Fat globule membranes in ewes' milk: the main enzyme activities during lactation

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Abstract

Studies on milk fat globule membranes (MFGMs) have primarily been carried out on human and bovine milk. An investigation of the proteins in sheep MFGM during lactation could provide information regarding the role of MFGM enzymes and milk quality. A trial was carried out on seven Massese ewes, from which individual milk samples were taken during lactation. All the enzyme activities investigated (oxidase activity of xanthine oxidoreductase, gamma-glutamyltranspeptidase, alkaline phosphatase and 5'-nucleotidase) were affected by the lactation phase, with the exception of the dehydrogenase activity of xanthine oxidoreductase (XOR). A higher oxidase activity (XO) of XOR was found when the diameter of milk fat globules was smaller. In addition, the absence of XO in colostrum led to the hypothesis that its antibacterial role is independent of its activity. The increase in alkaline phosphatase activity during lactation requires further research in order to better define the criteria for pasteurized sheep milk.

1. Introduction

Milk fat globules (MFGs) are enveloped by a triple membrane, called the milk fat globule membrane (MFGM). The MFGM consists of a single inner layer originating from the endoplasmic reticulum and a double layer, arising from the membrane of the secretory cell (Mather & Keenan, 1998). The amount of MFGM in milk changes depending on the number and diameter of globules (Martini, Salari, Pesi, & Tozzi, 2010). The MFGM supplies many proteins and enzymes, some of which have beneficial biological effects (Dewettinck, Rombaut, Thienpont, Le, Messens, & Van Camp, 2008), whereas others have been linked to human diseases (Riccio, 2004).

Research on MFGMs has mainly been carried out on cows' and human milk (Mather, 2000; Liao, Alvarado, Phinney & Lönnerdal, 2011), whereas there have only been a few studies on MFGM proteins in sheep (Martini, Salari, Pesi & Tozzi, 2010; Pisanu, et al., 2011).

To date, the exact physiological role of many MFGM enzymes has not been fully understood. The aim of this study was to investigate the activities of MFGM enzymes during

lactation in order to check their link with the size of the native milk fat globules secreted and with colostrum and milk nutritional and hygienic quality.

2. Materials and methods

2.1. Animals and sampling

A trial was carried out on seven Massese reared in Tuscany (Central Italy). All ewes were reared on the same farm and were homogeneous in terms of parity and feeding. The subjects were kept indoors at the tenth day before partum. The experiment lasted 120 days from 10 hours postpartum (day 0); 11 samples of milk were taken from each individual during lactation, producing a total of 77 samples.

2.2. Morphometric analysis of milk fat globules

The diameter and number of MFGs per mL were measured following the method of Scolozzi, Martini, & Abramo (2003). Our method enables the MFGs to be characterized directly, using an image analyser system, whereas other methods involve an indirect analysis using the refractive index (Michalski, Briard, & Michel, 2001). The detection limit of the microscope and the image analyser do not allow globules smaller than 0.4 μm to be measured. Although globules with a diameter <1 μm are numerous, and comprise 80% of the globules (Heid & Keenan, 2005) they only represent a few percent of the total milk fat volume (Walstra, 1969; Michalski, Ollivon, Briard, Leconte & Lopez, 2004).

2.3. Extraction MFGM proteins and enzyme assays

The cream was isolated from the skim following a macroversion of Patton and Huston's method (1986). This method, carried out on fresh milk, enabled us to isolate the MFGs, thus avoiding the denaturation of the MFGM proteins. To obtain an extract containing MFGM proteins, the cream was gently stirred for two hours in the presence of 1% triton X-100, and then centrifuged at 100,000xg for two hours at 10°C (Camici, Fini, & Ipata, 1985 modified). Three layer were obtained after the centrifugation: a pellet made up of MFGM lipids, a supernatant containing the MFGM proteins and an upper layer made up of the triglycerides of the core.

Total membrane proteins (TMP) were measured in the supernatant according to Bradford (1976). In the supernatant from each sample, an assay was made in duplicate of the dehydrogenase activity (XDH) of the enzyme XOR, oxidase activity (XO) of the XOR, gamma-glutamyltranspeptidase (γ-GT) alkaline phosphatase (AP), and 5' nucleotidase (5'-N) activities according to Benboubetra, Baghiani, Atmani, and Harrison (2004), Bergmeyer (1983), Huseby and Strömme (1974) and Fini, Camici, Minelli, Floridi, and Ipata (1986), respectively.

2.4. Statistical analysis

The results of the morphometric analysis of the MFGs and of the enzyme activities were analysed following ANOVA for repeated measurements, regarding the sampling time as a fixed effect and the subject as a random effect. To verify the relationships between enzymes and MFGs size, Pearson's correlation method was applied. Significant differences were considered at $P \leq 0.05$The statistical analysis was carried out using JMP software (2002).

3. Results and discussion

A greater amount of TMP was detected at 10 hours post partum (4.23 mg mL^{-1} of supernatant; Table 1). XO of the XOR, a protein that derives from the endoplasmatic reticulum (Bianchi et al., 2009), was not observed in the mammary secretion in the first few hours (day 0; Table 1). This was probably due either to the lower expression of XOR in colostrum as reported in the literature for cow and human milk (Reinhardt & Lippolis, 2008; Liao et al., 2011) or to the activation and deactivation of the enzyme during lactation as found by Benboubetra et al. (2004). In addition, XOR has been reported as having anti-bacterial properties by catalyzing chemical reactions (Harrison, 2006). In any case, we did not detect XO at the day 0. Thus, we hypothesized that the anti-bacterial properties of the enzyme are not due to this activity, at least at the beginning of lactation when the defence role of the newborn mainly involves other components such as immunoglobulins.

XO increased during lactation (Table 1) after the first 10 hours and 15th day ($P \leq 0.01$), reaching a climax on the 45th day (3.06 mU mg^{-1}), corresponding to a decrease in the average diameter of the measured MFGs (Figure 1). In addition, a negative Pearson's correlation between XO and the diameter of MFGs (-0.159; $P \leq 0.01$) was found, confirming the positive link between XO and smaller globules found in a previous study (Martini et al., 2010). This suggests that the link between XO and the diameter of MFGs is probably due to the fact that the enzyme XOR could be involved in the secretion of the globules (McManaman, Russell, Schaack, Orlicky, & Robenek, 2007).

In fact, XO is supposed to control MFGs secretion either by interactions with butyrophilin and adipophilin (Mather &Keenan, 1998) or through the generation of reactive oxygen species which work as signals for butyrophilin. The typical structure of receptor of the protein butyrophilin support this hypothesis (Ogg, Weldon, Dobbie, Smith, & Mather, 2004). Since the intracellular precursors of the MFGs (cytoplasmic droplets) increase in size by intracellular fusions, the higher XO would increase the speed of release of the globules by the mammary

cells, resulting in the secretion smaller MFGs. In contrast, XDH did not vary significantly during lactation, as previously described in bovine milk by Heinz and Reckel (1983), who reported that the mammary gland and milk contain the enzyme mainly as XO.

AP is the most important enzyme indicator for the correct pasteurization of milk (Rankin, Christiansen, Lee, Banavara, & Lopez-Hernandez, 2010). In our study, AP showed a progressive increase in activity during lactation, at 90 days reaching a climax that was about 40 times higher than the initial value of activity (day 0), as reported for ovine milk (Rankin et al., 2010). Since the legal limit for the test in terms of negativity for the activity of AP is currently defined only for cows' milk (Rankin et al., 2010) and since there is a considerable variation in AP activity with advancing lactation, further research is required in order to better define the extent of heat treatment in sheep milk.

Transmembrane glycoprotein 5'-N is an enzyme that plays a major role in the catabolism of mononucleotides and dinucleotides (Fini et al., 1986), however its role in milk has not yet been clarified. In our study, the activity of 5'-N showed an increasing trend during lactation, from a minimum of 4.77 to a maximum of 47.34 mU mg-1 of MFGM proteins on the 3rd and 60th day respectively (Table 1).

γ-GT is known to be one of the colostrum enzymes and its activity is positively correlated with the content of immunoglobulins in ruminants, therefore it is considered to be indicative of the quality of this initial secretion (Zarrilli et al., 2003). In fact, in our study, γ-GT showed the maximum activity in the colostrum phase, with peaks of activity between the third and tenth days (Table 1), decreasing, and then remaining constant thereafter.

4. Conclusions

All the enzyme activities assayed, with the exception of XDH were affected by lactation. A higher XO was found when the diameter of the milk fat globules is smaller. With regard to AP, the significant increase in its activity during lactation requires further research in order to better define the pasteurization criteria of sheep milk.

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210

211 Table 1 Activities of milk fat globules membrane enzymes (U mg^{-1} of milk fat globule membrane (MFGM) proteins) and total membrane proteins (TMP; mg mL^{-1}
212 of supernatant) in ewes' milk during lactation.

213

		Days in milk											SEM[a]
		10 h	3	6	10	15	20	30	45	60	90	120	
XO[b]	(mU mg^{-1})	ND	0.50B	0.70B	1.23B	0.62B	1.81A	1.75AB	3.06A	2.78A	1.87A	0.86B	0.001
XDH[c]	(mU mg^{-1})	0.76	0.60	0.18	0.78	0.11	0.52	0.92	1.15	0.87	0.66	0.25	0.001
γ-GT[d]	(U mg^{-1})	2.83b	4.91a	4.90a	4.74a	3.31ab	3.00b	2.77b	2.86b	3.64ab	3.19ab	3.51ab	1.656
AP[e]	(mU mg^{-1})	3.79C	7.54C	21.42C	22.73C	31.79BC	72.58BC	81.37B	128.33AB	148.83A	150.56A	140.76A	0.072
5'N[f]	(mU mg^{-1})	5.22B	4.77B	9.70B	12.51B	14.42B	19.82B	20.99B	28.30AB	47.34A	34.17A	22.84B	0.015
TMP	(mg mL^{-1})	4.23A	2.56B	2.15BC	3.10B	2.16B	1.92C	2.06C	2.18B	2.04C	2.32B	2.69B	0.771

214 [a] Standard error of the model

215 [b] Oxidase activity of the enzyme xanthine oxidoreductase

216 [c] Dehydrogenase activity of the enzyme xanthine oxidoreductase

217 [d] Gamma-glutamyl transpeptidase

218 [e] Alkaline phosphatase

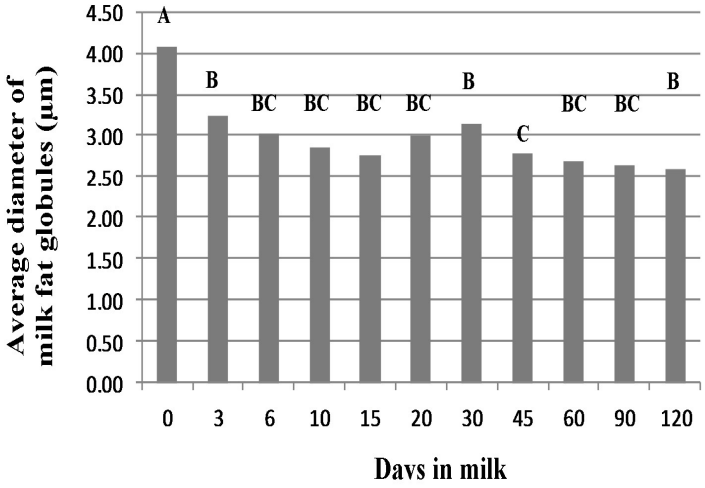
219 [f] 5'-nucleotidase

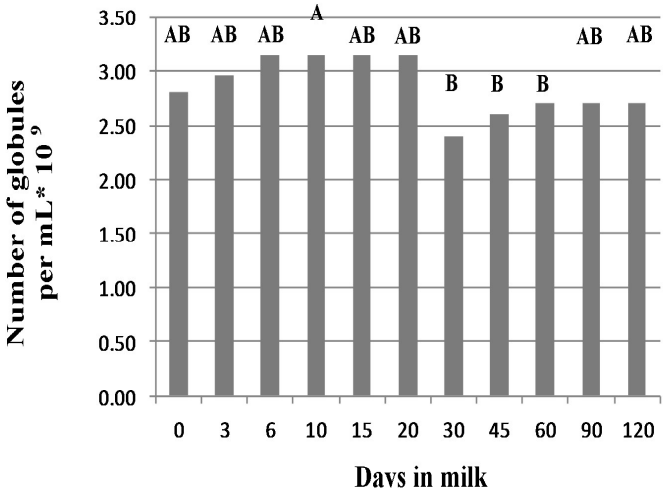
220 [g] Not determined.

221 Different letters indicate statistical differences across a row at $P \leq 0.01$ (A, B, C) and $P \leq 0.05$ (a, b).

222 Figure 1. Average diameter (µm) and number per mL of milk fat globules during lactation.

223 Different letters indicate statistical differences among bars at $P \leq 0.01$





224