

Purification and Properties of Alkaline Phosphatase in the Lactating Bovine Mammary Gland¹

ABSTRACT

Alkaline phosphatase has been purified 1400-fold from homogenates of lactating bovine mammary tissue. The purification procedure included subcellular fractionation, solubilization with butanol, fractionation with acetone, chromatography on concanavalin A-Sepharose, DEAE cellulose, DEAE-Sephadex, and gel filtration on Sephadex G-200. The enzyme activity was measured with the substrate *p*-nitrophenylphosphate in three buffers, and the maximum rate occurred at pH 10. For maximum activity, Mg²⁺ was required. Substrate specificity studies at three pH values indicated that the enzyme had broad specificity. It catalyzed the hydrolysis of aliphatic and aromatic phosphates and pyrophosphates, but the phosphoprotein β -casein was a poor substrate. Potent inhibitors of the enzyme were levamisole and sulfhydryl reagents (2-mercaptoethanol, dithiothreitol, and cysteine).

(Key words: enzymes, milk, secretion)

Abbreviation key: I₅₀ = concentration for 50% inhibition, *p*-NPP = *p*-nitrophenylphosphate.

INTRODUCTION

Alkaline phosphatase (EC 3.1.3.1) in mammary gland tissue was first observed more than 50 yr ago. Bailie and Morton (1) compared the mammary gland alkaline phosphatase to the

milk enzyme and concluded that both enzymes were associated with a microsomal fraction and were probably similar enzymes. Subsequently, the enzyme has been found in the mammary gland of rat, cow, cat, rabbit, goat, mare, dog, sheep, and human (14). Histological studies of the rat mammary gland provide evidence that alkaline phosphatase is associated with the plasma membranes of the myoepithelial cells, basal and lateral membranes of epithelial cells, and endothelial cells (4, 10, 16). Recently, Bingham and Malin (2) showed that alkaline phosphatase is covalently bound to mammary microsomal membranes through linkage to phosphatidylinositol. This type of attachment has also been found for alkaline phosphatase in the milk fat globule membrane (13) and in a variety of tissues and species (6, 11).

This paper deals with the biochemical characterization of alkaline phosphatase from the bovine lactating mammary gland. Our ultimate goal is to understand the role of alkaline phosphatase in lactation and the derivation of the enzyme in milk.

MATERIALS AND METHODS

Materials

Whole mammary glands from cows of known good health and productivity were obtained from cows in full lactation through the cooperation of John Keys, Beltsville Agricultural Research Center (Beltsville, MD). Following slaughter, the glands were trimmed of extraneous fat, cut into pieces (approximately 200 g), frozen, and stored at -80°C.

Sephadex G-200 and DEAE Sephadex were purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ); DEAE cellulose (DE 52) was from Whatman Chemical Separation

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¹Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

tion, Inc. (Clifton, NJ). 1-Butanol was obtained from Aldrich Chemical Co. (Milwaukee, WI). The substrates and buffers were from Sigma Chemical Co. (St. Louis, MO).

Alkaline Phosphatase

Alkaline phosphatase activity was measured at 37°C in a 1-ml reaction mixture containing 50 mM diethanolamine (pH 10.4), 50 mM NaCl, 2.5 mM MgCl₂, 5.0 mM *p*-nitrophenylphosphate (*p*-NPP) and alkaline phosphatase. Following an incubation period of 10 min, the reaction was stopped by the addition of 2 ml of .5 M NaOH, and the release of *p*-nitrophenol was determined spectrophotometrically at 410 nm. When phosphate esters other than *p*-NPP were used as substrates, phosphatase activity was determined by the phosphate released in a .5-ml aliquot of the incubation mixture. The phosphate was measured spectrophotometrically at 630 nm by the Malachite green reagent using the procedure of Chan et al. (3). A unit of enzyme was defined as the amount that liberated 1 μmol/min of product (either *p*-nitrophenol or phosphate).

Protein Determination

Protein concentration was determined by the method of Lowry et al. (12) using bovine serum albumin as the standard.

Subcellular Distribution

Subcellular fractions were prepared from frozen mammary tissue. Results obtained from frozen tissue were similar to those obtained from fresh tissue (data not shown). All procedures were carried out at 4°C. The tissue (100 g) was minced, suspended in 3 volumes of .25 M sucrose, and homogenized for 1 min (high speed) using a Polytron 10 ST homogenizer (Brinkmann, Westburg, NY). The homogenate was squeezed through fine cheesecloth 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 suspended in .25 M sucrose and recentrifuged. The washed pellets were suspended in .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 pellets and cytosol were tested for alkaline phosphatase activity.

Enzyme Preparation

Alkaline phosphatase was prepared by a procedure based on the method of Trepanier et al. (18). All operations were carried out at 4°C unless specified. Mammary tissue (500 g) was cut into small pieces and homogenized with a polytron for 1 min at high speed in 1 L of .25 M sucrose. The crude extract was filtered through coarse and then fine cheesecloth and centrifuged at 600 × *g* for 15 min. The resulting supernatant solution was filtered through cheesecloth to remove the fat and centrifuged at 12,000 × *g* for 30 min. Butanol (750 ml) at -20°C was added slowly to the supernatant solution, and the mixture was stirred for 30 min, followed by centrifugation at 10,000 × *g* for 30 min. Approximately 1 L of aqueous liquid was recovered. Acetone at -20°C was added to the solution while stirring to give a final concentration of 30% (vol/vol). The mixture was stirred for an additional 15 min and then centrifuged at 10,000 × *g* for 20 min. The supernatant solution was adjusted to 50% (vol/vol) acetone by the slow addition of more cold acetone. After centrifugation (10,000 × *g* for 30 min), the pellet was suspended in 60 ml of 100 mM Tris (pH 7.6), 1 mM MgCl₂, .02 mM ZnCl₂, 100 mM NaCl, 1 mM MnCl₂, and 1 mM CaCl₂ (buffer A).

The suspension of the acetone pellet (30 to 50%) was centrifuged and then applied to a column (2.5 × 10 cm) of concanavalin A-Sepharose that had been equilibrated with buffer A. The column was washed with buffer to elute fractions with absorbance at 280 nm. The alkaline phosphatase was eluted with 500 ml of buffer A containing mannose from 0 to .1 M at a flow rate of 60 ml/h. The samples with alkaline phosphatase activity were pooled and concentrated by ultrafiltration in a Filtron stirred cell (Filtron Technology Corp., Northborough, MA) using the Filtron Omega series membrane (nominal molecular weight limit of 80,000 Da). The enzyme solution was equilibrated with 10 mM Tris-HCl (pH 7.6), .1 mM MgCl₂, and .02 mM ZnCl₂ (buffer B) by repeated dilution and concentration.

TABLE 1. Subcellular distribution of alkaline phosphatase from bovine lactating mammary gland.

Fraction	Yield (%)	Specific activity of protein (U/mg)	Specific activity ratio ¹
Homogenate	100	.031	1.0
Nuclei	1.5	.037	1.2
Mitochondria	6.5	.032	1.0
Microsomes	20.7	.084	2.7
Cytosol	19.1	.014	.5

¹Ratio of each fraction to homogenate.

Following ultrafiltration, the alkaline phosphatase was applied to a DEAE-cellulose column (1.0 × 30) equilibrated with buffer B. Two column volumes of buffer B were pumped through the column. The enzyme was then eluted with 500 ml of buffer B, containing NaCl in a linear gradient from 0 to .2 M at a flow rate of 40 ml/h. The fractions containing alkaline phosphatase were combined and concentrated to 5 ml by ultrafiltration as described.

The alkaline phosphatase was filtered through a Sephadex G-200 column (1.5 × 90 cm) equilibrated with 100 mM Tris·HCl (pH 7.6), 100 mM NaCl, .1 M MgCl₂, and .02 mM ZnCl₂. The alkaline phosphatase fractions were combined and equilibrated with 10 mM MES [2-(N-morpholino) ethanesulfonic acid] 100 mM NaCl, .1 mM MgCl₂, .02 mM ZnCl₂, pH 6.0, (buffer C) by repeated dilution and concentration by ultrafiltration in a Filtron stirred cell. The ultrafiltered sample (6 ml) was ap-

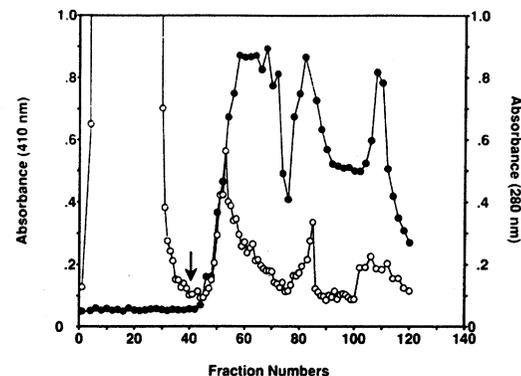


Figure 1. Chromatography of alkaline phosphatase on concanavalin A-Sepharose. Enzyme activity (O) and protein (●) were monitored at 410 and 280 nm, respectively. The arrow indicates the start of the linear gradient of mannose from 0 to .1 M.

plied to a column (1.5 × 15 cm) of DEAE-Sephadex equilibrated with buffer C. The alkaline phosphatase was recovered in the void volume, reduced to 4 ml by ultrafiltration, and stored at 4°C.

Electrophoresis

Native PAGE was performed with the PhastSystem (Pharmacia LKB Biotechnology, Inc.) using a 10 to 15% gradient gel. Alkaline phosphatase on the gel 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).

TABLE 2. Purification of alkaline phosphatase from bovine lactating mammary gland.

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Homogenate	14,400	596	.04	100	1
Supernatant (12,000 × g)	5500	276	.05	46	1
Butanol extract	1500	187	.12	31	3
Acetone precipitate (30 to 50%)	600	117	.19	20	5
Concanavalin A-Sepharose	34	47	1.38	8	34
DE-52 Cellulose	3.87	33.0	8.54	6	208
Sephadex G-200	.84	17.5	20.8	3	506
DEAE Sephadex G-50	.21	12.1	57.5	2	1402

RESULTS AND DISCUSSION

Subcellular Distribution of Alkaline Phosphatase

The alkaline phosphatase activity in subcellular fractions of lactating bovine mammary gland is shown in Table 1. Because most of the alkaline phosphatase was in the microsomes and cytoplasm, these fractions were used for further purification. The microsomal fraction yielded the enzyme with the highest specific activity.

Enzyme Preparation

Table 2 summarizes a typical purification procedure for the alkaline phosphatase of bo-

vine mammary gland. Approximately .2 mg of protein was recovered from 500 g of mammary tissue. Although the activity increased 1400-fold, the enzyme was not homogeneous by native PAGE electrophoresis. The supernatant solution obtained at $12,000 \times g$ contained only 46% of the alkaline phosphatase in the homogenate. The low recovery suggests that considerable activity resides in the nuclear and mitochondrial fractions and fat particles.

Affinity chromatography of the acetone precipitate (30 to 50%) on concanavalin A-Sepharose yielded three peaks of alkaline phosphatase activity (Figure 1). Because concanavalin A has a specific affinity for glycoproteins, only alkaline phosphatase and other glycoproteins were retained by the con-

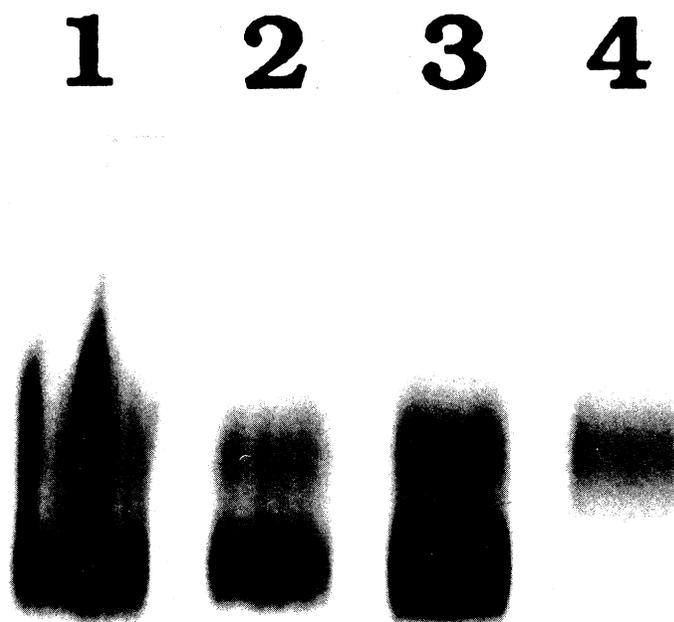


Figure 2. Native electrophoresis (10 to 15% gradient) of alkaline phosphatase fractions. The lanes represent the acetone precipitate that was applied to the concanavalin A-Sepharose column (1), peak 1 (2), peak 2 (3), peak 3 (4), which were eluted from the column with a mannose gradient.

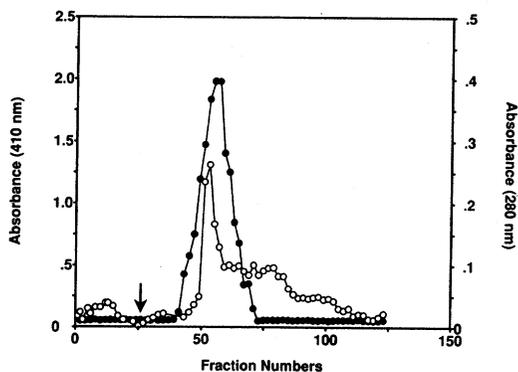


Figure 3. Chromatography of alkaline phosphatase on DEAE cellulose. Enzyme activity (○) and protein (●) were monitored at 410 and 280 nm, respectively. The arrow indicates the start of the linear gradient of NaCl from 0 to 0.2 M.

canavalin A-Sepharose column. The three alkaline phosphatase peaks were examined on electrophoresis (Figure 2) by staining for activity. Native gel (10 to 15%) revealed two activities. The faster moving component predominated in peaks 1 and 2 and in the material applied to the column (lanes 1, 2, and 3 in Figure 2). Peak 3, which represented less than 10% of the total alkaline phosphatase, contained only the slower moving component (lane 4, Figure 2). Because electrophoresis showed no clear separation of the isozymes

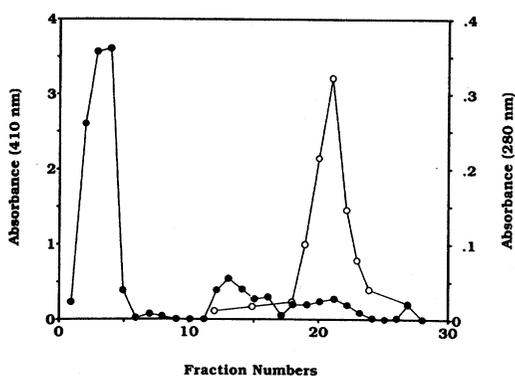


Figure 4. Gel filtration of alkaline phosphatase on Sephadex G-200. Enzyme activity (○) and protein (●) were monitored at 410 and 280 nm, respectively.

except for the third peak (representing only 10% of the total), the three peaks of alkaline phosphatase were combined.

Chromatography on DEAE-cellulose (Figure 3) resulted in a 6-fold increase in purity. Gel filtration on Sephadex G-200 separated the alkaline phosphatase peak from two protein peaks with a 500-fold overall increase in purity (Figure 4). Alkaline phosphatase was then chromatographed on a DEAE-Sephadex column at pH 6.0 and was eluted at the void volume, yielding a 3-fold increase in specific activity. This procedure resulted in an overall purification of 1400; the purification procedure was a modification of that followed by Trepanier et al. (18), who purified human liver alkaline phosphatase. Because the proteins in human liver differ from those of bovine mammary, the profiles in the chromatography show different elution patterns.

Effect of pH on Alkaline Phosphatase Activity

Figure 5 illustrates the effect of pH on alkaline phosphatase activity. The highest activity was obtained in ethanolamine buffer (pH 8.2 to 10.49). Over this pH range, the activity increased but reached no maximum. In CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer, activity was maximum between pH 10.5 and 11.1. The maximum activity in diethanolamine buffer occurred from pH 10.1 to 10.7. The optimal pH for alkaline phosphatase

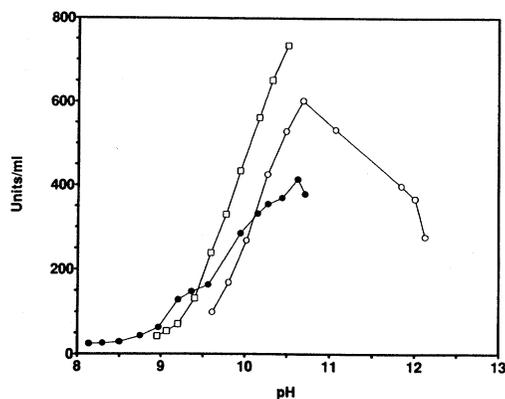


Figure 5. Effect of pH on alkaline phosphatase activity. Buffers were 50 mM ethanolamine (□), 50 mM diethanolamine (●), and 50 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] (○)

TABLE 3. Effect of divalent cations on alkaline phosphatase activity

Divalent metal ion concentration (mM)	MgCl ₂	ZnCl ₂	CaCl ₂	MnCl ₂	CoCl ₂
0	100	100	100	100	100
.01	112	18	100	165	115
.1	125	20	94	298	167
1	178	7	100	...	197

¹MgCl₂ (2.5 mM) was omitted from the assay mixture, and the indicated divalent cations were added.

tase activity is affected by a number of variables: the type of buffer, the specific substrate used, the concentration of the substrate, and the stability of the enzyme at high pH.

Effect of Divalent Metal Ions on Alkaline Phosphatase Activity

Table 3 shows the effect of divalent metal ions on activity. Activation of alkaline phosphatase is observed with Co²⁺, Mn²⁺, and Mg²⁺, whereas Zn ions are inhibitory. Calcium ions have little effect on activity. These results agree with studies of various tissue alkaline phosphatases (7).

Substrate Specificity

The release of phosphate from a number of substrates was measured at pH 10.1, 9.0, and 8.1 (Table 4). The highest activities were ob-

tained at pH 10.1 with four substrates containing aromatic groups: α -naphthyl phosphate, *p*-NPP, methylumbelliferyl phosphate, and phosphotyrosine. The alkaline phosphatase hydrolyzed 10 substrates with maximum activity at pH 9.0. β -Casein, which is a poor substrate, showed maximum hydrolysis at pH 8.1. The rate of hydrolysis of *p*-NPP is 400 times the rate obtained for β -casein. Results from Table 4 indicate that alkaline phosphatase catalyzes the hydrolysis of the pyrophosphatases ATP, ADP, and thiamine pyrophosphate. Several reports confirm that mammalian alkaline phosphatase has pyrophosphatase activity (5, 19, 20).

Inhibition

The concentrations for 50% inhibition (I_{50}) were determined by testing the activity in the presence of varying amounts of inhibitor and

TABLE 4. Relative reaction rates of alkaline phosphatase for substrates at various pH.¹

Substrate	pH 10.1	pH 9.0	pH 8.1
α -Naphthyl acid phosphate	174.5	13.9	1.2
<i>p</i> -Nitrophenylphosphate ²	100.0	7.7	3.5
4-Methylumbelliferyl phosphate	82.9	6.4	0
<i>o</i> -Phospho-DL-tyrosine	47.2	34.6	2.5
Adenosine-5'-triphosphosphate	25.4	33.6	24.3
Adenosine-5'-phosphate	18.8	42.4	6
Thiamine pyrophosphate	8.9	36.5	6.6
Pyridoxal-5'-phosphate	8.7	23.8	5.4
<i>o</i> -Phospho-DL-threonine	8.5	46.2	1.5
Adenosine-5'-diphosphate	6.4	32.4	8.7
Uridine-5'-monophosphate	6.1	38.1	1.9
<i>o</i> -Phospho-ethanolamine	5.3	25.3	8.9
<i>o</i> -Phospho-DL-serine	3.7	37.6	7.9
Ribose-5'-phosphate (barium)	1.4	15.2	7.1
β -Casein A ³	.02	.13	.25

¹Concentration of substrates was .5 mM, and the diethanolamine buffer was 50 mM.

²*p*-Nitrophenylphosphate at pH 10.1 was considered to be 100, and other activities were relative.

³ β -Casein concentration was .2 mM.

TABLE 5. The I_{50} values¹ of various inhibitors of alkaline phosphatase.

Inhibitor	I_{50} (mM)
Levamisole	.018
β -Mercaptoethanol	.023
DL-Cysteine	.048
Dithiothreitol	.143
NaF	No effect

¹Concentration of inhibitor for 50% inhibition.

extrapolating to 50% inhibition. Table 5 lists I_{50} values for several compounds. Sulfhydryl reagents (dithiothreitol), mercaptoethanol, and cysteine) at micromolar concentrations are effective inhibitors of alkaline phosphatase activity. The three peaks from the concanavalin A-Sepharose column were also inhibited more than 50% by 240 μ M cysteine (data not shown). Although the mechanism for this inhibition is not known, it has been suggested that these compounds chelate the Zn^{2+} that is an integral part of the alkaline phosphatase molecule (7).

Levamisole, an anthelmintic drug, was a potent inhibitor of alkaline phosphatase with an I_{50} value of 18 μ M. In addition, the three alkaline phosphatase peaks from the concanavalin A-Sepharose column were inhibited more than 70% by 90 μ M levamisole. Recent studies (9) showed that alkaline phosphatase, but not 5'-nucleotidase in milk fat globule membranes, is inhibited by levamisole. On the basis of levamisole inhibition, the alkaline phosphatase of bovine mammary gland can be classified as a bone-liver-kidney enzyme. Van Belle (19, 20) showed that rat and canine alkaline phosphatases from kidney, bone, placenta, heart, and liver were inhibited by levamisole, whereas the intestinal enzyme was unaffected.

A potent inhibitor of acid phosphatases, NaF (40 mM) has no effect on alkaline phosphatase.

CONCLUSIONS

Alkaline phosphatases from various mammalian tissues and bacteria have been studied extensively, but their physiological function remains unknown. Swarup et al. (17) provided

evidence that alkaline phosphatase dephosphorylates proteins containing phosphotyrosine and is therefore involved in the regulation of phosphorylation-dephosphorylation reactions. A role in bone calcification has also been suggested (14).

In the mammary gland, myoepithelial cells contain most of the alkaline phosphatase (10), which might suggest a function related to the milk ejection process. In comparison, much less activity is found in epithelial cells and in milk. The alkaline phosphatase in milk could be derived from cells that are sloughed off, whereas the origin of alkaline phosphatase in the fat globule membrane of cream may originate from a different source. Our results could support the concept of two alkaline phosphatases in milk, because two enzymes are present in the mammary gland (Figure 2). Previous research also indicated that mammary microsomes show two isozymes on electrophoreses; the slower moving band resembles the alkaline phosphatase from the fat globule membrane (2).

Several isozymes of alkaline phosphatase have been recognized: the intestinal enzyme, the human placental enzyme, and the isozyme found in bone, liver, and kidney and in most other tissues (8, 15). Based on the inhibition by levamisole and sulfhydryl reagents, the mammary alkaline phosphatases can be categorized as bone-liver-kidney enzymes. Histochemical evidence (10) suggests that alkaline phosphatase in secretory cells is associated in an inactive form with the interfacial layer of milk lipid droplets and milk fat globule membrane. Future research, particularly with regard to the association of alkaline phosphatase with myoepithelial cells, should delineate the role of alkaline phosphatase in lactation research.

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