

Purification and Properties of an Acid Phosphatase from Lactating Bovine Mammary Gland

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ABSTRACT

An acid phosphatase was partially purified from the cytosol of lactating bovine mammary gland by precipitation with ammonium sulfate and protamine, chromatography on carboxymethyl cellulose, and gel filtration on Sephadex G-75. The enzyme hydrolyzed aromatic phosphates but was less active toward alkyl phosphates, ATP, and phosphoproteins (casein and phosvitin). A sulfhydryl group seems to be essential for activity, since dithiothreitol and cysteine activated the enzyme; compounds that react with the sulfhydryl groups in proteins were inhibitory. Orthovanadate, phosphate, and zinc ions also inhibited the phosphatase. (Key words: acid phosphatase, mammary gland, lactation)

INTRODUCTION

Protein phosphorylation and dephosphorylation play important roles in cellular metabolism (2). Although some effort has been directed at characterizing the protein kinases in mammary gland (3, 10), relatively little is known about the phosphatases. A recent study compared an acid phosphatase in bovine lactating mammary gland to a similar enzyme in bovine spleen (8). Both enzymes acted on aromatic phosphates, casein, and ATP. Because these enzymes hydrolyzed phosphoproteins but not phosphomonoesters, they were designated as phosphoprotein phosphatases. The spleen enzyme has been purified to homogeneity (7). It is a purple molecule containing 2 atoms of iron

per molecule of enzyme. The phosphatase has a molecular weight of 40,000 and consists of two subunits. Although this enzyme has been studied extensively, its function has not been determined.

A similar enzyme has been found in bovine milk and has been characterized by several investigators (1, 4, 5), who showed that it resembles the phosphoprotein phosphatase of spleen. Both enzymes are active with the same type of substrates and are activated by ascorbic acid and other reducing agents. The object of this investigation was to reexamine the acid phosphatases of lactating mammary tissue with the ultimate objective of understanding their role in the regulation of mammary metabolism.

MATERIALS AND METHODS

Materials

β -Casein A was purified from the milk of an individual cow (9). *p*-Nitrophenyl phosphate, dicyclohexylammonium salt (pNPP) was purchased from Calbiochem-Behring.¹ Other substrates were from Sigma Chemical Company, St. Louis, MO. Carboxymethyl cellulose, CM-32, (CM-cellulose) was purchased from Whatman Lab Sales, Inc., Hillsboro, OR. Sephadex G-75 was from Pharmacia LKB Biotechnology, Piscataway, NJ.

Preparation of Phosphatases

Mammary glands were obtained from cows in full lactation through the cooperation of John Keyes from the Beltsville Agricultural Research Center. Following slaughter, the glands were trimmed of extraneous fat, cut into pieces (approximately 300 g), and stored at -80°C until needed. Mammary tissue (200 g) was cut into small pieces and combined with three volumes of .25 M sucrose containing the following protease inhibitors: 400 $\mu\text{g/L}$ of pepstatin, leupeptin, and antipain; 100 $\mu\text{g/L}$ of soybean

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¹Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

antitrypsin; .01 mM Na-*p*-tosyl-L-lysine chloromethyl ketone; .01 mM-tosyl-L-phenylalanine chloromethyl ketone; and .1 mM phenylmethyl-sulfonyl fluoride. The mixture was homogenized with a Polytron (Brinkmann, Westbury, NY) at full speed for 1 min, filtered through one layer of fine cheesecloth, and centrifuged for 15 min at $600 \times g$. The supernatant solution was filtered again through cheesecloth to remove the fat and centrifuged for 30 min at $12,000 \times g$. The resultant supernatant was centrifuged for 1 h at $100,000 \times g$. All operations were performed at 0 to 4°C.

The high speed supernatant was further purified. Solid ammonium sulfate was added slowly to a final concentration of 35%. After standing for 15 min, the mixture was centrifuged for 20 min at $10,000 \times g$. More ammonium sulfate was added to the supernatant to obtain a concentration of 70%, and the mixture was centrifuged for 20 min at $10,000 \times g$. The precipitate was dissolved in approximately 60 ml of the homogenation solution and then treated with 5 ml of 2% protamine sulfate (adjusted to pH 6.0). After 10 min, the solution was centrifuged for 15 min at $10,000 \times g$. The precipitate was discarded and the supernatant was dialyzed overnight against 4 L of .05 M of sodium acetate buffer, pH 5.0 (buffer A), with one change. The dialysate was clarified by centrifugation.

The enzyme solution was applied to a CM-cellulose column (2.5×20 cm), which had been equilibrated with buffer A, pH 5.0. The column was washed with buffer until the 280-nm readings of the eluate reached a baseline value. The enzyme was eluted with a linear salt gradient of 0 to .4 M KCl in buffer A with a total elution volume of 200 ml. Fractions of 3.5 ml were collected and monitored for phosphatase activity and protein concentration. The fractions containing acid phosphatase were pooled and concentrated in an Amicon ultrafiltration system (Amicon, Danvers, MA) with a PM-10 membrane. The enzyme was further purified on a Sephadex G-75 gel filtration column (2.5×88 cm) in buffer A containing .2 M KCl. The fractions with phosphatase activity were combined and reduced in volume by ultrafiltration. The enzyme was stored frozen in 50% glycerol.

Phosphatase Assays

Acid phosphatase activity was measured in a 1-ml reaction mixture containing 10 mM pNPP and 50 mM sodium acetate, pH 5.5, at 37°C. Assays were initiated with the addition of enzyme and terminated by addition of 2 ml of .5 M NaOH. Corrections were made for the non-enzymatic hydrolysis of pNPP. The amount of product, *p*-nitrophenol, was calculated from the absorbance at 410 nm using the molar extinction coefficient of $17,500 M^{-1}cm^{-1}$. Under the assay conditions used, acid phosphatase activity increased linearly with the amount of added enzyme and with the time of incubation. A unit of nitrophenyl phosphatase activity is defined as the amount of enzyme that releases 1 nmol of *p*-nitrophenol per minute.

When substrates other than pNPP were used, the acid phosphatase activity was measured by determining the phosphate released. The reaction was stopped by the addition of .2 ml 50% TCA, followed by centrifuging if needed. The phosphate was determined on a 1 ml aliquot by the method of Sumner (12). To each 1 ml of aliquot was added .3 ml of 7.5 N H₂SO₄, .3 ml of 6.6% ammonium molybdate, and 1.4 ml of freshly prepared ferrous sulfate (842 mg FeSO₄·7H₂O and .17 ml 7.5 N H₂SO₄ in 50 ml of water). The amount of phosphate was calculated from the absorbance at 650 nm using the molar extinction coefficient of $1295 M^{-1}cm^{-1}$.

Protein Estimation

Proteins were determined with the BCA protein assay reagent (Pierce Chemical Company, Rockford, IL); bovine serum albumin was used as the standard.

RESULTS

Purification of Mammary Phosphatase

A summary of the purification procedure is given in Table 1. The subcellular fractionation resulted in a yield of 47% in the cytoplasm. The remaining phosphatase activities were found in the pellets. The cytoplasmic enzyme was further purified by precipitation with (NH₄)₂SO₄ followed by precipitation with protamine. Chromatography of the protamine fraction on CM-cellulose yielded two peaks (Figure 1). The major peak was further purified by gel filtration on Sephadex G-75 (Figure 2). At this

TABLE 1. Purification of acid phosphatase from lactating bovine mammary gland.

| Fraction | Volume (ml) | Total protein (mg) | Total activity (units $\times 10^{-3}$) | Specific activity (units/mg) | Yield (%) |
|---|----------------|--------------------------|--|------------------------------------|--------------|
| Crude extract | 716 | 12,458 | 16.6 | 1.3 | 100 |
| Supernatant | | | | | |
| 600 \times g | 650 | 10,595 | 14.8 | 1.4 | 89 |
| 12,000 \times g | 630 | 10,017 | 8.6 | .9 | 52 |
| 100,000 \times g | 592 | 6453 | 7.8 | 1.2 | 47 |
| (NH ₄) ₂ SO ₄ , 35 to 70% | 620 | 4464 | 4.7 | 1.0 | 28 |
| Protamine treated | 85 | 680 | 3.3 | 4.9 | 20 |
| CM-Cellulose | 48 | 223 | 3.0 | 13.5 | 18 |
| Sephadex G-75 | 32 | 48 | 1.7 | 35.4 | 10 |

stage, the enzyme was very labile and lost considerable activity in a day at refrigeration temperatures. Activity could be retained by storing the phosphatase in 50% glycerol at 0°C.

Substrate Specificity

A survey of the substrates hydrolyzed by the purified enzyme is summarized in table 2. The enzyme showed a strong preference for aromatic phosphates (methylumbelliferylphosphate, pNPP, phosphotyrosine, and α -naphthyl acid phosphate). The pyrophosphates (ATP and ADF) and the phosphomonoesters (AMP, phosphoserine, phosphoethanolamine, and phosphothreonine) were hydrolyzed at a much lower

rate. The phosphoproteins, phosvitin and β -casein, were poor substrates compared to pNPP.

Effect of pH on Activity

The effect of pH on pNPP activity is shown in Figure 3. Maximum activity occurred between pH 5.5 and 6.2. The activity declined sharply at pH below and above these values.

Effect of Inhibitors on Activity

Micromolar amounts of N-ethylmaleimide and *p*-chloromercuribenzoate (PCMB) inhibited

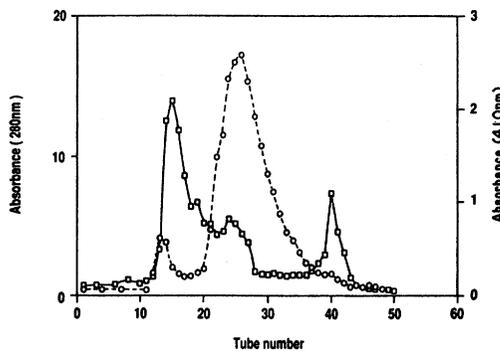


Figure 1. Separation of phosphatase by chromatography on CM-cellulose. The proteins were eluted with a linear salt gradient of 0 to .4 M KCl in .05 M acetate buffer, pH 5.0. Proteins were monitored by the absorbance at 280 nm (\square) and phosphatase was measured by the absorbance at 410 nm (O) with *p*-nitrophenyl phosphate as the substrate.

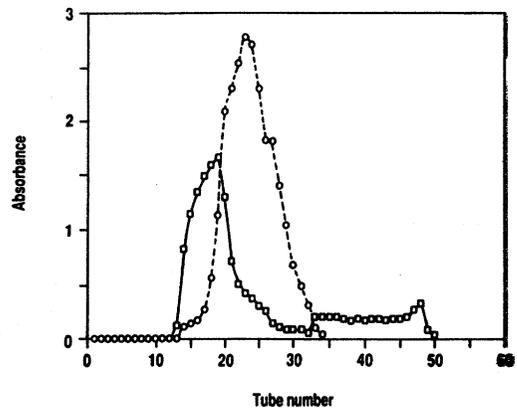


Figure 2. Gel filtration on Sephadex G-75 in .2 M KCl, .05 M acetate buffer, pH 5.0. Absorbance was measured at 280 nm to detect protein (\square) and at 410 nm for phosphatases (O) with *p*-nitrophenyl phosphate as the substrate.

TABLE 2. Substrate specificity of acid phosphatase.

| Substrate | Activity relative to <i>p</i> -nitrophenyl phosphate (%) |
|-----------------------------------|--|
| Methylumbelliferylphosphate | 159 |
| <i>p</i> -Nitrophenyl phosphate | 100 |
| Phosphotyrosine | 78 |
| α -Naphthyl acid phosphate | 61 |
| ADP | 14 |
| Phosvitin ¹ | 11 |
| AMP | 10 |
| <i>o</i> -Phosphoethanolamine | 7 |
| Phosphoserine | 6 |
| Phosphothreonine | 4 |
| β -Casein ¹ | 2 |
| ATP | 0 |

¹Concentration was 5 mg/ml. Other substrates were 10 mM.

mammary acid phosphatase (Table 3). Because these compounds react with sulfhydryl groups in proteins, the results suggest that the sulfhydryl groups of cysteine are essential for enzyme activity. Results obtained by testing the effect of sulfhydryl compounds on phosphatase activity were erratic. The following compounds were tested: 2-mercaptoethanol, dithiothreitol, reduced glutathione, and cysteine. The fully active enzyme was not affected by the addition of the reducing agents. However, when the enzyme lost activity, the activity could be partially or fully recovered by the addition of cysteine or dithiothreitol; the amount of reactivation varied considerably. This suggests that factors in addition to free sulfhydryl groups are required for activity. Reduced glutathione and 2-mercaptoethanol were ineffective.

Phosphate and orthovanadate (a phosphate analogue) are product inhibitors. Zinc ions at a concentration of 6.75 mM inhibited 50% of phosphatase activity, but other divalent cations (Ca^{2+} , Mg^{2+} , and Mn^{2+}) had no effect on enzyme activity (Table 4). A variety of compounds were tested (Table 4). The EDTA and EGTA (ethyleneglycol-bis-(β -aminoethyl ether)N,N'-tetraacetate), compounds that chelate divalent metal ions, were ineffective in altering activity. Because KCl (.2 M) had no effect on activity, ionic strength does not seem to be a factor. Calcium ions plus calmodulin, which is known to activate calcineurin, a phos-

TABLE 3. Inhibition of acid phosphatase by various compounds.

| Inhibitor | Concentration for 50% inhibition (mM) |
|---------------------------------|---------------------------------------|
| N-Ethylmaleimide | .66 |
| <i>p</i> -Chloromercuribenzoate | .013 |
| Orthovanadate | 1.0 |
| Phosphate | 3.75 |
| Zn^{2+} | 6.75 |
| Ascorbate | 25.0 |

phoprotein phosphatase (2), had no effect on activity. Fluoride ions, which inhibit a number of phosphatases, did not effect activity. Heparin, a potent noncompetitive inhibitor of phosphorylase phosphatase, had no effect on the mammary enzyme. The ATPase inhibitors, ouabain and oligomycin, were also ineffective.

DISCUSSION

Most of the acid phosphatase (47%) was found in the cytoplasm, and this enzyme was purified. However, considerable activity was distributed among the pellets: approximately 11% in the nuclei, 37% in the mitochondria, and 5% in the microsomes. Cytoplasmic acid phosphatase occurs in a number of mammalian tissues and species (6), and the total activity

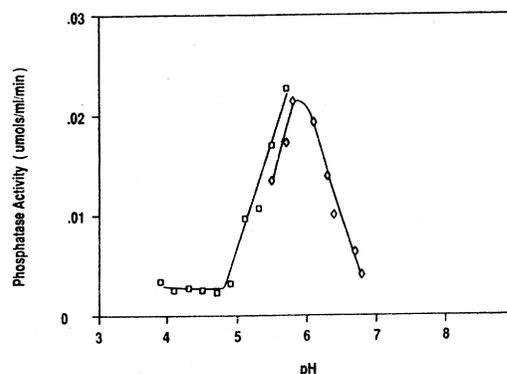


Figure 3. Effect of pH on phosphatase activity. Buffers were □, 50 mM acetate and ○, 50 mM 2-(N-morpholino)ethane sulfonate (Mes).

TABLE 4. Compounds that have no effect on phosphatase activity.

| Compound tested | Concentration used | |
|-------------------------------|-------------------------|-----------------|
| | (mM) | (mg/ml) |
| Sodium | 50 | |
| Oligomycin | | 1.0 |
| Heparin | | .04 |
| Cyclic AMP | .15 | |
| sodium fluoride | 40 | |
| Tartrate | 10 | |
| Ouabain | | 1.0 |
| Mn ²⁺ | 10 | |
| Mg ²⁺ | 10 | |
| Ca ²⁺ | 10 | |
| Calmodulin | | .01 |
| Ca ²⁺ + calmodulin | 10.0(Ca ²⁺) | .01(calmodulin) |
| KCl | 200 | |
| EDTA | 10 | |
| EGTA ¹ | 10 | |

¹Ethylenglycol-bis-(β -aminoethyl ether)N,N'-tetraacetate.

varies from 33% in rat lung to 96% in dog lens. Chen and Chen (6) presented evidence that cytoplasmic acid phosphatase has no link with lysosomes. However, its physiological role has not been defined.

The acid phosphatase described in this study differs from the mammary phosphoprotein phosphatase described previously (8). Although both phosphatases act on pNPP and other aromatic phosphates, only the phosphoprotein phosphatase can hydrolyze ATP, ADP, and casein, a phosphoprotein containing phosphoserine residues. Neither enzyme hydrolyzed appreciably the aliphatic phosphomonoesters.

The mammary phosphoprotein phosphatase is stimulated by reducing agents (thioglycollate, 2-mercaptoethanol, dithiothreitol, and ascorbate), whereas the mammary acid phosphatase from this study is stimulated by cysteine and dithiothreitol but not ascorbate. Revel and Racker (11) pointed out that the spleen phosphoprotein phosphatase, which is similar to the mammary enzyme (8), is stimulated by reducing agents and that the sulfhydryl groups in the enzyme are not involved. However, evidence presented in this paper clearly indicates that intact sulfhydryl groups are necessary for catalysis by the acid phosphatase.

Small amounts of phosphoprotein phosphatase have been found in bovine milk (1, 4, 5).

However, this enzyme differs in many respects from the acid phosphatase-described herein. The milk enzyme has a pH optimum of 4.9, is inhibited by fluoride ions, is unaffected by PCMB, and has high activity toward ATP and ADP. The mammary acid phosphatase has an optimal pH of 5.5 to 6.2, is unaffected by fluoride ions, is inhibited by PCMB, and does not catalyze the hydrolysis of ATP and ADP.

In the isolation of acid phosphatase, we examined various fractions for phosphoprotein phosphatase activity and were unable to locate the enzyme. In order to verify that the phosphoprotein phosphatase was absent, several attempts were made to purify the enzyme by known methods (8, 11). In both procedures, the phosphoprotein phosphatase is extracted from an ammonium sulfate precipitate by NaCl solubilization. When this procedure was tried, no phosphoprotein phosphatase was extracted. Amberlite CG-50, a weak cation exchange resin, has been successful in absorbing phosphoprotein phosphatase from milk (5) and mammary fractions (8), but we were unable to obtain activity by using this procedure. The absence of phosphoprotein phosphatase from our preparations cannot be explained. The cow used in this study was in full lactation when sacrificed. The presence of phosphoprotein phosphatase may be a function of the stage of lactation, the breed of cow, or other factors.

The function of mammary acid phosphatase is not known, although it has some properties of phosphotyrosine phosphatases, which are delineated by Ballou and Fischer (2). The enzyme was inhibited by orthovanadate and Zn²⁺, was stimulated by dithiothreitol, and showed no effect by divalent ions or metal chelators (EDTA and EGTA). Further work is in progress to understand the function of this enzyme and its relation to lactation. The source of the milk enzyme is also under investigation.

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