UDC 547.963.2:548.33:543.545
ISSN 1330-9862
(FTB-1452)

original scientific paper

Caprine α_{s1}-Casein Polymorphism: Characterisation of A, B, E and F Variants by Means of Various Biochemical and Molecular Techniques

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Received: November 16, 2004
Accepted: February 28, 2005

Summary

Considering a wide interest for the characterisation of caprine α_{s1}-casein variants and a large number of differently equipped laboratories, the objective of this study was to analyse and compare characteristics of caprine α_{s1}-casein variants by means of various biochemical and molecular techniques. The most frequent caprine α_{s1}-casein variants (A, B, E and F) were characterized by employing electrophoretic protein separation analyses (capillary zone electrophoresis, isoelectric focusing, and sodium dodecylsulfate polyacrylamide gel electrophoresis), chromatographic analysis (reversed phase-high performance liquid chromatography) as well as DNA analyses (ASA and real-time polymerase chain reaction approach). Further, we stressed weak and strong points for each method applied and provided information for the optimal and complementary use of those methods with respect to time, resolution and costs.

Key words: Capra hircus, α_{s1}-casein, electrophoresis, RP-HPLC, real-time PCR

Introduction

Factors affecting milk production generally belong to three categories: zootechnical, environmental and genetical. Until a few years ago, genetic selection and improvement of animals was based on indirect techniques of genotype assessment, relying in fact on measurements about phenotype observed in the individual or in particular groups of relatives. Then, progress on macromolecular research led to the advent of molecular biology. This discipline, applied to animal production, opened the possibility to plan selection directly on genotype rather than on phenotype, providing faster management and more accurate control capabilities due to the bypassing of the environmental effects.

The discovery of milk protein polymorphism (*1*) and its large potentials for the dairy industry have started numerous researches directed towards characterisation of different genetic variants (*2*), assessment of the relationship between genetic variants and milk production traits (*3*), assessment of the relationship between genetic variants and traits related to cheese production and milk processing (*4–6*) as well as towards gene biotechnology (*7,8*). Among the milk proteins studied, caprine

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α_{s1}-casein polymorphism is an excellent model for various disciplines in biology (*9*,*10*) and, at the same time, a good example of the application of milk protein research to dairy industry (*11*).

The great importance of caprine α_{s1}-casein polymorphism is due to its qualitative as well as quantitative implications. Grosclaude and Martin (*12*) described phylogenetic relationship among A, B (including B_1, B_2, B_3 and B_4), C, D, E, F, G and O (including O_1 and O_2) alleles. In the mean time, four additional alleles, I, H, L (*13*) and M (*10*), have been characterized. Quantitatively, these alleles are known as »strong« (A, B and C), »medium« (E), »weak« (D and F) and »null« (O) and are associated with high (3.6 g/L), medium (1.6 g/L), low (0.6 g/L) and null α_{s1}-casein content in milk, respectively (*12*). While the »strong« A and B alleles appear more frequently in the breeds from the Mediterranean, the »medium« (E) and »weak« (F) alleles are more frequent in French, Italian, Swiss and Spanish breeds (*12*,*14*,*15*). F and D variants differ from A variant in the deletion of 37 and 11 amino acids, respectively; both derive from an abnormal processing of the primary transcript. The more recently identified G variant (*16*) features internal deletions affecting the protein's middle or N-terminal region. At the DNA level, »null« allele is characterized by a deletion of 8.5 kbp starting in the 12th intron of CSN1S1 gene (*17*). Variants A, B, C and E differ from each other in the substitution of one amino acid only.

Polymorphism of this protein fraction does not seem to have a considerable effect upon the produced quantity of both milk and fat, meaning that these parameters do not vary with the protein content of milk (*18*), although some authors observed an influence of the genotype on fat quantity (*5*). The results of researches conducted on homozygous individuals for A, E and F alleles confirmed the effects of genotype on the casein content in milk; the cheesemaking yield observed in milk produced by A/A-genotype animals was 7 % higher in comparison with that of E/E genotype and 15 % higher in respect to that of F/F one (*19*). Quantitative differences between genetic variants of proteins, though usually quite low, can directly or indirectly affect technological and nutritional properties of milk, as pointed out in some studies on cheesemaking (*20–22*).

Numerous techniques have been employed for the characterisation of the caprine α_{s1}-casein polymorphic variants. In papers by Boulanger *et al.* (*23*), Russo *et al.* (*24*), Grosclaude (*3*), Addeo *et al.* (*25*) and Mahé and Grosclaude (*26*) techniques based on electrophoretic protein separation (electrophoresis on starch gel and urea, SUGE; polyacrylamide–agarose gel electrophoresis, PA-AGE; isoelectric focusing, IEF; sodium dodecylsulfate polyacrylamide gel electrophoresis, SDS-PAGE; two-dimensional electrophoresis, 2-D) were used. On the other hand, Mikkelsen *et al.* (*27*), Jaubert and Martin (*28*) and Iametti *et al.* (*29*) employed chromatographic analysis (reversed phase-high performance liquid chromatography, RP-HPLC).

A wide range of biochemical (mass spectrometry analysis, capillary electrophoresis) and molecular techniques (restriction fragment length polymorphism, PCR--RFLP; allele specific polymerase chain reaction, AS-PCR; amplification created restriction site polymerase chain reaction, ACRS-PCR) have also been applied (for different protein and DNA-based techniques see references *30–36*). More recently, the analysis of goat milk's protein polymorphisms was performed by mass-spectrometric (*37–39*) and molecular techniques (*10*,*17*,*40–43*). Each method applied, with respect to resolution, time, cost and effectiveness, has its strong and weak points.

The aim of this work is to underline the importance of the use of an integrated methodological approach in the analysis of goat CSN1S1 locus. Limitations and advantages of different biochemical and molecular techniques applied for the characterisation of the caprine α_{s1}-casein variants will be discussed, focusing on the benefits of the combined use of DNA-based and protein-based analytical techniques. To fulfil this objective we characterised the most frequent caprine α_{s1}-casein variants (A, B, E and F) using electrophoretic protein separation (IEF; capillary zone electrophoresis, CZE and SDS-PAGE), chromatographic analysis (RP-HPLC) and ASA-PCR; we also developed a new real-time PCR protocol as a tool for the discrimination of B and E alleles, in order to show the capabilities of this innovative technology in comparison with the other more common and well-established PCR techniques.

Materials and Methods

Sample collection

Individual milk samples were collected from Sarda (N=1200), Verzaschese (N=150), Frontalasca (N=50), Saanen (N=200) and Alpine (N=30) goat breeds. Milk samples from Sarda were from Sardinia, while the samples from all other breeds were collected in Lombardia (northern Italy) region. After collection, the milk samples were stored at –20 °C without any preservatives.

Work plan

All samples were screened by IEF. On the basis of IEF data, a pool of 50 samples containing a single allelic variant of interest (A, B/E or F) was selected. DNA was extracted from milk samples featuring B/E alleles and the discrimination of the two variants was carried out by PCR techniques. Subsequently, all 50 samples were analyzed by means of RP-HPLC to identify alleles in homozygous state (AA, BB, EE, FF). Limitations and advantages of CZE as a substitute for IEF and HPLC were evaluated.

Two reference samples for each of the AA, BB, EE, FF, AE, BE, BF, EF genotypes were then confirmed by sequencing.

Isoelectric focusing

Isoelectric focusing was performed in the presence of carrier ampholytes (CA-IEF) to resolve the α_{s1}-casein variants. Individual milk samples were defatted by centrifuging at 1000 × g for 15 min (4 °C) and diluted at a 1:3.5 volume ratio with a denaturing solution according to Krause and Belitz (*44*). Gel solution was prepared with 30 % acrylamide/bisacrylamide (37.5:1), 8 M urea and 12.2 % glycerol (87 %). The pH gradient gel was obtained by mixing 2.5–5 and 4–6.5 Pharmalyte ampho-

lytes (Amersham Biotech) volume ratio of 1:2, at a final concentration of 3 %, in a 124×258×0.4 mm polyacrylamide gel matrix supported on a GelBond PAG film (Amersham Biotech). Electrophoresis was carried out using a Multiphor II electrophoresis cell (Amersham Biotech) maintaining plate temperature at 10 °C. A volume of 15 μL per sample was loaded using application pieces (Amersham Biotech). The electrophoretic runs were performed at 4 mA, 250 V/cm max and 20 W for 90 min. Gels were stained overnight with a staining-destaining solution containing Coomassie brilliant blue G-250, then washed with water for 20 min and dried at 60 °C. The apparent isoelectric point of α_{s1}-CN A, B/E and F variants was determined by comparison with reference samples.

Capillary zone electrophoresis of milk samples

Electromigration was carried out using a BioFocus 2000 capillary electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) controlled by a BioFocus 5.2 operation and integration software. Separations were performed in a 60 cm × 50 μm ID hydrophilically coated fused-silica capillary column (Biocap Bio-Rad) installed in a liquid-cooled Cartridge with a distance between detection window and outlet of 4.6 cm, resulting in 55.4 cm effective length. Migrations were run at 38 °C with constant voltage at 20.00 kV and polarity from positive to negative. Sample solution was injected by a pressure of 17.9 kPa/s and UV-detection was performed at 214 nm. A 10 M urea solution was prepared by dissolving in 200 mL of double distilled water, 120 g of urea (Sigma, St Louis, MO), 166 mg of methylhydroxyethyl cellulose (MHEC 30 000, Serva Heidelberg, Germany) and 4 g of mixed-bed ion-exchange resin (AG501-X8, Bio-Rad). The solution was filtered over 540 filter paper (Whatman International, Maidstone, UK), after gently stirring overnight. Sample buffer at pH=(8.6±0.1) was prepared according to Recio *et al.* (*45*) by dissolving 505 mg of 2-amino-2-hydroxymethyl-1,3-propanediol (Tris; Merck, Darmstadt, Germany), 220 mg of 3-morpholinopropanesulphonic acid (MOPS; Sigma), 620 mg of ethylenediamine tetraacetic acid disodium salt (EDTA; Sigma) and 64 mg of DL-dithiothreitol (DTT; Sigma) in 25 mL of the 10 M urea solution. Before use the solution was filtered over 0.45-μm filter (Millex GV, Millipore). Electrophoresis buffer at pH=(3.0±0.1) was prepared by dissolving 148 mg of trisodium citrate dihydrate (Sigma), 1 g of citric acid (Sigma) and 6 mg of DL-dithiothreitol in 15 mL of the 10 M urea solution and 10 mL of double distilled water. Also, the solution was filtered over 0.45-μm filter before use. Defatted milk samples were diluted in volume ratio of 1:1.5 in sample buffer while isoelectric casein was dissolved at m/V=4 % in a sample buffer diluted (60 %) solution. Both milk and casein samples were incubated for 5 min at room temperature, centrifuged at 10 000 × *g* for 10 min and then the clear solution was used for CZE analysis. α_{s1}-CN variants were identified by comparison with reference samples.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Defatted milk samples were dissolved in reducing buffer (1:20) containing SDS according to Laemmli (*46*). Before electrophoresis the samples were boiled for 5 min to inactivate enzymes and denature proteins. The linear gel was run with SDS by the discontinuous buffer system. The stacking and separating gels were prepared with 4 and 15 % of acrylamide, respectively. Total monomer concentration and concentration of the crosslink relative to the total concentration was 30 and 3.3 %, respectively. For each sample 5 μL/lane were loaded. The run was performed in vertical Mini-Protein II electrophoresis cell (Bio-Rad, Richmond, CA). Electrophoresis was conducted with 200 V constant voltage for 50 min. The gels were stained with Coomassie R-250 for 1 h; destaining phase was performed with a solution containing acetic acid, methanol and double distilled water (volume ratio of 1:4:5) until the background was clear. α_{s1}-CN F was characterized by densitometric analysis.

The original SDS-PAGE was used to characterize casein fractions by computerized densitometry (Quantity One 1-D analysis software and GS-800 Imaging densitometer, Bio-Rad) and to verify the presence of α_{s1}-CN F. The variant was identified by comparing MW values with those of standard.

RP-HPLC analysis

The isoelectric casein (70 mg) was reduced with 1 mL of solution containing 8 M urea, 0.1 M Bis-Tris, 0.3 % β-mercaptoethanol, 1.3 % sodium citrate for 1 h at room temperature. Reduced casein was diluted in volume ratio of 1:5 with 6 M urea and 0.1 % TFA and filtered through a 0.45-μm filter (*28*,*47*,*48*). The HPLC equipment consisted of: dual cylinder pump model 515 (Waters), a manual sample injector (Rheodyne), 2487 UV detector (Waters). The equipment was linked to a data acquisition and processing system (Millennium 32, Waters). Protein separation was performed by a reversed-phase analytical column C4 Jupiter (250×4.6 mm), pore diameter 300 Å and particle size 5 μm (Phenomenex, USA). An injection loop of 20 μL was used. The solvent gradient programme for casein separation was linear gradient from 30 to 50 % solvent B in 40 min, then from 50 to 100 % B in 2 min, followed by an isocratic elution for 1 min and equilibration at the starting condition in 3 min. Solvent B was 0.1 % TFA in acetonitrile, while solvent A was 0.1 % trifluoroacetic acid in ultra pure water. Flow rate was 0.8 mL/min, and the eluted peaks were detected by absorbance at 220 nm. The column was kept at room temperature. Casein fractions were characterized by comparing retention time of reference samples genotyped at the α_{s1}-CN locus.

DNA extraction and purification

The DNA from somatic milk cells was extracted by phenol and chloroform (volume ratio of 1:1) (*49*), it was precipitated using 3 M sodium acetate, pH=5.2, absolute ethanol (volume ratio of 1:3.5) and washed with 70 % ethanol. For each sample, total DNA was pelleted by a speed vacuum and suspended in distilled water.

For a faster procedure, DNA was extracted and purified from 300 μL of milk by NucleoSpin silica membrane (NucleoSpin® Food, Macherey-Nagel). Depending on the origin of individual samples and on the milk conservation status, typical yields were in the range of 10–

30 μg DNA as measured in a GeneQuantPro spectrophotometer (Amersham Biosciences, Uppsala, SW) at 260/280 nm.

Primer sequences for ASA-PCR and real-time PCR

Primers were designed on the basis of the available goat α_{s1}-casein sequence (GenBank accession number X72221) in order to amplify the genomic region distinguishing the B and E variants (exon 19). The primers' sequences and positions were the following:

BE: forward 5′-CAACCTCAAATTGAAGGCACT-3′ (positions 808–828)

E: forward 5′-TGGTGTTTTTCTTTCTGGCTTA-3′ (positions 1267–1288)

R: reverse 5′-CAAGCTCTTAGGACAATTTCACTT-3′ (positions 1333–1356)

Allelic specific amplification by polymerase chain reaction (ASA-PCR)

Approximately 50 μg of genomic DNA were amplified in a 30-μL reaction by using 150 nM primers, 50 mM KCl, 10 mM Tris-HCl (pH=8.3), 2.5 mM $MgCl_2$, dNTPs each at 200 μM and 1.25 U Taq Gold DNA polymerase (Perkin Elmer, Norwalk, USA). The amplification was performed in a 9600 DNA thermal cycler (Perkin Elmer). Conditions consisted of an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of 95 °C (1 min), 51 °C (1 min) and 72 °C (2 min). Final extension was at 72 °C for 10 min. Specific primer (BE forward) amplified 90-bp (for B variant) and 550-bp (for E variant) fragments surrounding the nucleotide mutations of α_{s1}-CN region. Control amplifications were performed for each sample using a second specific primer (E forward), which amplified a 90-bp fragment only if E variant was present (*31*). The results of PCR were analysed by electrophoresis in 3 % agarose gel stained with ethidium bromide, followed by ultraviolet exposition. The identity of fragments was confirmed by sequencing.

Allelic specific amplification by real-time PCR

DNA amplification was performed by the DNA Engine Opticon™ system (MJ Research). Thirty microliters of DNA Master Brilliant™ SYBR Green® QPCR mixture (containing SureStart taq DNA polymerase 0.5 μL, core PCR buffer 3.0 μL, dNTPs 2.0 μL, $MgCl_2$ 0.9 μL, DMSO 0.9 μL, glycerol 4.8 μL, SYBR Green I dye volume ratio of 1:2000 3.0 μL and double-distilled water 12.9 μL; Stratagene) were added with 50 ng of genomic DNA and 150 nM primers.

Thermal cycling conditions were initiated with 1 cycle at 95 °C for 10 min (denaturation), 40 three-segment cycles for amplification, 95 °C for 30 s (denaturation), 51 °C for 30 s (annealing) and 72 °C for 30 s (extension), and then 1 cycle at 72 °C for 5 min (final extension). The specificity of the reaction is monitored by determining the product melting temperature (*50*).

Our optimized procedure involved two reactions per sample containing BE/R primer pair and E/R primer pair, respectively, along with no-template negative controls (H_2O control), to determine the characteristic melting curve profiles for B and E alleles. The positive control for B and E alleles was goat DNA isolated from peripheral blood. The amplicons were analyzed in agarose (2 %) gel to verify that the product of interest was reproduced to obtain the correlation between the gel and fluorescence data.

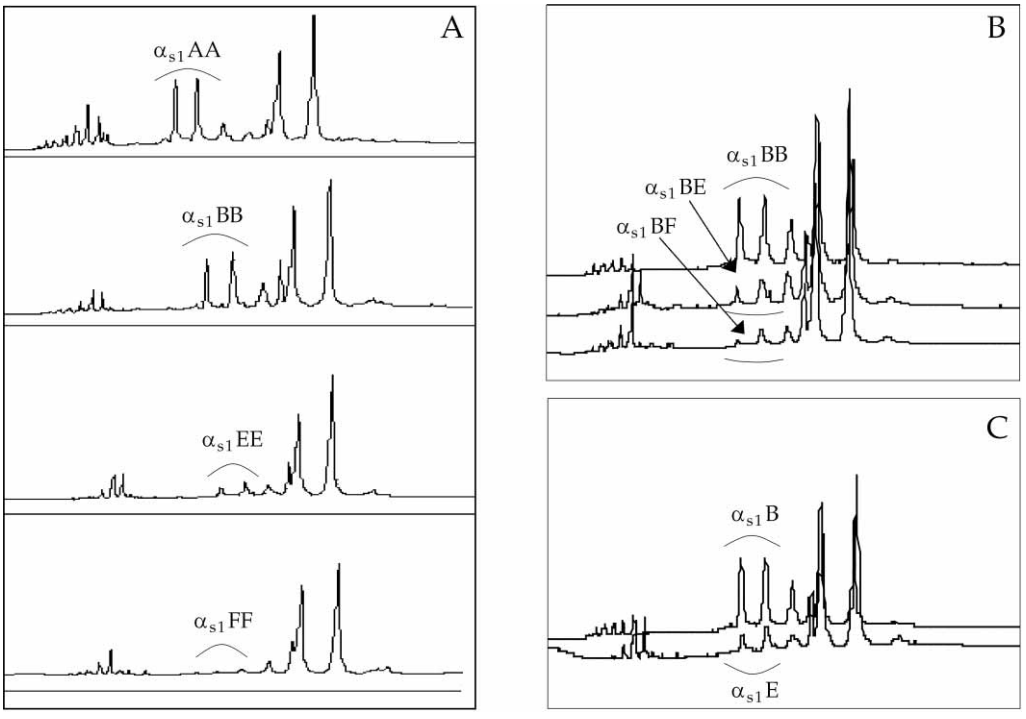

Fig.1. Capillary electropherograms of caseins from individual goat milk samples

Results

Electrophoretic protein separation analysis (CZE, IEF and SDS-PAGE)

The identification of the α_{s1}-CN variants by CZE, IEF and SDS-PAGE from individual milk samples is shown in Figs. 1, 2 and 3, respectively. The CZE method described here was found to be a suitable technique to perform a preliminary screening of α_{s1}-CN fractions. CZE method offered a direct resolution for α_{s1}-CN A variant in both homozygous and heterozygous state. This variant was characterized by two peaks with a migration time shorter than the migration time of the other α_{s1}-CN variants (Fig. 1A) (*45*), while a partial discrimination was obtained between variants B/E and E/F, which show similar electrophoretic properties but different α_{s1}-CN content (Figs. 1B and 1C). The B and E alleles co-migrated also when applying the IEF method, but the allele F was discriminated from the other alleles. Furthermore, we observed by the CZE method (Table 1) that when B variant was associated with F variant the calculated percent area was lower than the expected one on the basis of α_{s1}-CN content (*18*). A similar trend was also observed by RP-HPLC (Table 1). In comparison with the CZE method, IEF could resolve α_{s1}-CN F variant.

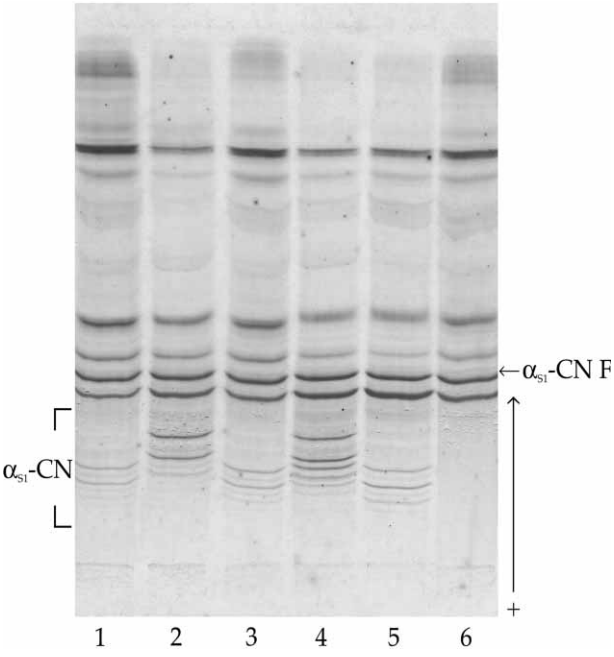

Fig. 2. Isoelectric focusing in pH gradient 2.5–5 and 4–6.5 of individual milk samples. Lanes 1, 3 and 5 show the α_{s1}-CN B and/or E (the two variants are not distinguishable only by IEF analysis). Lane 2 shows α_{s1}-CN A. Lane 4 shows α_{s1}-CN B or E in heterozygous state with α_{s1}-CN A. Lane 6 shows α_{s1}-CN F (between two bands of β-casein)

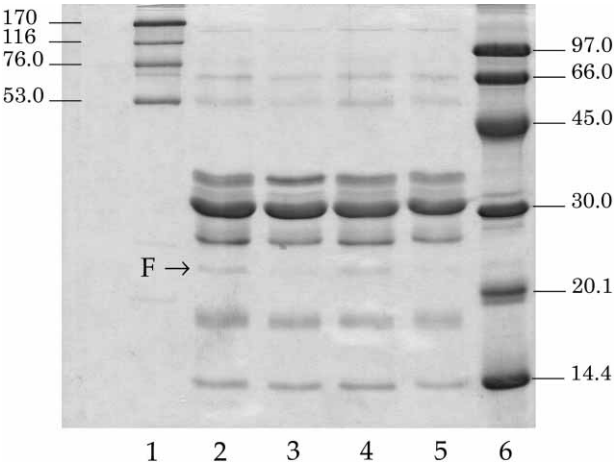

Fig. 3. SDS-PAGE of individual milk samples (lanes 2 to 5). A band characterizing α_{s1}-CN F is shown at ~23 kDa. Lanes 1 and 6: LMW and HMW SDS marker kits (Amersham Biotech)

We observed that the operator effect can negatively affect the resolution of the IEF analysis, in particular α_{s1}--CN F detection. Thus, to confirm the presence of α_{s1}--CN F we had to apply SDS-PAGE analysis (Fig. 3). However, it was not possible to distinguish other alleles by SDS-PAGE in the same analytical conditions and electrophoretic apparatus that allowed the recognition of the F allele.

Table 1. Percentage area values, calculated over total casein fraction, of known α_{s1}-CN genotype samples expressed as the mean of six determinations by CZE

Genotype	Area/%
AA	28.67
BB	31.67
EE	10.35
FF	4.20
AE	18.74
BE	8.12
BF	5.65
EF	6.77

Chromatographic analysis (RP-HPLC)

Caseins obtained from individual milk of homozygous and heterozygous samples at α_{s1}-CN locus were analyzed using the described RP-HPLC method. The chromatographic profiles are presented in Fig. 4. The results obtained by the HPLC method and electrophoretic methods were in good agreement. RP-HPLC gives rapid resolution of the most α_{s1}-CN variants, but the variants B and E co-eluted, thus they remained, as by CZE and IEF, indistinguishable according to Jaubert and Martin (*28*). The RP-HPLC method was suitable for routine separation and quantification of casein fractions. In Table 1 the percentage area values of α_{s1}-CN peak of samples previously analyzed by IEF and real-time PCR are shown. A, B, E and F variants in the homozygous state showed a double percent area value compared to their values in the heterozygous state (see Fig. 4, Table 2), while the genotypes BE and BF did not respect the expected proportion.

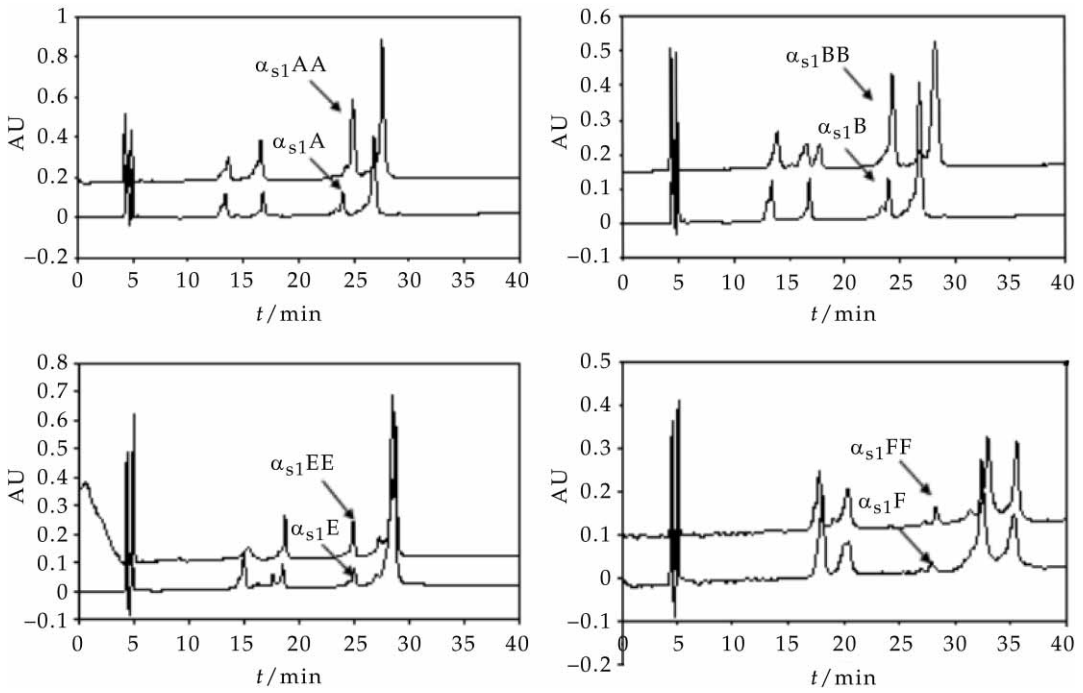

Fig. 4. RP-HPLC profiles of casein samples previously analysed by IEF and PCR. In each picture homozygous and heterozygous samples for A, B, E and F α_{s1}-CN are reported

Table 2. Percentage area values, calculated over total casein fraction, of known genotype samples at α_{s1}-CN locus expressed as the mean of six determinations analyzed in duplicate by RP-HPLC

Genotype	Area/%
AA	29.28
BB	28.67
EE	10.52
FF	2.28
AE	20.31[a]
BE	8.57
BF	5.81[b]
EF	5.55[c]

[a]Area peak α_{s1}A/ % + area peak α_{s1}E/%
[b]Area peak α_{s1}B/ % + area peak α_{s1}F/%
[c]Area peak α_{s1}E/% + area peak α_{s1}F/%

DNA analysis

ASA-PCR

Polymerase chain reaction technology is a revolutionary technique in genotypic selection and a wide variety of different methods has been described (*51*). In particular, the ASA-PCR strategy greatly facilitates the detection of point mutations. In this application target specificity is determined by the choice of short primers that are designed to hybridize to opposite DNA strands flanking specific sequence of E and B alleles. With the mismatched-primer design and reaction conditions applied we could discriminate between the two alleles, the PCR amplicons were analyzed by electrophoresis in 3 % agarose gel stained with ethidium bromide and examined under ultraviolet light (Fig. 5). α_{s1}-CN B profile showed the specific band of 90 bp while α_{s1}-CN E profile showed the specific band of 549 bp by using primer BE/R primer pair. The difference in length of the amplified fragment was determined by the specific insertion characterizing E allele (exon 19) according to Martin *et al.* (*52*).

To confirm the presence of E allele we performed a second PCR amplification by the use of specific primer E forward and the same reaction conditions as those applied in the previous amplification. In this case α_{s1}-CN E profile showed the specific band of 90 bp.

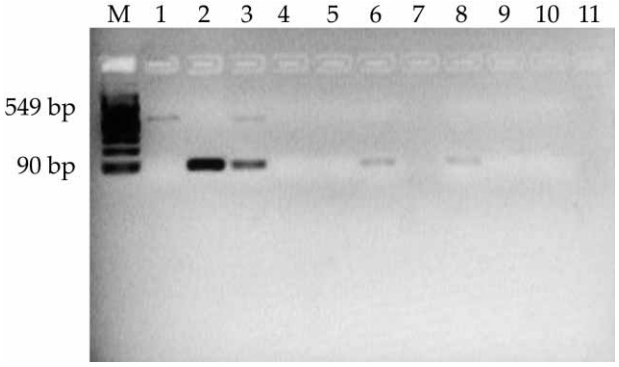

Fig. 5. Agarose gel electrophoresis of ASA-PCR products amplified with the B and E allele-specific BE/R primer pair from homozygous EE (lane 1), homozygous BB (lane 2) and heterozygous BE (lane 3) samples. Lanes 6 and 8 show the DNA fragments amplified by means of the E allele-specific E/R primer pair from homozygous EE (lane 6) and heterozygous BE (lane 8) samples. The absence of E allele is confirmed in homozygous BB samples (lane 7). Lanes 4 and 9: no template controls. Lanes 5 and 10: double distilled water controls

Real-time PCR

For discrimination of the B and E alleles we chose real-time PCR with product detection using generic SYBR Green I dye. This detection system collected fluorescence measurements during the PCR. SYBR Green I is a dye with a high binding affinity for double-stranded nucleic acids. Particularly, during the annealing and extension steps, after the primers hybridize on the target sequence, the SYBR Green I dye is bound and the fluorescence signal intensity increases. The increase of fluorescence signal depends on the initial concentration of target present in the PCR reaction. During the amplification the SYBR Green I dye is bound to any specific and non-specific double-stranded products, but even if SYBR Green I is not sequence-specific, specific products can be identified by melting curve (*53*). To increase the specificity of the target amplicon from the spurious PCR product, we used allele-specific primers and we optimized the reaction conditions. The results for each sample were recorded as the presence or absence of B and E alleles confirmed separately in individual wells (Fig. 6). The specificity of primers permitted to distinguish B and E target amplicon from the primer-dimer formations that usually melt at lower temperature (*54*). Melting curve acquisition was performed from 55 to 95 °C at 0.5 °C/s.

Discussion

IEF proved to be a direct, fast and low-cost method suitable for the α_{s1}-CN locus preliminary screening (*13*). It permitted simultaneous analysis of twenty samples per gel after 2.5 h of electrophoretic run, allowing to examine up to 40 samples per day using a single apparatus. IEF allowed the straightforward identification of A and F variants, whilst B and E variants were not resolved because of their identical isoelectric point. The SDS-PAGE was confirmed to be an easy and fast method to detect α_{s1}-CN F variant, useful to verify IEF results (*15*).

The results obtained by capillary electrophoresis confirmed this technique's applicability (*33*,*35*,*36*,*45*) for the characterization of α_{s1}-CN polymorphism in goat milk as a preliminary tool to easily distinguish high, medium and low content of α_{s1}-CN variants. In particular, the described CZE analysis allowed the identification of α_{s1}--CN A variant in both homozygous and heterozygous state and B variant in homozygous state, while the discrimination of E, F and B α_{s1}-CN variants in heterozygous state still remained difficult. The full automation of the technique, the absence of operator effect, and the chance to improve this protocol to reach a better resolution among α_{s1}-CN variants make this a reliable complementary electrophoretic method for the characterisation of caprine α_{s1}-casein polymorphism.

Analysis by HPLC was an essential step in the whole typing procedure, since it provided discrimination between alleles' homozygous and heterozygous states. In addition, the quantitative results were also related to the protein's expression levels in accordance with the strength of the variants (*12*,*26*,*55*). Routine phenotypic measurement of α_{s1}-casein variants therefore represents a fundamental parameter in the determination of milk quality expressed as cheesemaking yield.

DNA analysis was applied to the discrimination of B and E variants, whose presence was detected by IEF. The use of BE (forward) and R (reverse) primer pair sufficed to detect one or both alleles within the same sample by means of ASA-PCR. This protocol proved effec-

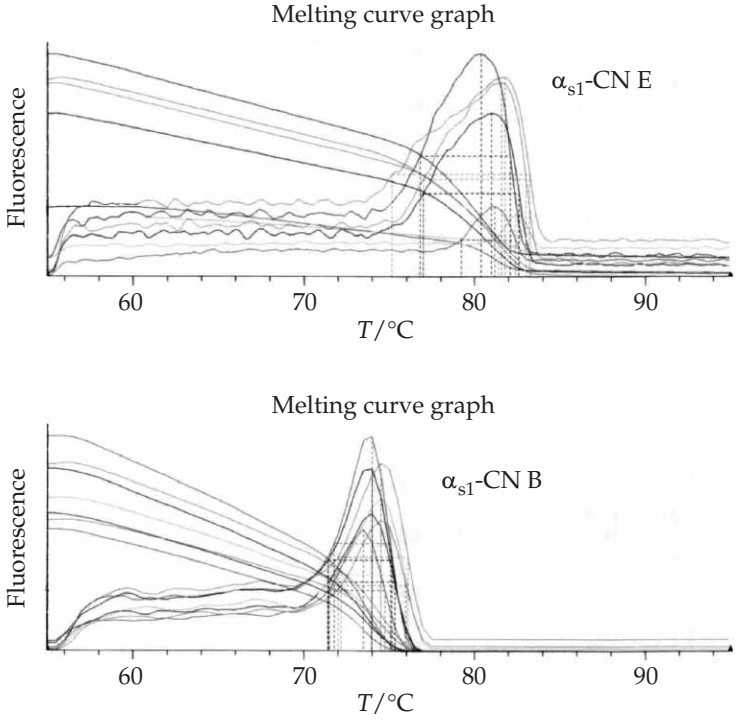

Fig. 6. Graphs of fluorescence *vs.* temperature. The relative intensities of the melting curve signals for individual wells are shown. The T_m difference between B and E allele was ~7 °C

tive and fast in allowing a clear distinction without the need for additional steps like restriction. The E forward primer was used as an additional control in case of ambiguous results. Conventional ASA-PCR genotyping was adapted and compared to the qualitative real-time PCR for allelic discrimination. The specific primer design was the most important factor in the method optimization. In real-time PCR, additional employment about casting and running gel and the use of toxic products like ethidium bromide were bypassed. The allelic discrimination method described here is one of the most rapid and easiest applications that can be carried out by real-time PCR; a different approach can be based on color probe-multiplex PCR quantification and allele typing (*50*). Both PCR techniques described allowed clear identification and discrimination of B and E alleles, which could not be achieved by any other technique applied in this work.

Conclusions

Knowledge on genetic polymorphism at goat casein loci allows setting up selection plans targeted to the improvement of milk production and cheesemaking yield. At present, the complete goat CNS1S1 locus sequence and functional structure are published (*56*). This will allow the development of more and more accurate typing methods and an in-depth study of the expression of casein genes in goat by means of highly innovative techniques like real-time PCR and DNA arrays (*57*). On the other hand, the need for routine genotype testing is steadily increasing. In consideration of this, we proposed a multiple approach to the characterisation of caprine α_{s1}--CN alleles, describing limitations and chances related to several analytical techniques suitable for routine control and confirming the advantage of applying complementary tools. The combined application of biochemical and molecular analytical techniques allowed an effective characterisation and quantification of the allelic variants at CSN1S1 locus and recognizing their effects on the expression level of this casein fraction, which was not possible only by means of DNA analysis. This approach also offered the possibility to verify the results by matching independent data, which represents another important aspect for routine control purposes.

Acknowledgements

This work was supported by MiPAF (Dietolat Project). We thank Dr. Ivan Bonizzi for editorial assistance.

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Polimorfizam α_{s1}-kazeina u koza: karakterizacija A, B, E i F varijanata različitim biokemijskim i molekularnim tehnikama

Sažetak

Budući da postoji veliki interes za karakterizaciju varijanata α_{s1}-kazeina u koza, a laboratoriji su različito tehnički opremljeni, svrha je ovoga rada bila analizirati i usporediti njihove značajke različitim biokemijskim i molekularnim metodama. Najučestalije varijante α_{s1}-kazeina u koza (A, B, E i F) utvrđene su elektroforetskim metodama odvajanja proteina (kapilarna zonalna elektroforeza, izoelektrično fokusiranje, poliakrilamid gel-elektroforeza s natrij-dodecilsulfatom), kromatografskim analizama (reverzna tekućinska kromatografija velike učinkovitosti) i DNA analizama (lančana rekcija polimerazom, i to alel specifična amplifikacija-ASA i »real-time« amplifikacija). Prikazane su pozitivne i negativne značajke svake primijenjene metode te navedene informacije za optimalnu i komplementarnu uporabu navedenih metoda s obzirom na potrebno vrijeme, rezoluciju i troškove.