Small Ruminant Research 124 (2015) 89–94

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Plasmin–plasminogen system and milk coagulation properties of two Greek dairy sheep breeds

P. Koutsouli[a,*], G. Patounas[a], Th. Massouras[b], I. Bizelis[a], I. Politis[a]

[a] Department of Animal Production and Aquaculture, Laboratory of Animal Breeding and Husbandry, Agricultural University of Athens, Iera odos 75, 11855 Athens, Greece

[b] Department of Food Science and Human nutrition, Laboratory of Dairy Science and Technology, Agricultural University of Athens, Iera odos 75, 11855 Athens, Greece

ARTICLE INFO

Article history:
Received 5 November 2014
Received in revised form 20 January 2015
Accepted 21 January 2015
Available online 28 January 2015

Keywords:
Plasmin
Plasminogen
Dairy sheep
Milk
Coagulation

ABSTRACT

The enzymatic activities of plasmin (PL), plasminogen-derived (PG) and plasminogen activator (PA) and their correlation with the milk coagulation properties and major milk components were studied in two Greek sheep breeds during their whole lactation. Individual milk samples were collected at 3-week intervals from 14 Karagouniko and 14 Chios sheep breed. The enzymatic activities of PL, PG and PA were measured by colorimetric assay. The milk coagulation properties (r_{CT} rennet clotting time, K_{20} curd firming time, A_{30} final curd consistency) were determined with a Formagraph. The results showed that PL, PG and PA activities and rennet clotting time (r_{CT}) did not differ between the two breeds. However, K_{20} and A_{30} differed significantly between Karagouniko and Chios milk. The stage of lactation (early, mid or late) significantly affected PL, PG and PA activities. PL and PA activity levels were lower during late lactation (after the 150 days of the lactation period) compared to the corresponding levels during early and mid lactation, while a significant reduction of PG activity was observed from mid to late lactation stage. Lactation stage also significantly affected milk coagulation traits: A decrease in r_{CT} occurred at mid lactation (from 100 to 150 days) while K_{20} increased from the early to the mid stage of lactation. The activities of PL and PA were significantly affected by the health status of the udder; milk with >300,000 SCC had 51% higher activity of PL and 86% higher activity of PA than milk containing <300,000 SCC. PG activity was 12% lower in milk with low SCC in comparison with milk with high SCC, but the results were not statistically significant. The coagulation properties of milk were not affected by SCC. A weak significant correlation exists between plasmin, PL + PG and K_{20}.

1. Introduction

Milk contains many endogenous enzymes with various activities (Silanikove et al., 2006). The role of endogenous enzymes in milk is important because during the storage of milk and before the manufacture of cheese these enzymes cleave caseins and affect negatively the rheological properties of milk. Recently, many studies have focused on the complex enzyme system of plasmin activator–plasminogen–plasmin, which is very important from technological point of view for milk and its products as it contributes to milk proteolysis. Proteolysis contributes during the cheese making process to highlight the desired taste and flavour while on the pasteurized milk may cause undesired gelation (Ismail and Nielssen, 2010). Plasmin (PL) is the main proteolytic enzyme in milk and its inactive

* Corresponding author. Tel.: +30 2105294440; fax: +30 210 5294442.
E-mail address: panagiota@aua.gr (P. Koutsouli).

http://dx.doi.org/10.1016/j.smallrumres.2015.01.015
0921-4488/

form is a zymogene named plasminogen (Politis, 1996; Silanikove et al., 2006). The most preferred substrate for PL in milk is β-casein and the dissociation of its three sensitive bonds results in the formation of γ1-, γ2-, γ3-caseins, which remain in the casein micelle and the corresponding *n*-terminal peptides (proteose-peptones), which move to the whey.

Factors controlling the PA–PG–PL system in cow's milk have been studied extensively while relative studies are limited for sheep milk. In cows a high level of somatic cell counts (SCC) in milk is associated with increased activity of PL, due to increased activity of PA (Politis et al., 1989b; Bastian et al., 1991). PL activity varied in different lactation stages and a correlation between PL levels with gradual involution of udder of cow at the declining phase of lactation was found (Politis et al., 1989a).

In ovine milk, the findings highlight the effect of udder health status and the stage of lactation on the plasmin–plasminogen system. From the study of Leitner et al. (2004) it is clear that infected glands in Assaf sheep had significantly higher activities of PL and PA. However, the information on variations in PL activity according to the stage of lactation is controversial. Albenzio et al. (2005) found that PL activity decreased from early to late stage of lactation in Comisana ewes while Bianchi et al. (2004) reported increased PL and PA activities in late lactation in Sardinian sheep milk. From the above it is apparent that breed differences exist and that the estimation of breed effect on the PA–PG–PL system was not considered so far.

The number of sheep in Greece is approximately 9.5 millions and virtually all the milk is processed into cheese, especially feta, a product of major economic importance in Greece. Karagouniko and Chios represent two important indigenous breeds. Karagouniko is a breed, which is well adapted to harsh environmental conditions, whereas Chios is characterized by higher milk yield and litter size, as albeit with greater susceptibility to mastitis (Hatziminaoglou et al., 1995). Theodorou et al. (2007) studied the PA–PG–PL system in milk of three Greek dairy sheep breeds, which differ in milk production capacity and concluded that the system is affected by breed, stage of lactation, and the health status of the udder. However there is no information regarding the correlations between coagulation properties with components of the PA–PG–PL system in dairy sheep breeds. The aim of this study is to evaluate, the activities of PL, PG, and PA and their correlation with milk yield, composition and rheological properties in milk of Karagouniko in comparison to the Chios breed.

2. Materials and methods

2.1. Animals and milk sampling

In this study, individual milk samples were collected from 14 Karagouniko and 14 Chios breed, which were free of clinical symptoms. The animals were kept in the premises of the experimental farm of the Agricultural University of Athens. The ewes were in their 2nd or 3rd lambing and lambed between December 2011, and January 2012. The experimental ewes completed their lactations by the 1st week of July to avoid the extremely hot conditions that occur in Greece during July and August. After weaning (42 days after lambing), the ewes were milked twice daily at 06:00 and 17:00 h by milking machine. Milk yield was recorded every 3 weeks and individual milk samples were collected during the morning and evening milking on the same date during the entire lactation. The individual samples were divided into two aliquots; the first one was immediately analysed for major milk components and Somatic cell counts (SCC) and the second was frozen and stored at −20 °C for PL, PG, and PA analysis.

2.2. Determination of chemical composition, SCC and milk coagulation properties

Milk composition (fat, total protein, lactose, and total solids) and the SCC of the bulk tank milk samples were determined using a CombiFoss 6000 FC apparatus (Foss Electric A/S, Hillerød, Denmark). This apparatus combines the MilkoScan FT 6000 Fourier transform infrared spectrometer and the Fosso-matic FC flow cytometry somatic cell counter. Before and during the experiments, the equipment was standardized for sheep milk according to FIL – IDF 141C (2000).

2.3. Analysis of milk coagulation properties

Milk coagulation properties (rennet clotting time – r_{CT}, curd firming time – K_{20} and firmness of the curd after 30 min – A_{30}) were estimated by a Foss Electric Formagraph (Foss Electric, Hillercd, Denmark). Samples (10 mL) were heated and maintained at 35 °C. Then, 200 μL of a rennet solution (Hansen Standard 160, with 80 ± 5% chymosin and 20 ± 5% pepsin; 160 international milk clotting units (IMCU)/mL) diluted to 1.6% (wt/vol) in distilled water was added at the beginning of analysis. The observation period last 60 min after rennet addition. Rennet clotting time, r_{CT}, (min) is defined as the time from addition of enzyme to the beginning of coagulation. K_{20} (min) is the interval from r_{CT} to the time at which the width of the graph attains 20 mm. A_{30} (mm) is a measure of the extent of curd firmness at 30 min after the addition of coagulant. Samples that did not coagulate within 30 min were classified as non-coagulating.

2.4. Determination of PL, PG, and PA activities

PL and PG were determined by a combination of the methods described by Politis et al. (1989a,b). Milk (3 mL) was mixed with 1 mL of 0.4 M sodium citrate and centrifuged at 27,000 × *g* for 20 min. The supernatant was recovered and assayed for PL and PG. Plasminogen-derived activity is defined as the PL activity generated after addition of urokinase (U0633; Sigma). The sum of PL + PG was calculated by summing the activity of PL plus the activity of PG. Both assays were performed in 250 μL of 0.1 M Tris–HCl buffer (pH 7.4) containing 0.6 mM Val-Leu-Lys-p-nitroanilide (V7127; Sigma Chemical Co., St Louis, MO), 30 plough units (2.5 μL) of urokinase, and 30 μL of the milk supernatant. All assays were analysed in duplicates. The reaction mixture was incubated at 37 °C and the absorbance at 405 nm was recorded at hourly intervals. A sample without supernatant served as a control for the detection of spontaneous breakdown of the substrate. In all cases, spontaneous hydrolysis was negligible. PL activity was measured in the same reaction mixture without added urokinase. PL and PG activities were determined from the linear part of the absorbance vs. time curve. One unit of PL was defined as the amount of enzyme that produced a change in absorbance of 0.1 at 405 nm in 60 min. A colorimetric assay was used to measure PA activity in the CN fraction (Gilmore et al., 1995). The principle of this methodology is that PA in the CN fraction converts exogenously added PG to PL. Plasmin activity is then determined as described above and the activity was corrected by reducing from the total activity, the activity in the non-treated samples.

2.5. Statistical analysis

The data were analysed using a linear mixed model, which is appropriate for repeated measurements per subject (animal) using the autoregressive order one [AR(1)] for modelling the error covariance structure. In order to meet ANOVA assumptions (i.e., normal distribution and homogeneity of variance within class effects) an animal with very high values for Plasmin (152.52 IU/mL) and Plasminogen Activator (297.56 IU/mL), which was substantially lower from the mean of PL activity (33.41 IU/mL) was excluded from the analysis. The fixed effects in the model included the effects of breed (2 levels, Karagouniko and Chios), the effects of lactation stage (3 levels, early: from 42 to 100 days, mid: from 101 to 145 days and late: from 146 to the end of milking) and the effect of udder health status (2 levels, above and below 300,000 cells/mL). The repeated measure factor was the individual sample of a given ewe. The