Journal of Food Technology 4 (2) : 128-134, 2006

Preliminary Evaluation of the Influence of Pasture Feeding on Proteolysis of Ragusano Cheese

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Abstract: The influence of native pasture for cows feeding on the levels and profiles of proteolysis in Ragusano cheese at different ages (1, 120 and 210 days) was evaluated through a comparison with the Total Mixed Rations (TMR) feeding. Eight experimental cheeses were produced from milk of cows consuming a TMR diet supplemented with native pasture and eight control cheeses from milk of cows fed with only TMR. Cows diet was found to have no significant ($p>0.05$) impact on the overall composition of Ragusano cheese, including the levels of primary and secondary proteolysis measured as soluble N in acetate buffer at pH 4.6 and in 12% trichloroacetic acid, respectively. Experimental and control cheeses showed similar extents of casein hydrolysis by urea-PAGE analysis and the densitometry of the electrophoretic profiles confirmed that nonsignificant differences ($p>0.05$) in bands intensity existed between experimental and control cheeses. These results suggested that the feeding strategy had no influence on the activity of proteases from rennet, milk and microflora on caseins and large peptides. Otherwise, cows diet had a qualitative impact on secondary proteolysis, influencing the composition of the 12% trichloroacetic acid-soluble peptide fraction of Ragusano cheese. Experimental and control cheeses showed different peptide profiles by reversed phase-HPLC analysis and the information provided by these profiles were useful in discriminating cheeses based on age and feeding treatment by principal components analysis. Proteases of microflora derived from local pasture probably produced soluble compounds that were specifically present in the peptide profiles of experimental cheeses. Further studies will concern the molecular characterization of the different peptide fractions of experimental and control cheeses.

Key words: Ragusano cheese, pasture, proteolysis

INTRODUCTION

Typical dairy products are historically linked to specific geographic regions and to well handed down traditional metodologies. They often have unique sensory properties that are deeply related to the environmental conditions of milk production[1]. Native microflora from the starting milk and from the animal and cheesemaking environment is known to play a role in determining the organoleptic quality of milk products[2]; cheeses made from raw milk ripen faster and develop a more intense flavour than pasteurized milk cheeses due to their highest levels of indigenous milk microflora[3,4]. However, the animal feeding and in particular the nature of pasture is considered to have the largest impact on sensory qualities of traditional cheeses. Many authors have reported significant differences in cheese flavour depending on the altitude of the pasture and botanical composition of the forage consumed by the animals[5-7]. Aromatic molecules present in the plant species of local pastures may potentially act as precursors of odorous compounds that the animal can elaborate within rumen and transfer to milk[8]. Alternatively, certain compounds (i.e., terpenes) may be directly transferred from forage to cheese via milk and thereby contribute to its aroma[9]. However, it is likely that pasture influence the sensory properties of cheese mainly by changes in the chemical composition of milk. Milk produced from cows fed on pasture had different fatty acid composition and volatile compounds than milk produced from cows fed with hay or grain[10]; casein composition in milk was also found to be affected by the nature of pasture[11]. Proteolysis is known to be a major event affecting the development of typical flavour and texture in most cheese varieties[12]; changes in the casein composition may therefore lead to a different production of aroma compounds and to variations in cheese quality.

Ragusano cheese is an hard, brine-salted pasta filata cheese produced in the Hyblean plateau of Sicily (Italy)

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according to a traditional technology[13]. The cheese has specific and well-characterised sensory attributes reflecting the characteristics of local pasture[14] and the contribution of natural microflora derived from raw milk. Cheeses produced according to the Protected Denomination of Origin regulamentation are traditionally obtained from milk of cows fed on native pasture[15]. However, changes in milk production practices and the demand for farms to remain economically competitive have recently increased the amount of conserved feeds and Total Mixed Rations (TMR) used for the animal feeding[16]. Cheeses produced from milk of cows fed with only TMR have been found to be less yellow[14] and rich in aroma-active compounds[16] than cheeses made from milk of cows consuming a TMR diet supplemented with local pasture. These results showed that compounds from native pasture plants changed the sensory characteristics of Ragusano cheese. The aim of this study was to evaluate the impact of native pasture on the levels and profiles of proteolysis in Ragusano cheese.

MATERIALS AND METHODS

Cheese manufacture and sampling: The feeding experiment was performed from February to May, when native pasture is available and has the highest quality. Twentysix Friesian cows were selected from a farmhouse producer of Ragusano cheese. The details of diet and cows management were previously described by Carpino *et al.*[14]. A group of thirteen cows consumed a TMR diet supplemented with native pasture (experimental group) while another group of thirteen cows was fed with only TMR (control group). Raw milks from experimental and control groups were separately collected and processed during four cheesemaking trials. Eight blocks of Ragusano cheese were produced from each milk type (for a total of 16 cheeses) according to the standard technology[13], as modified by Carpino *et al.*[14]. Conditions of cheese brine-salting, aging and sampling (1, 120 and 210 days) were as reported by Carpino *et al.*[16].

Compositional analysis: Grated cheese samples were analyzed in duplicate. Cheese analyses were as follows: total N by the Kjeldahl method[17], fat by the Gerber method[18] and NaCl by the Volhard method[19]. Nitrogen soluble in acetate buffer at pH 4.6 and in 12% trichloroacetic acid (TCA) were determined[20] and expressed as a percentage of the total N content. Total solids were determined, in four replicates, using a forced-air oven drying method at 100°C for 24 hrs[19].

Assessment of proteolysis in cheese

Analysis by urea-page: Cheese N fractions insoluble in acetate buffer at pH 4.6 were prepared and analyzed by urea-polyacrylamide gel electrophoresis (urea-PAGE) using a Protean IIxi vertical slab gel unit (Bio-Rad Labs Ltd., Watford, UK) as described by Fallico *et al.*[21]. The gels were stained using a modification of the method of Blakesley and Boezi[22] with Coomassie brilliant blue G250. The urea-PAGE gels were scanned using a GS-710 Calibrated Imaging Densitometer (Bio-Rad Labs Ltd., Hercules, USA) and the bands quantified using Quantity One v.4.1.1 (Bio-Rad Labs Ltd., Hercules, USA). Relative protein content in each band, expressed as percentage of the total optical density in a single lane, was estimated by automatic integration of the area of the corresponding peak in the densitogram.

Reversed phase-HPLC analysis of 12% TCA-soluble peptides: The 12% TCA-soluble N of the cheeses was prepared by suspending grated cheese at 10% (wt/vol) in 0.4 M-trisodium citrate buffer, pH 8. The suspension was pH-adjusted to 4.6 using 1 M-HCl and centrifuged at 3000 g for 10 min at room temperature. The fat layer was discarded and the pH 4.6-soluble N fraction recovered by filtration through Whatman No.42 filter paper. An appropriate volume of a 50% TCA (wt/vol) solution was added to the pH 4.6-soluble N fraction to give a 12% TCA final concentration. The 12% TCA-soluble N fraction was recovered by centrifugation at 3000 g for 10 min at room temperature, desalted eluting on a Sep-Pak Cartridge C18 (Waters Corporation, USA) and concentrated with a drying centrifuge to 1/3 of the initial volume. The 12% TCA-soluble N of the cheeses was filtered through 0.22 μm cellulose acetate filter paper (Millipore, Bedford, USA) and analyzed by reversed phase-HPLC (RP-HPLC) using a KONTRON equipment (Kontron Instruments, Milan, Italy). Peptides were separated on a reversed phase Vydac C18 (250 x 4.6 mm, 5 μm, 300 Å) analytical column (Vydac, Hesperia, USA) and detected at 220 nm using a Kontron variable-wavelenght detector (Mod. 430). Solvent A was 0.1% (vol/vol) trifluoroacetic acid (TFA) in deionized water and solvent B was 0.07% (vol/vol) TFA in acetonitrile. All chemicals were of HPLC-grade. 200 μL of the filtrates were applied to the column and eluted at a flow rate of 1 ml min^{-1} using a linear gradient from 0% to 50% of solvent B over 95 min.

Statistical analysis: One-way ANOVA was used to test (at p=0.05) the influence of the feeding treatment on chemical parameters at each sampling date. Data from RP-HPLC chromatograms were obtained by visually recognizing similar peaks in the profiles and using the

peak heights units (μAu) as variables for statistical analysis[23,24]. The peak heights were found by converting the chromatograms to ASCII files. Principal components analysis (PCA) on RP-HPLC data was performed standardizing the variables (mean = 0; SD= 1). Statistical analyses were performed by using SAS v.8.2 (SAS Institute, USA); PCA biplot was obtained by using XLSTAT for Windows v.7.5.2 (Addinsoft, USA).

RESULTS AND DISCUSSION

Compositional analysis: Cows diet was found to not influence the composition of Ragusano cheese made from milk of cows consuming a TMR diet supplemented with native pasture or fed with only TMR (results not shown). Nonsignificant ($p>0.05$) differences were found between experimental and control cheeses after 120 and 210 days of age in terms of pH, moisture, protein, fat-in-dry matter, salt-in-moisture and proteolysis levels. Values for compositional parameters in both cheese types were within the respective age-related ranges reported for Ragusano cheese[25]: however, S/M contents exceeded the maximum value (6%, w/w) required for obtaining the brand of Protected Denomination of Origin cheese[26].

Urea-PAGE analysis of primary proteolysis: Primary proteolysis in experimental and control cheeses at different ages (1, 120 and 210 days) was compared by urea-PAGE analysis of the cheese N fraction insoluble in acetate buffer at pH 4.6 (Fig. 1). The extent of casein degradation was not affected by cows diet, with the electrophoretic patterns of experimental and control cheeses being similar at all ages. Densitometric evaluation of the gel prints (results not shown) confirmed this finding by showing that the relative percent areas of bands separated by urea-PAGE were not significantly ($p>0.05$) different between cheese types at all ages. This result reflected the nonsignificant ($p>0.05$) differences found in the proportions of N soluble in acetate buffer at pH 4.6 between experimental and control cheeses (results not shown).

Electrophoretograms showed that 1 days-old experimental and control cheeses shared a low level of overall proteolysis featuring a moderate hydrolysis of casein (CN) α_{s1} and a more extensive degradation of β-CN. An increasing hydrolysis of α_{s1}-CN produced a number of peptides moving faster than native protein in profiles of 120 days-old cheeses: these fractions have been shown to be due to rennet action on α_{s1}-CN[27]. The main peptides produced in Ragusano cheese by the early and progressive activity of residual chymosin are typically α_{s1}-CN (f24-199) (α_{s1}-I) and α_{s1}-CN (f102-199)[21,23,28]. Major

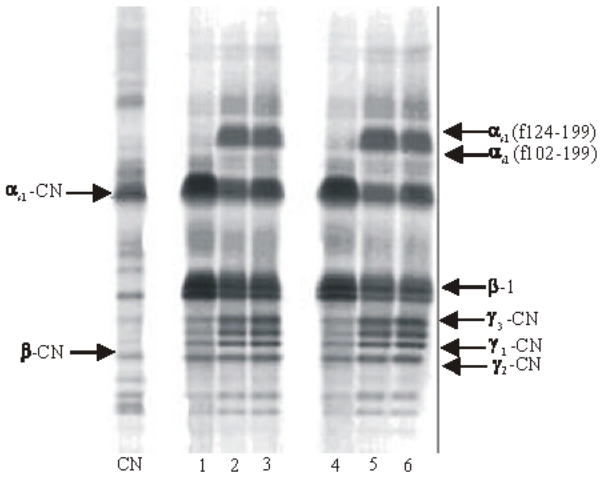

Fig. 1: Urea-polyacrylamide gel electrophoretograms (pH 8.6) of the acetate buffer at pH 4.6-insoluble N fraction of Ragusano cheese made from milk of cows consuming a TMR diet supplemented with native pasture (lanes 1-3) or fed with only TMR (lanes 4-6) after 1, 120 and 210 d of age, respectively. Isoelectric whole bovine casein (CN) was used as reference standard

changes were found for β-CN in both cheese types after 1 day of age. A couple of peptides migrated close to β-CN with a slightly higher mobility than native protein. These bands have been found to be typical of urea-PAGE profiles of Ragusano cheese[21,23,28]: the major one probably corresponds to β-CN (1-192) (β-I) fragment produced by rennet activity on β-CN[12]. Similar bands have been detected in Gouda cheese made with different amounts of rennet, increasing in intensity with coagulant concentrations[29]. The intensity of these bands decreased in experimental and control cheeses with the age suggesting a likely degradation to smaller peptides. The early action of plasmin on β-CN released theβ-CN (29-209) (γ_1-CN), β-CN (106-209) (γ_2-CN) and β-CN (108-209) (γ_3-CN) fragments[12] yet in 1-day old cheeses; the levels of these peptides regularly increased during aging of experimental and control cheeses. No further change in the overall level of casein hydrolysis and primary peptides production was found between the electrophoretic patterns of 120 and 210 days-aged samples in both cheese types. This finding related well with the trends for N soluble in acetate buffer at pH 4.6 (results not shown) and confirmed our previous reports: the early 4 months of cheese aging are essential to produce the peptide profile typical of Ragusano cheese and less variations occur between 4 to 7 months[21,23].

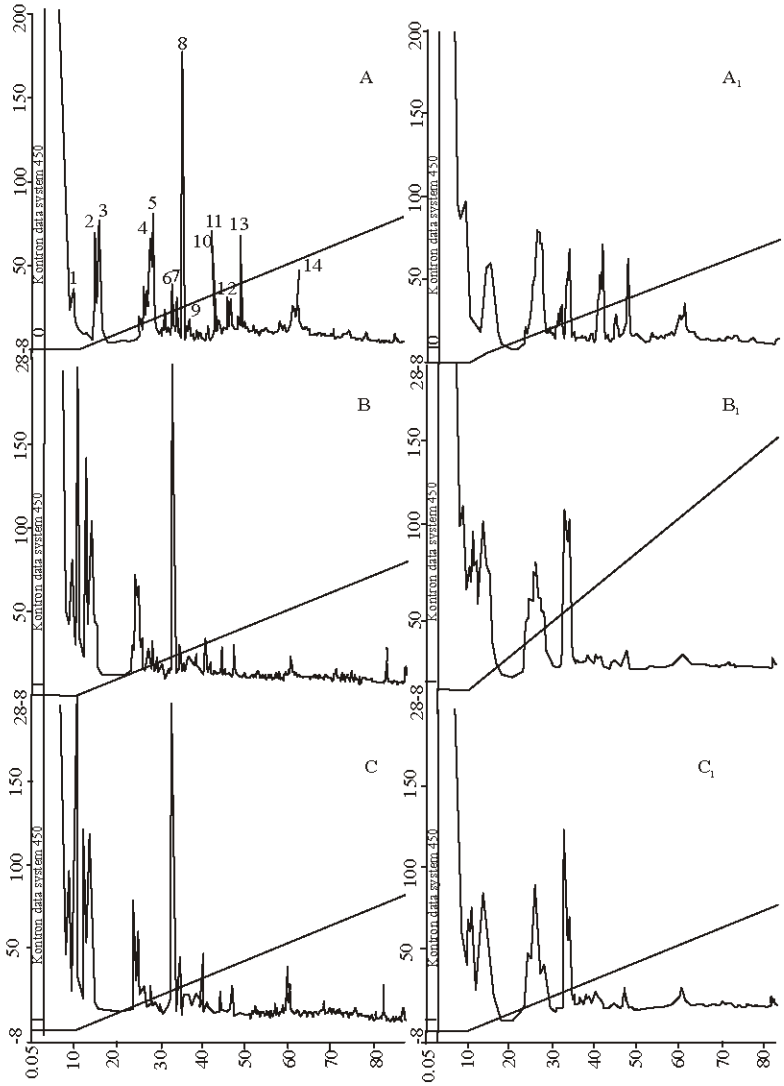

Fig. 2: Reversed phase-HPLC chromatograms of the 12% TCA-soluble peptides of Ragusano cheese made from milk of cows consuming a TMR diet supplemented with native pasture (left) or fed with only TMR (right) after 1 day (A, A_1), 120 days (B, B_1) and 210 days (C, C_1) of age, respectively. Numbers indicate matched peaks that were selected for principal components analysis

RP-HPLC and PCA analyses of secondary proteolysis: RP-HPLC analysis of 12% TCA-soluble peptides from experimental and control cheeses after 1, 120 and 210 days of age is shown in Fig. 2. Cows diet was found to have a nonsignificant impact on the quantitative composition of this peptide fraction, as measured by N soluble in 12% TCA (results not shown). Low molecular weight compounds in both cheese types were contained within the 10 to 60 min interval of the chromatogram. Large and medium sized peptides produced by the early action of proteinases from rennet, milk and microflora on casein matrix eluted in the medium hydrophobicity zone (40-60 min) of the peptide profiles (Fig. 2, A-A_1). These were degraded to small peptides and free amino acids by the activity of intracellular peptidases microflora as the cheeses aged, thus increasing the area of early eluting peaks (10-40 min) (Fig. 2, B-B_1 and C-C_1). Most of this soluble fraction was produced after 4 months of age and nonsignificant ($p>0.05$) changes occurred up to 7 months both in experimental and control cheeses: this finding was in agreement with previous results for Ragusano cheese[21,23].

RP-HPLC profiles of experimental and control cheeses showed that cows diet had a qualitative impact on

secondary proteolysis of Ragusano cheese, influencing the composition of the low molecular weight fraction. Peptide profiles of experimental cheeses displayed narrower and sharper peaks that conversely appeared wider and more displaced in those of control cheeses. PCA of data from chromatograms of experimental and control cheeses was therefore performed in order to confirm the influence of cows diet on the qualitative peptide profiles of Ragusano cheese. PCA was applied to a data set consisting of 14 matched peaks (Fig. 2, A); four principal components (PCs) were adequate to represent 82% of the total variance in the data set (eigenvalue>1). Scores of the first two PCs, that cumulatively accounted for 63.6% of the total variance, were plotted as shown in Fig. 3. Most of this variation was explained by PC1 (44.5%) that separated cheeses, independently from treatment, according to the aging time. A tendency for samples to group according to the cheese type was instead observed along the PC2 axis; however, the low percent of variance explained by PC2 (19%) suggested that cows diet was less influential than aging time in modifying the peptides profile of Ragusano cheese. Based on variables with high loading values (>0.8), a group of seven peaks was associated with the separation of these peaks eluted within 40 min in the HPLC profiles and

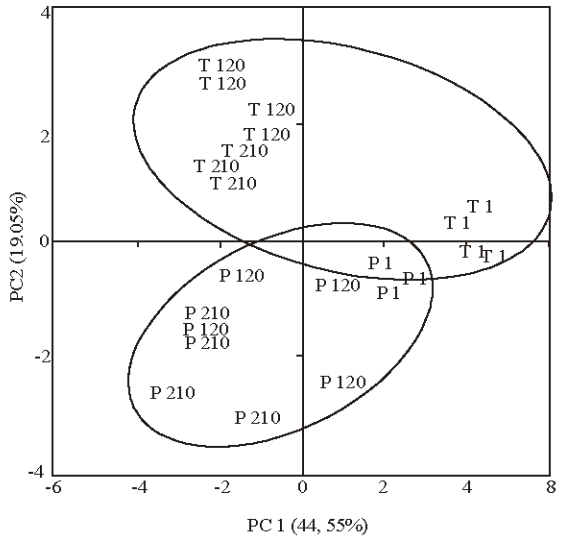

Fig. 3: Score plot of variables obtained by principal component analysis of data from RP-HPLC chromatograms of the 12% TCA-soluble peptides from Ragusano cheese made from milk of cows consuming a TMR diet supplemented with native pasture (P) or fed with only TMR (T) after 1 day, 120 and 210 days of age, respectively

then corresponded to hydrophilic compounds (small experimental and control cheeses within two clusters; peptides and free amino acids). It is well known that the proteolytic system of lactic acid bacteria mainly acts at a level of secondary proteolysis to produce small peptides, free amino acids and amino acids cathabolites: these compounds are directly related with the development of cheese flavour[12]. It has been also demonstrated that there may be a specific type of microflora in each production zone leading to significant variations in the sensory quality of the cheese[4,30]. Abondance cheeses made with milk from animals grazing different pasture areas were found to have different aroma profiles, with many of the distinguishing volatile compounds being of microbial origin; these results were associated to the contamination of starting milks with a different natural microflora[6]. Results from HPLC and PCA analyses suggested that diet someway affected the type of microflora present in milks and cheeses derived from different feeding treatments; this in turn affected the soluble compounds production in Ragusano cheese. A different proteases activity of microflora derived from local pasture probably produced low molecular weight compounds that were specifically present in the peptide profiles of experimental cheeses; this in turn gave rise to changes in the organoleptic attributes of these cheeses. Reports showing experimental cheeses as being more rich in aroma-active compounds, many of which deriving from amino acid cathabolism, than control cheeses[16] strongly support this hypothesis.

CONCLUSIONS

Results from this comparative study showed that cows diet had no effect on the overall composition of Ragusano cheese, including the levels of primary and secondary proteolysis. Experimental and control cheeses showed similar extents of casein hydrolysis by urea-PAGE analysis and the densitometry of the electrophoretic profiles confirmed that nonsignificant differences ($p>0.05$) in bands intensity existed between experimental and control cheeses. These results suggested that the feeding strategy had no influence on the activity of proteases from rennet, milk and microflora on caseins and large peptides. Otherwise, cows diet had a qualitative impact on secondary proteolysis, influencing the composition of the 12% trichloroacetic acid-soluble peptide fraction of Ragusano cheese. Experimental and control cheeses showed different peptide profiles by reversed phase-HPLC analysis and the information provided by these profiles were useful in discriminating cheeses based on age and feeding treatment by principal components analysis. Both results suggested that diet someway

affected microbial activities involved in oligopeptide and amino acid production in Ragusano cheese. Proteases of microflora derived from local pasture probably produced low molecular weight soluble compounds that were specifically present in the peptide profiles of experimental cheeses. Further studies will concern the molecular characterization of the different peptide fractions of experimental and control cheeses.

ACKNOWLEDGMENTS

The authors thank the technicians of Milk and Derivatives Lab of CoRFiLaC for technical assistance in cheese analysis and John Horne for statistical evaluation of chemical data. Financial support was provided by Assessorato Agricoltura e Foreste della Regione Siciliana, Palermo, Italy.

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