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BOVINE MILK ACID PHOSPHATASE

I. SOME KINETIC STUDIES AND OTHER PROPERTIES USING A PARTIALLY PURIFIED PREPARATION

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SUMMARY

A simple partial purification of bovine milk acid phosphatase is described using an ion-exchange resin followed by gel filtration on Sephadex G-100. An increase of 10^4 in specific activity compared to that of the original milk was obtained.

Gel filtration and polyacrylamide gel electrophoresis indicated that milk contains a single acid phosphatase isozyme with an isoelectric point close to pH 7.9 and a mol. wt of 42 000 $\pm$ 2000. Enzyme optimum pH was 4.9.

In general the enzyme was inhibited by most heavy metals and oxidising agents but among the most powerful inhibitors were Be^{2+}, Al^{3+} and F^-. The enzyme was not inhibited by *p*-nitrophenol, chelating agents or thiol reagents. Some reducing agents had activating properties.

With *p*-nitrophenyl phosphate as substrate kinetic data gave K_m values of 0.81 and 3.20 mM at pH 4.8 and 5.6, respectively. For pyrophosphate a K_m = 1.02 mM was obtained at pH 4.8 and for casein a K_m = 3.64 mM at pH 5.6. Binding appeared to be to the same active site *via* the phosphate group with all substrates. K_i values for F^-, Al^{3+} and P_i at pH 4.8 were 0.37, 0.12 and 1.52 mM, respectively. Data on the variation of K_m with pH suggested that a histidine residue may be involved in the active site and this was supported by observations on the inhibitory effects of iodoacetate.

INTRODUCTION

Acid phosphatases (orthophosphoric monoester phosphohydrolases, EC 3.1.3.2) have been identified in a very large number of plant and animal tissues (for reviews see Schmidt and Laskowski[1] and Hollander[2]). Many of these reports have been con-

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cerned only with activity in crude extracts or with the histochemical localization of the enzyme, but recently acid phosphatase has gained some interest as a result of apparent changes in total activity or distribution of isozymes in a number of pathological conditions[3–8]. More detailed studies point to two major conclusions. Firstly, many sources whether of plant, animal or microbial origin contain more than one enzyme with the ability to hydrolyze orthophosphoric monoesters at acid pH[3,4,9–16]. These may differ considerably in substrate specificity and behaviour towards inhibitors. Secondly, given the difficulties of interpretation which may result from the presence of several different enzymes, it has nevertheless been clearly demonstrated that highly purified preparations considered to consist of only one form of acid phosphatase still retain broad specificities[13,14,17–19]. It has been suggested[13] that this indicates a simple hydrolytic mechanism which may be of value in elucidating the methods by which the enzymes function. Possible roles for acid phosphatases have been proposed in the regulation of pyridoxal phosphate requiring enzymes[20], in steroid transport in vitamin B_6 metabolism[11] and in lipid metabolism[21].

Bovine milk has been reported[22,23] to contain acid phosphatase activity and some properties of a partially purified preparation have been described[24]. The enzyme has been shown to possess very broad specificity. In view of this it is of importance to establish the homogeneity of the milk enzyme with regard to the possible presence of isozymes, and to gain further insight into its properties, mechanism of action and the consequences of phosphatase activity on phosphoprotein substrates. The present paper reports some kinetic data and other findings obtained with a partially purified preparation.

MATERIALS AND METHODS

Acid phosphatase preparation

The first purification stage was similar to that previously described by Bingham and Zittle[24]. Untreated bulk skim milk was mixed in 10–12-l batches with 120 g Amberlite CG-50 Type II ion-exchange resin in the NH_4^+ form and stirred for 2 h at room temperature. After thorough washing of the separated ion-exchange resin the acid phosphatase was eluted with four 200-ml portions of 1 M NaCl and concentrated to approximately 50 ml by dialysis against polyethylene glycol (Carbowax 20 M, Union Carbide Co.). Extraction efficiency was similar when performed at 4, 20 or 37 °C.

Further purification was carried out by gel filtration on a 7.0 cm × 58.0 cm column of Sephadex G-100 made up in 0.1 M sodium acetate buffer, pH 4.8. Elution was with this same buffer at a flow rate of about 100 ml/h and fractions of 25 ml were collected. The absorbances at 280 nm were measured to localize protein-containing fractions. Enzyme-containing fractions were identified by mixing 0.5-ml portions of eluate with 0.5 ml *p*-nitrophenyl phosphate solution (1.0 mg/ml in 0.1 M sodium acetate, pH 4.8) and incubating for 10 min before addition of 0.25 M NaOH (3 ml) and reading the absorbance at 410 nm. These fractions were pooled and concentrated by dialysis against polyethyleneglycol. Lyophilization at pH 4.8 led to a 15% loss of activity with greater losses at pH 3.0 (about 60%) or pH 7.0 (about 25%) and frozen storage for 7 days led to a 30% loss. Sodium azide or thiomersalate appeared to be satisfactory preservatives for unfrozen solutions, but routinely the enzyme was prepared in small batches as above and used with the minimum of storage.

Enzyme assay

Routinely the method of Bingham and Zittle[24] was used with *p*-nitrophenyl phosphate as substrate for specific activity measurements, the only modification being the alteration of the pH of the 0.1 M sodium acetate buffer to 4.8. Units are expressed as μmoles *p*-nitrophenyl phosphate hydrolyzed/min under the assay conditions.

For experiments in which pyrophosphate or casein was used as the substrate, the enzyme activity was assayed by measuring the amount of phosphate liberated by the method of Sumner[25].

Polyacrylamide gel electrophoresis

Polyacrylamide gels containing 7.5% acrylamide were prepared according to Reisfeld *et al.*[26] using riboflavin as the polymerisation catalyst. Gels were poured into flat moulds to give slabs 3 mm in thickness and electrophoresis subsequently was carried out in a horizontal position at 6–12 V/cm for 16 h. Buffers employed throughout were of KOH–acetic acid in the gels and glycine–acetic acid in the apparatus[26] over the pH range 3.0 to 6.0 and 0.025 M Tris using 1 M HCl for pH adjustment in the gels with 0.2 M Tris–HCl in the apparatus over the pH range 7.0 to 9.0. After electrophoresis gels were stained for protein with a 0.1% solution of Amido black (Amido Schwartz 10B, Merck, Darmstadt, Germany) in methanol–water–acetic acid (5:13:2, by vol.) or for phosphatase activity using the following staining mixture. The substrate used was 5-chloro-3-hydroxy-2-naphtho-*o*-anisidine phosphate (naphthol AS-Cl phosphate, Koch-Light Laboratories Ltd, Colnbrook, England) and 10 mg was dissolved in approximately 2 ml dimethylformamide and made up to 25 ml with 1 M sodium acetate buffer, pH 4.8. The diazonium salt of *o*-aminoazotoluene (Fast Garnet BGC salt, Sigma Chemical Co., London, England) was used as the coupling dye and approximately 10 mg of this was added to the buffered substrate solution in which the gel slab was then immersed. Phosphatase activity leads to purple bands on a colourless background.

Molecular weight determinations

Estimation of molecular weight by gel filtration[27] was performed on 2.2 cm × 86 cm columns of Sephadex G-100 and G-200 using the following buffers: 0.1 M sodium acetate, pH 4.8; 0.1 M sodium acetate, pH 4.8, containing 0.15 M NaCl; 0.1 M sodium acetate, pH 4.8, containing 0.5 M NaCl; 0.1 M sodium acetate, pH 4.8, containing 0.020 M ascorbic acid; 0.1 M sodium acetate, pH 4.8, containing 0.050 M β-mercaptoethanol; 0.1 M sodium borate, pH 8.5, containing 0.12 M NaCl; 0.2 M NaCl; and 0.2 M ammonium bicarbonate, pH 8.6. Standard proteins of known molecular weight used for column calibration were cytochrome *c*, myoglobin, chymotrypsinogen, ovalbumin, bovine serum albumin and bovine γ-globulin.

Kinetic studies

Measurements of enzyme activity for Lineweaver–Burk plots were made with a minimum of ten different substrate levels over the range 0.20–5.0 mM for each plot, the intercept on the $1/S$ axis being $-1/K_m$ in the absence of inhibitors or $-1/K_p$ in the presence of a fixed level of inhibitor. Values for the inhibitor constants (K_i) were

calculated from values of K_p and K_m or v and v_p using the relationships reported[28] for competitive and for non-competitive inhibitors. Values of K_i were also obtained in some cases from plots[28] of $1/v$ *versus* i, the inhibitor concentration, a minimum of four different concentrations being used. Typical concentrations of inhibitors used were in the range 1.0–10.0 mM.

RESULTS

Purification of acid phosphatase

Extraction of raw skim milk with Amberlite CG-50 provided a very convenient and efficient means for the separation of the enzyme from the bulk of the milk protein. While careful control experiments showed that such treatment removed essentially all the acid phosphatase activity from the milk our experience has been that the levels of this enzyme present in many samples either of bulk milk or of the milk of individual animals collected at various times were in general almost an order of magnitude lower than those reported by Bingham and Zittle[24]. Thus the specific activity of acid phosphatase in milk was found to be approximately 0.00003 unit/mg protein if it is assumed that an absorbance at 280 nm of 1.00 was equal to a protein concentration of 1 mg/ml. With this same assumption after elution from the Amberlite resin the activity was approximately 0.01 unit/mg and after the gel filtration stage, 0.3 unit/mg. The action of EDTA was somewhat variable, but with most preparations of enzyme the addition of 5 mM EDTA to the assay mixture led to slightly enhanced specific activities of up to 0.6 unit/mg, presumably due to removal of traces of inactivating metal ions.

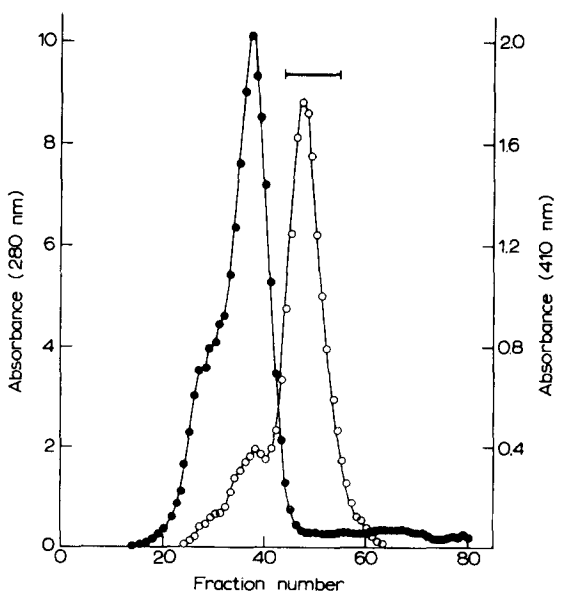

Fig. 1. Purification of bovine milk acid phosphatase by gel filtration on Sephadex G-100. ●—●, protein as measured by 280-nm absorbance; ○—○, enzyme activity (410-nm absorbance). For details see text. Enzyme-containing fractions were pooled as indicated (Fractions 44–55).

The considerable value of gel filtration on Sephadex G-100 as a purification stage is seen from Fig. 1 which demonstrates that the peak of enzyme activity was well separated from the bulk of other protein material. The apparent shoulder of enzyme activity in the region of Fraction 38 of Fig. 1 is due to interference in the absorbance readings at 410 nm by brown coloured lactoperoxidase eluted in the main protein peak and does not represent a true shoulder of acid phosphatase activity. Although the fractions with acid phosphatase activity (pooled as indicated on Fig. 1) represented a purification by a factor of over 10^4 compared with the specific activity in the original milk, using just two simple purification steps, the results of electrophoresis on polyacrylamide gels clearly showed that extensive further treatment is required to obtain the enzyme in a pure state, since several inactive protein-staining bands were observed (Fig. 2).

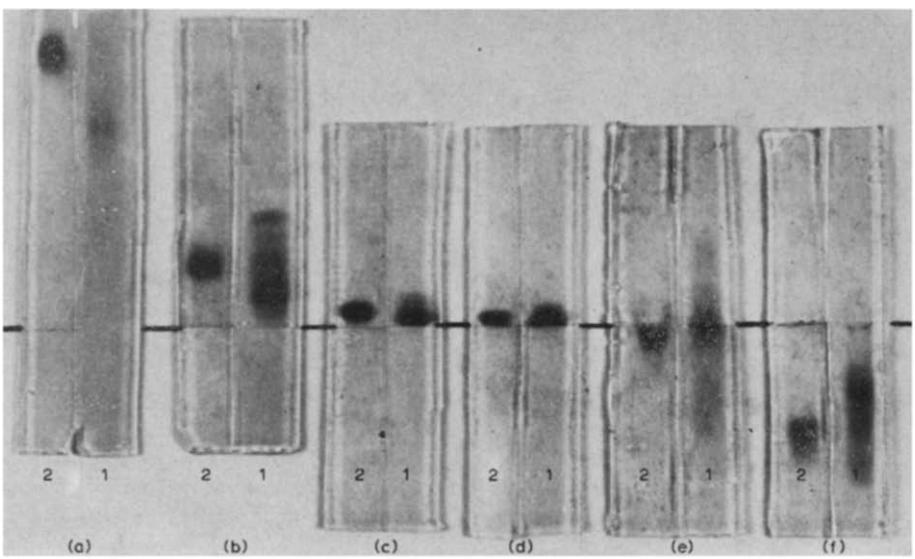

Fig. 2. Polyacrylamide gel electrophoresis of acid phosphatase at various pH values. a, 3.0; b, 4.3; c, 5.0; d, 7.0; e, 8.0; f, 9.0. Run at 6–12 V/cm for 16–18 h. 1, Amido Schwartz stain for protein; 2, enzyme activity. See text for details.

It may also be seen from Fig. 2 however that only a single band of enzyme activity was obtained when electrophoresis was conducted over the pH range of 3.0–9.0. Taken with the observation that a single symmetrical peak of activity was eluted from gel filtration columns (Fig. 1) this provided strong evidence that only a single form of enzyme with phosphatase activity at acid pH values was extracted from milk by the Amberlite resin. Since this apparently represented most, if not all, of such activity originally present in milk, it was concluded that bovine milk probably contains only one acid phosphatase. However isozymes formed by amino acid substitutions with no change in net charge would not be detected by the methods used.

Preliminary experiments on the mobility of the enzyme at a series of pH values suggested that the isoelectric point (pI) was at about pH 7.9.

Effect of pH

The variation with pH of acid phosphatase activity in the purified extract is shown in Fig. 3. Identical curves, with a single broad peak, were obtained using either 0.2 M sodium acetate or 0.1 M sodium citrate buffers. The pH optimum at 4.9 using *p*-nitrophenyl phosphate as substrate was in general agreement with the results obtained by other workers[24] using a partially purified preparation but not entirely in agreement with earlier results[23] obtained with crude preparations or unfractionated milk[22], although in the latter case a different substrate, phenyl phosphate, was used which would appear[23] to shift the pH optimum to a more acid value. In the present investigation no evidence was found for any alkaline phosphatase activity in our preparations.

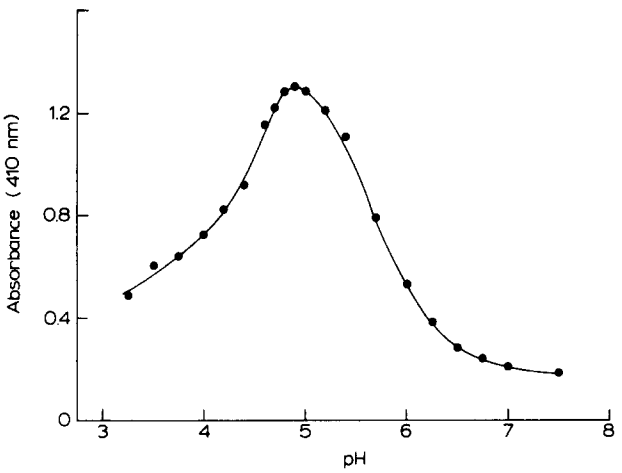

Fig. 3. Plot of pH *versus* acid phosphatase activity as measured by 410-nm absorbance due to nitrophenol liberated from *p*-nitrophenyl phosphate substrate. Buffer 0.2 M sodium acetate containing 0.005 M *p*-nitrophenyl phosphate. Reaction mixture (0.9 ml) incubated with 0.1 ml enzyme solution (0.1 I.U./ml) for 20 min prior to addition of 0.5 M NaOH (3.0 ml).

Molecular weight determinations by gel filtration

The results of gel filtration runs suggested that bovine milk acid phosphatase behaved as a single homogeneous component in terms of enzyme activity with an apparent mol. wt[27] of 44 000 $\pm$ 1000 in all of the buffer systems examined with columns of Sephadex G-100 and of 41 000 $\pm$ 1000 using Sephadex G-200 columns.

Inhibition by anions and cations

A list of anions and cations which were found to be inhibitory when added at a level of 0.010 M to the acid phosphatase assay mixture together with the approximate percentages of inhibition observed is shown in Table I, which also includes a number of substances tested which were not found to inhibit at this concentration. In general the most inhibitory substances were found to be either heavy metals or oxidizing agents but there were a few notable exceptions. For example, Be^{2+} and Al^{3+} were inhibitory, whereas Pb^{2+}, Mn^{2+} and surprisingly *p*-chloromercuribenzoate either were not inhibitory or were only weakly so. As has been shown for a number of other acid phosphatases[4,11,12,16,17,20] the bovine milk enzyme was strongly inhibited by F^-. No

TABLE I

INHIBITION BY ANIONS AND CATIONS

Portions (0.5 ml) of solution of the substances under investigation (0.020 M in water) were mixed with 1 M sodium acetate, pH 4.8 (0.2 ml), and enzyme solution (0.1 ml) in 0.1 M sodium acetate, pH 4.8, and preincubated for 10 min at 37 °C. Substrate solution, 0.025 M *p*-nitrophenyl phosphate in water (0.2 ml) was added and the mixtures incubated for a further 10–30 min. After addition of 0.25 M NaOH (3.0 ml) absorbances were recorded at 410 nm.

Cations	*Salt used*	*% Inhibition*	*Anions**	*% Inhibition*
Ag^+	Nitrate	64	F^-	83
Be^{2+}	Chloride	75	AsO_2^-	10
Cd^{2+}	Sulphate	53	BrO_3^-	22
Co^{2+}	Chloride	16	CrO_4^-	62
Cu^{2+}	Sulphate	83	IO_3^-	45
Fe^{2+}	Ammonium sulphate	28	IO_4^-	75
Hg^{2+}	Chloride	100	OCl^-	99
Hg^{2+}	Chlorobenzoate	5	VO_3^-	93
Mn^{2+}	Sulphate	28	S^{2-}	75
Ni^{2+}	Chloride	7	SO_3^{2-}	88
Sn^{2+}	Chloride	65	$S_2O_5^{2-}$	66
UO_2^{2+}	Acetate	100	$S_2O_8^{2-}$	35
Zn^{2+}	Acetate	89	SeO_3^{2-}	83
Al^{3+}	Chloride	84	WO_4^{2-}	75
Al^{3+}	Potassium sulphate	98	AsO_4^{3-}	40
Fe^{3+}	Sulphate	88	PO_4^{3-}	82
			$P_2O_7^{4-}$	73

Non-inhibitors

NH_4^+, Li^+, Na^+, K^+, Cs^+, Tl^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Pb^{2+}, Sb^{3+}, Tris

Cl^-, Br^-, I^-, NO_2^-, NO_3^-, CN^-, SCN^-, formate, acetate, propionate, *n*-butyrate, isovalerate, oxalate, succinate, malonate, DL-malate, maleate, sodium–potassium tartrate, citrate, urate, SO_4^{2-}, $S_2O_3^{2-}$, MoO_4^{2-}, BO_4^{3-}

EDTA; α,α'-dipyridyl; 1,10-*O*-phenanthroline; 8-hydroxyquinoline

D(+)-Glucose. D(+)-fructose, lactose, sucrose

* Na^+, K^+ or NH_4^+ salts in all cases.

evidence was found for activation by Mn^{2+} as has been reported[22] and in fact we found Mn^{2+} to be weakly inhibitory.

Addition of EDTA at a level of 0.020 M to portions of enzyme solution which had been inhibited by metal ions such as Al^{3+}, Hg^{2+}, Zn^{2+} showed that the inhibition was substantially reversed by this treatment, enzyme activity recoveries being 98, 66 and 63%, respectively. In the case of $HgCl_2$, inhibition was also reversed (98% regain of activity) by 0.040 M β-mercaptoethanol. This reagent was without effect on inhibition by zinc acetate or $AlCl_3$. In general, inhibition caused by oxidising agents was not reversed by reducing agents such as β-mercaptoethanol or $NaBH_4$.

Effect of reagents reacting with or protecting SH groups

Preincubation for 10 min at 37 °C of acid phosphatase solutions at pH 3.0, 4.8, 7.0, 8.0 and 9.0 with 0.010 M additions of iodoacetic acid, iodoacetamide, *N*-ethylmaleimide or *p*-chloromercuribenzoate before addition of substrate showed that under these conditions these compounds had little effect on acid phosphatase activity. Values within 5% of the controls were obtained in all cases and suitable controls showed that the enzyme was entirely stable over this pH range under the conditions

of these experiments. Under similar conditions at pH 4.8 dithiothreitol and β-mercaptoethanol were found to enhance enzyme activity by 41 and 58%, respectively. Activation effects (percentages of activation in parentheses) were also observed when dithiothreitol (53%), β-mercaptoethanol (160%), L-cysteine (43%), or thioglycollic acid (78%) were added to the enzyme at pH 4.8 at the same time as the substrate, the effect being considerably greater than reported by earlier workers[24]. Since activation was also found for ascorbic acid but not for compounds which titrate SH groups such as iodoacetic acid, iodoacetamide, *N*-ethylmaleimide or *p*-chloromercuribenzoate, it would appear that activation is a reflection of their properties as reducing agents.

In contrast to the above, very high levels (approximately 0.5 M) of β-mercaptoethanol and dithiothreitol were found to be inhibitory when added to the assay mixture at pH 4.8. When compared to suitable controls prolonged storage at pH 4.8 and 4 °C of acid phosphatase solutions containing lower levels (0.05 M) of β-mercaptoethanol, dithiothreitol or ascorbic acid also caused inhibition (20–50% in 18 days), as did lyophilisation of such solutions (20–90%).

In summary therefore it was concluded that bovine milk acid phosphatase was not susceptible to inactivation by a wide variety of SH-protecting or SH-blocking reagents and thus cannot be regarded as an enzyme in which SH groups play a significant role in catalysis. The activating effect of some SH-protecting reagents at low concentrations was considered to be related to their properties as reducing agents. At high concentrations, either as added or as may occur during lyophilization, or following prolonged exposure to these reagents this activation was replaced by inhibition which may suggest that they can exert an indirect influence on the catalytic process perhaps by producing conformational changes at a site removed from the actual active centre.

Kinetic studies

A typical Lineweaver–Burk plot for the determination of the K_m of acid phosphatase using *p*-nitrophenyl phosphate as substrate in 0.1 M sodium acetate buffer at pH 4.8 is shown in Fig. 4. Values of K_m at various pH values and with some alternative substrates are given in Table II, which also shows values of the inhibition constants (K_i) of a variety of inhibitors and alternative substrates using *p*-nitrophenyl phosphate as the enzyme substrate. Lineweaver–Burk plots in the presence and absence of the inhibitors showed that Al^{3+}, orthophosphate, pyrophosphate, hexametaphosphate and casein all behaved as competitive inhibitors, whereas KF was a powerful non-competitive inhibitor. The hydrolysis of *p*-nitrophenyl phosphate was not inhibited by the addition of *p*-nitrophenol when this was added to the reaction tubes at levels of 0.001, 0.002, 0.015 or 0.1 M.

The value of 0.81 mM for K_m obtained at pH 4.8 with *p*-nitrophenyl phosphate substrate is in agreement with the values of 1.0 and 0.51 mM at pH 5.0 reported[23] for crude preparations of the enzyme. With phenyl phosphate as substrate values of 2.0 mM at pH 5.0 for crude preparations[23] and 3.2 mM at pH 4.1 for the unpurified enzyme in milk[22] have been reported. The addition of 0.010 M ascorbic acid led to an increased K_m value (1.18) and also an increase in V, whereas the action of 0.010 M β-mercaptoethanol was to increase V with little change in the value of K_m.

The similarity between K_m values for *p*-nitrophenyl phosphate and for pyro-

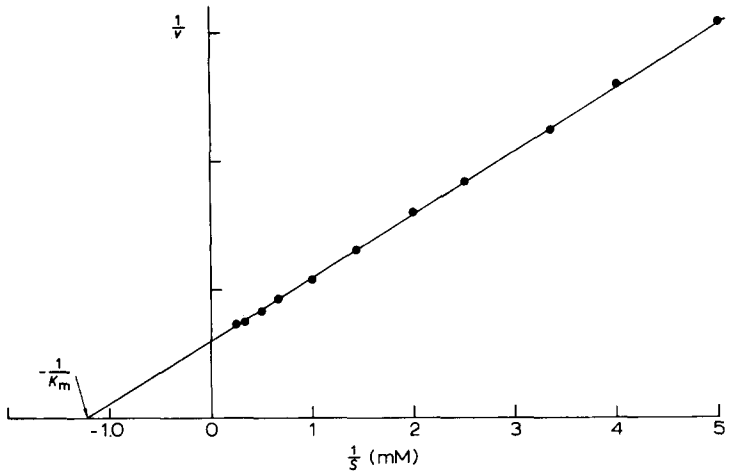

Fig. 4. Lineweaver–Burk plot of bovine milk acid phosphatase using *p*-nitrophenyl phosphate as substrate in 0.1 M sodium acetate buffer, pH 4.8.

TABLE II

SOME KINETIC CONSTANTS FOR BOVINE MILK ACID PHOSPHATASE

Substrate	*pH*	*Inhibitor**	K_m *(mM)*	K_i *(mM)*
p-Nitrophenyl phosphate	4.8	—	0.81	—
p-Nitrophenyl phosphate	5.2	—	1.41	—
p-Nitrophenyl phosphate	5.6	—	3.20	—
p-Nitrophenyl phosphate	4.8	KF	—	0.37**
p-Nitrophenyl phosphate	4.8	KF	—	0.26
p-Nitrophenyl phosphate	4.8	Al^{3+}	—	0.12
p-Nitrophenyl phosphate	4.8	P_i	—	1.52**
p-Nitrophenyl phosphate	4.8	P_i	—	2.16
p-Nitrophenyl phosphate	4.8	HMP	—	1.91
p-Nitrophenyl phosphate	4.8	PP_i	—	1.11
PP_i	4.8	—	1.02	—
p-Nitrophenyl phosphate	5.6	Casein	—	3.20**
p-Nitrophenyl phosphate	5.6	Casein	—	3.53
Casein	5.6	—	3.64	—
Casein	5.2	—	2.84	—

* Al^{3+} as aluminium potassium sulphate; P_i, orthophosphate; PP_i, pyrophosphate; HMP, hexametaphosphate.

** Values obtained from $1/v$ *versus* i plots.

phosphate at pH 4.8 and between those for *p*-nitrophenyl phosphate and for casein at pH 5.6 or 5.2 suggests that the factor common to all these substrates, namely the phosphate group, is the only important part of substrate molecules in terms of binding at the active site, and that all these substrates bind to the enzyme at the same site. Further evidence is provided by the general similarity of K_i values obtained using *p*-nitrophenyl phosphate as substrate and orthophosphate, hexametaphosphate, pyrophosphate and casein used as inhibitors with the K_m values obtained for *p*-nitrophenyl phosphate, pyrophosphate and casein used as substrates in the absence of inhibitors when measured at the same pH. The lack of inhibition of the

enzyme by *p*-nitrophenol is also strong evidence that this portion of the substrate is comparatively unimportant in binding to the active site. The wide range of possible substrates reported for this enzyme[24] suggests that substrate geometry at the active site is relatively uncritical.

When *p*-nitrophenyl phosphate was used as a substrate and K_i values obtained for the alternative substrates pyrophosphate and casein used as inhibitors, the K_i values obtained (Table II) were equilvalent to K_s. Since these are close to the K_m values obtained when they are used as substrates in the absence of *p*-nitrophenyl phosphate (Table II) it is clear that formation of the enzyme–substrate complex (ES) is rapid in relation to the breakdown of such a complex into products. Thus, the formation of ES from E and S approximates to a true equilibrium. With *p*-nitrophenyl phosphate (NPP) as substrate our data was consistent with the comparatively simple overall reaction mechanism:

$$E + \text{NPP} \overset{k_1}{\rightleftharpoons} E\text{–NPP} \overset{k_2}{\rightleftharpoons} E\text{–P}_i + \text{NP} \overset{k_3}{\rightleftharpoons} E + \text{P}_i$$

where NP is *p*-nitrophenol. The possibility of an intermediate isomerization step, namely the formation of E^*–NPP from E–NPP cannot be excluded so that positive identification of the rate limiting step was not possible on the basis of present evidence.

The variation of K_m with changing pH is indicated by Fig. 5. This suggested that there were inflections due to the ionisation of groups on the enzyme or substrate at about pH 4.9 and 6.9 which may possibly correspond to the pK_{a_2} of the *p*-nitrophenyl phosphate substrate and to the pK of a histidine residue, respectively, although Alvarez[29] has reported that the pK_{a_2} of *p*-nitrophenyl phosphate is 5.3–5.5. The involvement of histidine at the active site is supported by the known light-sensitivity of this enzyme[22]. The reaction of iodoacetic acid with histidine residues is generally slow which may explain the apparent lack of an inhibition effect with this reagent during the 10-min preincubation period, as used in the study of the action of SH reagents (see above). More prolonged incubation at pH 7.2 in the presence of 0.1 M iodoacetate however was accompanied by complete inactivation (Fig. 6) and this was consistent with the presence of an active site histidine residue. There was

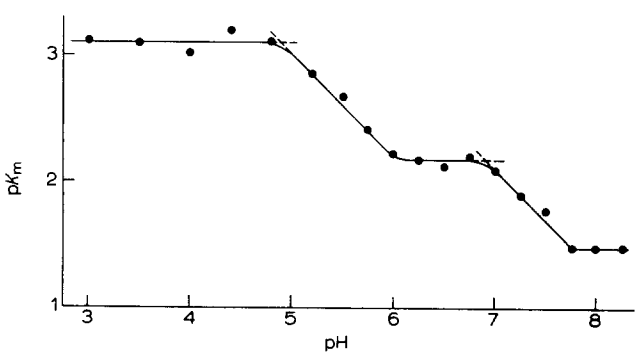

Fig. 5. Variation with pH of the K_m of acid phosphatase with *p*-nitrophenyl phosphate substrate obtained from Lineweaver–Burk plots. Buffers: 0.1 M sodium acetate over the pH range 4.0–6.25; 0.1 M sodium acetate–formic acid over the pH range 3.0–3.5 and 0.1 M Tris–HCl over the pH range 6.5–8.5.

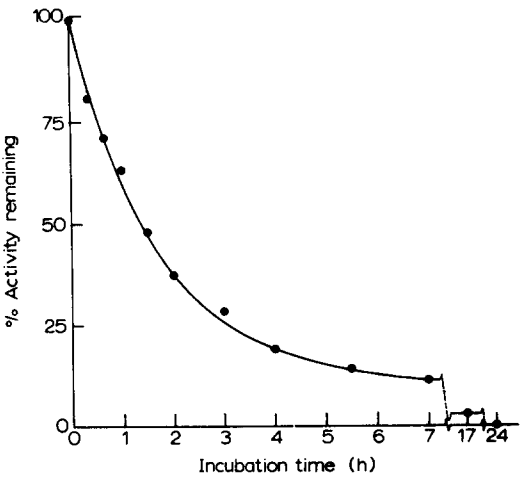

Fig. 6. Inhibition of bovine milk acid phosphatase by iodoacetate. Acid phosphatase solution adjusted to pH 7.2 was mixed with an equal volume of 0.2 M iodoacetic acid adjusted to pH 7.2 by the addition of solid Tris. The mixture was incubated at 37 °C in the dark and 0.1-ml aliquots were withdrawn after various time intervals for assay of acid phosphatase activity. Similar incubation of a control solution with no added iodoacetate showed that the enzyme itself was stable under these conditions.

also some evidence for further inflections in the pH *versus* K_m plot at about pH 2.3 and 8.3 which may correspond to the pK values of a carboxyl group and a sulphydryl group, respectively, but at these pH values the enzyme reaction was so slow that measurement of accurate K_m values was difficult and little reliance should be placed on these values.

DISCUSSION

The present results provide strong evidence that unlike most other sources of acid phosphatase, bovine milk contains only a single species of enzyme with phosphatase activity at acid pH. Since this is a basic protein and the initial extraction step was by adsorption onto a carboxylic ion-exchange resin, the possible presence of another unadsorbed isozyme remaining in the milk cannot be entirely excluded. Very low levels of phosphatase activity are difficult to determine in turbid milk solutions, but since the present procedure removes all detectable activity any other isozyme would clearly be a very minor component.

The properties of bovine milk acid phosphatase in terms of size, pH optimum, inhibition by anions and cations and the effect of thiol reagents showed that this enzyme was different from other known acid phosphatases of bovine tissues[13,19,30–33]. Although there are many similarities with the bovine spleen enzyme, the milk enzyme was distinguished from this by its higher molecular weight, more acid pH optimum, insensitivity to *p*-chloromercuribenzoate inhibition and the much smaller activating effects of various thiol and reducing reagents[31]. Fe^{2+} activated the spleen enzyme[31] but was found to be mildly inhibitory to the milk enzyme and molybdate has been reported[31] to be a potent inhibitor of the spleen enzyme, but was without effect on the milk enzyme. The behaviour towards chelating agents such as α,α'-dipyridyl;

1,10-*O*-phenanthroline and 8-hydroxyquinoline was also quite distinct since these inhibit the spleen enzyme[31] but were without effect on the milk enzyme. Taken with the lack of inhibition also by EDTA it was concluded that bovine milk acid phosphatase was not a metal requiring enzyme.

Bovine milk acid phosphatase has been reported to possess considerable stability to heat[22,23] and as a result may survive typical milk heat treatment processes. The enzyme is virtually unaffected by conventional pasteurization conditions and preliminary studies in our laboratory have suggested that approximately 15–20% of the initial activity remains after the ultra-high-temperature sterilization process. Such heat stability would be of considerable technological importance, firstly from the presence in the cream phase of acid phosphatase associated with the fat globule membrane[23], in which location it might play a role in the dephosphorylation of key lipid components or phosphoproteins, and secondly in the skimmilk phase where caseins provide a natural substrate. The distribution of the enzyme between the cream and aqueous phases has been examined[23,34] and it would appear that acid phosphatase is distributed throughout both phases. The importance of the phosphate groups of casein molecules in the binding of Ca^{2+} and in the formation of Ca^{2+} bridges for micelle formation is well established[35] and removal of such groups has been reported to have a considerable effect on micelle structure in casein solutions[36]. Changes in the isoelectric points of casein components arising from phosphatase action may disturb the delicately balanced stability of the milk system and play a role in coagulation processes, as indeed may interaction of the enzyme with fat globule membrane components, although such effects remain to be investigated. From a technological viewpoint enzyme-catalyzed reactions such as those outlined above may be of especial significance during the prolonged storage at ambient temperatures of ultra-high-temperature sterilized milk and milk products.

Studies on the further purification of this enzyme and amino acid residues involved in the active site and catalytic processes are in progress, together with an examination of the effects of acid phosphatases on the complex milk system.

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