Polymorphism of α_{S1}-casein in goat milk: identification of A, B, E and F variants by biochemical and genetic analysis

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RIASSUNTO – Polimorfismo al *locus* α_{S1}-caseina nel latte caprino: identificazione delle varianti A, B, E ed F mediante analisi biochimiche e genetiche – *Sono stati analizzati al locus α_{S1}-caseina latti caprini provenienti da razze italiane e serbe. Un primo screening analitico per l'identificazione delle varianti caseiniche A, B, E e F è stato realizzato mediante l'applicazione di due tecniche elettroforetiche classiche: l'isoelectric focusing e l'elettroforesi su gel di poliacrilammide in presenza di sodio dodecil solfato. Un secondo approccio analitico per la rapida risoluzione delle varianti genetiche B ed E, è stato basato sull'amplificazione allele-specifica mediante real-time polymerase chain reaction. Un terzo approccio analitico per identificare i soggetti omozigoti al locus α_{S1}-caseina è stato eseguito mediante cromatografia in fase inversa. La combinazione congiunta di queste tecniche analitiche permette una sicura ed economica caratterizzazione delle varianti genetiche A, B, E e F.*

KEY WORDS: *Capra hircus*, α_{S1}-casein, polymorphism

Abbreviation key: IEF = isoelectric focusing, **SDS-PAGE** = sodium dodecyl sulfate-polyacrylamide gel electrophoresis, **RP-HPLC** = reverse phase-high performance liquid chromatography, **PCR** = polymerase chain reaction.

INTRODUCTION – Many researches, related to genetic polymorphism of α_{S1}-casein in goat milk, showed a marked variability and implications in milk and dairy product traits. The genetic variants (from A to G) were associated with four levels of expression: 3,6 g/L per A, B and C ("strong" alleles); 1,6 g/L per E ("medium" allele); 0,6 g/L per F ("weak" allele) and 0 g/L per O ("null" allele). Differences existing among the A, B, C, D, E, F, G and O have been described (Grosclaude *et al.*, 1994). To analyze genetic polymorphism of caseins from goat milk, distinct electrophoretic (Russo *et al.*, 1986; Addeo *et al.*, 1988) and chromatographic techniques (Jaubert and Martin, 1992; Iametti *et al.*, 1999) were applied. In last years, the advancement in technological field is allowing the research of primary structure of protein variants (Ferranti *et al.*, 1997; Trujillo *et al.*, 2000) and basic sequence of new alleles (Martin *et al.*, 1999; Bevilacqua *et al.*, 2002; Ramunno *et al.*, 2002). In this work, we employed electrophoretic protein separation (IEF and SDS-PAGE), chromatographic analysis (RP-HPLC) and molecular biology techniques based on polymerase chain reaction (real-time PCR) to detect α_{S1}-CN genetic variants from goat milk. The utility of protein and DNA analysis combination was discussed.

MATERIAL AND METHODS – *Sample collection:* Individual milk samples were collected from Nera di Verzasca, Sarda, Alpine and domestical Serbian goat breeds. After collection the milk samples were stored at -20°C without any preservative. *IEF, SDS-PAGE:* The experimental procedures are as described by Enne *et al.* (1997) and Feligini *et al.* (2002). *RP-HPLC:* The equipment for HPLC consisted of two Waters 515 pump, an absorbance detector (Waters 2487) and system iterface data module

(Millenium 32). Separations were carried out on a 250 x 4.6 mm Jupiter C4 (Phenomenex). Solvents were: solvent A 0.1% (v/v) trifluoroacetic acid (TFA) in water; solvent B 0.1% TFA in acetonitrile. For casein separation, the eluition was achieved using a gradient from 30 to 50% solvent B for 40 min, 40-42 min from 50% to 100% B, 42-45 min 100% B, 45-46 30% B, at flow rate of 0,8 ml/min, and the absorbance at 220 nm was recorded. Injected volume was 20 μl. Before analysis, the isoeletric casein (70 mg) was reduced with 1 ml of solution urea 8 M, Bis-Tris 0.1 M, 0.3% beta-mercaptoethanol, 1.3% sodiocitrate for 1 h at room temperature. Reduced casein was diluited (1:5 v/v) with urea 6 M and 0.1% TFA and filtered through a 0.45 μm filter (Visser *et al.*, 1991). *DNA extraction and purification:* The DNA from somatic milk cells was extracted by phenol and chloroform (1:1 v/v). DNA was precipitated using 3M sodium acetate, pH 5.2, absolute ethanol (1:3.5 v/v) and washed with 70% ethanol. For each sample total DNA was pelleted by a speed vacuum and suspended in distilled water. The DNA was purified by Nucleo Spin silica membrane (Macherey-Nagel). Depending on the individual samples, typical yields were in the range of 5-10 μg DNA. *Real-Time PCR Conditions:* The 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, core PCR buffer, dNTP, $MgCl_2$, DMSO, glycerol and SYBR Green I dye; Stratagene) were added with 100 ng of genomic DNA and 150 nM each primer (Perez *et al.*, 1994):

as1E: dir 5'-TGGTGTTTTTCTTTCTGGCTTA-3'
as1notE: dir 5'-CAACCTCAAATTGAAGGCACT-3'
as1E/notE: rev 5'-CAAGCTCTTAGGACAATTTCACTT-3'

Thermal cycling conditions were initiated with 1 cycle at 95°C for 5 min (denaturation), 40 three-segment cycles for amplification (95°C for 30-s, 51°C for 30-s and 72°C for 30-s), 1 cycle at 72°C for 5 min (final extension). The specificity of the reaction is monitored by determination of the product melting temperature. The amplicon was analyzed on agarose gel to verify that the product of interest was reproduced to obtain the correlation between the gel and fluorescence data.

RESULTS AND CONCLUSIONS – First identification of phenotypic α_{S1}-CN A, B, E and F variants was performed by IEF in a pH range 2.5-6.5. This method offers a good separation and resolution of A and F variants. Moreover, the identity of F variant was confirmed by SDS-PAGE. B and E variants travelled to the cathode with a similar apparent pI by IEF. For B and E allelic discrimination we chose LightCycler-based real-time PCR with product detection using generic SYBR Green I dye. The specificity of primers permitted to distinguish B and E target amplicon from the primer-dimer formations and spurious PCR products. In real-time PCR, worrying about casting and running gel, gathering numbers, increasing variability were nullified. Last step was the RP-HPLC analysis. Optimized RP-HPLC method allows the complete separation of most of the α_{S1}-casein variants (Jaubert and Martin, 1992), but B and E variants remained, as with IEF, indistinguishables. On the other hand, this method permits the resolution of homozygous α_{S1}-CN AA, BB, EE and FF variants from samples previously analyzed by IEF and real-time PCR. The identification was performed comparing the percent area values of interest peak.

Described here is a convenient procedure to characterize a great number of samples as a routine analytical method.

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REFERENCES – **Addeo**, F., Mauriello, R., Di Luccia, A., 1988. J. Dairy Res. 55:413-421. **Bevilacqua**, C., Ferranti, P., Garro, G., Veltri, C., Lagonigro, R., Leroux, C., Pietrola, E., Addeo, F., Pilla, F., Chianese L., Martin, P., 2002. Eur. J. Biochem. 269:1293-1303. **Enne**, G., Feligini, M., Greppi, GF., Iametti, S., Pagani, S., 1997. Proc. IDF Seminar 'Milk Protein Polymorphism', Palmerston North, New Zealand, 275-279. **Feligini**, M., Cubric, V., Parma, P., Curik, I., Greppi, GF., Enne, G., 2002. Food

Technology and Biotechnology 4:293-298. **Ferranti**, P., Addeo, F., Malorni, A., Chianese, L., Leroux, C., Martin, P., 1997. Eur. J. Biochem. 249:1-7. **Grosclaude**, F., Ricordeau, G., Martin, P., Remeuf, F., Vassal, L., Bouillon, J., 1994. INRA Prod. Anim. 7:3-19. **Iametti**, S., Sessa, M., Feligini, M., Greppi, G.F., Enne, G., Pagani, S., 1999. Ital. J. Food Sci. 11:249-255. **Jaubert**, A. and Martin, P., 1992. Lait 72:235-247. **Martin**, P., Ollivier-Bousquet, M., Grosclaude, F., 1999. Int. Dairy J. 9:163-171. **Perez**, M.J., Leroux, C., Bonastre, A.S., Martin, P., 1994. Gene 2:179-187. **Ramunno**, L., Cosenza, G., Gallo, D., Ilario, R., Rando, A., Masina, P., 2002. Atti XV Cong. Naz. S.IP.A.O.C., Cagliari 11/14 settembre, 220. **Russo**, V., Davoli, R., Dall'Olio, S., Tedeschi, M., 1986. Zoot. Nutr. Anim. 12:55-62. **Trujillo**, A.J., Casals, I., Guamis, B., 2000. J. Dairy Sci. 83:11-19. **Visser**, S., Slangen, Ch.J., Rollema, H.S., 1991. J. Chromatogr. 548:361-370.