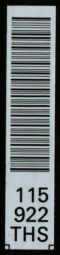

This is to certify that the

thesis entitled

THE PURIFICATION AND SOME PROPERTIES OF

PURPLE ACID PHOSPHATASE FROM BEEF SPLEEN

presented by

Sandy Shieufun Lin

has been accepted towards fulfillment
of the requirements for

~~Master~~ degree in ~~Science~~

Bruce A. Averill

Major professor

Date May 15, 1979

THE PURIFICATION AND SOME PROPERTIES OF PURPLE ACID PHOSPHATASE FROM BEEF SPLEEN

BY

Sandy Shieufun Lin

A THESIS

Submitted to

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ABSTRACT

THE PURIFICATION AND SOME PROPERTIES OF

PURPLE ACID PHOSPHATASE FROM BEEF SPLEEN

by

Sandy Shieufun Lin

The existing purification procedure for purple acid phosphatase from beef spleen was relatively inefficient and has been extended using cellulose phosphate chromatography. The recovery is much higher than that obtained with other techniques. The purity is increased by two and a half fold.

The Michaelis - Menten constant, K_m , for p-nitrophenyl phosphate has been found to be 2.0 mM. A plot of $1/V_o$ versus 1/(S) according to the Lineweaver - Burke equation was used to calculate the K_m .

The relationship between the structural features of inhibitors and the binding specificity of the enzyme is investigated through the inhibition studies of the structural analogues of the substrate, PNPP. The results suggest that the binding specificity is not related to the charge distribution on the aromatic moieties of the structual analogues, but related to the nature of phosphorus linkage.

A catalytic reaction pathway is postulated from the products inhibition studies.

ACKNOWLEDGEMENT

The author is indebted to Dr. B. A. Averill for his direction, assistance and encouragement in making this investigation possible. The author would also like to express thanks to Dr. Babcock for lending the Waring blendor used in the preparation of crude beef spleen homogenate.

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LIST OF ABBREVIATIONS

PNPP	p-Nitrophenylphosphate
PNP	p-Nitrophenol
PABP	p-Aminobenzylphosphonic acid
ASC	Ascorbic acid
MES	(2-(N-Morpholino) ethanesulfonic acid)
CM-52	Carboxymethyl 52
AEP	2-Aminoethylphosphonic acid
PABP*	p-acetamidobenzylphosphonic acid

I. INTRODUCTION

The enzymes that catalyze the hydrolysis of phosphate esters may be divided into three classes, namely, alkaline phosphatases, non-purple acid phosphatases and purple phosphatases. Most of the violet phosphatases are acid phosphatases, except the alkaline phosphatase isolated from Micrococcus sodonensis (1). These enzymes play a vital role in the regulation of the physiological level of inorganic phosphate and phosphorylated metabolites. A class of alkaline phosphatases containing zinc has been studied in great detail, as has the class of acid phosphatases, that do not contain any known prosthetic group and are not violet in color. Although a few isolated reports have appeared on a series of violet acid phosphatases, this class of enzymes remains poorly characterized as to the nature of the violet chromophore and the hydrolytic catalytic properties associated with an iron containing enzyme. This class of enzymes has been isolated from diverse sourses such as bovine spleen (2,3), porcine intrauterine fluids (4,5), sweet potato (6,7), kidney bean (8), the mold Neurospora crassa (9). All are reported to be basic glycoproteins. The violet acid phosphatase from beef spleen was first purified by Glomset in 1959 (3). The electronic spectrum of this enzyme is reported by them to have an intense absorption band at 550 nm and a shoulder at 310-320 nm similar to that reported by other workers. The enzyme has a pH optimum of about 5~6.

The enzyme from beef spleen showed activity toward a variety of

substrates including aromatic phosphate esters, phosphoproteins, neucleotides and inorganic pyrophosphate. Aliphatic phosphate esters such as glycerol phosphate were not hydrolyzed to any appreciable extent by the enzyme. In marked contrast, aromatic phosphate esters and phosphamides were hydrolyzed by the enzyme at a rapid rate. The enzyme was inhibited by various chelating reagents at low concentration with ATP and casein as substrates (10). Among these inhibitors molybdate was the most effective one. The enzyme was reported to be activated by reducing agents (11). Of these ascorbic acid and thioglycolic acid were the best activators. Furthermore the enzyme was inactivated by short exposure to reducing agents in the absence of substrates, casein and ATP, upon preincubation at room temperature. Ferrous ion protected against this inactivation (12). Although the existence of enzyme activity in diverse sources as described above has been known for over 20 years, only a few of these enzymes have been obtained in the purified preparation and demonstrated to contain tightly bound iron. They are those isolated from beef spleen (2), porcine uterine fluids (4), and kidney bean (8). The number of ferric ions per molecule of beef spleen acid phosphatase is reported by Revel and Racker (12) to be one and the molecule weight was 40,000 daltons. In 1978 the Australian group reported the presence of two iron atoms per molecule of enzyme from beef spleen (13). The equivalent weight per iron atom was 20,000 daltons. However the Swedish group had discounted the possibility of metal ion involvement on the basis of arc spectrography, EPR measurements and chemical analysis of certain metals (3).

There are two objectives of the research in our laboratory: (1) to determine the relationship of the structure of the prosthetic group to

the enzymatic activity; 2. to establish the nature of the unusual chromophore, including identification of the metal, its ligands and their geometrical arrangement in the violet phosphatases. No other iron containing enzymes have been demonstrated to have phosphatase activity. It is unique among iron containing enzymes in that it is hydrolytic whereas most iron-containing enzymes participate in electron transfer reactions or oxidation/reduction catalysis. In line with our laboratory's interest, the development of a separation technique in an attempt to improve the purity and recovery is described. In addition to this, the Michaelis-Menten constant, K_m , was determined for p-nitrophenyl phosphate as substrate. A catalytic reaction pathway is postulated through products inhibition studies. The activation of this enzyme by ferrous ion in the presence or absence of ascorbic acid (reducing agent) and substrate, PNPP , is described. The inactivation of this enzyme by ascorbic acid in the absence of substrate and ferrous ion is also described in this thesis.

II. EXPERIMENTAL

A. Materials and Methods

1. Reagents and materials

Bovine spleen was obtained from the local slaughter house at Okemos, Michigan. (2-(N-Morpholino) ethanesulfonic acid) was the Ultrol brand of Calbiochem, La Jolla, Ca; ascorbic acid was from Mallinckrodt Company, Paris Kentucky. Para-nitrophenylphosphate disodium salt hexahydrate was the product of Aldrich Chemical Company, Inc. Milwaukee Wis. Ammonium sulfate, enzyme grade, was the product of ICN pharmaceutical Inc; Life Science Group, Cleveland, Ohio.

2. Instrumentation

Conductivity and pH were measured with a digital electromark analyzer, Markson Scientific. The absorbance of UV & Visible light was measured by a Beckman DU spectrometer adapted with a Gilford photometer 252 and a Linear recorder. Protein solutions were concentrated through an Amicon micro-ultrafiltration system, 8 MC, and high performance thin-channel ultrafiltration system, TCF 10, under N_2 with Amicon diaflo PM 10 membranes, Dialysis tubing was the product of Fisher Scientific Company, Pittsburgh, Pa. 15219.

3. Preparation of column for chromatography

All the glass columns used were purchased from Fisher & Porter Inc. The column buffer was 0.1 M NaOAc buffer, pH 4.9 unless otherwise specified.

a. Ion exchange chromatography (CM52 cellulose)

CM-52 cellulose was purchased from Whatman LTD. Springfield Mill, Maidstone, Kent. 600 g of the above gel was added to 3 l of 0.1 M NaOAc buffer, pH 4.9 and gently stirred. The pH was adjusted to 4.9 and the fines were decanted. The above process was repeated until the wash solution was found to have a pH of 4.9 and the volume of the slurry was 3 l. The slurry was then added to a glass column 2.5 cm in diameter and gravity packed to a height of 62 cm. The column was then washed with one column volume of 0.1M NaOAc buffer, pH 4.9.

b. Gel permeation chromatography (Sephadex G75)

Sephadex G75 was purchased from Pharmacia Fine Chemicals, Division of Pharmacia Inc. Piscatawa, New Jersey. 20 g of medium mesh gel was added to 500 ml of 0.1 M NaOAc buffer, pH 4.9 and gently stirred. The pH was adjusted to 4.9 and the gel was treated in the same manner as described above. The volume of the resulting slurry was 300 ml. The slurry was then added to a glass column 1.25 cm in diameter and gravity packed to a height of 65 cm. The column was then washed with one column volume of 0.1 M NaOAc buffer, 0.1 mM in EDTA, pH 4.9, and kept at 4°C.

c. Cellulose phosphate chromatography

Monosodium cellulose phosphate was purchased from Whatman LTD Springfield Mill, Maidstone, Kent. 2.2 g of cellulose phosphate was added to 60 ml of 0.1 M NaOAc buffer, pH 4.9 and the gel was treated in the same manner as described above. The slurry was poured into a glass column 1 cm in diameter and gravity packed to a height of 7 cm. The column was then washed with one or two column volumes of column buffer and kept at 4°.

4. Enzymatic activity and protein concentration determination

a. Assay for acid phosphatase

The assay was performed either by the continuous method (2) or the fixed time interval method (5). The assay temperature was 25°. A Beckman DU spectrometer was used to record the absorbance at 410 nm. The apparent extinction coefficient, ε^*, of p-nitrophenol was determined to be 1266 l/mole at 25°, pH 6.0 with pK_a = 7.15. The assay mixture (final volume 1.25 ml) contained 100 mM MES buffer (pH 4.9), 10 mM ASC, 20 M ferrous ammonium sulfate, an aliquot of enzyme and 10 mM p-nitrophenyl phosphate (PNPP). The mixture, excluding PNPP, was equilibrated at 25°. PNPP was then added to the mixture and the reaction was started by addition of an aliquot of enzyme. In the routine continuous assay procedure the increasing absorbance of phenolate at 410 nm was followed at pH 6.0 with a recorder attached to the Gilford spectrophotometer. The rate of hydrolysis was then calculated from Beer's law. In the non-routine procedure (fixed time interval), the enzymatic reaction was terminated at fixed times by the addition of 6 N NaOH solution, and the absorbance at 410 nm was measured on the Gilford spectrometer. A Linear plot was obtained from the plot of the change in absorbance per minute versus time (minute). The extinction coefficient, ε ,(17,000 l/mole) was used to calculate the rate of hydrolysis.

b. Definition of a unit of activity and specific activity.

One unit of acid phosphatase activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1μmole of p-nitrophenyl phosphate per minute under the above specified conditions. Specific activity is defined as units/mg, i.e. units of enzymatic activity per milligram of protein.

c. Determination of protein concentration

The protein concentration was determined according to the Lowry method (14) or estimated from A_{280} by assuming a solution having A_{280} of 1 contains 1 mg/ml of protein. During the earlier stage of preparation, the fraction having A_{280} contains 4 mg/ml protein according to the Lowry method.

B. Protein purification

All chemicals used were reagent grade and all solutions were made with distilled, deionized water. All operations were performed at 4° C, unless otherwise specified. Fresh bovine spleen were obtained from a local slaughter-house, stored at -20°C, and thawed at 4° immediately prior to acid extraction. The acid extraction was carried out at 25°, the acid extract was stored for no more than 24 hours, and the ammonium sulfate fractionation was performed essentially according to Singer and Fructon (15). The fraction after salting out was dialyzed, then subjected to a series of chromatography steps, including CM-52 cellulose, G75 Sephadex and cellulose phosphate.

1. Acid extraction

1.65 Kg of frozen beef spleen was thawed at 4°C and ground with 3310 ml of cold water in a 1 gallon Waring blendor (2 mins at medium speed, 1 min at high speed). To the "homogenate" (5800 ml), 2 N H_2SO_4 (185 ml) was added with stirring until the pH was 3.0. After one hour the pH had risen to 3.5, and 40 ml 2 N H_2SO_4 was added to readjust the pH to 3.0. Toluene (26 ml) was then added, and the mixture was stirred at room temperature for 22 hours. It was then centrifuged at 2000 g for 30 minutes at 4°, and the supernatant fluid was filtered through glass wool to give 4000 ml of a brown-yellow solution (" acid extract "). The enzymatic activity and protein concentration were measured.

2. 60 - 90 % saturated ammonium sulfate fractionation

To 4000 ml of the acid extract, 1287 g of solid ammonium sulfate (60 % saturation) was added in small portions gently with stirring. The mixture was then centrifuged at 2500 x g for 30 minutes. The pellet, containing no activity, was discarded. To the supernatant fluid

(3600 ml), 817.0 g of $(NH_4)_2SO_4$ was added (90 % saturation) as described above. The mixture was centrifuged at 2500 x g at 4°. The precipitate collected was then suspended in a minimum amount of 0.1 M NaOAc buffer, pH 4.9, resulting in 300 ml of solution, which was then dialyzed for 48 hours at 4° versus 2 changes of 3 l of 0.1 M NaOAc buffer, pH 4.9. The conductivity of the resultant dialysate was near that of the acetate buffer alone. The total activity and concentration of the dialyzed protein were measured. The dialysis tubing was prepared by boiling it in a 0.001 M EDTA solution, and then boiling in distilled water. The tubing was washed with cold, deionized water and then followed by the addition of the aliquot enzyme.

3. CM-52 cellulose column

The dialyzed fraction of total volume 920 ml and activity of 552 units was loaded to a CM-52 cellulose column described previously on page 5.The bound protein was then washed by several column volumes of 0.1 M NaOAc buffer, pH 4.9, until the A_{280} was less than 0.05 and then eluted with a linear gradient consisting of 2.0 l of column buffer and 2.0 l of 0.6 M NaCl in column buffer, followed by 300 ml solution of 0.6 M NaCl in column buffer. The fractions were collected in an automatic fractionator, the A_{280} profile and the enzymatic activity profiles are shown in fig. 1. Fractions with a specific activity greater than 1.0 were pooled and the combined fractions were concentrated down to 5 ml by Amicon microultrafiltration (8 MC) under N_2 through Amicon UM-2 membrane.

4. Sephadex G75

The fraction with a volume of 5 ml from CM-52 cellulose column was then loaded on to a Sephadex G75 column as described on page 5. The

protein was then eluted with 0.1 M NaOAc buffer, pH 4.9. The protein concentration and the enzymatic activity profile were plotted (fig. 2). Fractions with a specific activity greater than 14.0 were pooled and concentrated to 1 ml by ultrafiltration as above. The concentrated enzyme was then frozen in liquid nitrogen and kept at 4°. A summary of the purification scheme is presented in Table I with calculated yields.

5. Further purification by cellulose phosphate column

A sample containing 149 units of activity was loaded on to the cellulose phosphate column as described on page 5, and then eluted with a linear gradient containing 100 ml of 0.5 M NaCl in 0.1 M NaOAc buffer, pH 4.9 and 100 ml of 2.0 M NaCl in 0.1 M NaOAc buffer, pH 4.9. Fractions with a specific activity greater than 19.0 were pooled and concentrated as described above. The average specific activity was 35.00 units/mg. The elution profile is shown in fig. 3. A summary of the purification scheme is presented in Table I.

Figure 1

Elution Profile of Beef Spleen Acid Phosphatase from CM-52 Cellulose Chromatography Column.

Closed circles : A_{280}. Open circles : Enzymatic activity. The volume of each fraction was 12 ml.

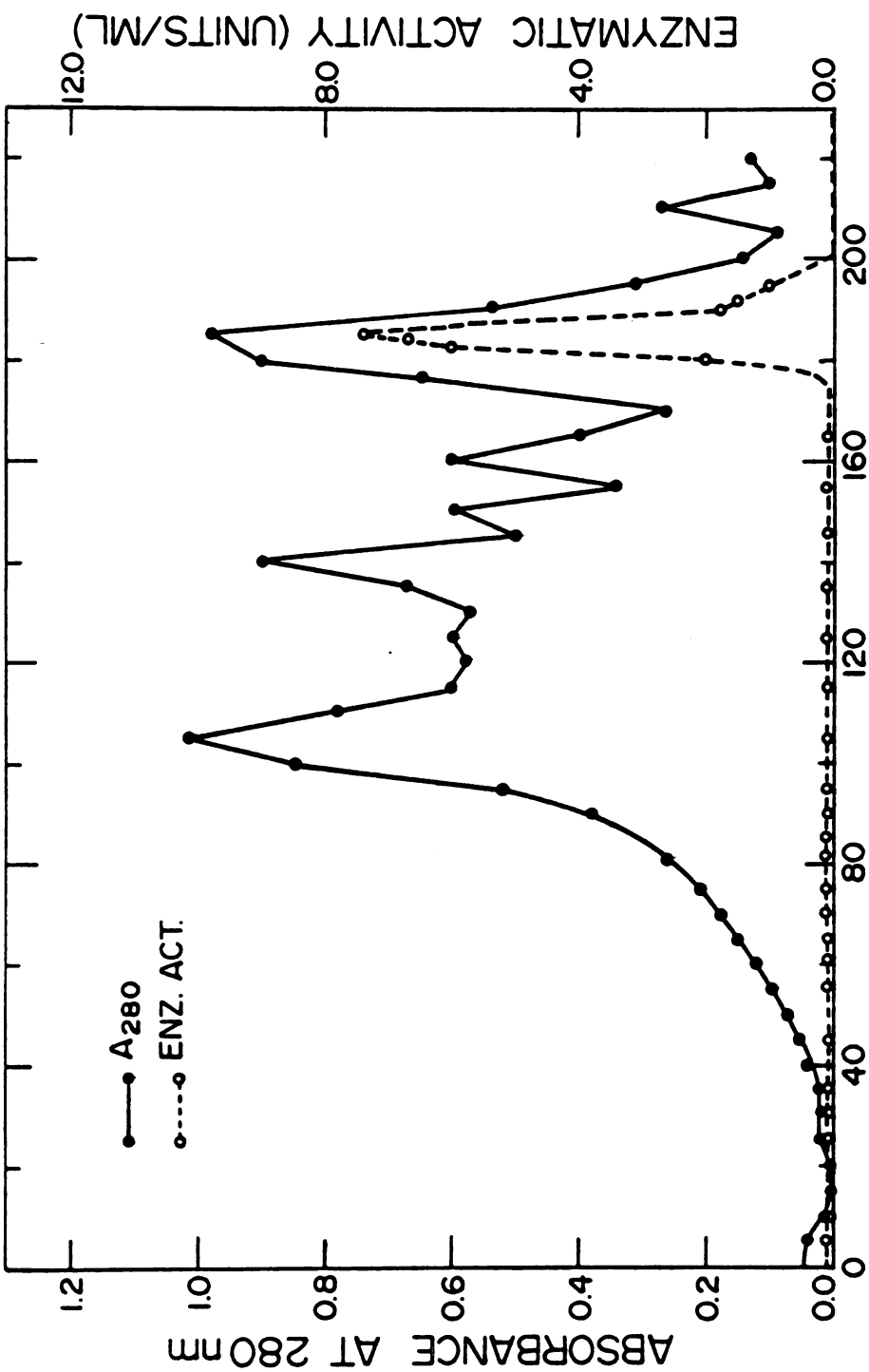

ABSORBANCE AT 280 nm
ENZYMATIC ACTIVITY (UNITS/ML)
FRACTION NUMBER
A280
ENZ. ACT.
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0.0
4.0
8.0
12.0
0
40
80
120
160
200

FIG. I

Figure 2

Elution Profile of Beef Spleen Acid Phosphatase from Sephadex G75 Chromatography Column

Closed circles : A_{280}. Open circles : Enzymatic activity. The volume of each fraction was 3 ml.

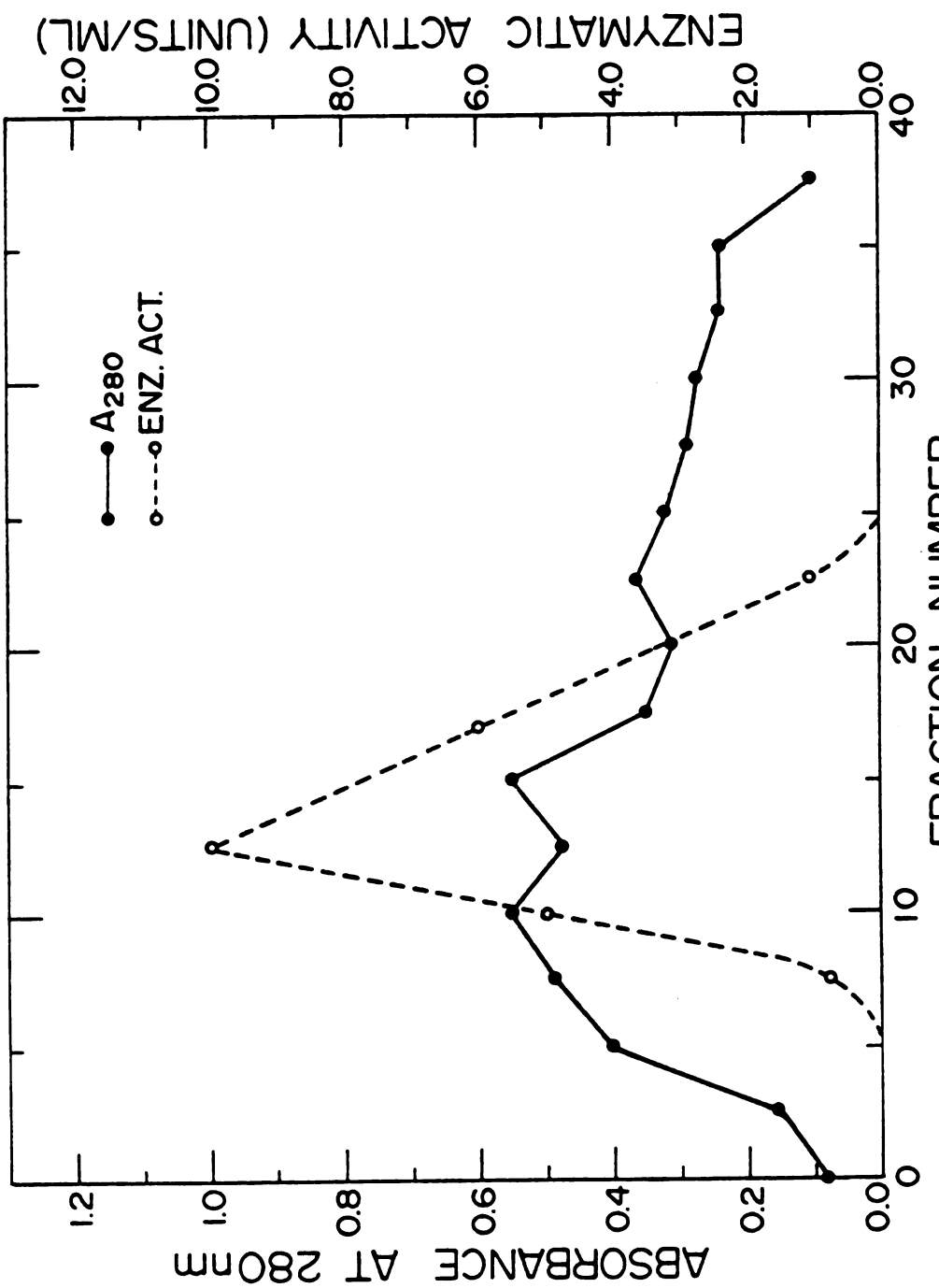

ABSORBANCE AT 280nm
ENZYMATIC ACTIVITY (UNITS/ML)
FRACTION NUMBER
A280
ENZ. ACT.
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0.0
2.0
4.0
6.0
8.0
10.0
12.0
0
10
20
30
40

FIG. 2

Table I

Purification of Beef Spleen Acid Phosphatase

step	total volume (ml)	total activity (units)	total protein (g)	specific activity (units/mg)	% yield
acid extarction	4000	3600	49.60	0.07	100.0
60 - 90 % satd $(NH_4)SO_4$	920	552	1.93	0.28	15.0[b]
CM-52 cellulose	700	700	0.24	2.80	19.0
Sephadex G-75	45	300	0.01	28.00	8.3
cellulose[a] phosphate	24	115	0.0033	35.00	70.0[c]

[a] A fraction containing 149 units of acid phosphatase activity with a specific activity of 14.0 units/mg was used.

[b] 90 % in small scale

[c] in this step, others are overall

Table II

Acid Extraction

pH	% toluene	total activity (units)	total protein (mg)	specific activity (units/mg)
3.0[a]	0.5	425	5358	0.08
3.5[b]	0.5	341	7134	0.05
3.0[c]	1.0	270	4244	0.06

a Extraction of 85 g beef spleen

b Extraction of 90 g beef spleen

c Extraction of 80 g beef spleen

Table III

Saturated Ammonium Sulfate Fractionation

% ammonium sulfate	total activity (units)	total protein (mg)	specific activity (units/mg)	% yield
55 - 80[a]	140	795	0.17	90
60 - 90[b]	128	475	0.27	90
65 - 100[c]	70	229	0.28	50
< 55[a]	16	-	-	9
< 60[b]	7	-	-	.5
< 65[c]	4	-	-	3

a Acid extract containing 175 units of acid phosphatase activity was used.

b Acid extract containing 140 units of acid phosphatase activity was used.

c Acid extract containing 140 units of acid phosphatase activity was used.

The specific activity of acid extract in each case was 0.08 units/mg.

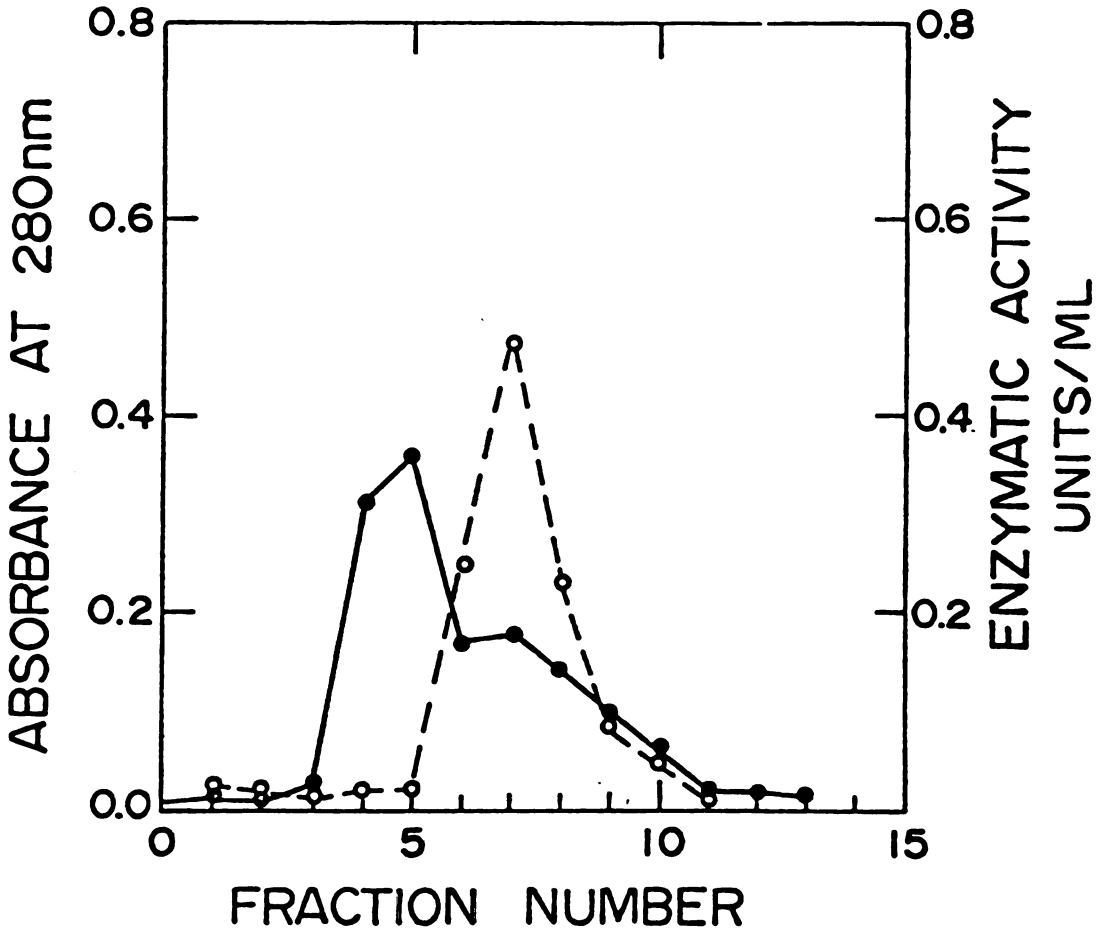


FIG. 3

Elution Profile of Beef Spleen Acid Phosphatase from Cellulose Phosphate Column

A linear gradient of 0.5 M - 1.0 M NaCl in NaOAc buffer (pH 4.9) was used for elution. The volume of each fraction was 5 ml. Closed circle : A_{280}. Open circles : Enzymatic activity.

Figure 4

Determination of K_m for p-nitrophenylphosphate

V_o is the initial velocity of the catalytic reaction. (S) is the concentration of p-nitrophenylphosphate. The standard method was perfomed at 25°, pH 6.0 with various concentration of substrate, (S), : 0.5 mM, 1.0 mM, 2.0 mM, 4.0 mM, 5.0 mM.

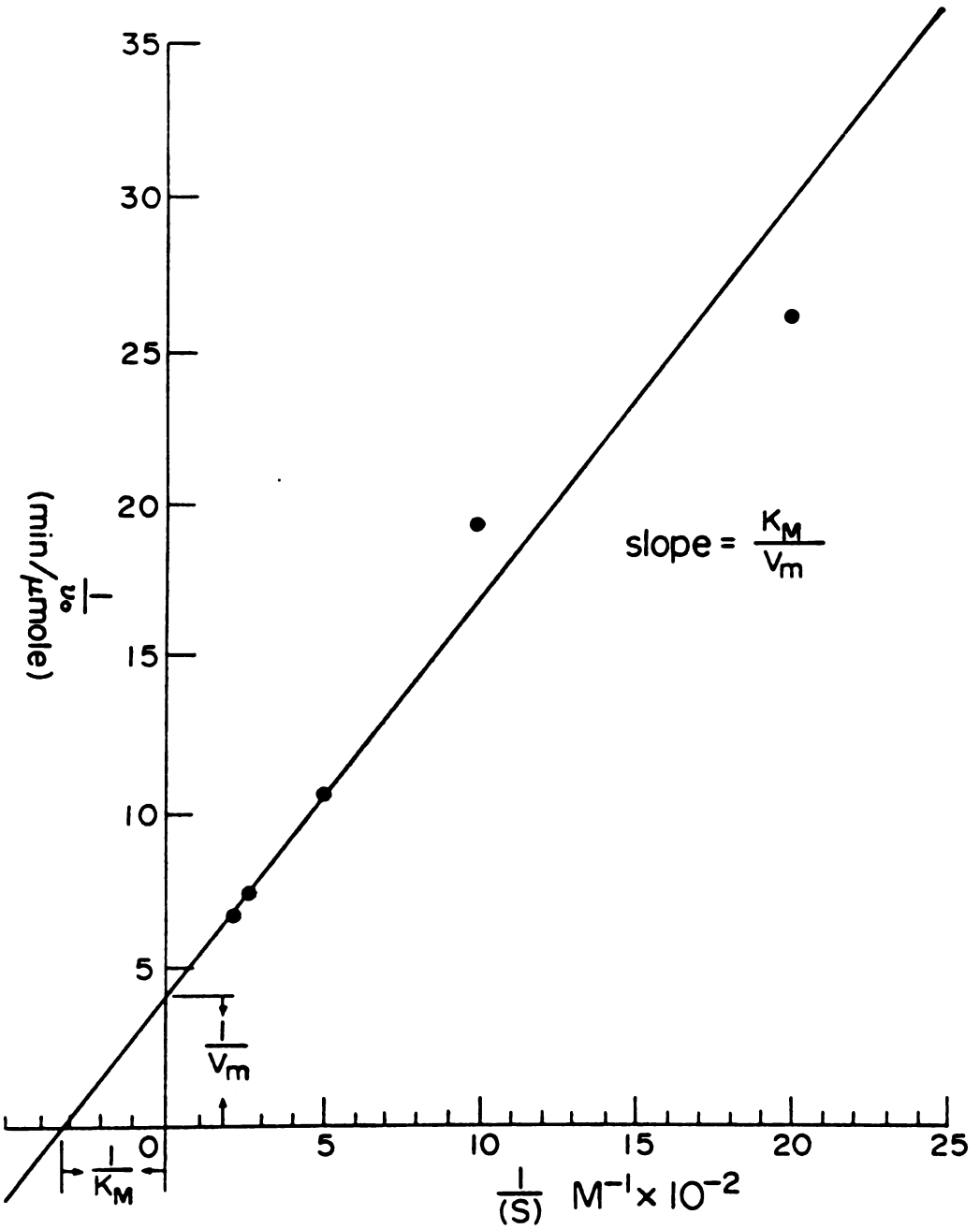

35
30
25
20
15
10
5
0
5
10
15
20
25
slope = $\frac{K_M}{V_m}$
$\frac{1}{V_m}$
$-\frac{1}{K_M}$
$\frac{1}{(S)}$ $M^{-1} \times 10^{-2}$
$\frac{1}{v_0}$ (min/μmole)

FIG. 4

Table IV

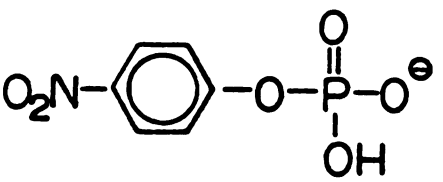
Inhibition Study with PABP & PABP*

	(A) PNPP alone	(B) 4 mM PABP added	(C) 8 mM PABP* added
Enzymatic activity	0.063	0.064	0.065

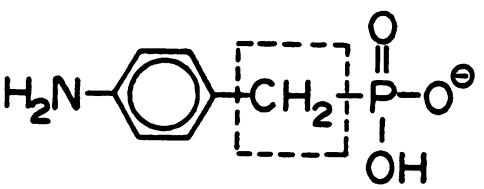
Standard assay procedure was used except that 0.5 mM PNPP was used in all cases.

Figure 5

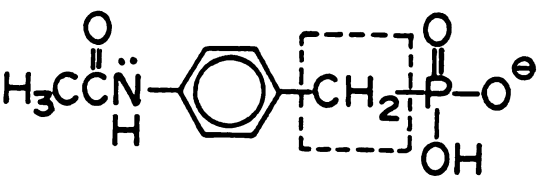
The Chemical Structures of PNPP, PABP and PABP*



PNPP



PABP



PABP*

FIG. 5

Table V

Inhibition Studies with Hydrolysis Products, p-Nitrophenol and P_i

	Enzymatic activity	% inhibition
Sample I, 10 mM P_i	0.002	99.00
Control I [a]	0.195	-
Sample II, 1.5 mM p-nitrophenol	0.118	0.00
Control II [b]	0.120	-

[a] Containing the same assay components as that in sample I except 10 mM P_i.

[b] Containing the same assay components as that in sample II except 1.5 mM p-nitrophenol.

1.0 mM PNPP was used in all cases.

Figure 6

Enzymatic Reaction Pathway

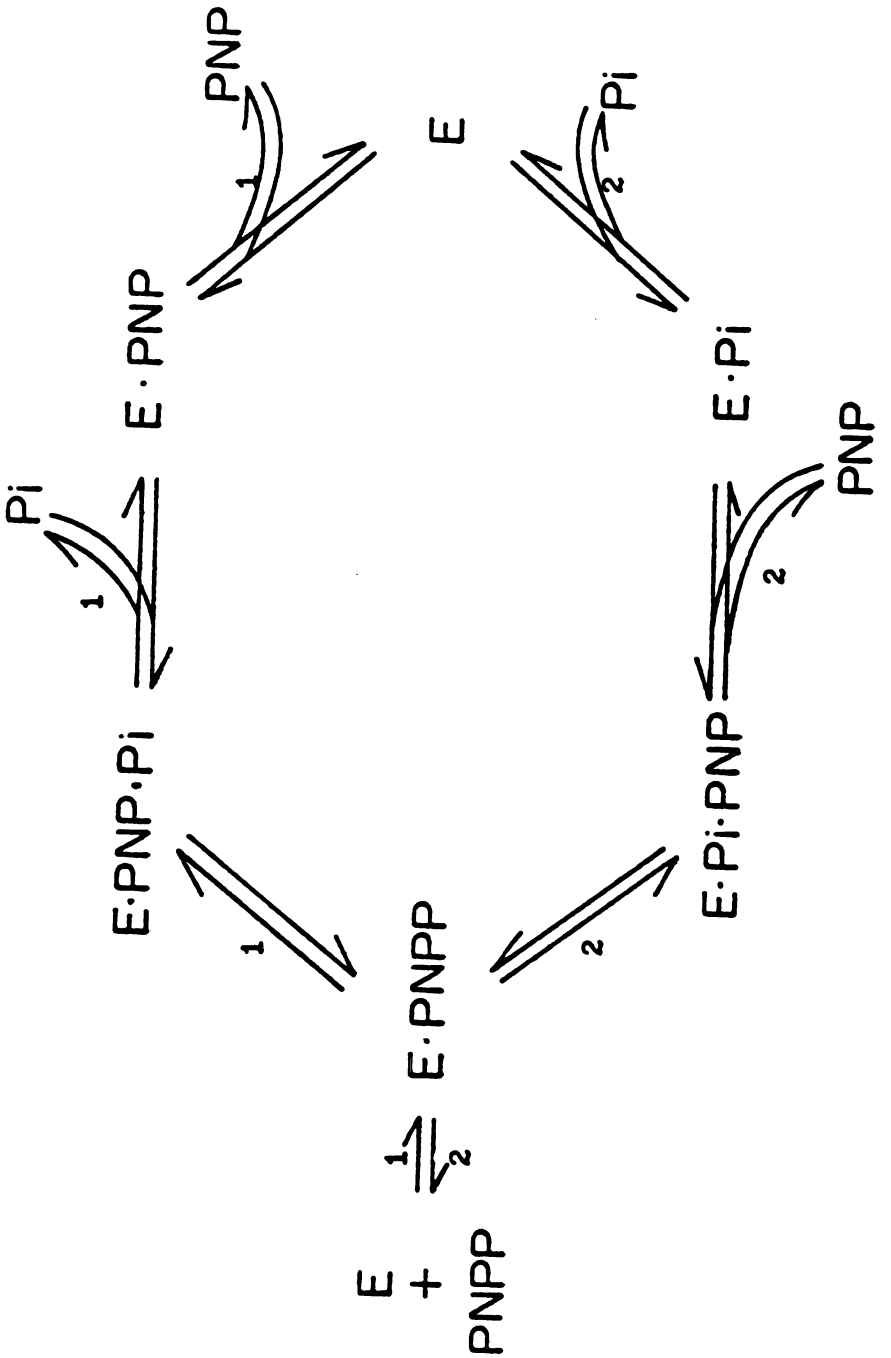


FIG.6

Table VI

Activation of Beef Spleen Acid Phosphatase by Ferrous ion

and Inactivation by Ascorbic Acid in The Absence of PNPP

preincubation period (mins)	(A)[a] 0.02 mM Fe^{+2} Δ A/min	(A)[a] 0.02 mM Fe^{+2} % activation	(B)[a] 0.4 mM Fe^{+2} Δ A/min	(B)[a] 0.4 mM Fe^{+2} % activation	(C)[b] 0.4 mM Fe^{+3} Δ A/min	(C)[b] 0.4 mM Fe^{+3} % activation	(D)[c] control Δ A/min	(E)[d] ASC Δ A/min	(F)[e] ASC Fe^{+2} Δ A/min
5	0.14	19.0	0.20	73.0	0.00	0.0	0.115	0.00	0.04
10	0.12	4.3	0.19	65.0	0.00	0.0	0.115	0.00	-
15	0.12	0.0	-	-	-	-	0.115	-	-

a
Standard assay mixture except 5mM PNPP was preincubated in various periods at 25 ,pH6.0 in the abscence of ascorbic acid . The reaction mixture was started by the addition of substrate,PNPP

b
The same procedure in A and B was performed here, the same assay mixture as that in A was used except the substitution of ferric ion for ferrous ion.

c
All components but ferrous ion in A and B was used,the same procedure in A or B was performed.

d
Ascorbic acid was added to the assay mixture in D,the same procedure in A or B was performed.

e
0.02 mM ferrous ion was added to sample E, 0.05 mM PNPP was used in this case.

III. DISCUSSION

The enzyme prepared by the published procedure (2), which involves acid extraction, ammonium sulfate fractionation, CM-52 cellulose chromatography (Table I), was obtained in poor yield and in a relatively impure form. As seen in Table I, the specific activity (0.07 units/mg) of the crude acid extract of beef spleen was relatively low compared to that obtained by Campbell and Zerner (which was 0.37 u/mg) (2). Presumably this could be due to the nutritional difference between the beef spleen used or could be due to the effect of pH value and toluene concentration in the acid extraction step. The recovery obtained in the ammonium sulfate fractionation step (Table I) was only half that of Campbell and Zerner (2). Singer and Fructon's method was used in both cases. The 4 fold purification obtained from 60 - 90 % ammonium sulfate fractionation (Table I) is relatively low. In general the average purification in this step was on the order of 25. The 40 fold purification obtained by Campbell and Zerner seems to be remarkably high. The effect of the pH of acid extract on the purity and the effect of the per cent ammonium sulfate fractionation on the recovery were therefore examined in an attempt to obtain a highly purified enzyme. Table II lists the specific activity of the acid extract obtained at different pH value and with different toluene concentrations. We conclude that the pH value of acid extract and the concentration of toluene does not make much difference in the enzymatic purity of acid extract. The results in Table III

show that the ammonium sulfate fraction giving the maximum recovery is the same as that used by Campbell and Zerner (2), i. e. 60 - 90 %. Slight change in the per cent ammonium sulfate used over the range of 60 - 90 % does not make much difference in the recovery and purity. The recoveries in both the CM-52 step and the Sephadex G75 step were quite low, both in our preparation and in that of Campbell and Zerner. The cellulose phosphate chromatography was thought to be potentially an efficient technique in increasing the yield and degree of purification. As seen in Fig. 3, a fraction containing 149 units per milligram was eluted as a single peak corresponding to a position equivalent to about 0.6 M NaCl in NaOAc, pH 4.9 and towards the trailing edge of the major protein peak (Fig. 3). A 2 1/2 fold purification (Table I) is obtained, and a high recovery (70 %) is obtained in this technique. Purification by cellulose phosphate column is simple and can be used to advantage for the production of large quantities of enzyme.

As seen in Fig. 5, PABP and PABP* are both structural analogues of substrate, PNPP, in that they both have an aromatic moiety and a phosphorus containing group similar to that of PNPP. Consequently they were thought to be potential inhibitors of PNPP. In line with this, an inhibition study was then carried out in an attempt to establish the relationship between the structural feature and binding specificity of the beef spleen acid phosphatase toward these two compounds. First of all the inhibition study was done on PABP, the results indicate that PABP is not an inhibitor of PNPP (Table IV). Two possible factors are proposed to account for the difference of binding specificity between PABP and PNPP. One is the charge distribution, the other is the nature of the phosphorus linkage. The second pK_a of the phosphonic acid group on PABP was estimated to be 8.5 (16), the pK_b of amino group on PABP was esti-

mated to be 4.0. The fact that PABP is not very water soluble at the assay pH 6.0 suggests that some of it may exist as zwitter ion with a positive charge being localized on amino group and a negative charge being localized on the phosphorus containing group, whereas PNPP has mononegative charge on the phosphate group (pK_{a2} of phosphoric acid is close to 7.0), and no charge on the aromatic moiety. Should the enzyme be able to differentiate between the difference in charge distribution on the aromatic moieties of PABP and PNPP, then the enzyme should be inhibited by the derivative of PABP formed after the charge on aromatic moiety is removed. PABP*, a derivative of PABP formed by acylation of the amino group, was then subjected to an inhibition study (Table IV). The result indicates that PABP* is not an inhibitor either. This implies that the binding specificity has nothing to do with the charge distribution factor, but has to do with the nature of phosphorus linkage. It seems that phenolic linkage is crucial for binding. The enzyme, however, also shows activity toward a class of phosphoamidates (17), hence the phenolic linkage can not be the only factor toward the binding specificity of beef spleen acid phosphatase. Perhaps more weight has to be given to the nature of C-P bond in phosphonates as analogues of natural phosphates as regard to the binding specificity. During the past 15 years there has been developed significant interest in the preparation and investigation of phosphonic acids and their derivatives which appear as the analogues of naturally occurring phosphates. Crofts and Kosolapoff have measured the second pK_a's for a series of phosphonic acids and found them to be in the range 7.7 - 8.2 when a primary alkyl group is attached to phosphorus (16). This is to be compared with the corresponding monoalkyl phosphates (18). A typical decrease in acidity

of phosphonic acids was found upon substitution of an alkyl group for the phenolic oxygen in the phosphorus containing group (19). Dittredge and Roberts demonstrated the existence of a glycerol ester of 2-aminoethylphophonic acid in the lipid extract of anemones, which yields AEP on hydrolysis (20). The resistance of AEP to the action of hydrolytic enzymes which are present in sea anemones suggests that substances with C-P bonds provide groups which are similar to phosphate group in the dissociation constants, but which are more likely to be stable to the action of phosphatases or other hydrolytic enzymes. Only a few reports have appeared on the nature of C-P bond in phosphonic acid and its derivatives. Zeleznick (21) and Mastalerz (22) have demonstrated the biological cleavage of the C-P bond by several species of bacteria. No attempt has been made here to cover the literatures dealing with the use of many synthetic phosphonic acid analogues of naturally occurring substances as potential inhibitors, which may be used as immobolizing agents coupled to the solid matrix support of the column used in affinity chromatography, a potentially powerful separation technique.

Little has been done on the investigation of catalytic reaction mechanism on the violet acid phosphatases over last 20 years. From the inhibition studies on the catalytic reaction products, P_i and p-nitrophenol, we were able to propose a catalytic reaction pathway for the beef spleen acid phosphatase. Two reaction pathways were considered primarily (Fig. 6). According to microscopic reversibility, if route 1 is the actual pathway then inhibition by p-nitrophenol should occur. If route 2 is the actual one, then inhibition by inorganic phosphate should occur. Table V indicates that the enzyme is inhibited by phosphate but not by p-nitrophenol to any extent. This implies that route 2 is the major

catalytic reaction pathway in which the p-nitrophenol diffuses away first, followed by the loss of P_i once the intermediate E P_i PNP was formed.

The Lineweaver Burke equation is very useful in enzyme kinetic studies. A straight line is obtained from the plot of $1/V_o$ versus 1/(S) from this equation. The Michaelis Menten constant, K_m , can thus be determined from the slope of the plot; it is an approximate value for the intracellular level of the substrate. It is a constant for a given enzyme, at fixed pH, ionic strength and temperature. It provides a mean to tell whether two enzymes from different source are the same or not. The K_m of beef spleen acid phosphatase for p-nitrophenyl phosphate has not ever been determined before. It is determined to be 2.0 mM approximately (Fig. 4), a relatively high value compared to the normal level (less than 1 mM) of intracellular substrates, Perhaps it is because that PNPP is not a physiological substrate.

In agreement with the observation of previous workers (12), it is found that enzyme activity is considerably enhanced by ascorbic acid in the presence of substrate, PNPP. The enzyme is inactivated by short exposure to ascorbic acid in the absence of substrate, PNPP (column E in Table VI). A hypothesis to explain these observations is that the enzyme is isolated from beef spleen as a ferric - enzyme complex (resting form), which is not very active and is of considerable stability to the heat. For activity, it is visualized that the ferric - enzyme has to be reduced to form a ferrous - enzyme complex which is more readily dissociable. Ascorbic acid is found to be the most effective reducing agent. The ferrous - enzyme complex is stablized in the presence of substrate. Without substrate the ferrous ion dissociates from the enzyme, resulting in the loss of activity. Addition of ferrous ion in the presence or

absence of substrate and / or ascorvic acid simply favors the equilibrium condition for the ferrous - enzyme complex. The result from column E & F in Table VI supports this hypothesis. The addition of ferric ion in the absence of ferrous ion and ascorbic acid does not activate the enzyme to any extent (column C of Table VI). Preincubation of the enzyme in buffer and ferrous ion in the absence of ascorbic acid for variable time intervals before starting the reaction by the addition of substrate results in variable activity. The results from column A & B in Table VI shows that the activity decreases as the preincubation period is increased. This is due to the oxidation of ferrous ion upon exposure to air. In addition to that, the degree of activation by ferrous ion is also dependent on the concentration. As the concentration of ferrous ion increases, the degree of activation increases.

IV. SUMMARY

1. The inefficient purification of beef spleen acid phosphatase by the published procedure (2) has been improved by further purification on a column of cellulose phosphate to advantage of increasing the recovery by 6 times that of CM-52 cellulose column and Sephadex G75 gel permeation column. A two and half fold purification is obtained on this column.
2. The K_m of beef spleen acid phosphatase toward substrate p-nitrophenyl-phosphate was determined to be approximately 2.0 mM from the plot of the Lineweaver Burk equation.
3. Inhibition studies with two analogues of substrate, PABP and PABP*, revealed that the binding specificity of enzyme was not related to the type of phosphorus linkage in the compound. Further work has to be done in order to establish a clear relationship between the structural features of the substrate or inhibitor and the binding specificity of beef spleen acid phosphatase.
4. The enzyme was activated by reducing agent (ascorbic acid) in the presence of substrate, PNPP. However, reducing agents such as ascorbic acid inactivated the enzyme in the absence of PNPP and ferrous ion. Ferrous ion prevents this inactivation. As the concentration of ferrous ion increases, the degree of activation increases. In contrast to that, ferric ion was found to have no effect of activation toward this enzyme.

5. An enzymatic reaction pathway is postulated from the result of the inhibition studies with the catalytic reaction products, p-nitrophenol and inorganic phosphate. The pathway involves the formation of an intermediate, $E \cdot P_i \cdot PNP$, followed by the sequential release of p-nitrophenol and inorganic phosphate.

BIBLIOGRAPHY

(1) R. H. Glew and E. C. Heath, J. Biol. Chem; 246, 1556 (1971)

(2) H. D. Campbell and B. Zerner, Biochem. Biophys. Res. Commun; 54, 1498 (1973)

(3) J. Glomset and J. Porath, Biochim. Biophys. Acta; 39, 1 (1960)

(4) D. C. Schlosnagle, E. G. Sander, F. W. Bazer, and R. M. Roberts, J. Biol. Chem. 251, 4680 (1976)

(5) D. C. Scholsnagle, F. W. Bazer, J. C. M. Tsibris and R. M. Robert, R. M. Roberts, J. Biol. Chem. 249, 7574 (1974)

(6) K. Vehara, S. Fujimoto and T. Taniguchi, J. Biochem. (Tokyo), 75 627 (1974)

(7) K. Uehara, S. Fujimoto, T. Taniguchi and K. Nakai, J. Biochem. 75, 639 (1974)

(8) S. Nochumson, J. J. Orangers, and N. V. Dimitrov, Fed. proc., 33, 1378 (1974)

(9) M. M. Jacobs, J. F. Nyc, and D. M. Brown, J. Biol. Chem., 246, 1419 (1971)

(10) T. Roche, N. V. Thoai, and P. Pin, C. R. Soc. Biol., 147, 409 (1953)

(11) T. A. Sundararajan and P. S. Sarma, Bioch. J., 56, 125 (1954)

(12) H. R. Revel and E. Racker, Biochim. Biophy. Acta, 43, 465 (1960)

(13) H. D. Campbell, D. A. Dionysius, D. T. Kiough, B. E. Wilson, J. D. Jersey and B. Zerner, Biochem. Biophy. Res. Commun; 82, 615 (1978)

(14) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biol. Chem. 193, 265 (1951)

(15) M. F. Singer & J. S. Fructon, J. Biol. Chem. 229, 111 (1957)

(16) P. C. Crofts and G. M. Kosolapoff, J. A. C. S. 75, 337 (1953)

(17) K. M. Moller, Biochim. Biophy. Acta , 16, 162 (1955)

(18) J. R. Vanwazer,"Phosphorus and its compounds", Vol. 1, Interscience, New York, N.Y., 1958, pp 364ff

(19) E. Robert, Chemical Reviews, 77, 349 (1977)

(20) J. S. Kittredge and E. Roberts, Science, 164, 37 (1967)

(21) L. D. Zelezni , T. C. Myers, E. Titchener, Biochim. Biophys. Acta, 78, 564 (1963)

(22) R. D. Harkness, J. Bacteriol. 92, 623 (1966)