ALKALINE PHOSPHATASE IN THE LACTATING BOVINE MAMMARY GLAND AND THE MILK FAT GLOBULE MEMBRANE. RELEASE BY PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C

Abstract—1. Alkaline phosphatase is covalently bound to bovine mammary microsomal membranes and milk fat globule membranes through linkage to phosphatidylinositol as demonstrated by the release of alkaline phosphatase following treatment with phosphatidylinositol-specific phospholipase C.

2. The release of alkaline phosphatase from the pellet to the supernatant was demonstrated by enzyme assays and electrophoresis.

3. Electrophoresis of the solubilized enzymes showed that the alkaline phosphatase of the microsomal membranes contained several isozymes, while only one band with alkaline phosphatase activity was seen in the fat globule membrane.

4. Levamisole and homoarginine were potent inhibitors of the alkaline phosphatase activities in both membrane preparations and in bovine liver alkaline phosphatase, but not in calf intestinal alkaline phosphatase.

INTRODUCTION

Alkaline phosphatase (EC 3.1.3.1) has been detected in the mammary gland of cows (Bailie and Morton, 1958) and rats (Leung *et al.*, 1989; Soloff *et al.*, 1980) and has been studied extensively in milk (Dapper *et al.*, 1987; Freudenstein *et al.*, 1979; Khodaparast-Sharifi and Snow, 1989; Malin and Basch, 1990; Zittle and Bingham, 1959; Zittle and Bingham, 1960). Bailie and Morton (1958) compared the enzymatic properties of alkaline phosphatase in cow's milk to those of mammary gland and concluded that the two enzymes were similar. On the other hand, several studies have shown that the enzymatic properties of the milk enzyme differ from those of the intestinal alkaline phosphatase (Zittle and Bingham, 1959; Morton, 1955). Two distinct forms of alkaline phosphatase occur in most mammalian species, a liver/bone/kidney form and an intestine form (Goldstein *et al.*, 1980). Mammary alkaline phosphatase in the rat has been classified as the liver/kidney/bone isozyme by Leung *et al.* (1989).

Alkaline phosphatase in the fat globule membrane is linked to phosphatidylinositol and can be released by incubation with phosphatidylinositol-specific phospholipase C (PI-PLC) (Malin and Basch, 1990). The present study deals with the release of alkaline phosphatase from mammary microsomes using PI-PLC. The enzymatic properties of the solubilized enzyme are compared with those of the alkaline phosphatase of bovine liver, calf intestine and the PI-PLC-treated enzyme from bovine fat globule membrane.

MATERIALS AND METHODS

Materials

Mammary glands were obtained from cows in full lactation through the cooperation of John Keys, Beltsville Agricultural Research Center. Following slaughter, the glands were trimmed of extraneous fat, cut into pieces (approximately 200 g), frozen, and stored at −80°C. Fresh, raw cream was obtained from a local dairy. PI-PLC purified from *Bacillus thuringiensis* was a gift from Dr Sidney Udenfriend, Roche Institute of Molecular Biology (Nutley, NJ). *p*-Nitrophenyl phosphate and other biochemicals were obtained from Sigma Chemical Company (St Louis, MO).

Preparation of membranes

Fat globule membranes were prepared from washed raw cream by extracting acylglycerols of the fat droplets with dimethyl sulfoxide using an adaptation of the method of Dapper *et al.*, 1987. Centrifugation at 90,000 *g* for 60 min and 2°C yielded pellets of fat globule membranes, which were stored at −20°C.

Mammary tissue was minced and mixed with 0.25 M sucrose at a concentration of 25% (w/v). All procedures were carried out at 4°C. The mixture was homogenized using a Polytron 10 ST homogenizer (Brinkmann, Westburg, NY), then squeezed through fine cheese cloth to remove unbroken cells and connective tissue. Pellets were prepared by successive centrifugations at 600 *g* for 10 min (nuclei), 12,000 *g* for 30 min (mitochondria) and 100,000 *g* for 60 min (microsomes and cytosol). The pellets were resuspended in 0.25 M sucrose and recentrifuged. The pellets were then suspended in 0.05 M Tris buffer, pH 7.5 at a volume approximately 10% of the original volume and then

homogenized, using a Potter–Elvehjem homogenizer. The subcellular fractions were stored at −20°C. To obtain a microsomal pellet, the fraction was thawed and centrifuged at 100,000 *g* for 1 hr.

PI-PLC treatment

The microsomal and fat globule membranes were homogenized in 0.05 M Tris buffer (pH 7.5) at a concentration of 8.8 mg/ml (based on solids content). The procedure of Malin and Basch (1990) was followed. Leupeptin (3 μM), pepstatin (3 μM) and phenylmethylsulfonyl fluoride (30 μM) were included in the homogenate to inhibit endogenous proteolysis. The membranes were incubated for 60 min at 37°C with 5 U/ml of PI-PLC. Identical samples without PI-PLC served as controls. After incubation, samples were centrifuged at 100,000 *g* for 1 hr at 2°C. Pellets were resuspended by homogenization with buffer volumes equivalent to those of the supernatant removed. Supernatants and pellets were analyzed for alkaline phosphatase, proteins and electrophoretic patterns.

Alkaline phosphatase activity

Alkaline phosphatase activity was measured at 37°C in a 1 ml reaction mixture containing 50 mM diethanolamine (pH 10.2), 100 mM NaCl, 2.5 mM $MgCl_2$, 5.0 mM *p*-nitrophenyl phosphate and alkaline phosphatase. Following an incubation period of 10 min, the reaction was stopped by the addition of 2 ml 0.5 N NaOH and the released *p*-nitrophenol was determined spectrophotometrically at 410 nm. The extinction coefficient of *p*-nitrophenol was taken to be 16,000 M^{-1} cm^{-1}. One unit of enzyme is defined as that amount that liberates 1 μmole of product per min.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Electrophoresis

Native polyacrylamide gel electrophoresis was performed witht the PhastSystem Pharmacia (Piscataway, NJ) using 8–25% gradient gels. Alkaline phosphatase on the gels was located by using 5-bromo-4-chloro-3-indolyl phosphate as the substrate and detecting the product with nitroblue tetrazolium (phosphatase conjugate substrate kit, Bio-Rad Laboratories, Richmond, CA).

RESULTS

Subcellular fractionation of alkaline phosphatase

The alkaline phosphatase activities in subcellular fractions of lactating bovine mammary gland are shown in Table 1. Although alkaline phosphatase activity was found in all subcellular fractions, the microsomal fraction yielded the largest amount of enzyme with the highest specific activity.

Table 1. Subcellular distribution of alkaline phosphatase in the mammary gland

Fraction	Yield (%)	Specific activity (units/mg protein)	Specific activity (ratio of each to homogenate)
Homogenate	100.0	0.61	1.0
Nuclei	1.5	0.72	1.2
Mitochondria	6.5	0.62	1.0
Microsomes	20.7	1.64	2.7
Cytosol	19.1	0.28	0.5

Subcellular fractions were prepared as described in Materials and Methods. Each sample was assayed for alkaline phosphatase activity and protein concentration.

Table 2. Release of alkaline phosphatase from fat globule membranes and mammary microsomes

	Alkaline phosphatase activity (total units)	Protein (mg)	Specific activity (units/mg)
Fat globule membranes	5.6	4.0	1.4
Control*			
Pellet	3.6	2.5	1.4
Supernatant	0.0	1.1	0.0
PI-PLC-treated			
Pellet	2.8	3.5	0.8
Supernatant	2.8	0.13	21.5
Mammary microsomes	10.9	5.7	1.9
Control*			
Pellet	13.9	5.8	2.4
Supernatant	0.2	0.4	0.5
PI-PLC-treated			
Pellet	1.8	3.0	0.6
Supernatant	6.8	0.55	12.4

*PI-PLC was omitted from the controls.

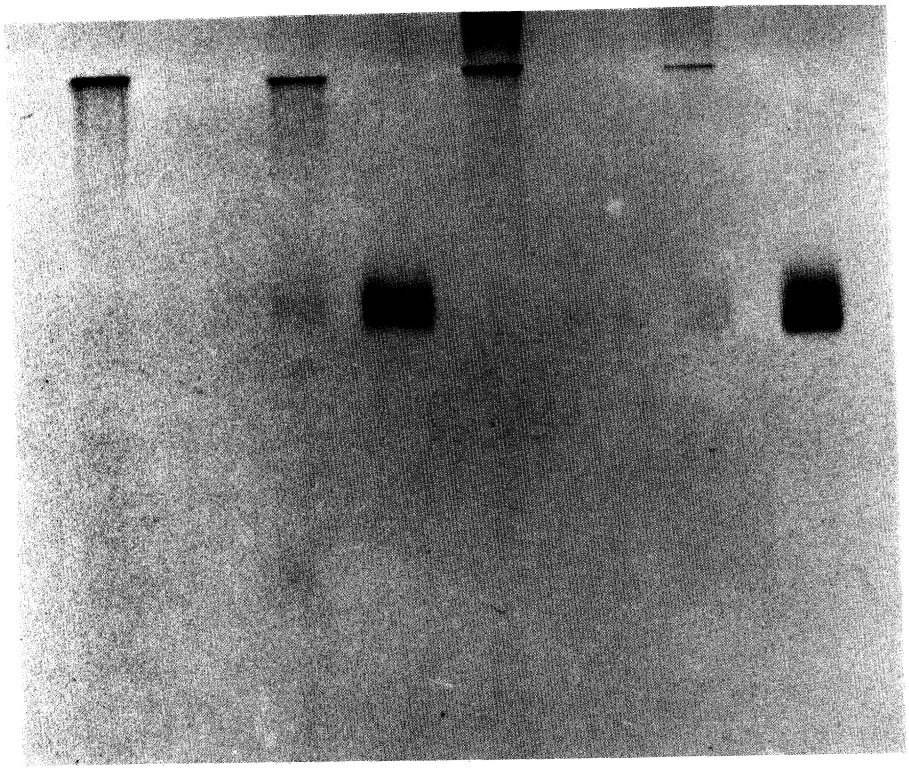
Fat globule membranes and mammary microsomal membranes were incubated with and without PI-PLC. Following an incubation period of 60 min at 37°C, the mixture was centrifuged. The pellet, the supernatant and the original preparation were tested for protein concentration and alkaline phosphatase activity. Details of the procedures are described in Materials and Methods.

Treatment of microsomes and fat globule membranes with PI-PLC

Previous research in this laboratory by Malin and Basch (1990) indicated that treatment of fat globule membranes with PI-PLC released alkaline phosphatase into the supernatant. Our results in Table 2 confirm this finding. In untreated fat globule membranes, all the alkaline phosphatase activity is found in the pellet. Following PI-PLC treatment, 50% of the alkaline phosphatase is released to the supernatant. Similar results were obtained with mammary microsomes except that 79% of the alkaline phosphatase was released to the supernatant. Several experiments confirmed that more alkaline phosphatase was released from the microsomes than from the fat globule membranes, following incubation with PI-PLC. Table 2 shows that relatively little protein was released from the membranes following PI-PLC treatment: 4% for the fat globule membranes and 15% for the mammary membranes. In both preparations, the specific activities of the PI-PLC-treated supernatants were 7–15-fold higher than the original membranes.

Electrophoresis

Samples in Table 2 were subjected to electrophoresis on native polyacrylamide gels and stained for alkaline phosphatase activity (Fig. 1). The membrane-bound enzymes were easily distinguished from those released from the pellet by PI-PLC treatment. In lanes F1 and M1, the alkaline phosphatases of the control pellets are bound to the membranes and remain in the stacking gel. The alkaline phosphatases in the supernatant from the PI-PLC-treated samples are soluble enzymes and move into the separating gel (lanes F4 and M4). Alkaline phosphatase activity was not detected in lanes F2 and M2 (control supernatants). Lanes F3 and M3 (the PI-PLC-treated pellets) show some residual membrane-bound



F1 F2 F3 F4 M1 M2 M3 M4

Fig. 1. Native electrophoresis (8–25% gradient) of fat globule membranes (lanes F1–F4) and mammary microsomes (lanes M1–M4). The lanes represent the control pellets (F1 and M1) and supernatants (F2 and M2) and the PI-PLC-treated pellets (F3 and M3) and supernatants (F4 and M4). Gels were stained for alkaline phosphatase activity as described under Materials and Methods.

alkaline phosphatase that was not released by PI-PLC treatment.

The differences between the two alkaline phosphatase preparations can be seen in lanes F4 and M4. The fat globule membrane enzyme (lane F4) is a single band, while the microsomal enzyme (lane M4) shows a broad band that probably represents two or more isozymes.

Effect of levamisole on alkaline phosphatase activity

Levamisole is a potent inhibitor of mammalian alkaline phosphatases found in liver, kidney and bone. However, it has little effect on calf intestinal enzyme (Harris, 1989; Van Belle, 1972). Figure 2 shows the effect of increasing concentrations of levamisole on four alkaline phosphatases. The PI-PLC-treated enzymes from fat globule membranes and microsomal membranes and the bovine liver enzyme were all inhibited by levamisole. The concentrations needed for 50% inhibition (I_{50}) were 30, 17 and 4 μM, respectively. Whether the differences in I_{50} values among the three enzymes are significant cannot be determined without further studies. However, the three alkaline phosphatases are all inhibited by levamisole while calf intestinal alkaline phosphatase is unaffected.

The inhibition by levamisole was confirmed by electrophoretic experiments. Figure 3A shows the alkaline phosphatase activity of PI-PLC-treated fat globule membranes (lane 1), phospholipase-treated microsomal membranes (lane 2), bovine liver alkaline phosphatase (lane 3), and calf intestinal alkaline phosphatase (lane 4). While both sides of the gel (A and B) were stained for alkaline phosphatase activity, the alkaline phosphatase incubation mixture for side

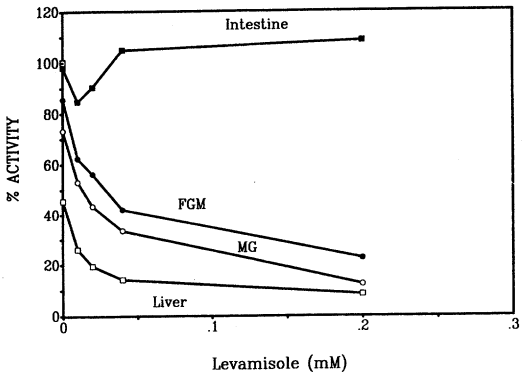


Fig. 2. Effect of levamisole concentration on the alkaline phosphatase activity of fat globule membranes (●), mammary gland microsomes (○), calf intestine (■) and bovine liver (□). Alkaline phosphatase activity was measured as described under Materials and Methods.

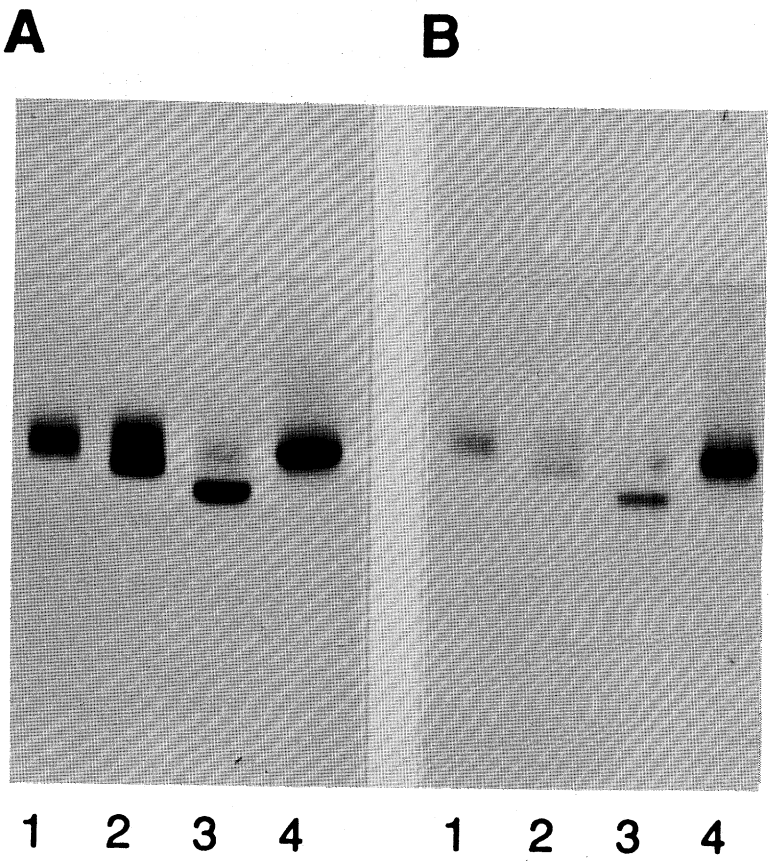


Fig. 3. Effect of levamisole on alkaline phosphatase activity in gels. Following electrophoresis, the gels were cut in half. Side A was stained for alkaline phosphatase activity. Side B was stained for alkaline phosphatase using an assay mixture containing 0.2 mM levamisole. Samples were PI-PLC-treated fat globule membrane (1), PI-PLC-treated microsomal membrane (2), bovine liver alkaline phosphatase (3), and calf intestinal alkaline phosphatase (4). The assay for detecting alkaline phosphatase on gels is described in Materials and Methods.

B contained 0.2 mM levamisole. Although some residual activity can be seen in the membrane preparations incubated with levamisole (1B and 2B), the bands were considerably lighter than the preparations without levamisole (1A and 2A). The intestinal enzyme was not affected by levamisole (4A and 4B), which confirms the previous experiment (Fig. 2). An interesting finding was that liver alkaline phosphatase (without levamisole) shows two bands (3B). When levamisole is added the slower band is abolished (3B). A possible explanation for the liver alkaline phosphatase activity remaining in the gel after levamisole treatment may be related to the substrate used. The gels were incubated with 5-bromo-4-chloro-3-indolyl phosphate, while in Fig. 2 *p*-nitrophenyl phosphate is used as the substrate. The electrophoresis experiments were repeated 4 times—with identical results.

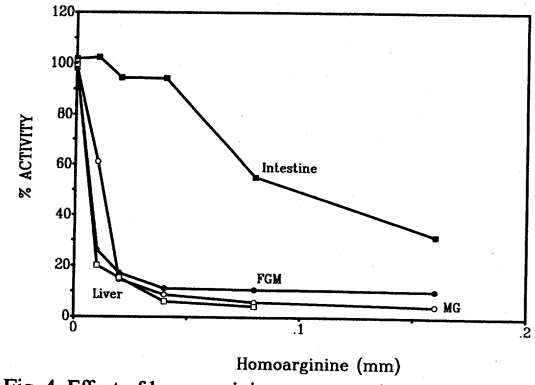


Fig. 4. Effect of homoarginine concentration on the alkaline phosphatase activity of fat globule membranes (●), mammary gland microsomes (○), calf intestine (■), and bovine liver (□). Assay conditions are described under Materials and Methods.

Effect of homoarginine on alkaline phosphatase activity

Figure 4 shows the effect of increasing concentrations of homoarginine on four alkaline phosphatases. Homoarginine inhibits the alkaline phosphatase from fat globule membranes, microsomes and liver to a much greater extent than the intestinal alkaline phosphatase. I_{50} values for the four enzymes are 5, 12, 5 and 75 μM, respectively.

DISCUSSION

Research on membrane structure has shown that alkaline phosphatase is one of the enzymes which is

linked to membranes by attachment to phosphatidylinositol (Ferguson and Williams, 1988; Kominami *et al.*, 1985; Low, 1987; Low and Saltiel, 1988). Release of the protein by PI-PLC is regarded as convincing evidence of the involvement of phosphatidylinositol in membrane anchoring (Low, 1987). By this criteria both fat globule membrane and microsomal alkaline phosphatases are linked to the membrane through phosphatidylinositol, since both enzymes were released by PI-PLC-treatment.

Fat gobule membranes are composed of plasma membrane (Patton and Keenan, 1975) plus an inner layer of unknown origin (Freudenstein *et al.*, 1979). The alkaline phosphatase released from fat globules shows one band on electrophoresis and is probably derived from the plasma membranes. Patton and Keenan (1975) pointed out that fat globule membrane enzymes with high specific activities, such as alkaline phosphatase, are associated with the mammary apical plasma membranes. On the other hand, the mammary microsomal membranes, which include organelles as well as plasma membranes, are more diversified; the appearance of multiple bands is not unexpected and indicates the presence of isozymes.

Levamisole is a potent inhibitor of the liver/kidney/bone alkaline phosphatases but has much less effect on the intestine enzyme (Van Belle, 1972). Enzyme assays (Fig. 2) and gel electrophoresis (Fig. 3) show that alkaline phosphatases of milk fat globule membranes and bovine mammary microsomal membranes are inhibited by levamisole and therefore resemble the liver alkaline phosphatase more than the intestinal enzyme. No inhibition of the calf intestinal enzyme was noted under the conditions used. On gel electrophoresis (Fig. 3) bovine liver alkaline phosphatase migrates farther than the other enzymes. The difference may be a function of how the liver alkaline phosphatase was isolated. Electrophoretic differences have been noted for the liver enzyme. Liver alkaline phosphatase, solubilized by butanol or deoxycholate, migrates farther than the enzyme released by PI-PLC (Malin and Basch, 1990). The substrate used in the assays of Fig. 2 was different from that used on the gel (Fig. 3). As stated previously, one liver isozyme on the gel was less affected by the levamisole and may be related to the fact that different substrates were used for the two experiments.

Mammary gland alkaline phosphatase was reported to be inhibited 74% by homoarginine at a 10 mM concentration (Colston *et al.*, 1988). Our results show homoarginine inhibition, but at much lower concentrations. Significantly, mammary and fat globule membrane alkaline phosphatases and liver alkaline phosphatase were inhibited by similar concentrations of homoarginine, while the calf intestinal enzyme required almost ten times as much homoarginine for a similar inhibition.

Our findings suggest that more than one alkaline phosphatase exists in the microsomes of lactating bovine mammary gland. Further research would be needed to localize the isozymes on specific membranes since the microsomes contain a mixture of endoplasmic reticulum, plasma membranes, ribosomes and other less defined particles. Histochemical studies of rat mammary gland have localized alkaline phosphatase on the basal secretory membrane, and the plasma membrane of myoepithelial cells as well as the outer membrane of nascent fat globules (Leung *et al.*, 1989). Thus the mammary microsomes may contain two distinct enzymes, one possibly associated with myoepithelial cells and one with secretory function.

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