PURIFICATION OF ALKALINE PHOSPHATASE

BY AFFINITY CHROMATOGRAPHY

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ABSTRACT

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Purification of Alkaline Phosphatase by Affinity Chromatography

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Affinity chromatography has been successfully employed for the purification of alkaline phosphatase (APase). A potential inhibitor of APase, 3-aminopropylphosphonic acid (3-APPAl, was coupled to agarose, Affi-Gel 10, to form an affinity adsorbent for APase. Phosphocellulose was used as a second adsorbent. A partially purified extract from C57BL mice was applied to both adsorbents in 0.1 M acetate buffer, pH 5.0, and eluted with 1.0 M KCl in 0.05 M tris buffer, pH 7.4. Both adsorbents were effective in adsorbing the enzyme, and the specific activity of the enzyme recovered from both adsorbents was greatly enhanced. The relative yields of the adsorbents differed greatly. Approximately 57% of the activity which was bound to the 3-aminopropylphosphonic acid-Affi-Gel 10 adsorbent was
recovered in a 140-fold enrichment relative to the extract which was applied to the column. Only 13% of the bound activity could be eluted from the phosphocellulose adsorbent in a 125-fold enrichment. Because of the poor yield, the phosphocellulose adsorbent has been evaluated as noneffective for affinity chromatography purification of APase. Conversely, the 3-aminopropylphosphonic acid-Affi-Gel$^\text{TM}$ 10 adsorbent provides a rapid, one-step procedure for APase purification.
ACKNOWLEDGMENTS

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CHAPTER I
INTRODUCTION

Alkaline phosphatase (APase) from \textit{E. coli} is a dimer (Lazdunski \textit{et al.} 1971) with a molecular weight of 86,000. It has been demonstrated that there is only one structural gene for the protein, indicating that the enzyme is made of identical subunits (Rothman and Byrne, 1963). It is a zinc-metalloenzyme (Plocke \textit{et al.} 1962). It has been shown that the enzyme mechanism involves the intermediate formation of a phosphoryl-phosphatase and that the active center contains a serine residue (Monod \textit{et al.} 1965).

Several reports have linked APase activity with mouse leukemia. In AKR mice, a strain showing a high rate of spontaneous thymic lymphoma, clusters of APase-positive thymic lymphocytes are present whereas the thymic lymphocytes in C57BL mice, a low leukemia strain, are consistently negative (Smith, 1961) except in embryos less than 17 days old in which a low level of activity may be demonstrated (Lagerlöf and Kaplan, 1967).

Histochemical studies have shown the location of APase in tumors to be on the membrane of lymphoma cells. Some is also found in endothelial cells or vessels and in the cytoplasm of reticuloendothelial cells. In the normal spleen it is seen in the cells surrounding the germinal
follicles. In the thymus of the 16-day embryo, it appears in mitotically active cells presumed to be precursor cells of the thymic lymphocytes (Lagerlöf and Kaplan, 1967).

A biochemical characterization of APase from chemical- and viral-induced thymic lymphomas of C57BL mice (Lumb and Doell, 1970) showed the major component of the tumor APase to have an electrophoretic mobility similar to that of the normal spleen. A minor component of the tumor APase activity was found to have a slower mobility than any component of the enzyme from normal tissues. On the basis of these findings and other biochemical criteria such as pH optimum, substrate specificity, heat inactivation, and inhibitor and activator effects, it was concluded that the major component of the tumor APase activity is similar to the APase activity of the spleen.

More recently, a comparative study of lymphoma, spleen, placental, fetal thymus, and whole fetus APases from C57BL mice, showed that the APase activity of lymphomas is distinct from that of the normal adult liver and duodenum and from 16-day whole fetus but is similar by most biochemical criteria to the 16-day fetal thymus, adult spleen, and 16-day placental APase (Floyd, 1973, Tisdale 1974, Yahya, 1974). The only difference
observed between the placental and lymphoma APase activities was an additional slower-moving isozyme in the lymphoma extract.

The present research was an outgrowth of a much broader research which was initiated to determine the role of derepression of embryo functions in malignant transformation. Many of the experiments have been performed with crude APase extracts. However, there are inherent inaccuracies associated with a crude extract. This research was therefore undertaken to develop an affinity chromatography procedure for the purification of APase. Purification of APase will allow information to be obtained with respect to kinetic constants, inhibition constants, antigenic determinants, amino acid composition, and molecular weight.

Affinity chromatography is a relatively new procedure which has been shown to give higher yields and a higher degree of purification than conventional methods. The technique exploits the property of enzymes to bind ligands specifically and reversibly (Cautrecasas and Anfinsen, 1969). The basic principle is to immobilize one of the components of the interacting system, i.e., the ligand, to an insoluble, porous support which can then be used to selectively adsorb that component (the enzyme) of the bathing medium with which it can selectively interact (Figure 1). Impurities are then washed away while the enzyme is bound to the matrix. Elution
Fig. 1  Schematic representation of affinity chromatography
LIGAND
M Matrix

SPECIFIC ADSORPTION

WASH

ELUTION

PURE ENZYME

EXTRANEOUS PROTEIN
of the bound enzyme is achieved by changing such parameters as salt concentration, pH, ionic strength, or by the addition of a competing molecule such as a substrate.

According to Cautrecasas and Anfinsen (1969), successful application of affinity chromatography depends in large part on how closely the particular experimental conditions chosen permit the ligand interaction to simulate that observed when the components are free in solution. Careful consideration must be given to the nature of the matrix, the dependence of the interaction on the structure of the ligand, the means of attachment, and conditions selected for adsorption and elution of the enzyme. In other words, the conditions chosen for the purification of a particular enzyme must reflect the specificity of that enzyme.

There are several general considerations which serve as guidelines in the preparation of an affinity column. An ideal matrix should interact weakly with APase to minimize nonspecific adsorption. It should exhibit good flow properties which are retained after coupling, possess chemical groups which can be activated or modified to allow chemical linkage, be mechanically and chemically stable to the conditions of coupling and elution, and form a porous network to permit uniform and unimpaired entry and exit of large macromolecules.
Cautrecasas (1970) has described the preparation, stability and handling of a number of derivatives. Commercially available, cross-linked dextran derivatives possess most of the desirable features except for their low degree of porosity. Cellulose derivatives, because of the fibrous and nonuniform character, impede proper penetration of large protein molecules. Beaded derivatives of agarose have nearly all the properties of an ideal matrix. The beaded agarose derivatives have a very loose structure which allows molecules of high molecular weight to diffuse readily. They also readily undergo substitution reactions by activation with cyanogen halides (Porath et al. 1967), are very stable, and have a moderately high capacity for substitution (Cautrecasas, 1970).

The molecule to be linked to the matrix must be one that displays affinity for the enzyme to be purified. It can be a substrate analog, effector, cofactor, and in some cases, substrate. The ligand must possess chemical groups that can be modified for linkage to the matrix (Cautrecasas and Anfinsen, 1969). It is also important that the ligand be sufficiently distant from the matrix to minimize steric interference. Steers et al. (1970), have shown that a distance of 20 to 25 Å is ideal. This separation of ligand and matrix can be accomplished
by selecting a ligand with a long hydrocarbon arm attached to it which can in turn be attached to the matrix. Alternatively, such a hydrocarbon extension arm can be first attached to the solid support (Cautrecasas, 1970).

With respect to adsorption and elution, Cautrecasas and Anfinsen (1971) report that the specific conditions for adsorption are dictated by the properties of the protein to be purified, and that elution generally requires changing the pH, ionic strength, or temperature of the buffer. Elution can also be achieved by adding a competitive inhibitor or substrate.
CHAPTER II

REVIEW OF LITERATURE

Perhaps the first example of purification of an enzyme by what is now called affinity chromatography was the demonstration by Lerman (1953) of adsorption of mushroom tyrosinase on an adsorbent prepared by reacting diazotized aminophenol to cellulose that contained resorcinol residues in ether linkage. Powdered cellulose was partially esterified by one of two procedures: (a) refluxing in benzene with phosphorus tribromide followed by refluxing in alcohol with an excess of sodium m-hydroxyphenoxide or (b) refluxing with sodium ethylate and p-nitrobenzyl bromide. Coupling of the diazonium salts was indicated by color change. Demonstration of adsorption by the cellulose derivatives, tests on specificity, and rough estimation of the capacity of the adsorbents were carried out by frontal analysis. The enzyme was eluted with 0.02 M pyrophosphate at pH 8.3, 0.1 M glycine at pH 9.6, or other buffers of similar pH.

Dramatic purification results have been reported in the past few years with various agarose derivatives. Steers et al. (1970) purified β-galactosidase from
the constitutive strain of *E. coli*, K-12. Comparisons were made of the adsorption of this enzyme to derivatives of agarose and of polyacrylamide which contained the competitive inhibitor p-aminophenyl-β-D-thiogalactopyranoside attached to the matrix backbone by arms differing in length. This was one of the first studies which indicated that the distance of the ligand from the matrix influenced results. Complete recovery of enzymatic activity was obtained when the ligand was placed about 20 to 25 Å from the matrix, and elution was done with borate buffer, pH 10. More recently, it has become increasingly clear that for successful purification by affinity chromatography, the ligand groups critical in the interaction with the macromolecule to be purified must be sufficiently distant from the backbone of the solid matrix to minimize steric interference with the binding process.

A covalent affinity column for the purification of electric eel acetylcholinesterase has been described by Ashani and Wilson (1972). A potential inhibitor of the enzyme, 2-aminoethyl p-nitrophenyl methylphosphonate, was coupled to extended sepharose. The acetylcholinesterase was trapped on the column by covalently bonding with the insoluble matrix. The enzyme was recovered by using the reactivator 2-(hydroxyaminomethyl)-1-methyl-
pyridinium iodide or 1,1'-trimethylene bis (4-hydroxamino-
pyridinium) dibromide. Kalderon et al. (1970) have also
purified eel acetylcholinesterase using the competitive
inhibitor aminocaproyl-p-aminophenyl-trimethylammonium
bromide linked to agarose. Small amounts of the enzyme
were adsorbed to relatively large columns. More than
one third of the total enzyme activity was unexplainedly
unretarded. The material adsorbed was not removed after
washing with a volume of starting buffer equal to five
times the column volume. It was eluted, however, by
increasing the ionic strength (1 M NaCl) of the buffer.
The enzyme was eluted in rather dilute form achieving
a seventeen-fold purification. Purification of soy-
bean lipoxygenase has been described by Grossman et al.
(1972). The purification of the enzyme was achieved
by passing the crude extract through a linoleyl aminoo-
yethyl agarose column. Lipoxygenase was eluted from
the adsorbent with 0.2 M acetate buffer, pH 5.0. Both
the yield and the purity of the lipoxygenase were
facilitated by prior removal of the contaminant proteins
on CM-cellulose. These proteins were eluted with 0.005 M
acetate buffer, pH 5.0.

Staphylococcal nuclease was inhibited by 5'-phos-
phonucleosides (Cautrecasas and Anfinsen, 1971) and
bound very tightly to columns containing agarose to
which p-T-p-aminophenyl was covalently attached. It was possible to purify this enzyme to homogeneity in a single step, even from the crude culture medium. Elution was obtained in a small volume in solutions of acetic acid (0.1 M) or of ammonium hydroxide (0.1 M).

An oligonucleotide affinity column for RNA-dependent DNA polymerase from RNA tumor viruses was prepared by Gerwin and Milstein (1972). Columns of (dT)_{12-18} cellulose provided a one step enrichment procedure for RNA-dependent DNA polymerase. The enzyme from RD114 cells, as well as that from Rauscher murine leukemia virus, was purified this way. The eluting agent was a linear KCl gradient.

Kristiansen et al. (1970) described the purification of L-asparaginase from *E. coli*. Direct attachment of D-asparagine to agarose resulted in an ineffective adsorbent, and it was necessary to insert an aliphatic extension arm to achieve a satisfactory adsorbent. Elution was achieved with 1 mM D-asparagine.

Chorismate mutase from *Claviceps paspali* was purified by Sprossler and Lingens (1970) by attaching the allosteric activator L-tryptophan to agarose by the cyanogen bromide procedure. Virtually all the activity from a 9 mg sample of DEAE-cellulose purified
sample adsorbed strongly to a small column. Elution of more than 80% of the activity resulted with phosphate buffers supplemented with 1 mM L-tryptophan.

Lefkowitz et al. (1972) have purified β-adrenergic receptor protein by affinity chromatography. When solutions of the crude extract were passed through columns of norepinephrine-agarose, 85% of the receptor-binding activity was adsorbed to the column. Elution was achieved with 0.1 M epinephrine at pH 3.8.

A hemoglobin-Sepharose column prepared by the cyanogen bromide procedure was used by Chua and Bushuk (1969) to purify proteases from crude extracts of malted wheat flour. High recoveries (90%) of the proteolytic activity were achieved on elution with 0.1 N acetic acid, and virtually all the nonproteolytic components were separated by this procedure.

Aminophenyl glycosides were covalently attached to polyacrylamide beads by way of a histamine-coupled Bio-Gel intermediate by Truffa-Bachi and Wofsy (1970). The column selectively bound cells that produced anti-hapten antibodies of corresponding specificity, while cells without the specific antihapten sites passed through freely and were recovered quantitatively in the eluate. The binding of the antihapten specific cells was inhibited by hapten.
CHAPTER III
MATERIALS AND METHODS

Source of Tissue

Tissue was obtained from inbred C57BL/KA mice originally obtained from Dr. Henry Kaplan at Stanford University. The inbred mice were maintained by brother-sister matings and placentae were used at 16 days of gestation.

Preparation of Enzyme Extract

The tissue was first homogenized in a glass homogenizer in 0.015 M NaCl. The enzyme was then solubilized with 1% Shell Noniodet-P40 (NP40) detergent. The precipitate was removed by centrifugation at 75,000 x g. The solubilized enzyme was pooled and then treated with 30% butanol at 4°C to remove extra protein. The butanol-extracted material was then dialyzed against 10 volumes 0.01 M Tris-HCl at pH 7.4.

Assay for Enzyme Activity

Enzyme activity was determined by either of two methods: (A) One tenth milliliter of the sample and 0.1 ml of the substrate para-nitrophenyl phosphate
(pNPP) at a concentration of 33.8 mM were added to 0.8 ml of 0.05 M ammediol (2-Amino-2-methyl-1,3-propanediol), pH 10. The mixture was incubated at 37°C for 30 min, then 2 ml of a 1:1 ratio of 1 N NaOH and 0.2 M EDTA was added to stop the reaction. The absorbance at 400 nm was measured on a Turner spectrophotometer. (B) One tenth ml pNPP and 0.1 ml of the sample were added to 0.8 ml of 0.05 M diethanol amine, pH 10, containing 5 mM magnesium chloride. The reaction was allowed to proceed for 4 min at 37°C and absorbance at 400 nm was recorded on a model 102 Hitachi spectrophotometer (Nissei Sangyo Instruments, Inc.). A unit of enzyme activity is defined as the amount which liberates 1 μM pNP/min/ml under either condition.

Protein Determination

Protein was determined either by the method of Lowry et al. (1951) with bovine serum albumin as standard or by measuring the absorbance at 280 nm.

Inhibitor Studies

Theoretically, a compound having a moiety structurally similar to phosphate should bind APase. On this premise, 3-aminopropyl phosphonic acid (3-APPA) was selected
as a ligand for the affinity column. This compound is an analog of α-butyric acid and has the following structural formula:

\[
\text{O} \\
\text{NH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{P}-\text{OH} \\
\text{OH}
\]

Two concentrations of 3-APPA, 10 and 20 mM, were prepared in 1.0 M diethanol amine, pH 10, which contained 5 mM magnesium chloride. The buffer without any 3-APPA was used as the control. These solutions (0.8 ml) were pipetted into test tubes containing 0.1 ml of the enzyme extract. The reaction was started by adding 0.1 ml pNPP which was prepared in final concentrations of 3.38, 0.85, 0.338, and 0.169 mM. Each inhibitor concentration was tested with each substrate concentration in triplicate. The reaction was allowed to proceed for 4 minutes at 37°C and the velocity (μM pNP/min/ml) was measured. To determine the mechanism of inhibition, a Lineweaver-Burke plot was used.

**Preparation of Affinity Adsorbent**

Affi-Gel 10 was purchased from Bio-Rad Laboratories, Richmond, California. This is a highly purified agarose gel that has aliphatic side arms (10 Å long)
which are terminated by N-hydroxysuccinimide esters. Compounds containing primary aliphatic or aromatic amino groups couple with Affi-Gel 10 in a one step reaction to form peptide bonds. The coupling reaction is as follows:

\[
\text{OCH}_2\text{CH}_2\text{CH}_2\text{NCH}_2\text{O-N} + \text{R-NH}_2 + \text{pH 6.5 to 8.5 Buffer} \rightarrow \text{OCH}_2\text{CH}_2\text{CH}_2\text{NCH}_2\text{CH}_2\text{O-N-R + HO-N}
\]

To prepare the adsorbent, 25 ml of 0.05 M borate buffer, pH 8.5, containing 50 mM 3-APPA was added to a bottle containing 1 gram of Affi-Gel 10. The bottle was shaken gently, then intermittently shaken at 4°C for 24 hours. After 24 hours, the slurry was poured into a Pharmacia 45 x 1 cm column and continuously washed with 0.05 borate, pH 8.5. As the gel was washed, 25 ml fractions were collected, and the adsorbance at 260 nm was read until it reached zero. N-hydroxysuccinimide that is released during the coupling reaction adsorbs strongly at 260 nm, and its removal served as an indication of completed washing. When the column had been washed free of N-hydroxysuccinimide, 1.0 mg/ml
bovine serum albumin was passed through so that any remaining active bonds would be saturated.

Preparation of Phosphocellulose Adsorbent

Two grams of cellulose phosphate cation exchanger, fine mesh, were dissolved in 50 ml 0.1 M tris (hydroxy-methyl aminomethane), pH 7.0. The mixture was allowed to stand at room temperature for 2 hours. The slurry was poured into a 45 x 1 cm column and continuously washed with 0.1 M tris at pH 7.0.

Adsorption and Elution of Enzyme to Affinity Adsorbent

Several attempts were made to retain the enzyme on the 3-APPA-Affi-Gel adsorbent. The extract was first layered onto a column which was equilibrated with either 0.05 M borate or ammediol buffer, pH 10. The column was washed until no further protein could be detected, then changed by the addition of 0.05 M tris buffer, pH 7.5. In a second series of experiments, the extract was applied to a column which was equilibrated with 0.05 M tris, pH 9.0, containing 0.1 M KCl. To elute, the column was washed successively with 0.05 M tris, pH 9.0, in which the KCl molar concentration respectively was varied from 0.2, 0.1, 0.05 to zero. In a final experiment, the
extract was applied to a column which was equilibrated with 0.1 M acetate buffer, pH 5.0. To facilitate elution, the column was changed to 0.05 M tris containing 1.0 M KCl, pH 7.4.

Adsorption and Elution of Enzyme to Phosphocellulose Adsorbent

The extract was applied to a column which was equilibrated with 0.1 M acetate buffer, pH 5.0. The column was eluted with 0.05 M tris containing 1.0 M KCl, pH 7.4.

Determination of Enzyme Activity Bound to Adsorbents

Throughout this study, the presence of activity bound to the adsorbents was routinely determined by adding 33.8 mM pNPP to the column and by looking for a yellowing of the gel, which would be indicative of pNP liberation.
CHAPTER IV

RESULTS

A Lineweaver-Burke plot of the inhibitor experiments performed with 3-APPA (Fig. 2) shows that APase is non-competitively inhibited by 3-APPA. This is an indication that the inhibitor binds at a locus on the enzyme other than the active site.

Figure 3 shows the elution pattern obtained when the enzyme was applied to a column equilibrated with 0.05 M borate, pH 10.0 and subsequently was eluted with 0.5 M tris, pH 7.5. All of the activity passed through the column unretarded (Fig. 3). No further activity was eluted with the addition of tris buffer.

The column which was equilibrated with 0.05 M ammediol, pH 10.0 was also ineffective in adsorbing APase (Fig. 4). All of the activity which was applied to the column passed through in a single peak. Addition of tris buffer, pH 7.5, did not elute any further activity from the column.

The elution pattern shown in Figure 5 was obtained when the enzyme activity was applied to a column equilibrated with 0.05 M tris, pH 9, containing 0.1 M KCl. Again, the adsorbent was ineffective in binding the enzyme which passed through in a single peak with a
Fig. 2. Lineweaver-Burke plot of 3-APPA and placental alkaline phosphatase.
Fig. 3. Chromatographic pattern obtained when enzyme was passed through a column of 3-APPA-Affi-Gel 10. The column was equilibrated with 0.05 M borate, pH 10.0, and eluted with 0.05 M tris, pH 7.5.
Fig. 4. Chromatographic pattern obtained when enzyme was passed through a column of 3-APPA-Affi-Gel 10. The column was equilibrated with 0.05 M ammediol, pH 10.0, and eluted with 0.05 M tris, pH 7.5.
Fig. 5. Chromatographic pattern obtained when enzyme was applied to 3-APPA-Affi-Gel 10. The column was equilibrated with 0.05 M tris, pH 9, containing 0.1 M KCl, and eluted with a KCl gradient.
major protein fraction under these conditions. When the column was developed with a KCl gradient of decreasing molar concentrations, no further activity was eluted.

When the enzyme was applied to the 3-APP-Affi-Gel 10 adsorbent with 0.1 M acetate, pH 5.0, most of the activity was retained on the column. The elution pattern shown in Figure 6 shows some activity coming off in the initial effluent. This is superfluous activity which was intentionally added in excess to insure that all available binding sites would be utilized. When the column was developed with 1.0 M KCl in 0.05 M tris, approximately 57% of the bound activity was eluted. Purification was about 140-fold relative to the extract which was applied to the column (Table 1). Since the yield of the affinity column was somewhat smaller than expected, a 5% Shell NP40 detergent solution was passed through the column to facilitate the recovery of additional activity. No further activity or protein could be eluted. To determine if any activity remained on the column, 10.0 ml of 33.8 mM pNPP was added to the column. The column did not turn yellow.

The phosphocellulose adsorbent was effective also in adsorbing the enzyme when it was applied with 0.1 M
Fig. 6. Chromatographic pattern obtained when enzyme was applied to 3-APPA-Affi-Gel 10. The column was equilibrated with 0.1 M acetate, pH 5.0, and eluted with 1.0 M KCl in 0.05 M Tris, pH 7.4.
### TABLE 1

Purification of Alkaline Phosphatase

<table>
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<tr>
<th>Treatment of Tissue</th>
<th>Specific Activity (μM pNP/min/ml)</th>
<th>Yield (%)</th>
<th>Enrichment</th>
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<td>Solubilization-NP40 and Butanol</td>
<td>3.2</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3-APPA-Affi-Gel 10</td>
<td>453</td>
<td>57</td>
<td>140</td>
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<tr>
<td>Phosphocellulose</td>
<td>400</td>
<td>13</td>
<td>125</td>
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</tbody>
</table>
acetate, pH 5.0. Figure 7 shows the chromatographic elution pattern of the column which was also developed with 1.0 M KCl, pH 7.4. Only about 13% of the activity could be eluted in a 125-fold enrichment relative to the extract which was applied to the column. No further activity was eluted upon the addition of a 5% Shell NP40 solution, and the column did not turn yellow when 33.8 mM pNPP was added.
Fig. 7. Chromatographic pattern obtained when enzyme was applied to phosphocellulose adsorbent. The column was equilibrated with 0.1 M acetate, pH 5.0, and eluted with 1.0 M KCl in 0.05 M tris, pH 7.4.
CHAPTER V
DISCUSSION AND CONCLUSIONS

Affinity chromatography, as depicted in Figure 1, exploits the property of enzymes to bind to ligands specifically and reversibly. Ideally, the enzyme is selectively adsorbed from the bathing medium, washed to remove extraneous protein, then eluted in purified form. The aim of this work was to develop an affinity chromatography purification procedure for APase utilizing these principles.

In the course of this investigation, several combinations of adsorption and elution systems were employed. While each of these in some way reflected the specificity of APase, and adhered to the basic principles of affinity chromatography, their effectiveness varied greatly:

The noncompetitively inhibitory substrate analog, 3-APP A, bound to an agarose matrix was able to adsorb the enzyme when it was applied at pH of 5.0. This result seems reasonable since APase of *Escherichia coli* takes up phosphate at this pH. The use of a non-competitive ligand for APase affinity purification is also a reasonably precedentented procedure. Doellgast and Fishman (1974) used L-phenylalanine, also a non-competitive APase inhibitor, coupled to Sepharose to

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prepare a highly purified human placental APase. These researchers used an extract with an initial specific activity of 0.184 μM/min/mg and employed several intermediate purification techniques including DEAE-Sephadex in the presence of Triton X-100 detergent. Retention of the enzyme was greatest at the highest concentrations of (NH₄)₂SO₄. By using decreasing concentrations and changing the types of salts, elution was effected achieving a 1000-fold purification in 22% yield. The investigators reported that the (NH₄)₂SO₄-mediated binding retention appeared to be related to the hydrophobic character of the substituted Sepharose rather than any specific binding site of the enzyme. This conclusion was based primarily on the fact that the elution profile of the enzyme on D-phenylalanine-Sepharose was identical with that on L-phenylalanine-Sepharose. Further, the enzyme was also retained on L-leucine-Sepharose and on aniline-Sepharose.

It is believed that the binding of APase to 3-APPA-Agarose is also a nonspecific interaction. Two findings lead to this conclusion. First, the binding is not reversed by substrate and secondly, elution is not effected by borate which is reported to be a competitive APase inhibitor (Prioleau, 1974). In spite of this
apparent non-specificity, the 3-APPA-Affi Gel 10 adsorbent does bind the enzyme allowing the removal of extraneous protein, and thus, permitting some degree of purification. Changing the ionic strength of the column with 1.0 M KCl effects elution of about 57% of the activity in a 140-fold enrichment.

The phosphocellulose adsorbent also binds the enzyme at pH 5.0. Again, elution could not be effected with substrate or a competitive inhibitor. Thus this binding is also believed to be nonspecific. Further, that the binding of APase to Phosphocellulose was a hydrophobic interaction was somewhat dispelled when no additional activity could be eluted with the NP-40 detergent solution. Elution from this adsorbent was also achieved by changing the ionic strength of the column with 1.0 M KCl. Only 13% of the activity applied could be recovered in a 125-fold enrichment. This poor yield was obtained consistently and must be inherent to this adsorbent under the conditions evaluated.

Of the two procedures developed, the one which employs the 3-APPA-Affi Gel 10 adsorbent appears to be the most expedient for APase purification. This technique will allow the rapid purification of relatively large
quantities of APase. Conversely, when large yields are mandatory, the phosphocellulose procedure would not be ideal. About the same degree of purification is achieved with both adsorbents, and both may be used several times without appreciable impairment of the binding capacity.
CHAPTER VI

SUMMARY

1. 3-Aminopropylphosphonic acid is an inhibitory substrate analog of APase.
2. Mechanistic studies show that this inhibition is noncompetitive.
3. 3-Aminopropylphosphonic acid can be coupled directly to Affi-Gel™ 10 agarose to form an effective affinity chromatography adsorbent for the purification of APase.
4. Phosphocellulose is also an effective adsorbent for APase purification.
5. APase binds to both adsorbents when it is applied with 0.1 M acetate, pH 5.0.
6. Elution is effected from both adsorbents by changing the ionic strength with 1.0 M KCl, pH 7.4.
7. The yield of the 3-APPA-Affi-Gel 10 adsorbent is approximately 57% in a 140-fold enrichment.
8. Phosphocellulose yields about 13% of the bound activity in a 125-fold enrichment.
9. Both adsorbents can be used repeatedly without loss of binding ability.


Lefkowitz, R., E. Haber, and D. O'Hara. 1972. Identification of the cardiac β-adrenergic receptor


