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IRON-CONTAINING ACID PHOSPHATASES: COMPARISON OF THE ENZYMES FROM BEEF SPLEEN AND PIG ALLANTOIC FLUID

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Summary: The iron-containing violet acid phosphatases from beef spleen and pig allantoic fluid have been purified to homogeneity. Molecular weight determinations by zonal gel filtration, SDS-gel electrophoresis, and ultracentrifugation support values close to 40,000 for both enzymes, necessitating reappraisal of literature values. Similarly, the equivalent weight for iron is close to 20,000 for both enzymes, indicating the presence of two iron atoms per molecule of enzyme. The enzymes also have very similar ultraviolet and visible spectra, with λ_{max} values close to 550 nm, and ϵ_{550} values (in terms of iron) of 2.04×10^3 and 2.00×10^3 for the beef spleen and pig allantoic fluid enzymes respectively.

Campbell and Zerner (1) showed the presence of iron in highly purified preparations of the violet acid phosphatase ("phosphoprotein phosphatase") from beef spleen. Their data indicated a 1:1 molar ratio of iron to enzyme, based on the molecular weight (23,000) and $A_{1cm}^{1\%}$ [1] at 280 nm (15.0) reported by Glomset and Porath (2), and assuming that the ratio A_{280}/A_{550} for the pure enzyme is 17.8. Subsequently, Roberts and his co-workers (3) found that a violet progesterone-induced glycoprotein from pig uterine washings is also an iron-containing acid phosphatase. This protein is present in large amounts in pig allantoic fluid, its content peaking just after mid-term in pregnancy (4).

We undertook a study of the enzyme from pig allantoic fluid for two reasons: (i) much larger amounts of highly purified enzyme could apparently be obtained for mechanistic and structural studies than from beef spleen; and (ii) the molecular weight and stoichiometry of iron binding reported for the pig enzyme (3) differ substantially from our data on the beef spleen enzyme. In this communication, we report comparative data on the molecular weights, equivalent weights for iron binding, and spectra of the two enzymes. Our results for both enzymes strongly support a molecular weight of ~40,000, with two iron atoms per molecule of enzyme.

Experimental Section

All chemicals used were reagent grade if not specified, and all solutions were made with distilled, deionized water. Measurements of pH were made at 25° on a Radiometer pH meter 4c. Spectral measurements and enzyme assays were carried out at 25°, using Cary 14 and Cary 17 recording spectrophotometers.

[1] Abbreviations used: $A_{1cm}^{1\%}$, the absorbance of a 1% solution (10 mg/ml); A_{280}, absorbance at 280 nm; SDS, sodium dodecyl sulfate.

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Two assay procedures for acid phosphatase activity were used, both with *p*-nitrophenyl phosphate (Calbiochem) as substrate. In assay I (1), the increase in absorbance at 410 nm was measured at pH 6.00 in the presence of 10 mM ascorbate and 20 μM ferrous ammonium sulfate to ensure that the enzymes are completely in the reduced (active) form. In assay II, 0.1 M sodium acetate buffer, pH 4.90 (3 ml), was equilibrated in the cell compartment of the spectrophotometer. *p*-Nitrophenyl phosphate (100 μl; 0.152 M in water) and an aliquot of enzyme (usually 20 μl) were added, and the increase in absorbance at 390 nm measured on the 0 - 0.1 absorbance scale with buffer and substrate in the reference beam (final pH 4.96; ϵ_{390} *p*-nitrophenyl phosphate = 120.4; ϵ_{390} *p*-nitrophenol = 463). The increase in absorbance is linear for at least one chart width (0.1 absorbance units). This assay was carried out under similar conditions (pH, absence of reducing agents) to those described (3), but has the major advantage of being a continuous assay.

The beef spleen enzyme was prepared by modification of the method described previously (1). The modifications involve concentration and purification of the enzyme in the acid extract by batch adsorption to CM-cellulose rather than by ammonium sulfate precipitation, and an extra chromatographic step on cellulose phosphate. Details of this procedure will be published elsewhere. In one large-scale preparation starting with 40 kg of spleen, 35 mg of enzyme was obtained with the following properties: A_{280}/A_{550} = 15.1; specific activity in assay I, 13.0 (mkat/l)/A_{280}; $\geq$95% pure by polyacrylamide gel electrophoresis in pyridine-HCl buffer, pH 4.9.

The enzyme from pig allantoic fluid was prepared by a modification of the published procedure (4), which involves chromatography on CM-cellulose followed by gel filtration, where it was found that Sephadex G-75 gave better resolution than Sephadex G-100. The large-scale preparation used in the present work had as starting material the allantoic fluid collected from the concepti of four sows at about 70 days of pregnancy. Figure 1 presents the elution profile from the Sephadex G-75 column, and shows that the enzyme is partially resolved into a faster moving violet form [fraction 90, λ_{max} 550 nm; specific activity in assay II, 0.63 (mkat/l)/A_{280}] and a slower moving pink form [fraction 120, λ_{max} 515 nm; specific activity in assay II, 5.45 (mkat/l)/A_{280}]. The constancy of specific activity across the A_{280} peak in the presence of ascorbate (assay I) supports the proposition that the enzyme as isolated is a mixture of oxidized and reduced forms, but is essentially free of non-enzyme protein. The A_{280}/A_{530} ratio decreases slightly across the peak, as expected from the ratios for the reduced and oxidized forms of the enzyme. Thus, enzyme reduced by treatment with 0.1 M β-mercaptoethanol and 1 mM ferrous ion has λ_{max} 510 nm and A_{280}/A_{530} = 13.8. Enzyme oxidized by treatment with 1.2 mM potassium ferricyanide has λ_{max} 560 nm and A_{280}/A_{530} = 15.3. The pooled peak (Figure 1, fractions 81 - 124) was concentrated by ultrafiltration (Diaflo PM-10 membrane) to give an enzyme solution with the following properties: λ_{max} 530 nm; A_{280} = 19.44; A_{280}/A_{550} = 15.65; A_{280}/A_{260} = 1.56; specific activity in assay I, 1.16 (mkat/l)/A_{280}; specific activity in assay II, 2.17 (mkat/l)/A_{280}; $\geq$95% homogeneous by polyacrylamide gel electrophoresis in Tris-citrate buffer, pH 4.9. Pretreatment of an aliquot of this enzyme with 0.1 M β-mercaptoethanol and 1 mM ferrous ion in 0.1 M sodium acetate buffer, pH 4.9, followed by assay II, gave a maximum specific activity of 5.65 (mkat/l)/A_{280}. Repeated assays (assay II) of the enzyme solution (stored at 4°) over a period of 3 months showed no change in specific activity.

Determination of sedimentation and diffusion coefficients was carried out at 20° in a Spinco model E ultracentrifuge using the schlieren optical system. Details of these and other experimental procedures used are included in Table I.

Results and Discussion

Acid phosphatases have been prepared from beef spleen and pig allantoic fluid in essentially pure form. We have confirmed that a slight modification of the simple literature procedure (4) yields essentially homogeneous pig enzyme in large amounts (550 mg from four pregnant sows in the preparation described). Comparison of the accessibility of the two enzymes leaves no doubt that the pig enzyme is the enzyme of choice for further work on enzymes of this class. Two major differences are revealed by comparing the catalytic properties of the purified enzymes. When assayed in the presence of ascorbate at pH 6, the specific activity of the beef enzyme is about ten times that of the pig enzyme. This may of course reflect different pH depend-

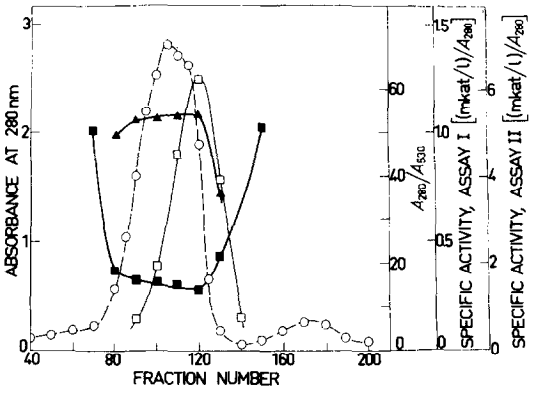

Figure 1. Elution profile of pig allantoic fluid acid phosphatase on Sephadex G-75 (column dimensions 74 × 5.2 cm; flow rate, 210 ml/h; 14-ml fractions): A_{280} (○); A_{280}/A_{530} (■); specific activity in assay I (▲); specific activity in assay II (□).

encies and/or K_m values for *p*-nitrophenyl phosphate. The other difference is in the state of activation of the enzymes as isolated, as shown by the effect of reducing agents on specific activity. With the beef enzyme (assay I), the rate in the presence of ascorbate is ~40 times the rate in its absence. With the pig enzyme (assay II), prior reduction of the pooled enzyme with β-mercaptoethanol and ferrous iron leads to a maximum activation of 2.5-fold. In contrast, the pig acid phosphatase isolated by Schlosnagle *et al.* (5) is almost all in the oxidized form, the activity being stimulated by up to 15-fold by treatment with β-mercaptoethanol or ascorbate.

Table I shows a comparison of some of the properties of the two enzymes. Perusal of the data shows the great similarity between molecular weights, equivalent weights and spectral properties of the two enzymes. The oxidized and reduced forms of the pig enzyme clearly have slightly differing elution volumes on zonal gel filtration. This small difference may be accounted for by a change in shape of the molecule upon reduction (see, for example, ref. 8).

On SDS-gel electrophoresis, the beef enzyme is fractionated into two polypeptides whose combined molecular weight (41,000) agrees well with the estimated molecular weight of the single polypeptide chain of the pig enzyme (40,000) and with the other estimates of the molecular weight of the beef enzyme. The presence of two polypeptide chains in the beef enzyme is probably due to proteolysis during the prolonged acid extraction (1). Chicken liver carboxylesterase, homogeneous by other criteria, gives three polypeptides on SDS-gel electrophoresis, one with a molecular weight ~65,000 and two with molecular weights of about 30,000 (8). This behavior is consistent with the partial cleavage of a peptide bond either *in vivo* or during the extraction. Similarly, tetanus toxin isolated from within the *Clostridium tetani* cells has a molecular

TABLE I

Molecular Properties of the Iron-Containing Acid Phosphatases from Beef Spleen and Pig Allantoic Fluid

Property	Spleen Enzyme	Allantoic Fluid Enzyme
Molecular weight		
(i) by zonal gel filtration[a]	38,000	38,000[b]; 36,000[c]
(ii) by SDS-gel electrophoresis[d]	15,000; 26,000	40,000
(iii) by ultracentrifugation	37,200[e]; 35,000[f]	41,000[g]
Equivalent weight for Fe	19,400[h]	21,000[i]
Spectral data		
(i) $A^{1\%}_{1cm}$[j]	15.9	14.2
(ii) A_{280}/A_{550}	15.1	15.6[k]
(iii) ϵ_{550} (per Fe)	2.04×10^3	2.00×10^3
(iv) A_{280}/A_{260}	1.58	1.56

[a]On Sephadex G-75, in 0.1 M acetate buffer, pH 4.90, containing 0.2 M NaCl; ribonuclease A, chymotrypsinogen A, pepsin, ovalbumin, and bovine serum albumin as molecular weight standards. [b]Pooled enzyme treated with ferricyanide (2.44 mM) to convert it to the oxidized form. [c]Pooled enzyme treated with ferrocyanide (2.50 mM) to convert it to the reduced form; the column buffer was O_2-free, and contained 1 mM ferrocyanide. [d]Proteins dialyzed against oxygen-free 0.1 M phosphate buffer, pH 7.1, containing 1% w/v SDS and 1% w/v β-mercaptoethanol at 38°, then against 0.01 M phosphate buffer, pH 7.1, 0.1% in SDS and β-mercaptoethanol, and run in 7% polyacrylamide gels at 28-30° in 0.1 M phosphate buffer, pH 7.1, 0.1% in SDS and β-mercaptoethanol for 6 h at 7 mA/gel; standard proteins, pepsin, chymotrypsinogen A, ribonuclease A, cytochrome *c* and myoglobin; estimated from a plot of mobility relative to bromophenol blue against log M_r. [e]In 0.1 M acetate buffer, pH 4.90, containing 0.2 M NaCl; calculated using the Svedberg equation from the values of *s* and D extrapolated to zero protein concentration (2.85 S and 7.30×10^{-7} cm^{2}s^{-1}), and a $\bar{v}$ of 0.737 ml g^{-1} (from the amino acid composition). [f]As for *e*, but using $\bar{v}$ of 0.725, assuming the presence of 10% w/w carbohydrate. [g]Calculated from the sedimentation equilibrium data of Chen *et al.* (6), using $\bar{v}$ = 0.72 ml g^{-1}, estimated from the amino acid analysis and 12% carbohydrate content. [h]Estimated by atomic absorption spectroscopy. [i]Estimated as the complex with bathophenanthroline disulfonate as described by Campbell and Zerner (1). [j]Determined by the dry weight procedure of Blakeley and Zerner (7). [k]Determined on pooled enzyme from large scale preparation, with ~40% of maximum activity (before activation); conversion to the inactive form produces a small change in this ratio. The A_{280}/A_{530} of this enzyme = 15.2.

weight of 150,000, and is a single polypeptide chain, whereas toxin isolated from culture filtrates consists of two non-identical polypeptide chains with molecular weights of 100,000 and 55,000 (9,10).

The molecular weight reported for the beef spleen enzyme (2) was based on a sedimentation coefficient of 2.4 S and a diffusion coefficient of 9.2×10^{-7} cm^{2}s^{-1} at a protein concentration of 7 g/l in 0.1 M sodium acetate buffer, pH 5.6, at 20°. The extrapolated values of $s_{20,buffer}$ and D (Table I, footnote *e*) differ considerably from the literature values which took no account of concentration dependence. These

values, together with a $\bar{v}$ calculated from the amino acid composition, yield estimates of molecular weight much higher than the previous estimates (1,2), and fully consistent with the values obtained by other methods. The molecular weight of 32,000 reported for the pig enzyme on the basis of sedimentation equilibrium experiments (6) depends on a $\bar{v}$ of 0.64 ml/g! A $\bar{v}$ of this magnitude is characteristic of a polysaccharide (11). From the amino acid composition and the 12% carbohydrate content (6), a $\bar{v}$ of 0.72 ml/g may be calculated. Substitution of this value of $\bar{v}$ yields a molecular weight of 41,000, in agreement with the results obtained by the other procedures.

The data on the stoichiometry of iron binding clearly indicate the presence of two iron atoms per molecule of enzyme. As discussed in the introduction, the previous suggestion of a 1:1 stoichiometry in the beef enzyme (1) was based on the best estimates then available of molecular weight, $A^{1\%}_{1\,cm}$ and A_{280}/A_{550}. As shown in Table I, these values have been revised, most notably the molecular weight. Comment is also required on the estimate of 32,000 for the equivalent weight for iron of the pig enzyme (3). The reported value (6) of ϵ_{545} in terms of the molar concentration of iron (1.76×10^3) is only about 10% lower than the value reported in Table I. The major discrepancy between the two equivalent weights (21,000 *vs* 32,000) results from the different methods used to determine protein concentration. A method based on an independently determined property of the pure protein, such as the gravimetrically determined $A^{1\%}_{1\,cm}$ at 280 nm used in this work, is essential if an accurate estimate of protein concentration is to be obtained (7). Schlosnagle *et al.* used the Lowry method with bovine serum albumin as standard protein (12). Colour development in this method depends on the number of tyrosine and tryptophan residues, and bovine serum albumin is deficient in tyrosine and/or tryptophan compared with pig allantoic fluid acid phosphatase: $A^{1\%}_{1\,cm}$ at 280 nm ~6.5 for bovine serum albumin (13). Hence, the actual concentration of the pig enzyme was considerably less than that estimated by the procedure used, leading to the high value of 32,000 for the equivalent weight.

As has been discussed in earlier reports (1,14), other acid phosphatases have been purified which resemble more or less closely the enzymes characterized in this work. The number of similar enzymes continues to grow (see, for example, references 15-20), making it clear that this class of acid phosphatase is widely distributed. These enzymes continue under active investigation in this laboratory, especially with respect to their biological significance and the role of iron in catalysis.

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