# Alkaline Phosphatase, Acid Phosphatase, Lactoperoxidase, and Lipoprotein Lipase Activities in Industrial Ewe's Milk and Cheese

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Alkaline phosphatase activity in raw, industrial ewe's milk increased steadily >2-fold between January [1.7 units (U)/mL] and June (3.75 U/mL), whereas acid phosphatase increased 4-fold in January and February (17 mU/mL) and then remained constant until the end of lactation. By contrast, lipoprotein lipase exhibited a downward trend and lactoperoxidase decreased 2-fold during lactation. When assayed at cheese-ripening temperatures, acid phosphatase retained 16% of its activity at 37 °C, whereas lactoperoxidase retained between 30 and 45% of its activity at 20 °C. The rate of hydrolysis of model triacylglycerols by lipoprotein lipase was highest for tricaprylin. Although alkaline phosphatase in raw milk cheeses was variable from 1 to 180 days of ripening, no apparent reactivation was observed. The activity of acid phosphatase increased 2-fold during the 180 days of ripening in the cheeses made in summer, whereas in winter and spring much smaller increases were observed. Both raw milk cheeses made in summer and all pasteurized milk cheeses had very low levels of lactoperoxidase throughout ripening.

**Keywords:** *Alkaline phosphatase; acid phosphatase; lactoperoxidase; lipoprotein lipase; ewe's milk; ewe's milk cheese; ovine cheese; lactation period; cheese ripening.*

## INTRODUCTION

The quality of sheep's milk cheeses tends to be more variable than that of cow's milk cheeses due to a number of factors such as the characteristics of the milk from different breeds of sheep, stage of lactation when cheese is made, or use of various renneting agents (commercial calf rennet, artisanal and commercial lamb rennet pastes), among others. Sheep's milk has been characterized with respect to fat and protein content (Anifantakis, 1986; González-Llano and Ramos, 1989; Muir et al., 1993) with occasional references to the levels of some enzymic activities. Although some 60 enzyme activities have been identified in milk, their significance to cheese quality has not been well studied (Fox et al., 1996).

Alkaline phosphatase, acid phosphatase, lactoperoxidase, and lipoprotein lipase are four enzyme activities of technological significance in the dairy industry. The alkaline phosphatase test has been widely used for a long time (Aschaffenburg and Mullen, 1949) as a control for the efficiency of pasteurization. A positive result with this test is the accepted parameter to characterize a raw milk product, whereas it is taken as a sign of improper pasteurization (or contamination with raw milk) in a pasteurized milk product. However, positive alkaline phosphatase results have been reported in blue-veined cheeses (Rosenthal et al., 1996) and in fresh, unripened, Hispanic-style cheeses (Pratt-Lowe et al., 1987) made from pasteurized milk. In both cases this enzyme activity was of microbial origin. Its behavior in raw milk cheeses has not been well characterized, including how long it could be detected. For regulatory purposes, the determination of its level of activity during cheese maturation could provide useful information. Thus, it was of interest to follow this activity in both raw and pasteurized milk cheeses of long ripening periods.

Acid phosphatase has a greater thermal stability and it is most active at pH values typical of cheese ripening. Its activity could be of major importance in the cheese-ripening process as it is very active against phosphoprotein substrates such as the casein of milk (Andrews, 1991). Phosphopeptides have been reported to be resistant to proteolytic attack, although not bitter (Dulley and Kitchen, 1972). Thus, a high acid phosphatase activity during ripening could result in excessive proteolysis and flavor defects.

The lactoperoxidase system (lactoperoxidase/thiocyanate/hydrogen peroxide) is a natural antimicrobial system present in milks from many species (Perraudin, 1991). The enzyme lactoperoxidase catalyzes the oxidation of thiocyanate by hydrogen peroxide, the antimicrobial effect being due to intermediate reaction products (Pruitt and Reiter, 1985). Thiocyanate is widely distributed in animal tissues, with levels in bovine milk ranging between 1 and 10 ppm (Wood, 1975). The third component of the system could be generated in milk by leucocytes and by lactic acid bacteria. The literature refers primarily to the bovine, porcine, caprine, guinea pig, and human enzymes (Wolfson and Sumner, 1993; Zapico et al., 1990), but little information has been found about the level of this activity in sheep's milk as well as in any kind of cheese.

The natural lipolytic enzyme in milk is lipoprotein lipase. Its level of activity is usually low because of the difficult access of the enzyme to its substrates in milk. Unlike in milk, in which lipolytic activity causes rancidity (Deeth, 1993), the presence of this lipoprotein lipase or other lipases in dairy products (such as cheeses) would be interesting as their reaction products, free

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fatty acids and their derivatives, could impart specific tastes and flavor to the processed product (Kim and Lindsay, 1990; Chilliard, 1982).

Idiazabal cheese is a raw ewe's milk cheese, typical of the Basque Country region of Spain. Most of the production is commercialized within Spain between 90 and 180 days of ripening, with very few cheeses being sold after that time. Although the Denomination of Origin does not allow the use of pasteurized milk, trial batches were made to study its characteristics for potential export. The work presented herein is part of a larger study to characterize the main biochemical and microbiological aspects of the ripening of Idiazabal cheese, relating them to sensorial parameters in the finished product. Knowledge of how these enzymic activities vary along the cheese-making period, both in milk and during ripening of cheeses, may allow the cheesemaker to introduce appropriate modifications in the fabrication process.

## MATERIALS AND METHODS

**Milk and Cheese Samples.** Bulk ewe's milk was from several commercial flocks of *latxa* sheep used in the Basque Country region of Spain to manufacture Idiazabal cheese. Milk from an individual flock was a gift of Dr. L. Oregi (Basque Government Department of Agriculture, Experimental Station, Arkaute, Alava, Spain). The lactation period for this flock extended from early March until June. Milk samples were taken after the milk from all ewes had been pooled and were kept on ice. Enzyme activities were assayed on the day milk was collected.

The lactation period for an individual flock extends ≈4 months between November and July, depending on the altitude at which the flock is located, with flocks on lower elevations starting lactation earlier. Industrial cheese-making (using mixed milk from different flocks) extends from January until the end of June. Thus, industrial milk in January contains a large percentage of milk from flocks early in their lactation stage, while industrial milk in June corresponds to the late lactation period of most flocks. In April milk could be a mixture (of unknown composition) of milk from late, mid, and early lactation period flocks. Milk had been refrigerated for up to 48 h in commercial farmhouses and pasteurized upon receipt (when needed) in a local cheese factory (Queserías Araia, S.A., Araia, Spain) prior to cheese-making. Pasteurized milk samples were immediately stored on ice until enzymic activities were assayed. Cheeses were made according to the traditional method for the industrial production of Idiazabal cheese approved by its Denomination of Origin (Basque Government, 1986), using commercial calf rennet (Ch. Hansen, Madrid, Spain), in 200 L vats. Cheeses (approximately 1 kg and 15 cm diameter) were made in three consecutive weeks in January, April, and June (total of nine fabrications), as indicated in figure legends. Three different types of cheeses were made for each fabrication: (C) control cheese made from raw milk with no starter added; (R) cheese made from raw milk with a starter culture added (90% *Lactococcus lactis* sbsp. *diacetylactis* and 10% *Lactobacillus lactis* sbsp. *lactis*, isolated from Idiazabal cheese, Pérez-Elortondo, unpublished results); (P) cheese made from pasteurized milk with the same starter culture as for cheese R. For each fabrication and type of cheese, samples from two different cheeses were taken for analysis after 1, 90, and 180 days of ripening. Cheese extracts were prepared immediately, and enzyme assays were carried out on the same day. Other cheese samples were wrapped in plastic film and aluminum foil and frozen at −80 °C to determine enzyme stability.

**Enzymic Assays.** Enzyme activities were determined in quadruplicate both in milk samples and in cheese extracts. Milk was used either whole or defatted by centrifuging it at 5000*g* for 20 min. Cheese extracts were prepared as follows: 5.0 g of cheese (taken uniformly along the radius of the piece) were homogenized in a Potter-type homogenizer on ice with 25.0 mL of 0.12 M Tris-HCl, pH 8.0, buffer and centrifuged at 5000*g* for 20 min at 4 °C. After the fat layer was removed, the aqueous layer was filtered through Whatman No. 1 filter paper and the filtrate was used for lactoperoxidase and alkaline and acid phosphatase determinations. For lipoprotein and esterase determinations, the filtrate was concentrated with a PM10 membrane in an Amicon ultrafiltration unit on ice.

Alkaline phosphatase was determined as described by McComb et al. (1979) in 0.9 M 2-amino-2-methyl-1-propanol (AMP, Sigma Chemical, Madrid, Spain), pH 10.45, with *p*-nitrophenyl phosphate (pNPP, Sigma Chemical) as substrate (assay concentration was 15.8 mM), at 35 °C. Total volume was 2.0 mL, and reaction rates were determined at 400 nm. The molar absorptivity of *p*-nitrophenol at 400 nm was taken as 19 000 $M^{-1}$ $cm^{-1}$. The AMP buffer system was used instead of the carbonate buffer used by other workers (Kitchen et al., 1970) to ascertain that if any reactivation of the alkaline phosphatase occurred in pasteurized cheeses during cheese maturation, it would easily be detected. Values for alkaline phosphatase activity obtained in the presence of AMP buffer were ∼2-fold higher than those obtained in the traditional carbonate buffer, due to the activating effect of AMP for phosphatases (Tietz, 1979). No $Mg^{2+}$ was added to assay mixtures because no increase in activity was observed in its presence.

Acid phosphatase was determined essentially as described by Larsen and Parada (1988). Milk (80 μL) or cheese extract (0.3 mL) was added to an appropriate volume of 0.1 M sodium acetate buffer, pH 4.4 (total assay volume was 1.0 mL), containing pNPP (final concentration in the assay mixture was 7.5 mM). Mixtures were incubated at 37 °C for 1 h, and reactions were stopped with 1.0 mL of 12% (w/v) trichloroacetic acid. After the precipitated proteins had been separated by centrifugation at 16000*g* for 10 min, 0.7 mL of the clear supernatant was mixed with 1.5 mL of 2 M NaOH. Absorbance was read at 405 nm.

Lactoperoxidase was determined as described by Pruitt et al. (1990) in 0.1 M phosphate buffer, pH 6.0, and at 20 °C. The assay concentrations of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and $H_2O_2$ were 3.88 mM and 0.1 mM, respectively, 2.5 mL being the total assay volume. Reaction rates were determined at 415 nm, and the molar absorptivity coefficient of ABTS was taken as 32 400 $M^{-1}$ $cm^{-1}$.

Lipoprotein lipase was adapted from the method of Egelrud and Olivecrona (1972). The substrate emulsion was prepared as follows: 0.86 g of tricaprylin was sonicated with a solution of 1.3% (by weight) bovine serum albumin in 17.8 mL of 0.37 M Tris-HCl buffer, pH 8.6, containing 0.66 M NaCl and 20 μL of heparin (1000 international units/mL). Assay mixtures (2.0 mL total volume) containing 0.4 mL of decomplemented human serum and 0.8 mL of substrate emulsion in 0.37 M Tris-HCl buffer, pH 8.6, were incubated at 37 °C for 1 h (milk) or 4 h (cheese extracts). Reactions were stopped with 5.0 mL of hexane/2-propanol/10 N sulfuric acid (1:4:0.1 by volume). Free fatty acids were extracted with 3.0 mL of water and 3.0 mL of hexane and titrated with 0.005 M methanolic KOH, using phenolphthaleine as indicator. When ewe's milk fat [extracted according to the modified method of Folch, as described by Hamilton et al. (1992)] was used as substrate, the free fatty acids produced were extracted and analyzed by gas−liquid chromatography as described (Chávarri et al., 1997).

All enzyme units are reported in international units (U), 1 U being the amount of enzyme that catalyzes the production of 1 μmol of product/min under the described conditions. Activity in milk is given in units per milliliter and in cheese as units per kilogram of cheese. All determinations were done in quadruplicate, and the results presented are the mean ± standard deviation.

Thiocyanate concentration in milk samples was determined as described by Gaya et al. (1991).

**Statistical Analysis.** The BMPD statistical package (Dixon, 1983) was used for statistical treatment of the results.

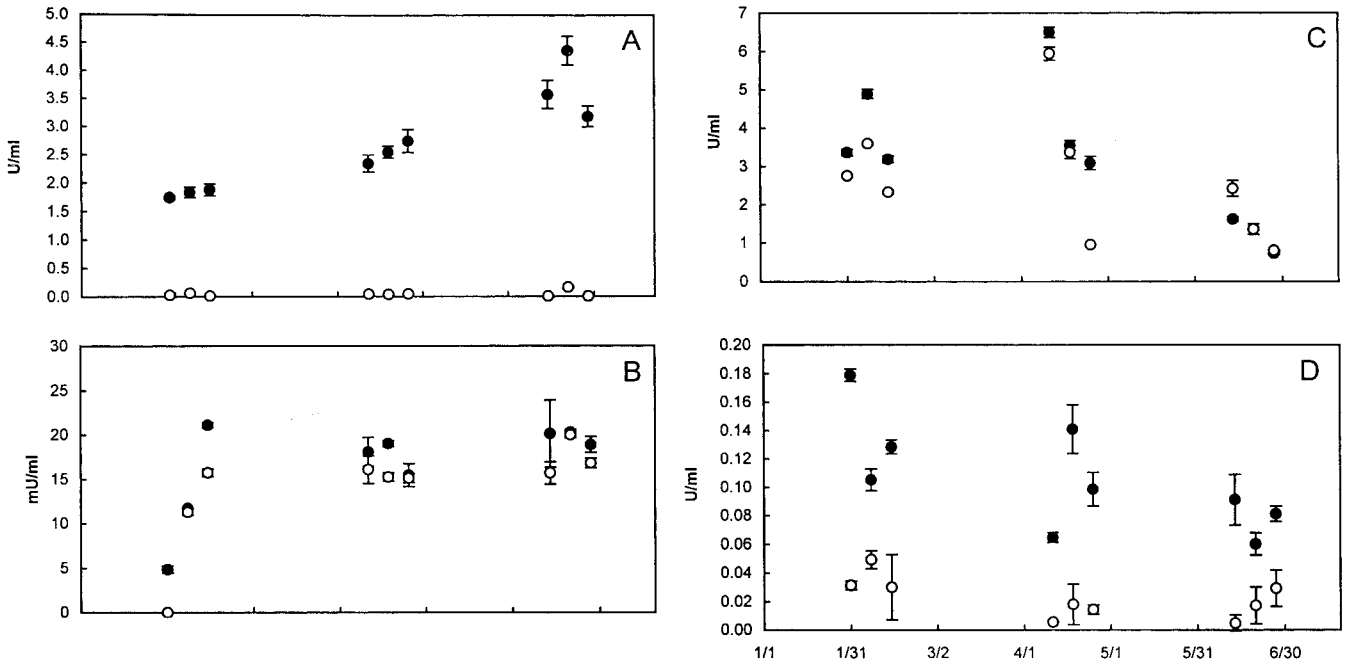


**Figure 1.** Enzyme activities in ewe's crude (●) and pasturized (○) milk throughout lactation period: (A) alkaline phosphatase; (B) acid phosphatase; (C) lactoperoxidase; (D) lipoprotein lipase. Values reported represent the mean of four determinations with the error bars indicating the standard deviation. When no error bars appear, the magnitude of the standar deviation is smaller than the symbol used.

**Table 1. Enzyme Activities in Raw Ewe's Milk from a Single Flock**[a]

| date | alkaline phosphatase, U/mL | acid phosphatase, mU/mL | lactoperoxidase, U/mL | lipoprotein lipase, U/mL |
|---|---|---|---|---|
| March 28 | $1.819 \pm 0.135^{a}$ | $29.29 \pm 0.45^{a}$ | $9.854 \pm 0.127^{a}$ | $0.160 \pm 0.014^{a}$ |
| May 5 | $2.257 \pm 0.086^{b}$ | $25.99 \pm 0.10^{b}$ | $10.565 \pm 0.340^{b}$ | $0.243 \pm 0.021^{b}$ |
| June 9 | $2.653 \pm 0.204^{c}$ | $21.61 \pm 0.05^{c}$ | $8.653 \pm 0.382^{c}$ | $0.117 \pm 0.003^{a}$ |

[a] Enzyme activities were determined as described under Materials and Methods. Pooled milk samples from all ewes were taken on the dates indicated after the morning milking. Values reported represent the mean ± standard deviation of four replicates. For each enzyme activity different letters indicate statistically significant ($p < 0.05$) differences.

Analysis of variance was employed to test for statistically significant differences ($p < 0.05$) in the levels of the various enzyme activities over the lactation period. Bonferroni's paired *t* test was used to test for statistically significant differences ($p < 0.05$) between values for any two of the three different times in the lactation period, winter, spring, and summer.

## RESULTS AND DISCUSSION

**Enzyme Activities in Industrial Milk.** Alkaline phosphatase activity in industrial ewe's milk was observed to increase >2-fold between January (early lactation, average of 1.75 U/mL) and June (late lactation, average of 3.75 U/mL) (Figure 1A), in agreement with the 2-fold variation in activity reported for bulk bovine milk collected during different seasons (Andrews, 1991). Differences among the three lactation stages were statistically significant ($p < 0.05$). When the milk from a single flock was analyzed, a similar trend was observed, although the actual values were lower (Table 1), in contrast with the results reported by Anifantakis and Rosakis (1983) for three Greek flocks in which the activity was maximal in mid-lactation, with lower values in both early and late lactation. The level of this enzyme in ewe's milk (when determined in the more frequently used carbonate buffer) was comparable to that reported by Anifantakis and Rosakis (1983) for ewe's milk but >2-fold higher than that reported for bovine milk (Kitchen et al., 1970) and ~5-fold higher than that reported for goat's milk (Williams, 1986). The optimum pH was 10.5 (Figure 2), and the optimum

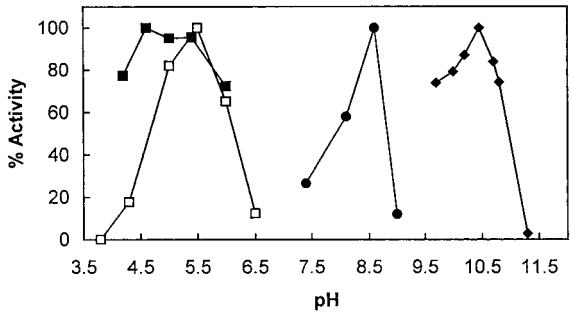


**Figure 2.** Variation of enzyme activity in industrial, raw milk with pH: alkaline phosphatase (◆); acid phosphatase (■); lactoperoxidase (□); lipoprotein lipase (●). Assay conditions (except pH) were as described under Materials and Methods.

temperature was 40 °C. As expected, pasteurization completely inactivated this enzymic activity. The residual activity observed for one of the June samples could be due to improper pasteurization, because total viable counts for this sample were reduced by 25% upon pasteurization, whereas in the rest of the samples an average 34% reduction was observed (de Vega, 1996). Between 70 and 80% of the alkaline phosphatase activity of whole milk was found in skim milk. When cheese was made, only 40−45% of alkaline phosphatase was lost in the whey.

Acid phosphatase activity (Figure 1B) increased 4-fold in January and then remained constant (17 mU/mL) until the end of June. This value is ≈1 order of

**Table 2. Thiocyanate Concentration in Bulk Raw Ewe's Milk throughout Lactation Period[a]**

| date | [SCN], ppm | date | [SCN], ppm | date | [SCN], ppm |
|---|---|---|---|---|---|
| Jan 31 | 18.26 ± 2.64 | April 11 | 9.18 ± 0.15 | June 13 | 1.64 ± 0.22 |
| Feb 7 | 5.70 ± 0.22 | April 18 | 2.12 ± 0.05 | June 20 | 2.08 ± 0.18 |
| Feb 14 | 4.13 ± 0.16 | April 25 | 2.12 ± 0.08 | June 27 | 1.55 ± 0.16 |

[a] Milk samples were taken on dates specified. Determinations were made as described under Materials and Methods. Values are the mean ± standard deviations of four replicates.

magnitude higher than that reported for bovine milk (Kitchen et al., 1970), although no indication is given in this publication as to variations between early and late lactation. The activity of acid phosphatase in the milk from a single flock was somewhat higher than that observed in bulk milk (Table 1) and it decreased toward the end of the lactation period, the differences between periods being statistically significant ($p < 0.05$). This activity had a broad pH optimum between 4.5 and 5.5 (Figure 2). When the assay temperature was lowered to 10 °C (approximate ripening temperature for Idiazabal cheese is between 8 and 12 °C), the enzyme retained 16% of its activity at 37 °C. Both the pH profile and the fact that it was active at low temperatures indicate that it can be active under cheese-ripening conditions. Pasteurization did not inactivate it to any significant extent, as expected. The activity found in skim milk was between 65 and 80% that of whole milk. Between 30 and 34% of the milk acid phosphatase was found in the whey after renneting.

Lactoperoxidase activity in industrial milk (Figure 1C) was variable during the winter and spring months, with an average value of 4 U/mL, decreasing by an average factor of 2 by the end of June ($p < 0.05$). This value is ≈3-fold higher than the average value of 1.4 U/mL reported for bovine milk (Stephens et al., 1979). By contrast, lactoperoxidase activity in the milk of a single flock gave a mean value of 9.7 U/mL, 2-fold higher than the mean value reported by Medina et al. (1989) for Manchega sheep's milk at mid-lactation, which could indicate differences due to the breed of sheep. The lactoperoxidase pH optimum was 5.5 (Figure 2). When the enzyme was assayed at cheese-ripening temperatures, it retained between 30 and 45% of the activity found at 20 °C, suggesting that the enzyme could also be active during cheese ripening. Over 90% of the lactoperoxidase activity of whole milk was found in the skim milk. After renneting, 65% of this enzyme activity appeared in the whey. Pasteurization reduced the level of activity by an average 10−30% in winter and spring, with one sample exhibiting a 70% reduction in activity. Pasteurization did not inactivate any of the June samples, which initially had very low activity. Thiocyanate content in milk depends on the composition of the pastures, with cows on natural pastures containing clover giving milk with up to 15 ppm of thiocyanate (Pruitt and Reiter, 1985). The concentration of thiocyanate (Table 2) was very high at the end of January, when most of the sheep would be receiving dry fodder. The lowest values were observed in spring and summer. Considering that the levels of lactoperoxidase were not limiting and that approximately 15 ppm of thiocyanate and 8.5 ppm of hydrogen peroxide would be adequate for the lactoperoxidase system to be activated (Björck et al., 1979), addition of these compounds could help improve the microbiological quality of refrigerated milk.

Lipoprotein lipase activity (Figure 1D) was routinely assayed with tricaprylin as model substrate. This activity, although variable, exhibited a slight downward trend as lactation progressed, with the differences between January and June being statistically significant ($p < 0.05$). When model substrates were used, the hydrolysis of tricaprylin proceeded at the highest rate, with the rates for tricaprin, trilaurin, and olive oil being comparable (Figure 3A). Results with tripalmitin were highly variable (not shown) due to difficulties in obtaining a stable emulsion at the assay temperature of 37 °C. When ewe's milk fat was used as substrate, caprylic and capric acids appeared as products at an apparently lower rate than palmitic acid (typical results are shown in Figure 3B). However, taking into consideration that palmitic acid represented 24% of the total fatty acids in this ewe's milk fat and caprylic and capric acids represented 3 and 9%, respectively (de Renobales et al., unpublished observations), these results did not contradict those obtained with model substrates. Our results were consistent with those reported for purified bovine lipoprotein lipase (Deckelbaum et al., 1990), which showed that this enzyme exhibited a higher rate of hydrolysis toward triacylglycerols containing medium-chain fatty acids than toward those containing long-chain fatty acids. Results for oleic acid (which represented 24% of the total fatty acids in this ewe's milk fat) were highly variable. Lipoprotein lipase activity in milk from a single flock presented slight variations with an increase toward mid-lactation (Table 1). Pasteurization caused an average 73−95% inactivation of this enzyme. The low percent inactivation (45%) observed in the second June sample was most likely due to improper pasteurization of this batch, as mentioned above.

**Enzyme Activities during Cheese Ripening.** The amount of alkaline phosphatase determined in raw milk cheeses with no starter culture added showed a general trend to increase from January to June (Figure 4A), most likely due to the increase reported above for ewe's milk. Within each fabrication, and as ripening progressed, the levels of alkaline phosphatase were variable, in some cases decreasing by different amounts from 1 to 180 days, whereas in others, after a decrease at 90 days, levels rose again at 180 days. This apparent "reactivation" observed in some cases was most likely due to sample variation because of the following reasons: (1) none of the cheeses made with pasteurized milk showed any alkaline phosphatase activity except those made with improperly pasteurized milk (Figure 1A, second June fabrication), in which a low and constant level was observed throughout ripening (data not shown); (2) different cheeses (rather than samples of the same cheese) were analyzed at different ripening times. Therefore, it is reasonable to conclude that no microbial alkaline phosphatase was produced in these cheeses, as has been reported in some cases (Pratt-Lowe et al., 1987; Rosenthal et al., 1996) or that reactivation of the milk enzyme occurred (Lyster and Aschaffenburg, 1962; Linden, 1979). In cheeses made with raw milk and starter culture added (group R) the activity levels and the variability within each fabrication were not different from those reported for the control fabrication in Figure 4A. When the activity was measured at various distances from the outer rind to the center of the cheese, a slight decrease in activity was observed in the center, but it was not statistically significant. This was most likely due to the small cheese size and to the fact that the temperature is never >35 °C during the

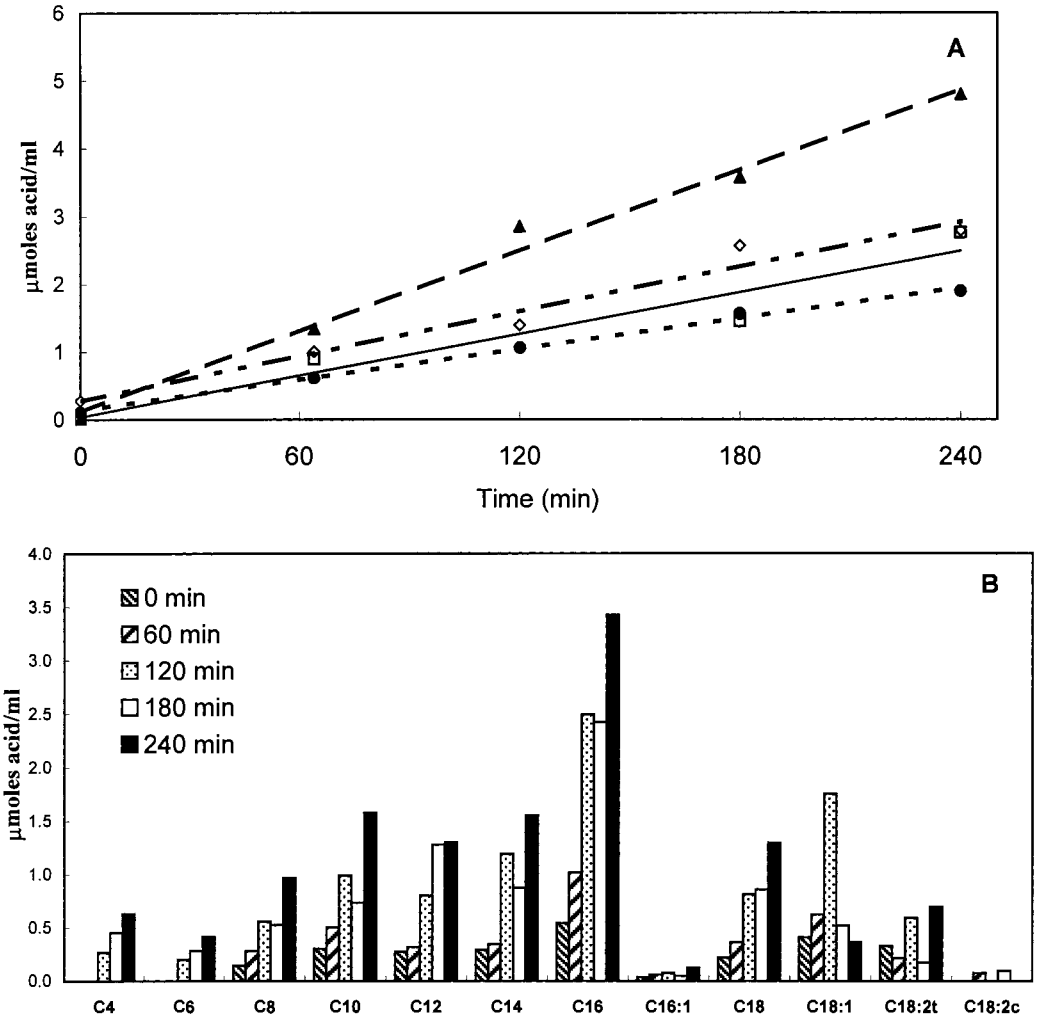


**Figure 3.** Lipoprotein lipase activity in industrial, raw milk with model substrates (A) and ewe's milk fat (B): tricaprilyn (▲); tricaprin (●); trilaurin (◇); olive oil (□).

manufacturing process, thus avoiding a partial thermal inactivation of the enzyme. By contrast, in Grana-Padano and Parmigiano-Reggiano cheeses (28 cm diameter), which are cooked at 53 °C, a decreasing gradient of alkaline phosphatase activity from the outer rind to the center of the piece has been found (Pellegrino et al., 1995; Resmini and Pellegrino, 1996). Alkaline phosphatase levels were unaltered in cheese samples kept at −80 °C for as long as 2 years.

The levels of acid phosphatase measured on the first day of ripening in raw milk cheeses were comparable regardless of the time of the year (Figure 4B). However, cheeses made in June exhibited a 2-fold increase in acid phosphatase activity between days 1 and 180, whereas those made in winter and spring showed more modest increases. Cheeses made with raw milk and starter culture added (group R) gave essentially the same results as the control group reported in Figure 4B. Cheeses made with pasteurized milk exhibited a similar pattern, except that the level of acid phosphatase activity in cheeses made in June was 17% lower than that reported for raw milk cheeses in Figure 4B. An increase in acid phosphatase activity during ripening could result in a higher degree of hydrolysis of the phosphopeptides (Andrews, 1991), which would be rendered more susceptible to proteolytic attack (Dulley and Kitchen, 1972). This is in agreement with the sensorial analysis of the raw milk cheeses, which correlated flavor defects in cheeses made in June with excessive proteolysis, whereas pasteurized cheeses with a starter culture added made in June had less proteolysis and significantly ($p < 0.05$) better sensorial scores (Mendía, 1998). The possibility that this increase in acid phosphatase is due to certain microbial populations is currently being investigated. Acid phosphatase activity remained constant in cheese samples kept at −80 °C during 2 years.

The levels of lactoperoxidase activity (Figure 4C) in day 1 cheeses closely reflected the levels found in the milk with which cheeses were made (Figure 1C). The activity of lactoperoxidase in cheeses made in winter and spring with raw milk was ≈1 order of magnitude higher than that in cheeses made in summer. Group R cheeses made with raw milk and added starter culture gave somewhat higher values for lactoperoxidase activity than those reported in Figure 4C, although the differences were not significant. However, in group P cheeses made with pasteurized milk the level of lactoperoxidase activity was an average 50% lower than in raw milk cheeses throughout lactation, in contrast with the relative thermal stability of this enzyme observed in pasteurized milk (Figure 1C). This could be due to a gradual loss of activity of the heat-treated enzyme during cheese-making when the enzyme is at temperatures between 20 and 38 °C for over 1.5 h. The fact that lactoperoxidase activity decreased by 50% in cheese

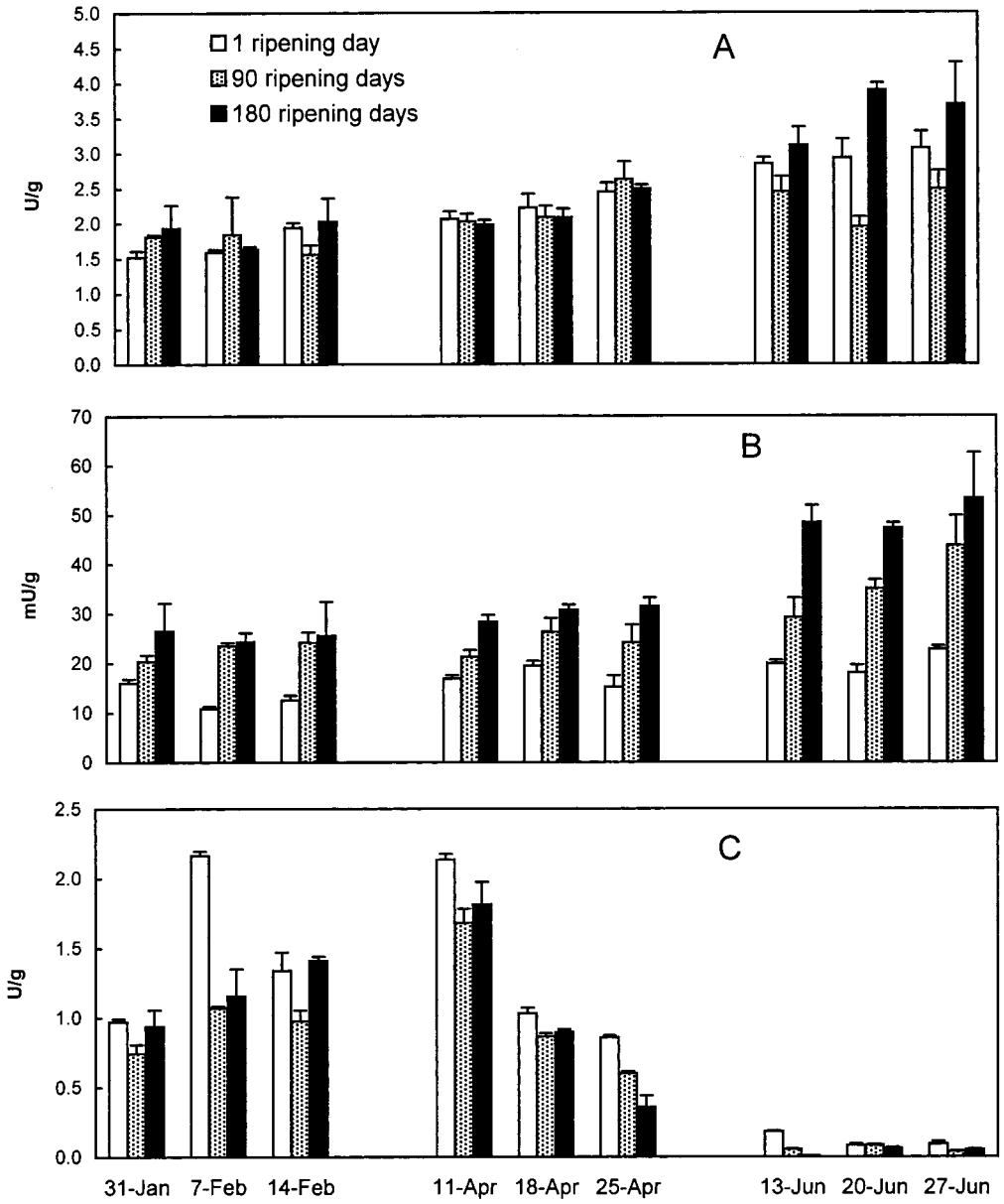


**Figure 4.** Enzyme activities during ripening of raw ewe's milk cheeses manufactured at the specified times in the lactation period: (A) alkaline phosphatase; (B) acid phosphatase; (C) lactoperoxidase. The bars correspond to, from left to right in each grouping, 1 day of ripening, 90 days of ripening, and 180 days of ripening.

samples kept at −80 °C after 5 months also indicates that this enzyme is not as stable as acid phosphatase despite its behavior during pasteurization.

Several attempts were made to determine both lipoprotein lipase and esterase activities in cheese extracts at various times during ripening. Results were inconclusive due primarily to the very low levels of each activity found, which were difficult to reproduce in a systematic manner. Lipoprotein lipase activity tended to be associated with the fat layer in variable amounts.

Results presented in this paper indicate that the level of alkaline and acid phosphatases, lactoperoxidase, and lipoprotein lipase in raw ewe's milk changed significantly ($p < 0.05$) between early and late lactation. Lactoperoxidase levels were sufficiently high in winter and spring to allow the activation of the lactoperoxidase system as a means of improving the bacteriological quality of the milk. This system could potentially be activated in raw milk cheeses made in winter and spring to improve their hygenic quality. Due to the much lower levels of lactoperoxidase found in pasteurized milk cheeses, or in raw milk cheeses made in summer, the activation of the lactoperoxidase system in these cases could be questionable. The increase in acid phosphatase levels in cheeses made in June needs to be further investigated to identify its origin in view of the apparent correlation with increased proteolysis and flavor defects. The observed increase in alkaline phosphatase levels in raw milk at the end of lactation did not cause positive responses to the alkaline phosphatase test in pasteurized cheese samples over the 180 day ripening period, except for a particular fabrication that was not properly pasteurized. Thus, pasteurization would inactivate this enzyme in ewe's milk, as expected, despite the higher levels found at the end of lactation.

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