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Genetic Polymorphism of β-Lactoglobulin in Native Sheep from the Island of Pag

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Summary

Milk samples from 248 Pag ewes, belonging to 14 different flocks and located through the Pag Island (Croatia), were analyzed by isoelectrofocusing and PCR-RFLP. Two genetic variants (A and B) and three genotypes (AA, AB and BB) of β-lactoglobulin have been identified. According to the allele frequency (A=0.48, B=0.52) and occurrence of genetic variants, the Pag breed is similar to other Mediterranean dairy sheep breeds. The observed genotype frequencies at the β-lactoglobulin locus (AA=0.185, AB=0.589 and BB=0.226) were significantly different from those expected from Hardy-Weinberg equilibrium. In comparison with 12 different studies related to Mediterranean dairy sheep populations, the significant departure from Hardy-Weinberg was also obtained in Valle del Belice sheep. However, the sample size required for detection of the same amount of departure from the Hardy-Weinberg as it was observed in Pag sheep was only sufficient in four studies (including Pag sheep). The superiority of the AB β-lactoglobulin genotype for milk production could be one of possible reasons for the observed excess of heterozygotes and allele frequencies at intermediate level.

Key words: β-lactoglobulin polymorphism, Pag sheep, Pag cheese

Introduction

Reported relationship between genetic variants of the β-lactoglobulin (β-LG) and milk yield, milk composition and cheese-making ability in cattle (*1,2*) have raised interest for the establishment of the relationship between β-LG polymorphism and milk production traits in dairy goat and sheep populations. Although in recent years the effects of β-LG polymorphism on sheep milk production and manufacturing properties of the cheese have been extensively studied, the results are conflicting, indicating either superiority of a given β-LG variant or absence of relationship (*3*). However, there is an ongoing interest, especially in the Mediterranean countries where sheep are mainly kept for the production of cheese, concerning the relationship between the genetic

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variants of β-LG and traits related to milk and cheese production. The Pag sheep is native breed from the Pag Island (Northern Dalmatia) where 35 000 ewes are kept solely for the production of a well-known (in Croatia) autochthonous Pag cheese.

The aim of this paper was to study genetic polymorphism of the β-LG locus in native Pag sheep and to compare the results with other Mediterranean dairy sheep populations kept in similar conditions and oriented toward cheese production.

Materials and Methods

Sample preparation

Individual milk samples were collected from 248 Pag ewes belonging to 14 flocks located through the Pag Island. After collecting, samples were stored at –20 °C, without any preservatives. Later, the samples were thawed at room temperature and centrifuged at 27 000 rpm for 90 min at 4 °C to obtain whey proteins. Isolated whey proteins were diluted in denaturing solution (1:3.5) according to Krause and Belitz (*4*).

Electrophoresis of milk samples

Isoelectric focusing was performed in the presence of carrier ampholytes, the 4–8 pH gradient was obtained by mixing 4.0–6.5 and 5.0–8.0 Pharmalyte (Pharmacia Biotech) in a 1:1 volume ratio according to Feligini *et al.* (*5*). Protein separation was carried out in 124 x 258 x 0.4 mm polyacrylamide gel. Gel stock solution was prepared with N,N'-methylene-bis-acrylamide (30 % T/2.6 % C), 8M urea (w/v) and 12.2 % (w/v) glycerol. Proteins were electrophoresed using Multiphor II Electrophoresis Cell (Pharmacia Biotech, Sweden) at 4–5 °C. In each lane 15 μL sample was loaded. Gels were stained overnight with Coomassie Brilliant Blue G-250. The apparent isoelectric point of β-LG variants was determined by reference samples for which genotypes were resolved by DNA analysis.

DNA extraction from milk and blood

DNA was extracted from milk somatic cells and blood samples. The individual milk was frozen at –20 °C without preservative. After thawing milk samples were centrifuged at 1000 rpm for 10 min at 4 °C. The supernatant was eliminated and the remaining cell-pellets were resuspended in 2 M Tris-HCl, pH=7.5, 40 mM EDTA, 3 M NaCl, 10 % SDS and 2 mg/100 μL proteinase K (Sigma). Samples were shaken overnight at 42 °C and mixed with 1 mg/100 μL RNase (Boehringer Mannheim) for 1 h at room temperature. DNA was extracted by phenol and chloroform (1:1 volume ratio).

The blood samples were taken from jugular vein into tubes with 5 % EDTA and stored for one week at 4 °C. The DNA was extracted from blood lymphocytes (*6*).

PCR and restriction enzyme analysis

Allele discrimination was based on size differentiation of β-LG genetic markers. PCR conditions were performed according to Feligini *et al.* (*7*). Primers LGB1 (5'-CAA CTC AAG GTC CCT CTC CA-3') and LGB2 (5'-CTT CAG CTC CTC CAC GTA CA-3') were used for amplification of DNA. Restriction analysis was carried out using *Rsa*I enzyme (Boehringer). The amount of digestion mixture was 15 μL containing 9.7 μL of DNA template, 1.5 μL of 10x buffer (SuRE/Cut Buffer L, Boehringer Manheim, enzyme type dependent), 0.3 μL of *Rsa*I (5 units μL^{-1}) and 3.5 μL of distilled water. The reaction mixture was digested at 37 °C for 1 h and then 8 μL of sample were mixed with 2 μL of loading dye and then samples were loaded to the 3 % agarose gel stained with ethidium bromide and examined by ultraviolet light.

Statistical analysis

Allele frequencies, standard errors (SE), disequlibrium coefficients ($\hat{D}$) as well as Hardy-Weinberg equilibrium (HWE) tests were derived from the genotypic frequencies obtained from the literature or from the present study (Pag sheep). Standard errors of the allele frequencies (SE) and disequilibrium coefficients ($\hat{D}$) were calculated according to formulae /1/ and /2/, as suggested by Weir (*8*);

$$SE = \sqrt{\frac{1}{2N}\tilde{p}_A(1-\tilde{p}_A)} \qquad /1/$$

$$\hat{D} = \tilde{p}_A\tilde{p}_B - 0.5\tilde{p}_{AB} \qquad /2/$$

where N is the number of sampled genotypes; $\tilde{p}_A$, $\tilde{p}_B$ are allele frequencies of variants A and B, respectively; $\tilde{p}_{AB}$ is genotypic frequency of the phenotype AB. For each population, HWE was tested by two-sided chi-square tests with degree of freedom equal to one and were, thus, related to hypotheses concerning excess or a deficiency of heterozygosity. The critical values of the disequilibrium coefficients required for the rejection of the HWE hypothesis ($\hat{D}_r$), with sample size used in the analysis (see Table 1) and at the significance level $\alpha = 0.05$ and the power of test $1\text{-}\beta = 0.80$, were calculated according to Weir (*8*). All calculations and statistical tests were done by SAS software package (*9*) and used algorithms, which are available on request.

Results and Discussion

Whey fractions were obtained from Pag ewes' milk and then were analysed by isoelectrofocusing to obtain electroforegram for β-LG. Figs. 1 and 2 show two different variants, A and B, which we detected for Pag ewes' milk. Identification of β-LG variants cannot just rely on a simple comparison of isoelectrofocusing patterns. When we analysed samples by isoelectrofocusing, 60 samples were not clear and it was difficult to decide if it is A or B variant. Hence, to confirm β-LG variants of milk of 60 Pag ewes, we analysed samples by PCR-RFLP. One size of band occurred, which was 120 bp, after amplifying the DNA. As variant A has two *Rsa*I sites, after digestion three fragments are produced (66 bp, 37 bp and 17 bp). The PCR product from variant B has just one *Rsa*I site and digestion produces two fragments (103 bp and 17 bp). In Fig. 2, 17 bp fragment is very weak or not visible on the gel.

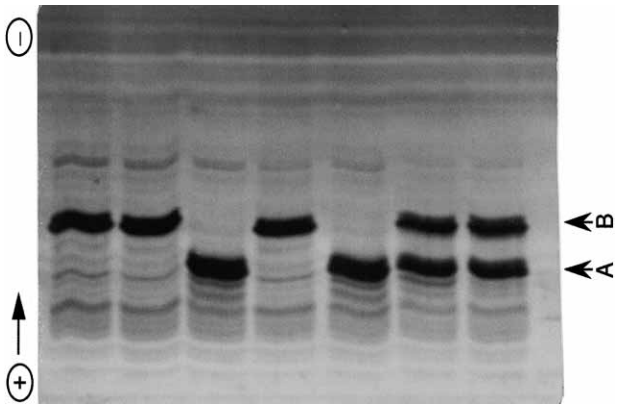

Fig. 1. Isoelectric focusing (pH gradient 4-8) of individual whey samples from Pag sheep; the β-LG variants A and B are showed

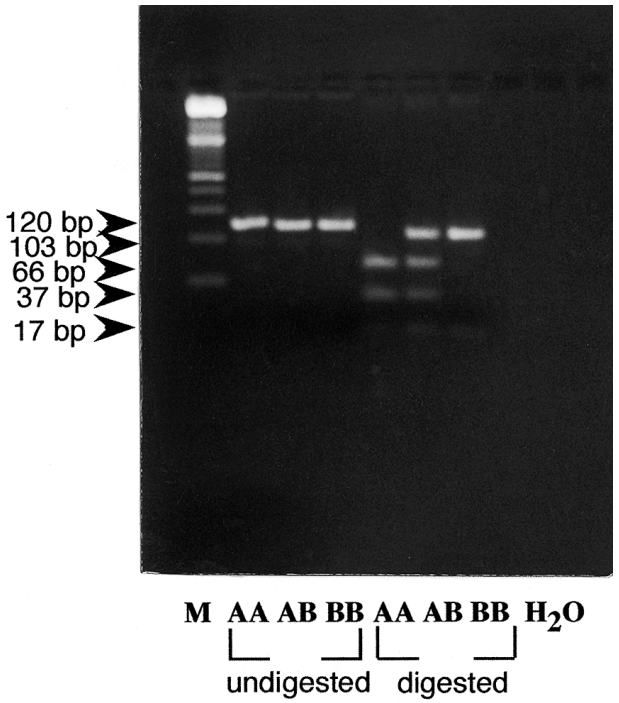

Fig. 2. Agarose gel (3 %) shows the results of PCR (120 bp) and genetic markers of AA (66 bp, 37 bp and 17 bp), AB (103 bp, 66 bp, 37 bp and 17 bp) and BB (103 bp and 17 bp) genotypes obtained after digestion by *Rsa*I. (In all genotypes 17 bp fragment is weak or not visible). M: MWM 50 bp ladder (Boehringher Mannheim).

Isoelectrofocusing is a good method for the identification of different variants of ewe's milk but sometimes results can have many artefacts and in that case it is advisable to confirm the analysis by PCR-RFLP. PCR-RFLP is a more expensive method but, on the other side, it is more precise.

By combining information from analysis of isoelectrofocusing patterns and PCR-RFLP we estimated the distribution of β-LG variants in whey fraction. Out of the 248 samples, the heterozygous genotype AB occurred most frequently (146 samples) while the other two homozygotes, AA and BB, appeared in 46 and 56 samples, respectively. Thus, with respect to the gene frequency and the occurrence of genetic variants the Pag sheep is similar to other Mediterranean dairy sheep breeds where alleles A and B of β-LG locus occur at the intermediate allele frequency (Table 1). In the majority of populations, the B allele is more frequent than A. The slight exceptions are two Spanish (Manchega and Segureña) and two Italian (Barbaresca-siciliana and Massese) breeds where A variant is the most frequent one. Why two genetic variants of the β-LG (A and B) are maintained at the intermediate gene frequency in the Mediterranean dairy sheep populations is still an open question. Continuous week selection performed by farmers and potential superiority of the AB β-LG genotype for milk and/or cheese production might be possible expla-

Table 1. Allele frequencies and Hardy-Weinberg equilibrium tests for the β-LG locus in the Mediterranean dairy sheep populations

Breed	N	A	B	SE	$\hat{D}$	$\hat{D}_r$	$\chi^2_{[v]}$	P_{HWE}	Authors
Massese	54	0.53	0.47	0.05	0.04	0.11	1.14	0.29	V. Russo *et al.* (*10*)
Sarda	72	0.47	0.54	0.04	0.01	0.07	0.04	0.85	V. Russo *et al.* (*10*)
Comisana	250	0.50	0.50	0.02	0.02	0.04	1.30	0.26	L. Chiofalo *et al.* (*11*)
Siciliana-pinzirita	72	0.50	0.50	0.04	–0.04	0.08	2.00	0.16	L. Chiofalo and P. Micari (*12*)
Barbaresca-siciliana	62	0.62	0.38	0.04	0.00	0.14	0.00	0.96	L. Chiofalo and P. Micari (*12*)
Sopravissana	135	0.58	0.42	0.03	–0.02	0.08	0.53	0.47	S. Dall'Olio *et al.* (*13*)
Sarda	144	0.51	0.49	0.03	–0.02	0.06	0.70	0.40	G. Bardin *et al.* (*14*)
Manchega	486	0.68	0.32	0.02	0.00	0.06	0.00	0.99	G. López-Gálvez *et al.* (*15*)
Segureña	50	0.67	0.33	0.05	0.05	0.18	2.67	0.10	G. López-Gálvez *et al.* (*15*)
Lacha	234	0.47	0.53	0.02	0.01	0.04	0.55	0.46	I. Recio *et al.* (*16*)
Massese	128	0.63	0.37	0.03	–0.04	0.10	3.07	0.08	M. Rampilli *et al.* (*17*)
Valle del Belice	155	0.35	0.65	0.03	–0.04	0.03	4.23	0.04	P. Giaccone *et al.* (*18*)
Pag sheep	248	0.48	0.52	0.02	–0.05	0.04	7.98	0.01	Present study

N = sample size; A and B = β-LG variants; SE = Standard error of the allele frequency; $\hat{D}$ = Disequilibrium coefficient; $\hat{D}_r$ = The absolute value of the disequilibrium coefficient that, considering the sample size used in the analysis, would lead to the rejection of the Hardy-Weinberg equilibrium at the significance level $\alpha = 0.05$ and power of test $1\text{-}\beta = 0.80$; $\chi^2_{[v]}$ = Calculated χ^2 value with corresponding degrees of freedom [v]; P_{HWE} = Significance level /P-values/ determined by $\chi^2_{[v]}$ test.

nation for the observed phenomenon. For the Pag sheep, the observed genotype frequencies were significantly different from those expected from HWE (Table 1). Among other Mediterranean dairy sheep breeds, the deviation from HWE was observed only for Valle del Belice sheep. However, it should be noted that out of 12 other studies taken from the literature only in the studies of Chiofalo *et al.* (*11*), Recio *et al.* (*16*) and Giaccone *et al.* (*18*), the sample size was sufficient to detect disequilibrium of the same or lower magnitude (for tests with significance level α=0.05 and the power of test 1-β=0.80) as was observed for the Pag sheep. The observed excess of heterozygotes suggests, also, the superiority of the AB β-LG genotype for milk and/or cheese production and our future work will refer to the relationship between β-LG genetic variants and traits related to milk and cheese production.

Conclusions

By combining information from analysis of isoelectrofocusing patterns and PCR-RFLP we calculated the frequency of β-LG variants in the sampled whey fractions. With respect to the gene frequency (A=0.48, B=0.52) and the occurrence of genetic variants at the β-LG locus, the Pag breed is similar to other Mediterranean dairy sheep breeds. The observed genotype frequencies of β-LG genotype (AA=0.185, AB=0.589 and BB=0.226) were significantly different from those expected from HWE, suggesting the superiority of the AB genotype for milk and/or cheese production. The significant departure from HWE was also obtained in Valle del Belice sheep. The sample size required for detection of the same amount of departure from the HWE, as observed in this study, was only sufficient in four studies (out of 13 studies taken from literature).

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References

1. E. Jakob, Z. Puhan, *Int. Dairy J. 2* (1992) 157–178.
2. F. Grosclaude, *Productions Animales, INRA 1* (1998) 5–17.
3. L. Amigo, I. Recio, M. Ramos, *Int. Dairy J. 10* (2000) 135–149.
4. I. Krause, H. D. Belitz, *Lebensmittelchem. Gerichtl. Chem. 39* (1985) 29–46.
5. M. Feligini, A. Nudda, G. Pulina, G. Enne, *International Symposium of Animal Production*, Milano, Italy (1997) pp. 309–315.
6. J. Sambrook: *Molecular Cloning: A Lab Manual, 2nd ed.*, Cold Spring Harbour, New York (1989).
7. M. Feligini, P. Parma, R. Aleandri, G. F. Greppi, G. Enne, *Anim. Genet. 29* (1998) 460–477.
8. B. S. Weir, In: *Genetic Data Analysis II*, Sinauer Associates, Inc. Publishers, Sunderland (1996).
9. SAS Institute: *SAS/STAT User's Guide*, Release 6.12 edn. Cary (1996).
10. V. Russo, R. Davoli, L. Migliori, *Zoot. Nutr. Anim. 7* (1981) 421–428.
11. L. Chiofalo, P. Micari, A. Girmenia, *Zoot. Nutr. Anim. 12* (1986) 73–80.
12. L. Chiofalo, P. Micari, *Scienza e Tecnica Lattiero-casearia, 38* (1987) 104–114.
13. S. Dall'Olio, R. Davoli, P. Bosi, *Scienza e Tecnica Lattiero-casearia, 40* (1989) 186–194.
14. M. G. Bardin, P. Bolla, A. Caroli, F. Ponti, *Atti Societa Italiana Scienze Veterinari, 46* (1992) 1705–1707.
15. G. Lòpez-Gàlvez, L. Amigo, M. Ramos, *Milchwissenschaft, 49* (1994) 123–124.
16. I. Recio, A. Fernandez-Fournier, P. J. Martin Alvarez, M. Ramos, *Le Lait, 77* (1997) 259–265.
17. M. Rampili, F. Cecchi, L. Gilett, T. M. P. Cattaneo, *Proceedings of the IDF Seminar*, Palmerston North, New Zealand (1997) pp. 311–315.
18. P. Giaccone, L. Di Stasio, P. Fiandra, R. Finocchiaro, *Atti XII Congresso Nazionale* ASPA, Pisa (1997) pp. 271–272.

Genetski polimorfizam β-laktoglobulina u paške ovce

Sažetak

Uzorci mlijeka od 248 paških ovaca iz 14 različitih stada lociranih po cijelom otoku Pagu analizirani su tehnikama izoelektričnog fokusiranja i PCR-RFLP. Identificirane su dvije genetske varijante (A i B) i tri genotipa (AA, AB i BB) β-laktoglobulina. Po frekvenciji alela (A=0.48, B=0.52) i prisutnosti genetskih varijanti paška je ovca slična ostalim mediteranskim mliječnim pasminama ovaca. Dobivene genotipske frekvencije *β*-laktoglobulinskog lokusa (AA=0.185, AB=0.588 i BB=0.226) pokazale su signifikantno odstupanje od očekivanog Hardy-Weinbergove ravnoteže. U 12 različitih istraživanja koja se odnose na mediteranske mliječne pasmine ovaca, signifikantno odstupanje primijećeno je još samo u pasmine Valle del Belice. Međutim, samo u četiri istraživanja (obuhvaćajući i istraživanje autora) veličina uzorka bila je dovoljna za detekciju istog odstupanja od Hardy-Weinbergove ravnoteže. Superiornost AB genotipa *β*-laktoglobulina za proizvodnju mlijeka mogući je uzrok veće prisutnosti heterozigote te intermedijarne frekvencije alela.