The presence of a Zn^{2+} -dependent acid *p*-nitrophenyl phosphatase in bovine liver

Isolation and some properties

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1. The presence of a Zn^{2+} -dependent acid *p*-nitrophenyl phosphatase (EC 3.1.3.2) in bovine liver was described. 2. The enzyme was purified to apparent homogeneity and migrates as a single band during electrophoresis on polyacrylamide gel. 3. The enzyme requires Zn^{2+} ions for catalytic activity, other bivalent cations have little or no effect. 4. The enzyme, of M_r 118 000, optimum pH 6–6.2 and pI 7.4–7.5, was inhibited by EDTA, tartrate, adenine and ATP, but not by fluoride. 5. The common phosphate esters are poor substrates for the enzyme, which hydrolyses preferentially *p*-nitrophenyl phosphate and *o*-carboxyphenyl phosphate. 6. The Zn^{2+} -dependent acid *p*-nitrophenyl phosphatase of bovine liver was different from the high- M_r acid phosphatases previously detected in mammalian tissues.

INTRODUCTION

Acid phosphatases are ubiquitous enzymes, which have been studied in plants and animals (see Hollander, 1971). Mammalian liver contains at least two acid phosphatase forms, of M_r 90 000–102 000 and 13 400–30 000, which can be separated by gel-exclusion chromatography (Heinrikson, 1969; Campbell *et al.*, 1973; Rehkop & Van Etten, 1975; De Araujo *et al.*, 1976). The effect of inhibitors, substrate requirements, optimum pH and kinetic parameters also distinguish between the enzymes (Shibko & Tappel, 1963; Heinrikson, 1969; Baldijao *et al.*, 1975; De Araujo *et al.*, 1976; Saini & Van Etten, 1978a; Taga & Van Etten, 1982).

Most of the acid phosphatase activity in bovine liver was associated with the low- M_r enzyme, which was purified to homogeneity (Heinrikson, 1969; Lawrence & Van Etten, 1981). In the present paper I describe the presence in bovine liver of a high- M_r form of acid phosphatase which requires Zn^{2+} ions for activity. The enzyme has been purified and partially characterized.

EXPERIMENTAL

Materials

Substrates, modifiers, Folin reagent and M_r markers were purchased from Sigma. Sephadex G-200 was from Pharmacia, and DEAE- and CM-cellulose were from Whatman. Other chemicals were obtained from Carlo Erba. All reagents were made with demineralized and double-distilled water.

Methods

Enzyme assays. Phosphatase activity was determined at 37 °C in 50 mM-sodium acetate buffer, pH 6, containing 2.5 mM-*p*-nitrophenyl phosphate and 4 mM- ZnCl_2 . The reaction was started by addition of enzyme solution in a final volume of 0.5 ml. After 5–10 min the reaction was stopped by adding 1–2.5 ml of 1 M-NaOH, and the A_{415} of the liberated *p*-nitrophenol was read ($\epsilon_{415} = 1.78 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$). One enzyme unit is defined

as the amount of enzyme that produces 1 μmol of *p*-nitrophenol/min. Specific activity was expressed as enzyme units per mg of protein, determined as described by Lowry *et al.* (1951).

The pH/activity curves were obtained in 50 mM-acetate (pH 4–6.2), 25 mM-barbital (pH 6–9) or 50 mM-glycine/NaOH (pH 8.8–9.6).

The effect of metal ions was assayed at fixed 2.5 mM-*p*-nitrophenyl phosphate concentration, with different metal ion concentrations. The effect of modifiers was tested at various concentrations in the presence of 4 mM- ZnCl_2 .

K_m was obtained by the statistical method of Wilkinson (1961), including S.E.M., with ten substrate concentrations ranging from 0.2 to $2.5 \times K_m$. The hydrolysis of various phosphate esters (5 mM final concn.) was assayed at 37 °C in 50 mM-acetate buffer, pH 6, as described by Saini & Van Etten (1978a). The ionic strength, I , was maintained at 0.15 M in all enzyme assays by addition of NaCl (Van Etten & McTigue, 1977).

Analytical polyacrylamide-gel electrophoresis was carried out on 7.5% acrylamide gels at pH 8.3 (Davis, 1964). The gels were cut into 2 mm slices and eluted overnight in 200 μl of 0.2 M-sodium acetate buffer, pH 6. Enzyme activity was assayed by adding 400 μl of 5 mM-*p*-nitrophenyl phosphate and 4 mM- ZnCl_2 . After 1 h at 37 °C the reaction was stopped by 400 μl of 1 M-NaOH, and the A_{415} of each fraction was read. The proteins were stained as described by Malik & Berrie (1972).

The pI was determined by isoelectric focusing on polyacrylamide gel as described by Drysdale *et al.* (1971), by using ampholytes in the pH range 5–8 or 7–9. Electrophoresis was run at 1 mA/tube until the voltage reached a value of 400 V (about 80–90 min), and then a constant voltage was maintained for another 1 h. Enzyme activity was assayed on sliced gel as described above. Control gels were sliced and eluted in 1 ml of deionized distilled water for pH profiles.

The apparent M_r was estimated on a calibrated Sephadex G-200 column (1.8 cm $\times$ 85 cm) by comparing the elution volumes of the following standards: bovine

Fig. 1. Elution profile of Zn^{2+} -dependent acid *p*-nitrophenyl phosphatase from the CM-cellulose column equilibrated in 10 mM-acetate buffer, pH 6

The sample was loaded and the proteins were eluted with a linear gradient of 0–0.3 M-NaCl in equilibration buffer; 6 ml fractions were collected at a flow rate of 45 ml/h. $\square$, A_{280} (protein); $\blacksquare$, enzyme activity.

γ -globulin (M_r 158000), bovine serum albumin (67000), ovalbumin (45000) and lysozyme (14000). The void volume was determined with Blue Dextran. The correlation coefficient of a plot of the mobility of standards against $\log M_r$ was 0.998.

Enzyme purification. The data reported in this paper refer to 50 g of fresh bovine liver. All operations were performed at 4 °C. The tissue was washed twice in cold 0.25 M-sucrose/1 mM-EDTA/5 mM-Tris/HCl buffer, pH 7.4, and then immersed in 0.3 M-acetate buffer, pH 6, and homogenized in a Potter–Elvehjem homogenizer. The homogenate was made up to 130 ml with acetate buffer and gently stirred for 4 h. The extract was centrifuged at 45000 *g* for 90 min and the pellet discarded. The supernatant (100 ml) was dialysed against 20 vol. of 50 mM-acetate buffer, pH 6, and then fractionated with $(\text{NH}_4)_2\text{SO}_4$. The fraction obtained between 30 and 70% saturation was resuspended in 20 ml of 50 mM-acetate (pH 6) and adjusted to pH 4.5 with cold 1 M-acetic acid. After centrifugation at 45000 *g* for 20 min, the supernatant was dialysed against 50 mM-acetate buffer, pH 6, containing 0.1 M-NaCl. The sample (about 25–30 ml) was divided into two batches, and each was loaded on to a Sephadex G-200 column (3 cm $\times$ 65 cm) equilibrated with the dialysis buffer; 10 ml fractions were collected at a rate of 15 ml/h and assayed for enzyme activity. The pooled fractions containing more than 0.1 unit/ml were concentrated by 70% saturation with $(\text{NH}_4)_2\text{SO}_4$, and resuspended in 0.1 vol of 10 mM-Tris/HCl buffer, pH 7.4, and dialysed in the same buffer. The enzyme solution (about 14–16 ml) was poured on to a DEAE-cellulose column (2 cm $\times$ 20 cm), equilibrated in 10 mM-Tris/HCl buffer, pH 7.4, and the proteins were eluted with a linear NaCl gradient (0–0.1 M, in 600 ml of Tris buffer). Fractions (6 ml) were collected at 45 ml/h and assayed as described above. The fractions containing

more than 0.1 unit/ml were pooled, dialysed against 10 mM-acetate buffer, pH 6, and poured directly on to a column (2 cm $\times$ 10 cm) of CM-cellulose equilibrated in the same buffer. The proteins were eluted with a linear NaCl gradient (0–0.3 M; 500 ml) in equilibration buffer, and 6 ml fractions were collected at 45 ml/h. The phosphatase activity was eluted as a single protein peak of constant specific activity after a protein peak with no phosphatase activity (Fig. 1). The fractions containing activity were pooled (about 80–90 ml) and used for biochemical analysis or concentrated by dialysing the enzyme solution against 10 mM-acetate buffer, pH 6, and then adsorbing it on to a micro-column of CM-cellulose (1 cm $\times$ 1.5 cm). The enzyme was eluted in a single step with 0.5 M-NaCl. This operation gives a 40–50-fold concentration of the enzyme preparation.

RESULTS AND DISCUSSION

Acid phosphatases originating from many sources have been found to need metal ions for their activity. In the chicken, Wang (1969) and Strittmatter (1972) demonstrated the presence of a soluble form of acid *p*-nitrophenyl phosphatase, stimulated by Zn^{2+} , in the liver and duodenum during embryonic development. A Zn^{2+} -stimulated acid phosphatase has also been reported in rat testicular tissues (Vanha-Perttula, 1970) and rabbit spermatozoa (Gonzales & Meizel, 1973). These enzymes, however, were studied only as crude preparations (Wang, 1969) or in a partially purified form (Strittmatter, 1972; Gonzales & Meizel, 1973). In the present paper I demonstrate the presence of Zn^{2+} -dependent acid *p*-nitrophenyl phosphatase in bovine liver. The enzyme was purified to apparent homogeneity and migrates as a single protein band on polyacrylamide-gel electrophoresis (Fig. 2). A summary of a typical preparation is presented in Table 1. The purified enzyme, with a specific activity of 10 units/mg of protein, is stable for 1 week at 4 °C, and no loss of activity was observed when it was stored in dilute solution at –20 °C for several months.

The effect of various concentrations of ZnCl_2 , MnCl_2 and CoCl_2 on *p*-nitrophenyl phosphate hydrolysis (Fig. 3) indicates that Zn^{2+} was necessary for stimulating the catalytic activity of the enzyme, the optimal concentration being 3–5 mM. Mn^{2+} could substitute for Zn^{2+} , but gave only 15–20% activation. Co^{2+} and other bivalent cations, including Mg^{2+} , Ca^{2+} , Ba^{2+} , Be^{2+} , Sn^{2+} , Pb^{2+} , Ni^{2+} and Fe^{2+} , showed no effect. The optimal pH for the enzyme was about 6–6.2 in the presence of either 4 mM- ZnCl_2 or 4 mM- MnCl_2 (Fig. 4). No shift in the pH optimum was produced by varying the metal concentration.

The Zn^{2+} -dependent acid *p*-nitrophenyl phosphatase of bovine liver was insensitive to NaF, and 50% inhibition was observed with 5 mM-L(+)-tartrate, 2 mM-EDTA, 1 mM-adenine and 2.5 mM-ATP. The enzyme is strongly inactivated by 0.5 mM-*p*-chloromercuribenzoate or 1 mM- Ag^+ or Hg^{2+} .

The rates of hydrolysis of various phosphate esters indicate that *p*-nitrophenyl phosphate and *o*-carboxyphenyl phosphate were hydrolysed rapidly by the bovine liver enzyme. The activity towards phenyl phosphate was only 15% of that measured for *p*-nitrophenyl phosphate. Other commonly used phosphate esters, including α -naphthyl phosphate, β -glycerophosphate, *O*-phospho-L-serine, ATP, ADP and AMP are not hydrolysed at a

Fig. 2. Polyacrylamide-disc-gel electrophoresis of the Zn²⁺-dependent acid *p*-nitrophenyl phosphatase from bovine liver

A sample (10–15 µg) of the purified enzyme was subjected to electrophoresis for 2.5 h in 7.5%-acrylamide gels at pH 8.3. One gel (b) was stained for protein with Coomassie Brilliant Blue R-250. A duplicate gel (a) was sliced into 2 mm sections, and each was assayed for enzyme activity with *p*-nitrophenyl phosphate as substrate.

measurable rate. The enzyme preparation is devoid of diphosphatase and pyrophosphatase activities.

The Michaelis constant for *p*-nitrophenyl phosphate determined at pH 6 is 0.38 ± 0.04 mM. The M_r determination gives a value of 118000; the variation of three independent experiments was about 5%. Electrofocusing on polyacrylamide gel shows a single peak of enzyme activity with a pI of 7.45–7.50.

Fig. 3. Effect of metal ions concentration on activity of purified acid *p*-nitrophenyl phosphatase from bovine liver

Assays were carried out at 37°C with 2.5 mM-*p*-nitrophenyl phosphate, 50 mM-acetate buffer, pH 6, and metal ion at the desired concentration. Each point was an average of three experiments. The maximum activity was expressed by taking the highest activity in any fraction as 100%, and the other activities were expressed as a percentage of the maximum activity. ■, Zn²⁺; □, Mn²⁺; ▲, Co²⁺.

The Zn²⁺-dependent acid *p*-nitrophenyl phosphatase of bovine liver seems to be very similar to that of chick-embryo liver and duodenum with respect to Zn²⁺ stimulation, optimum pH and inhibition by adenine nucleotides (Wang, 1969; Strittmatter, 1972). The negligible activation by Mn²⁺, Co²⁺ and Mg²⁺, the apparent M_r and substrate specificity differentiate this enzyme from that of rat testicular tissue (Vanha-Perttula, 1970) and rabbit spermatozoa (Gonzales & Meisel, 1973).

The M_r of 118000 is similar to that reported for the high- M_r acid phosphatases from mammalian liver (Igarashi & Hollander, 1968; Campbell *et al.*, 1973; Saini & Van Etten, 1978a,b), prostatic (Luchter-Wasyl & Ostrowsky, 1974; Saini & Van Etten, 1978b) and placental tissue (Di Pietro & Zengerle, 1967). These enzymes, however, were not stimulated by Zn²⁺, showed an optimal pH in the more acidic region (pH 4.5–5.5) and were strongly inactivated by NaF.

Table 1. Summary of purification of the Zn²⁺-dependent acid *p*-nitrophenyl phosphatase of bovine liver

Procedure	Protein (mg)	Activity (units)	Specific activity (units/mg of protein)	Recovery (%)
Liver extract	5800	168	0.029	100
(NH ₄) ₂ SO ₄ fractionation	3650	120	0.033	71
pH fractionation	2200	96	0.043	57
Sephadex G-200	420	54	0.128	32
DEAE-cellulose	28	38	1.36	22
CM-cellulose	1.2	12	10.00	7

Fig. 4. Effect of pH and metal ions on activity of acid *p*-nitrophenyl phosphatase from bovine liver

The metal ions (■, Zn²⁺; □, Mn²⁺) were added to give a final concentration of 4 mM. Assays were carried out at 37°C with 2.5 mM-*p*-nitrophenyl phosphate in the following buffers: 50 mM-acetate (pH 4–6), 25 mM-barbital (pH 6–8.8) and 50 mM-glycine/NaOH (pH 8.8–9).

On the basis of these findings, I conclude that bovine liver contains an acid *p*-nitrophenyl phosphatase form, which requires Zn²⁺ ions for its activity, and is different from those previously detected in mammalian tissues.

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