ultra pure grade. Formic acid (FA) ≥ 98%, ammonium bichcarbonate, 1,4-dithiothreitol, iodoacetamide, acrylamide/bis-acrylamide 30% solution, urea and Coomassie Brilliant Blue G-250, were from Sigma-Aldrich (St. Louis, MO, USA). Modified trypsin (porcine) and chymotrypsin (bovine) were from Princeton Separations (Adelphia, NJ, USA). Water and acetonitrile (ACN), LC-MS CHROMASOLV®, ≥ 99.9%, were from Fluka.

Samples

Seven Silter cheeses were examined, four of them produced in the valley area and three from alpine mountains. In Figure 1 is reported a schematic process of Silter cheese production according to its PDO disciplinary.

Extraction of casein from Silter cheese

A total of 7 Silter cheese samples were taken after ripening for about 9-10 months. Three samples were produced from cows reared in Alpine sites and four in the valley. Each sample was prepared according to the method of Krause et al. [18], with slight modifications. Cheese (10 g) was grated, dissolved in 0.5 M sodium citrate (1:3 w/v) and shaken for 10 minutes. Then it was heated at 40°C for 60 min and subsequently 2 N HCl was added to reach a final pH of 4.6, followed by centrifugation at 10.000xg for 10 min at 4°C. The supernatant was discarded and the fat was manually removed. The casein precipitate was washed three times with distilled water performing each time a centrifugation at 10.000xg for 12 min. Caseins were finally washed two times with cold acetone and recovered for electrophoretic analysis.

Alkaline polyacrylamide gel electrophoresis in the presence of urea (Urea-PAGE)

Each casein sample (0.02 g) was dissolved in 1.0 mL of urea sample buffer (0.06 M Tris-HCl, pH 8.6, 8 M urea, traces of bromphenol blue sodium salt) containing 2% DTT. Samples (4 μL) were loaded onto a 7-4% polyacrylamide gel mini in the presence of 8 M urea. Urea-PAGE analysis was conducted in a discontinuous gel as reported by Andrews [19]. Briefly, electrophoresis was performed with a 7% Acrylamide/Bis-acrylamide separating gel (from a 30% solution), in a buffer 1.5 M Tris-HCl pH 8.6 and 8 M urea; the stacking gel was prepared with 4% Acrylamide/Bis-acrylamide, (from a 30% solution) in a buffer 0.5 M Tris-HCl pH 6.8 and 4 M urea. The electrode buffer was prepared with 0.024 M Tris and 0.19 M glycine and the pH adjusted to 8.6. Separation was performed using a Mini-PROTEAN Tetra Cell electrophoresis chamber (Bio-Rad Hercules, California, Stati Uniti) at 200 V for 1 hour.

After the electrophoretic separation the gels were stained with Coomassie Brilliant Blue G-250, according to Candiano et al. [20] and destained with deionized water. The casein fractions were identified according to Mayer [21].

Two-dimensional gel electrophoresis (2-DE)

An aliquot of 100 μL of isoelectric caseins dissolved in a denaturing solution containing 9 M urea to a final concentration of 0.5% (w/v), was added with 150 μL of DeStreak rehydration solution (GE-Healthcare Bio-Sciences, Little Chalfont, U.K.), adding to the solution carrier ampholyte (2%, pH 3-10) and DTT (1%), then loaded onto immobilized pH gradient (IPG) strips, pH 3-10, 13 cm long (Biorad, Hercules, California, Stati Uniti). Following rehydration (16 hours), isoelectrofocusing (IEF) was carried out at 20°C using an Ettan IPGphor (GE-Healthcare Bio-Sciences, Little Chalfont, U.K.); the voltage was increased stepwise to 8000 V, reaching a total of 13000 Vhrs. Afterwards, the IPG strips were reduced and alkylated using buffers containing 6 M urea, 50 mM pH 8.8 Tris-HCl, 2% SDS, 30% glycerol and 0.002% BPB, successively supplemented with 1% (w/v) DTT and 2.5% (w/v) iodoacetamide for 20 min each.

The SDS-PAGE separation was performed using 15% polyacrylamide gels (15 × 18 cm) and placed in a Bio-Rad Protean II xi Cell (Bio-Rad, Hercules, California, Stati Uniti) electrophoresis chamber. The electrophoretic separation was performed at 30 mA for gel at 10°C. The gels were then stained with Coomassie Brilliant Blue G-250 (Sigma Aldrich, Steinheim, Germany) and digitalized using an

Figure 1: Process of Silter cheese production according to its PDO disciplinary.
Protein identification by nano-LC-ESI-IT-MS/MS

Enzymatic hydrolysis: The protein spots identified by software image analysis were manually excised from the gel and subjected to destaining, reduction, alkylolation, washing and, finally, in-gel enzymatic digestion with trypsin/chymotrypsin [22]. Briefly, the gel-spot destaining was performed by alternating washing cycles of 15 min each with 50 mM NH₄HCO₃ in 50% aqueous acetonitrile and acetonitrile. Reduction and alkylolation were performed by 10 mM dithiothreitol (45 min at 56°C) and 55 mM iodoacetamide (30 min at room temperature in the dark), respectively. After washing cycles with 50 mM ammonium bicarbonate and acetonitrile, the enzymatic mixture trypsin-chymotrypsin in 25 mM ammonium bicarbonate was added at the ratio of 1:50 (w/v). The mixture was incubated overnight at 30°C, then the enzymatic digestion was stopped by the addition of few microliters of 5% formic acid. The resulting peptide mixture was stored at -20°C until used.

Liquid Chromatography and Mass Spectrometry

Chromatographic analysis was performed by a nano-LC apparatus, Ultimate 3000 (Dionex LC-Packings, Amsterdam, The Netherlands) which consisted of an autosampler, a low pressure gradient micro-pump series, equipped with flow managers, a column thermostat and an UV detector set at 214 nm. The UV flow cell was connected to an ESI-Ion Trap HCTultra-ETD II Basic System (Bruker Daltonics Srl, Bremen, Germany). The nanoLC-ESI-ITMS/MS system was controlled by the softwares Chromelope CHROM-1 (Dionex) and Hystar 2.3 (Bruker Daltonics). A PepMapTM C18 nano trap column (300 µm i.d. × 5 mm, 5-µm particle size, LC Packings) was used for concentrating and desalting the injected sample. Chromatographic separations were carried out by a PepMap™ C18 analytical column (15 cm length × 75 µm i.d., 3-µm particle size, 100-A pore diameter; LC Packings). The mobile phase consisted of 0.1% FA in water (eluent A) and ACN/0.1% FA in water (80:20 v/v) (eluent B). Sample elution through the trap column and no multiple injections for each protein digest were carried out by a PepMap ™ C18 analytical column (15 cm length × 75 µm i.d., 3-µm particle size, 100-A pore diameter; LC Packings). The linear gradient to 10% A and 90% B; 10 min isocratic step at 10% A and 90% B; 1 min to the initial mobile phase composition (96% A and 4% B), at which the system was re-equilibrated for 23 min, for a total pre-concentration and desalting. Chromatographic separations were cross-checked and only peptides corresponding to the normalized delta scores (e.g. the difference in scores of the 1st and the 2nd ranked peptides, divided by the score of the 1st rank peptide) were examined. In order to assess the confidence of peptide identification, the minimal requirements of ion scores, rank, number and abundances of experimental product ions in the MS/MS spectrum were considered. In order to assess the confidence of peptide identification, the normalized delta scores were monoisotopic and carbamidomethylated at cysteine residues. A maximum number of 2 missed cleavages were allowed and a peptide tolerance of 0.3 Da, both for precursor peptide ion and MS/MS tolerance was set, for the window of error for matching the peptide mass values. The option "automatic error tolerant" search was checked to discover unsuspected chemical and post-translational modifications, sequence variants and non-specific cleavage products.

Protein identification was accepted when MASCOT search results delivered scores higher than the identity threshold (p<0.05) for each sample injection, and with the same protein identification as the top hit for the multiple injection (at least twice) of the same sample. Peptide matches above the identity threshold were submitted to a post-database search validation by a manual inspection of the corresponding MS/MS spectra. Therefore, the sequence assignments were cross-checked and only peptides identified in each replicate that satisfied the minimal requirements of ion scores, rank, number and abundances of experimental product ions in the MS/MS spectrum were considered. In order to assess the confidence of peptide identification, the normalized delta scores were included in the peptide list only if a series of at least 3 continuous fragment ions were observed and if the relevant normalized delta scores was higher than 0.30.

Results and Discussion

The proteolysis occurred in Siler cheeses from valley or alpine pastures, was investigated by alkaline Urea-PAGE analysis, two-dimensional gel electrophoretic with image analysis followed by single-spot analysis via mass spectrometry.

Alkaline urea-PAGE electrophoresis

In Figure 2 are shown the electrophoretic patterns of three alpine pasture cheeses (APC) and four valley cheeses (VC). The former were characterized by a lower intensity or absence of β-CN bands and more intense γ-CN bands than the latter did. In addition the APC patterns showed more bands related to the αs1 fragments, in particular the αs1-CN bands were more marked than αs1-CN ones which were present in all the samples with a comparable band intensity. The formation of these peptides was detected in other cheeses, e.g., in...
Grana cheese [23,24], Abundance cheese [4,6], where the authors essentially identified two αs1 fragments (24-199, αs1-I-CN; 24/25-169, αs1-II-CN) as consequence of the chymosin action [15] and the three γ-CN originated by plasmin. Marcos et al. [25] comparing proteolysis on several cheese varieties reported that, in general, αs1-casein was degraded more extensively than β-casein. These authors reported that in cheeses in which β-casein was degraded less extensively (e.g., Parmesan, Emmental, Gruyere and Tilsit), the concentrations of γ1- and γ2-caseins were high, while in cheeses where almost all β-casein had been degraded (e.g. Roquefort), less γ1-casein and more γ2-and in particular, γ3-casein were present, indicating more extensive action of plasmin. Therefore, greater amount of γ-CN and αs1 fragments observed in APC suggested the occurrence of a more intense hydrolysis at level of plasmin and rennet, respectively.

**Figure 2:** Alkaline discontinuous gel electrophoresis in presence of urea (UREA-PAGE) at pH 8.6, of insoluble caseins at pH 4.6. The electrophoregram shows cow’s milk casein in the lane 1; Silter cheese caseins from valley pastures in the lanes 2-3-5-7 and Silter cheese caseins from alpine pastures in the lanes 4-6-8.

**Proteomic analysis of Silter produced from Alpine and valley milks**

To better investigate the nature of products from primary proteolysis 2DE gels were fitted out. The typical 2DE maps of Silter cheeses are shown in Figure 3; panel A shows the 2DE map of Silter obtained by alpine milk while panel B shows the 2DE map of Silter obtained by valley milk. The 2DE map of APC showed more spots, which on the basis of spot coordinates (molecular weight and isoelectric point) and tandem mass spectrometry, were identified as fragments of αs1-CN, β-CN and κ-CN. In 2DE map of Silter caseins from alpine milk we observed that many spots migrated from the acidic area to the neutral area (spots 1, 4, 5, 7; and 9; 13, 15 and 17; 19; 21 and 23) and few spots were distributed from the neutral to the basic area (11; 25; 27). This distribution was expected because the caseins have an average acid (pH 4.6) isoelectric point (pI) on average of pH 4.6. In fact proteomic studies of milk proteins 2DE was performed in the pH range 4.0-7.0 to reach a better resolution [26,27]. Nevertheless, in the case of cheese it is more useful to analyse casein fraction in the pH range 3.0-10.0 in order to detect milk proteins and fragments from primary proteolysis of casein. In a recent proteomic study of six months ripened “Caciocavallo cheese” [28], and proteolysis in mozzarella cheese [29], performed in pH range 3.0-10.0, spreading spots were observed on the entire gel surface. The most of the spots were identified as αs1-CN and β-CN fragments from primary proteolysis due to the enzymatic activity of plasmin and chymosin.

**Figure 3:** Two-dimensional gel electrophoresis (2DE) was performed in the first dimension by IPG strip 3-10 and in the second dimension, orthogonal to the first, SDS-PAGE was performed using 15% polyacrylamide gels. The 2DE maps show Silter cheese caseins from milk cow fed at alpine (A) and valley (B) pastures. Identification of the numbered spots is reported in Table 1.
submitted to proteolysis before LC-MS/MS analysis. Hence, we suggested that the amino acidic ranges reported in Table 1 are a good approximation of the effective proteins, also confirmed by the correspondence between the gel-spots localization and the calculated MW and pl values. Our results confirmed that the in-gel digestion with the combined use of trypsin-chymotrypsin provided an accurate identification of cheese proteins from milk produced in alpine farms. Protein bands, corresponding to the main milk proteins, were identified, i.e. αs1 casein (spot N. 1,4,5,7,19), αs2 casein (spot N. 11), β-casein (spot N. 9,13,15,17,21,23,25) and k-casein (spot N. 27). Multiple observations of an individual protein that could be isoforms or fragments have been localized in different 2-DE gel regions. A significant correlation between abundance of some polypeptides and ripening process of cheese from milk produced in alpine and valley farms was found. The comparison of 2DE maps showed the casein fractions and the relevant fragments generated by the enzymatic action of plasmin and chymosin. As a matter of fact, peptides from the C-terminal portion of β-casein and polypeptidic fragments associated with αs1-casein at different intensity were found in several protein spots. The 2DE map of Siler cheese from alpine dairy showed a higher number of spots at level of γ-CN [γ2 β-casein (106-209) and γ3-CN, β-casein (108-209)] associated to the spots 21, 23 and 25 f(114-209). Moreover, in cheese samples from alpine pasture relatively high levels of fragments of k-casein were also found, such as spot N. 27, f(17-105) that can be identified as para-k-CN.

<table>
<thead>
<tr>
<th>Gel Spot</th>
<th>Protein</th>
<th>Entry Name</th>
<th>Protein Code</th>
<th>MASCOT Score</th>
<th>No. of Matched Peptides</th>
<th>Sequence Coverage (%)</th>
<th>Amino acidic range</th>
<th>MW (Da)</th>
<th>pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>αs1-casein</td>
<td>CASA1_BOVIN</td>
<td>P02662</td>
<td>291</td>
<td>21</td>
<td>53</td>
<td>24-193</td>
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<td>P02662</td>
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<td>18</td>
<td>44</td>
<td>53-209</td>
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<td>CASK_BOVIN</td>
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<td>7</td>
<td>39</td>
<td>17-105</td>
<td>10273</td>
<td>9.76</td>
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</table>

Table 1: Proteins from Siler cheese identified by MASCOT MS/MS ion searching against Swiss Prot database.

It is noteworthy that in the 2DE map was observed the absence of spot related to β-CN and several spots related to γ-CN fragments, confirming the highest activity of plasmin in Alpine cheese and the highest intensity of the spot identified as para-k-CN, indicated a higher activity of rennet, probably due to a high amount of rennet used to coagulate Alpine milk.

**Image analysis**

The image analysis data are shown in Table 2. In order to establish a relationship between the number of spots and their volume intensities, which showed unbalanced trends in almost all samples, the two datasets were related by computing the ratio between the total number of spots and the total volume intensity, according to Di Luccia et al. [30]. In mathematical terms, this ratio represents a density number defined as spot number density (ds):

$$ds = n/V_i$$

where n is the total spot number and V_i is the total volume intensity. Considering that primary proteolysis, which is the first action of endogenous and exogenous (rennet) enzymes on casein fractions, which in turn generate large protein fragments that are still detectable by gel electrophoresis. An increasing of ds should be expected throughout the ripening period. The ds values calculated for the SA and SV are shown in Table 2 and Figure 6 as histograms. A significant difference (P<0.05) between the ds values of SA and SV cheeses was observed, indicating a more intense proteolysis activity in the SA. These results establish that a more intense action of plasmin and rennin occurred in SA, and probably a higher amount of rennet was used to coagulate milk from alpine pasture to balance time of coagulation and curd firmness [31,32] in alpine milk. These results reconcile the outcomes of Buchin et al. [4], Bugaud et al. [6] that reported more β-CN fragments in milk accounted for higher plasmin activity in alpine and those of Leiber et al. [8] that found a diminution of protein content and k-CN glycosylation, factors that influence negatively the coagulation aptitude of alpine milks. Moreover, Siler is a hard cheese and during ripening the action of endogenous and exogenous enzymes, including microbial enzymes, could further modify alpine and valley milk raw matter.
Figure 4: NanoLC-ESI-MS/MS analysis, UV profile (black line) and Base Peak Chromatogram (gray line) of the tryptic-chymotryptic digest from the gel spot No. 19, identified as αs1-casein.

Figure 5: MS/MS spectrum of the triple charged ion at m/z 596.9 from the phosphopeptide KVPQLEIVPNpSAEER of αs1-casein (spot No. 19).

Table 2: Number and volume of spots detected by 2-DE gel electrophoresis and image analysis of casein fractions of Silter cheese produced from alpine (SA) and valley (SV) milk.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Number of total spot (ns)**</th>
<th>Total volume (Vt)</th>
<th>Density spot x 103 (ds)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>66 ± 3</td>
<td>17164 ± 2140</td>
<td>3.85 ± 0.47</td>
</tr>
<tr>
<td>SV</td>
<td>42 ± 7</td>
<td>14916 ± 2596</td>
<td>2.95 ± 0.63</td>
</tr>
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</table>

*P<0.05; **P<0.01

Conclusion

The proteomic study and image analysis demonstrated unequivocally that Silter cheese obtained by alpine pasture milk (SA) was subjected to a more intense proteolysis than that of Silter cheese manufactured with valley milk (SV). The primary proteolysis occurred in SA produced more β-CN fragments in particular γ-CN ascribable to a higher activity of plasmin in SA samples, according to the preliminary results of the cited literature. In this respect, the notable reduction of β-CN or its absence could be used to trace Alpine cheese. Different dosage of rennet, instead, to make up for a lower coagulation property of alpine milk is probably responsible for the higher concentration of αs1-CN fragments since the alpine location (sojourn) acted unfavourably on renneting properties and cheese yield for a minor content of milk proteins and minor glycosylation of κ-CN. Finally, amongst the fragments from primary proteolysis it was remarkable the identification of spots with an approximate pI 4.5 and molecular weight of 15 kDa as a fragment of αs1-CN, which, hitherto, not yet well defined by literature in the ripening of cheeses.

References

1. The Denomination of Protected Origin. Designation of the protected designation of origin.


