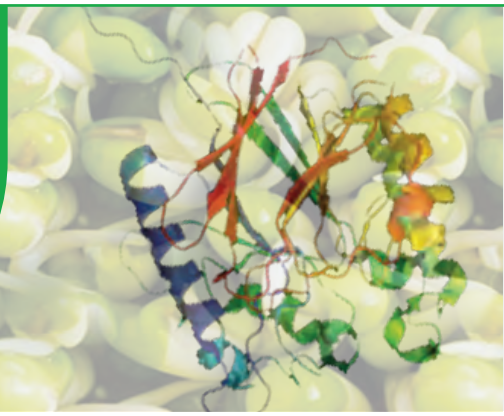
PURIFICATION OF ACID PHOSPHATASE FROM SPROUTED MOONG



A. Preparation of crude extract from sprouted moong beans and assay of enzyme activity.

B. Partial purification of acid phosphatase using ammonium sulphate fractionation.

C. Purification of acid phosphatase by ion exchange chromatography.

A. Preparation of crude extract from moong beans and assay of enzyme activity.

A.1. Aim

To prepare crude extract from sprouted moong beans and assay of enzyme actrivity.

A.2. Introduction

Acid phosphatase is a phosphohydrolase. It is active at acidic pH. The enzyme commission number for acid phosphatase is 3.1.3.1. The molecular mass of wheat germ acid phosphatase is 58 KDa. It is a fairly nonspecific enzyme. The substrate for acid phosphatase in many bean sprouts is polyphosphoinositol which is present in the seed coat. Acid phosphatases (APases) catalyze the hydrolysis of inorganic phosphate (P_i) from a broad range of P_i-monoesters with an acidic pH optimum. The liberated P_i is reassimilated into cellular metabolism via mitochondrial or chloroplastic ATP synthases of respiration or photosynthesis, respectively. Eukaryotic APases exist as a wide variety of tissue- and/or cellular compartment-specific isozymes that display marked differences in their physical and kinetic properties.Increases in intracellular (vacuolar) and secreted APase activities are useful biochemical markers of plant nutritional P_i deficiency. The protocols for protein extraction, APase activity determination and measurement of soluble protein concentration from plant tissues or cell suspension cultures are presented.

$$\text{polyphosphoinositol} \xrightarrow{\text{Acid phosphotase}} \text{Inositol} + P_i$$

For enzyme assay, the substrate used is para nitro phenol phosphate

$$\text{PNPP} \xrightarrow[\text{0.1 M (pH 5.5) Acetate buffer}]{\text{Acid phosphatase}} \text{PNP} + \text{0.5 N KOH} \longrightarrow \text{read at 405 nm}$$

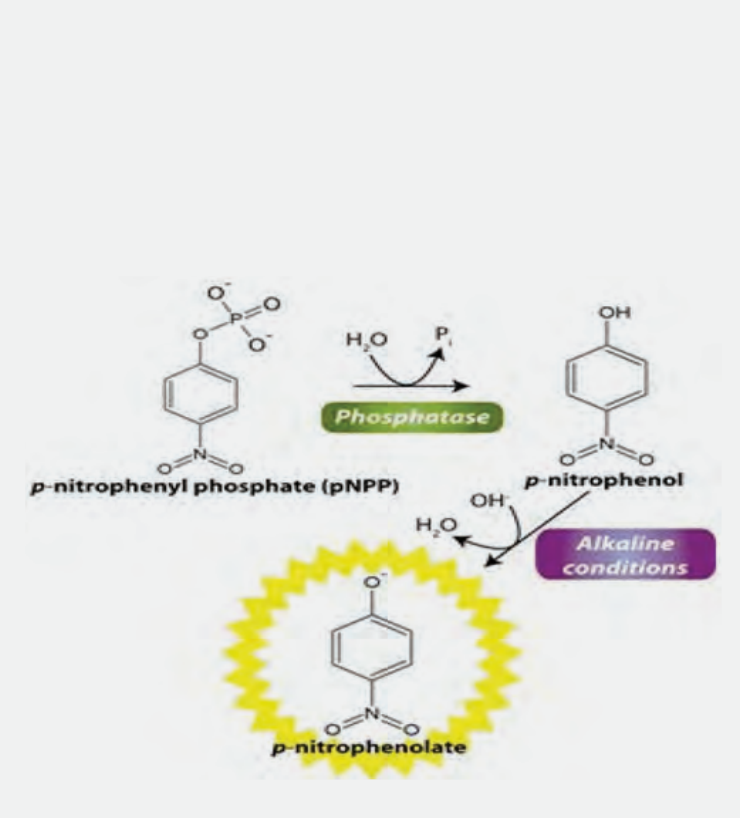


Fig. 1: Diagram showing steps taking place during reaction source (https://www.gbiosciences.com/Bioassays/Phosphatase-Assay)

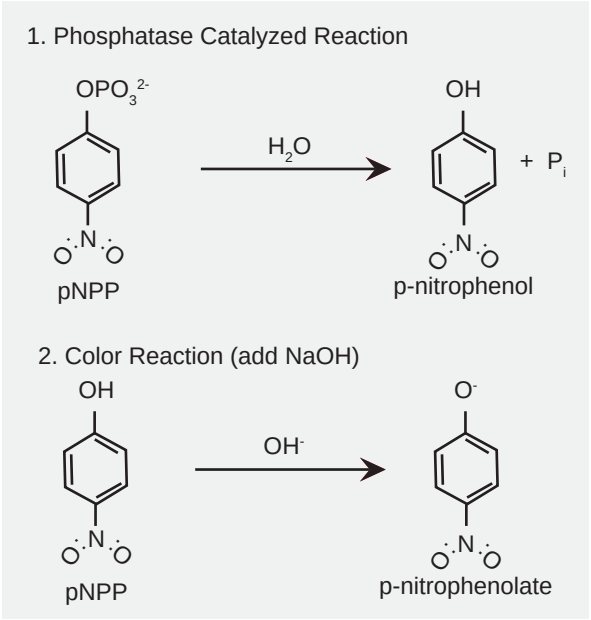


Fig. 2: (1) APase activity is often assayed spectrophotometrically at 405-410 nm by determining the amount of pNP produced following the hydrolysis of Pi from pNPP.
(2) Addition of KOH/NaOH after a specified assay time (e.g., 10 min) serves to stop the APase reaction while simultaneously converting the product p-nitrophenol into the yellow colored p-nitrophenolate (λmax = 405-41
(Source: http://www.bio-protocol.org/e889)0 nm)

2.1 Enzyme Assays:

A useful enzyme assay must meet four criteria:

i. Absolute specificity
ii. High sensitivity
iii. High precision
iv. Convenience

Enzyme assays are of two types:

i. Continuous assay
ii. Discontinuous assay

2.2 Continuous Assays:

Continuous assays are most convenient, with one assay giving the rate of reaction with no further work necessary. Here, the reaction is not terminated, however the absorbance is measured at regular intervals. The cuvette with enzyme and substrate is placed in the spectrophotometer and the absorbance is measured at λ_{max}.

2.3 Discontinuous Assays:

Here, the reaction is terminated at one step by adding inhibitor after incubation and then the absorbance is measured. The concentration of product formed can be calculated using the standard curve.

2.4 Protein Purification:

The deep study of many biological systems has involved purification of one or more of the sytem components. The five basic steps of purification are as follow:

i. Development of suitable procedures.
ii. Selection of the best source from which the molecule may be purified.
iii. Solubilization of the desired molecule.
iv. Stabilization of the molecule repeatedly at each stage of its purification.
v. Development of a series of isolation and concentration procedure.

2.5 Method of Solubilization:

Solubilization is required for any protein to be purified, because all the isolation procedures commonly used operate only in aqueous solution. A wide range of solubilization methods exist, and the one chosen must take into account the characteristics of the cells to be broken and the nature of the proteins to be subsequently isolated. A second consideration that strongly influences the choice of solubilization procedures is the sample size. The methods used to break a few milligrams of cultured animal cells are likely to be of little use in processing several pounds of yeast or beef liver.

2.8 Ultrasonic waves:

Sonic oscillators provide a very efficient means of breaking cells or organelles. They are effective against bacteria and yeast at sufficiently long periods of application. The times may be shortened, however by including glass beads in the cell suspension. Sonicators are usually composed of 2 main units: an electric generator which produces an ultrasonic signal of high intensity and a transducer which transmits these waves into the solution in contact with it. It is the shock and vibration set up by these sound waves that brings about tissue destruction or moves the five glass beads. The major drawback is the large amount of heat generated at the transducer.

2.9 Presser:

There are 3 major types of presses that may be used to break open microorganisms or cells. They are the Hughes, French and Eton presses. All 3 of these instruments function by placing a cell suspension of small volume (5-50 mL) under 4000-8000 lb pressure and forcing it through a small opening. Breakage is brought about by shearing of the cells as they pass through the small onfice. This method is both gentle and thorough, but its application is restricted by its limited sample size range.

A.3. Materials Required

3.1 Biological Material: Moong beans for crude extract.

3.2 Chemicals/Reagents: Acetate buffer 1 M (stock solution) and 0.1 M (working) pH 5.5, 0. 5 M KOH, PNPP (p-nitrophenylphosphate).

3.3 Equipment: Centrifuge, UV- Visible spectrophotometer, pipettes.

3.4 Glassware/Plastic ware: Cuvette, test tubes, beaker, conical flask.

3.5 Miscellaneous: Tissue paper, mortar and pestle.

A.4. Procedure

4.1 Enzyme extract preparation:

i. Prepare the 0.1 M acetate buffer (pH 5.5) from stock of I M acetate buffer.

ii. Prepare the enzyme extract in 0.1 M acetate buffer (pH 5.5) by grinding the moong beans sprouts using a mortar and pestle.

iii. Homogenize the moong beans in acetate buffer and centrifuge it at 5000 rpm for 10-15 min.

iv. Separate the pellet and the supernatant. The supernatant is collected as crude extract, as supernatant contains enzyme.

4.2 cid phosphatase assay:

i. Add 2.7 mL of Acetate buffer 0.1 M concentration in test tubes along with 0.1 mL of enzyme and add 0.2 mL of substrate.

ii. Add 2 mL 0.5 M KOH in the control test tube prior to incubation to cease enzyme activity.

iii. Incubate for 10 minutes 37 ^{O}C.

iv. Add 2 mL of 0.5 M KOH solution after incubation to each test tube labeled Test.

v. Use 1:10 and 1:100 dilutions of the enzyme. Make 1: 100 dilution of enzyme in both acetate buffer and water. Observe variation enzyme activity.

vi. Measure the absorbance and calculate the enzyme activity.

A.5. Observation

Observation table:

Blank solution = 3 mL buffer + 2 mL KOH

S.No	Sample	Acetate Buffer (mL)	Substrate (PNPP) (mL)	Enzyme (mL)	Incubate at 37° C for 10 min	0.5 M KOH	Absorbance for PNP (at 405 nm)	Absorbance T-C
1	Blank	2.7	0.2	0.1		2 mL		
2	Neat Test Control							
3	Dilution in buffer 1:100 (T) 1:100 (C)							
4	Dilution in buffer 1:10 (T) 1:10 (C)							

* T= Test, C= Control

A.6. Calculation

$$\text{Enzyme Activity} = \frac{\text{Optical Density (T - C) x Dilution factor x Assay volume x } 10^3}{\text{Molar extinction coefficient x Time x Vol. of enzyme}}$$

Activity is measured in µM/min= 1 IU (international Unit)

1 IU is the amount of enzyme required to convert 1 µM concentration of substrate into the product per minute under optimum conditions (of pressure, pH and temperature).

SI unit = Katal = $\frac{\text{(moles of substrate)}}{\text{second}}$

Where 1 Katal = 6×10^7 I.U.

A.7. Result

i. The enzyme activity of crude sample of mung beans was found to be_____ µmoles ml^{-1} min^{-1}.

ii. Enzyme activity for 1: 100 dilution in acetate buffer was found to be_____ µmoles $ml^{-1}min^{-1}$

iii. Enzyme activity for 1: 10 dilution in acetate buffer was found to be _____ µmoles $ml^{-1}min^{-1}$

A.8. Precautions

i. Clean the cuvette thoroughly after every reading.

ii. Pipetting should be done carefully.

iii. Set the appropriate filter of the Spectrophotometer.

iv. Calibration must be done using the blank solution.

B. Partial purification of acid phosphatase using ammonium sulphate fractionation.

B.1. Aim

To partially purify acid phosphatase from crude extract using ammonium sulphate fractionation and dialysis of fractionated samples.

B.2. Introduction

2.1. Salt Precipitation:

The solubility of the protein varies with the ionic strength of the solution. Ionic strength is related to the concentration of salt in the solution.

On addition of salt two phenomena can take place:-

i. Salting in

ii. Salting out

2.2. Salting In:

At very low concentration of salt, the salt ions interact with the protein molecules. This way, they shield the counter ions on protein molecules and prevent aggregation of the protein, thus increasing the solubility of the protein in the solvent. This process is known as salting in.

2.3. Salting Out:

In the interaction between the polar amino acids and the solvent can be prevented, thus destabilizing the solution (solvation shell), then the protein molecules will aggregate and this way, the protein can be precipitated out. Salt like ammonium sulphate will quench the solvent molecules and thus, there will not be enough solvent for protein molecules to dissolve.

Hydrophobic amino acids that are present in the core are exposed on the surface and hydrophobic interactions between the protein molecules cause aggregation which leads to precipitation of the protein. This process is referred to as salting out.

2.4. Desalting:

Once the protein has precipitated out, the salt molecules need to be removed before proceeding to the next step of purification. Removal of salt molecules refers to desalting. If a solution of proteins is separated from bathing solution by a semipermeable membrane, small molecules and ions can pass through the semipermeable membrane to equilibrate between protein solution and bathing solution, called dialysis bath or dialysate. This method is useful and therefore helpful in desalting. For desalting, the solution should be kept overnight and the buffer should be charged every 4-5 hours.

2.5. Hofmeister Series:

The Hofmeister series or lyotropic series is a classification of ions in order of their ability to salt out or salt in proteins. The effects of these charges were first worked out by Franz Hofmeister, who studied the effects of cations and anions on the solubility of proteins. Anions appear to have a larger effect than cations, and are usually ordered as follows:

$F- \approx SO^{2-}_4 > HPO^{2-}_4 >$ acetate $> Cl^- > NO^-_3 > Br^- > ClO^-_3 > I- > ClO- > SCN^- > NH^+_4 > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} >$ guanidinium

2.6. Nomogram:

It is a table that gives an idea of the amount of salt that has to be added in per liter of solution to obtain desired concentration for fractionation of proteins. It is an optimized table. Most proteins get precipitated in 30%-40% range of $(NH_4)_2SO_4$. (See Annexure Table 1: for nomogram)

2.7. Determination of Enzyme Activity:

Ammonium sulphate fractionation can be used for purification of enzymes. The protiens which are unwanted other than acid phosphatase will be removed by precipitating with different concentrations of ammonium sulphate. After centrfugation the pellets which are obtained are named as P_1 and P_2.

Before fractionation enzyme activity = E

Ideally, assuming no loss of enzyme activity during fractionation,

$E = P_1 + P_2 +$ supernatant

However, this never happens as some amount of enzyme is always lost during purification process.

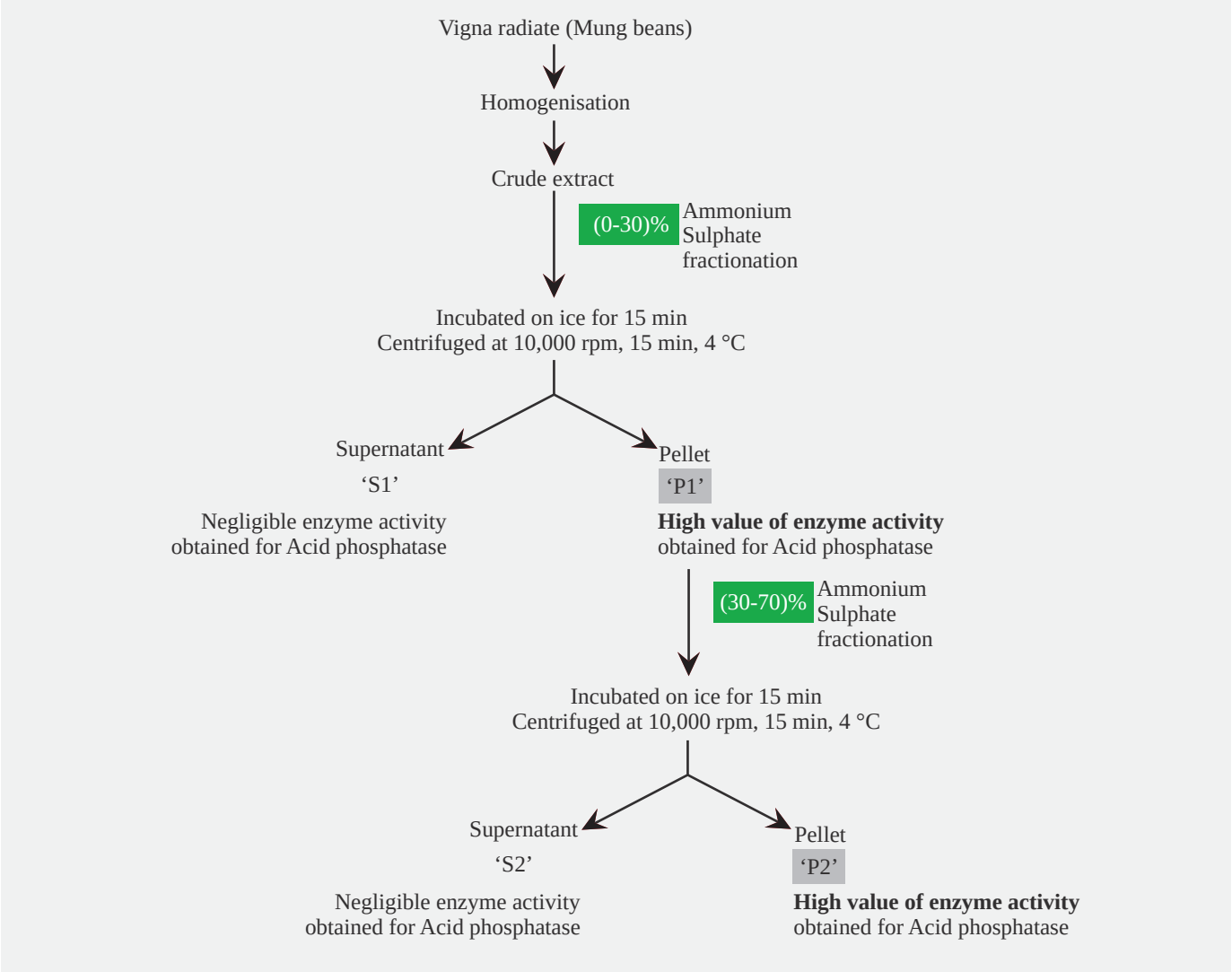


Fig. 3: Flow chart for salting out

2.8. Specific Activity:

It is the activity of enzyme per milligram protein in the sample.

$$\text{Specific activity} = \frac{\text{Total activity } \mu\text{mol min}^{-1}\text{mg}^{-1}}{\text{mg protein in the sample}}$$

i. In every step of purification, contaminating proteins are eliminated, so the amount of protein decreases.

ii. Therefore, specific activity would increase at each step as some proteins are being removed.

iii. Increasing specific activity

2.9. Protein Estimation (Lowry's Method):

Protein is estimated from samples by Lowry's method.

2.10. Dialysis:

Dialysis is a technique to remove the salt ions present in protein sample which are employed during purification process. It is the random, thermal movement of molecules in solution that leads to the net movement of molecules from an area of higher concentration to a lower concentration until equilibrium is reached.

In dialysis, a sample and buffer solution (called dialysate), separated by a semi permeable membrane that causes differential diffusion patterns, thereby permitting the separation of molecules in both the sample and dialysate. Due to the pore size, large molecules in the sample cannot pass through the membrane, thereby restricting their diffusion from the sample chamber. To enhance the separation of the proteins in the bag (tube) from other impurities such as salt we can also take advantage of the equilibrium constant. In an equilibrium environment, the salt will flow through the membrane, until the concentration outside the dialysis tube is equal to the concentration inside. At this point there is no net flow of the salt through the membrane because equilibrium reached. But if we add in a new solution buffer, then the remaining amount of salt will flow out of the dialysis tube until the concentration of salt in the new buffer equals the concentration in the tube. If we keep replacing the buffer, their will enhance the purification of the proteins inside.

B.3. Materials Required

3.1. Biological Material: Moong beans extract, samples for dialysis.

3.2. Chemicals/Reagents: Ammonium sulphate, acetate buffer 0.1 M, 1 M (for dialysis).

3.3. Equipment: Centrifuge,UV- Visible spectrophotometer, pipettes, magnetic stirrer.

3.4. Glassware/Plastic ware: Cuvette, test tubes, beaker, conical flask, glass rod, dialysis tube.

3.5. Miscellaneous: Tissue paper, water, scissors.

B.4. Procedure

4.1. Ammonium sulphate fractionation:

i. Prepare the enzyme extract as given in the step “Enzyme extract preparation”.

ii. Place the crude sample on ice and add $(NH_4)_2SO_4$ gradually for 0-30% precipitation.

   a. For fraction with 0-30% salt saturation 176 g of $(NH_4)_2SO_4$ in 1 L solution (from nomogram), hence 1 mL= 0.176 g of $(NH_4)_2SO_4$

   For desired vol. (mL) of crude sample = 0.176 × vol. =_____g.

   b. For fraction with 30-70% salt saturation 273 g of $(NH_4)_2SO_4$ in 1 L solution (from nomogram), hence 1 mL= 0.273 g of $(NH_4)_2SO_4$

   For desired vol. (mL) of S1 (Supernatant) = 0.273 × vol. =_____g.

NOTE: See Annexure Table 1: Nomogram, at the end of the protocol.

iii. Add respective amount of $(NH_4)_2SO_4$ to crude extract of acid phosphatase. Stir properly. Incubate on ice for 15 min.

iv. Centrifuge the solution at 10,000 rpm at 4 °C. Separate the supernatant (S_1). Collect the pellet (P_1) as well in acetate buffer.

v. Add the required amount of salt for 30%-70% concentration to the supernatant (S_1). After adding the salt, centrifuge the solution. Collect S_2 and P_1 separately.

vi. Once all the fractions are obtained, calculate the enzyme activity for each fraction.

vii. Carry out the protein estimation using Lowry’s method and determine the amount of protein in each fraction

viii. Determine the specific activity and percentage yield and recovery of enzyme and protein.

4.2. For Dialysis:

i. Wash the dialysis tube thoroughly with distilled water and boil in H_2O for 30 mins.

ii. After boiling, let the tube to cool down at normal temperature and wash with acetate buffer thoroughly to pretreat the tubes.

iii. After washing the tube with acetate buffer for 3-4 times tie the tube from one end tightly using thread.

iv. Check the knot for leakage using blotting paper.

v. Then transfer the sample in the tube using autopippettes.

vi. After transferring the samples from previous experiment in separate tubes, tie the other end also tightly

vii. Cross check the tube to prevent any leakage of the samples.

viii. Label the tubes as P_1, P_2, S_1, and S_2.

ix. Tie the dialysis tubes with glass rod to suspend the tubes in 0.1 M buffer on the magnetic stirrer for dialysis over night.

x. Next morning, remove the samples (tubes) from the 0.1 M acetate buffer and place them in 1 M acetate buffer, leave for dialysis for another 5 hrs on magnetic stirrer.

xi. Take the samples out from dialysis tubes and transfer them in the eppendorfs, label them.

B.5. Observations

5.1. Ammonium Sulphate Fractionation:

i. Volume of crude = ---------mL

For 0-30% fractionation, $(NH_4)_2SO_4$ required = 0.176g/mL of extract

For --------mL, $(NH_4)_2SO_4$ required = ----------g

ii. Volume of S_1 (supernatant) = ----------mL

For 30%-70% fractionation, $(NH_4)_2SO_4$ required = 0.273g/mL of extract

For ----------mL, $(NH_4)_2SO_4$ required =----------g

5.2. Lowry's method for Protein Estimation:

BSA stock: - 100µg/mL

S.No	BSA conc. (µg/mL)	Vol. (mL)	Vol. of H_2O (mL)	Analytical Reagent (mL)	Incubate at room temperature for 10 min	Folin's Reagent (mL)	Incubate at room temperature for 30 min	Abs. (660 nm)	Ab. (660 nm) Sample
1	Blank	0		2.5		0.25			Crude___
2	10								Crude___
3	20								P1_____
4	40								P2_____
5	60								S1_____
6	80								S2_____
7	100								

5.3 Enzyme Activity of Acid Phosphatase

S.No	Sample	Acetate Buffer (mL)	Subsrate PNPP (mL)	Enzyme (mL)		0.5 M KOH (mL)	Absorbance (405 nm)	Enzyme Activity (µmoles ml^{-1} min^{-1})
1	Blank	3						
2	Crude(T)							
3	Crude(C)							
4	P1 (T)							
5	P1 (C)							
6	S1 (T)							
7	S1 (C)	2.7	0.2	0.1	Incubate At 37o CFor 10 minutes	2.0		
8	S2 (T)							
9	S2 (C)							
10	P2 (T)							
11	P2 (C)							

*1:10 Dilution of P_2 may be used and other samples may be used in neat form according to the need.

*In control tubes, add KOH before incubation

$$\text{Enzyme Activity} = \frac{\text{O.D.} \times \text{Dilution factor} \times \text{Assay volume} \times 10^3}{\text{Molar extinction coefficient} \times \text{Time} \times \text{Vol. of enzyme}}$$

$$\text{Percentage yield} = \frac{\text{Total Activity} \times 100}{\text{Total activity of crude}}$$

$$\text{Percentage recovery} = \frac{\text{Total Protein} \times 100}{\text{Crude Protein}}$$

$$\text{Fold Purification} = \frac{\text{Specific Activity}}{\text{Specific activity of crude}}$$

5.4. Calculation Table:

Sample	Total Volume	Activity	Total Activity	Protein $mgmL^{-1}$	Total Protein	Specific Activity	% yield	% Recovery	Fold purification
Crude									
(0-30%) P1 S1									
(30-70%) P2 S2									

5.5. Table for dialyzed samples:

Sample	Total Volume	Activity	Total Activity	Protein $mgmL^{-1}$	Total Protein	Specific Activity	% yield	% Recovery	Fold purification
Undialyzed									
Dialyzed									

B.6. Result.

6.1. Ammonium sulphate fractionation is carried out to purify acid phosphatase from moong beans extract. Following observations can be made over the course of the experiment:-

i. In the crude, total protein is _____ mg and total activity is _____ µmoles $mL^{-1}min^{-1}$.

ii. In the 1st round of Ammonium Sulphate Fractionation (0-30%), however the total protein decreases to _____mg and the total activity decreases to _____µmoles $mL^{-1}min^{-1}$.This indicates that acid phosphatase is not present in the fraction.

iii. In the 2nd round of Ammonium Sulphate Fractionation, the total activity was found to be _____µmoles $mL^{-1}min^{-1}$. The high activity P_2 indicates that Acid phosphatase has precipitated in this fraction. The fold purification is _____for this round of precipitation.

iv. Also, before dialysis, the total protein in P_2 was estimated to be _____mg whereas after dialysis, it decreases to _____mg. This implies that a major loss of protein takes place during dialysis.

6.2. The samples were successfully dialyzed to remove salt ions present in them, and comparative analysis was made between dialyzed and undialyzed samples.

i. Undialyzed sample:

Yield = _____.

Recovery = _____.

Fold purification = _____.

ii. Dilyzed sample:

Yield = _____.

Recovery = _____.

Fold purification = _____.

B.7. Precautions:

i. Addition of Ammonium Sulphate must be slow and gradual.

ii. All dilutions must be made accurately.

C. Purification of acid phosphatase by ion exchnage chromatography

C.1. Aim:

To purify acid phosphatase by ion exchange chromatography from fractionated samples

C.2. Introduction:

Ion exchange may be defined as the reversible exchange of ions solution with ions electrochemically bound to some sort of insoluble support medium. The ion exchanger is the inert support medium to which a covalently bound positive (in the case of anion exchanger) or negative (in the case of cation exchanger) functional groups are bound. Any ion electrostatically bound to the exchanger is referred to as a counter ion. The value of this technique in the isolation and separation of charged compound is that conditions can be found under which some compounds are electrochemically bound to the ion exchanger whereas other are not.

At a specific pH, the isoelectric point, the molecule contains an equal number of positive and negative charges. This amphoteric nature of proteins may be manipulated to great advantage when this method is used for purification purpose. For example, the pH of a protein mixture may be lowered to the point where the desired protein behaves as a cation. If the preparation is chromatographed on a cation exchange column, under these conditions of pH, many of the anionic protein species are lost. If this is followed by an increase in pH to convert the desired protein to its anionic form, the preparation may be chromatographed on an anionic exchanger column with the concomitant loss of many of the cationic species. It should be pointed out that anion and cation exchange chromatography used sequentially often afford a large degree of purification even if the pH of preparation cannot be varied.

The selection of an exchanger that is best suited to a particular application is largely an empirical process. The nature of the supporting matrix usually determines its flow properties, ion accessibility, chemical and mechanical stability. Charged groups that are covalently bonded to the matrix determine the type and strength of binding. The size of the resin/ beads determines the flow rate, equilibration time and capacity of the exchanger. The larger the mesh size (decrease in the bead size), the larger the capacity and counterion equilibration time but lower the flow rate.

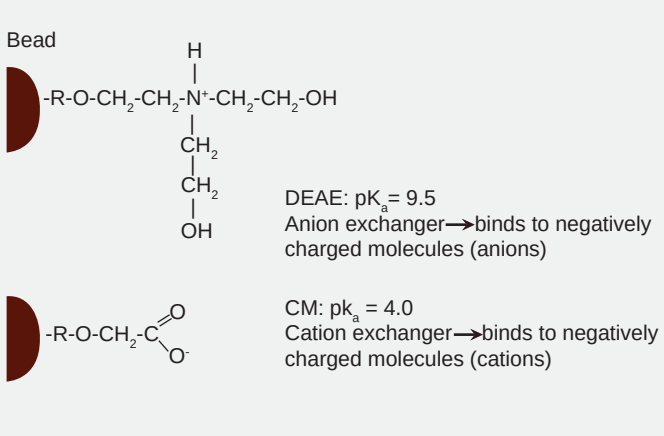


Fig. 4: Showing the charges on DEAE cellulose and CM beads

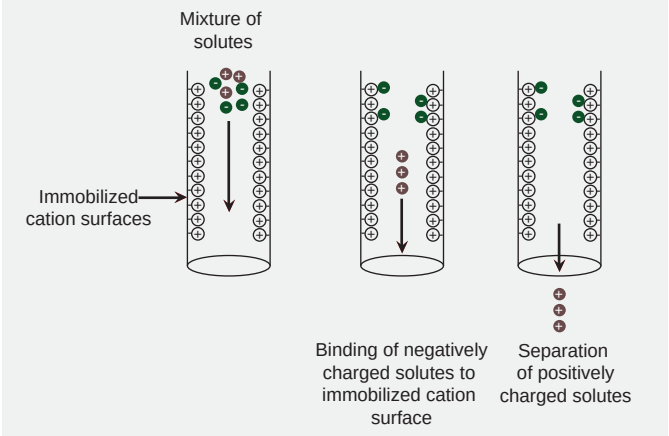


Fig. 5: Separation of charges in the column

(Source: Taken from Pranav Kumar (2014). Fundamentals and techniques of biophysics & molecular biology. New Delhi: Pathfinder publication)

Diethylaminoethyl (DEAE) has positive charge and carboxymethyl (CM) has negative charge on them. Positive charge of DEAE attracts negatively charged molecules. CM is suitable for binding with positively charged molecules (Fig. 4).

Column materials used for ion exchange chromatography contain charged groups covalently linked to the surface of an insoluble matrix. The charged groups of the matrix can be positively or negatively charged. Anion exchangers have positively charged groups that will attract negatively charged anions. When a mixture of solutes is loaded into the anion exchanger, negatively charged solutes bind to the exchanger. Beads have positive charged functional groups hence after loading the mixture of the sample (solutes) into the column separation of the solutes take place according to the charges on them as shown in (Fig. 5).

When the mixture of solutes is loaded into the column contaning the beads which have covelently attached charged groups with them, seperation of the solutes takes place according to the charge present on the solutes. If the bead is DEAE which is an anion exchanger that binds to negatively charge molecules seperation of anions take place depending upon the buffers of different concentration that is used for the elution of solutes (Fig. 6).

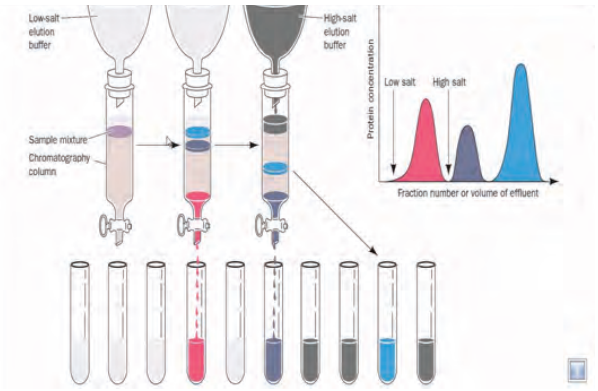


Fig. 6: Showing the elution of different solutes by applying buffers of different salt concentration for the successive elution of solutes.

(Source: https://www3.nd.edu/~aseriann/CHAP6B.html/sld005.htm)

2.1 Preparation of the Exchange medium:

There are four basic procedure involved in preparing an exchanger:-

i. Removal of impurities that result from incomplete purification of the exchanger by the manufacture.

ii. Swelling the medium (termed precycling) so that a greater percentage of the exchangers charged groups are exposed to the suspending solution.

iii. Removal of the fines (very small particles of exchanger).

iv. Conversion of the counter ion of the exchanger to that desired for a given application.

The process of precycling an exchanger is to expose the charged groups that are bound to the matrix. At the molecular level, cellulose exchangers are carbohydrate polymers that have substituted hydroxyl groups. During the drying step of the manufacturing process, water is removed from the exchanger and extensive intermolecular hydrogen bonding of the hydroxyl groups occurs. Such bonding results in very dense packing of the carbohydrate polymers, which in turn results in burial of many of the charged functional groups. Suspending on exchanger in water breaks a small volume of these hydrogen bonds but much stronger treatment is required to fully swell the matrix and hence break the remaining hydrogen bonds. In the case of DEAE, an anion exchanger, treatment with HCL results in the conversion of all the diethylamino ethyl groups to their charged species (C_2H_4N+H $(C_2H_5)_2$). Placing like positive charges on the functional groups results in mutual repulsion and hence maximum amount of swelling.

Failure to precycle an exchanger results in drastically reduced capacity and resolution. The loss in resolution becomes more acute as the size and charge densities of molecules being separated increases. There are a large number of buffers that may be selected to maintain the pH of a chromatographic medium. Four general considerations pertinent to this condition are given below.

i. Cation buffers should be used with anion exchangers and vice versa.
ii. The pKa of the chosen buffer should lie within $\pm$ 0.7 units of the pH at which the system will be buffered.
iii. The pH of the buffer system should be chosen so that the ions to be separated possess the same charge as the counter ions of the exchanger.
iv. The buffer system selected must be such that it does not interfere with analysis of the fractions yield.

Four things must be considered in the chromatography of a group of ions, have given below:

i. The volume and shape of the column.
ii. The shape of the gradient to be employed.
iii. The rate of elution.
iv. The size of the fractions to be collected.

Water Regain:

It is the volume upto which 1g of resin will swell when kept in water overnight.

Water regain for DEAE = 10 -15ml/g

Binding Capacity:

It is the mass of solute to which the resin can bind.

Binding capacity of DEAE = 0.6 mg/g

Bed Volume:

It is the volume occupied by the gel in the column.

C.3. Materials Required

3.1. Biological Material: Sample of acid phosphatase.

3.2. Chemicals/Reagents: Equilibration buffer, 0.1 M acetate buffer (pH 5.5), 0.5 N HCL, 0.5 N NaOH, DEAE cellulose, 50 mM NaCl, 100 mM NaCl, 200 mM NaCl.

3.3. Glassware/Plastic ware: Beaker, eppendorfs.

3.5. Miscellaneous: Cuvette, wash bottle, pipettes, stand, dropper.

C.4. Procedure

4.1 Column Preparation:

i. Swelling in water: - weigh the required amount of DEAE and keep in water overnight for swelling to take place.

ii. Precycling: - This is done to expose the charged groups. This is done by washing first with 0.5 N HCL and then with 0.5 N NaOH.

iii. Packing into column: - Place the glass wool first at the bottom/mouth of the column so that gel beads do not come out. Then equilibrate with a suitable equilibration buffer.

4.2 Equilibration and Elution:

i. Equilibrate the column by pouring 0.1 M acetate buffer (pH 5.5) through the column. Run the equilibration buffer till the buffer eluting out of the column has the same pH i.e. 5.5.

ii. Set the flow rate for elution.

iii. Load 750 µL of the sample.

iv. Keep adding the equilibration buffer for the elution of first 10 fractions.

v. Obtain the next 10 fractions with buffer of 50 mM NaCl.

vi. Obtain the subsequent fractions with solutions of ionic strength of 100 mM and 200 mM NaCl respectively.

4.3 Detection

i. Measure the absorbance of all fraction at $\lambda = 280$ nm. Plot the graph of fraction number vs. absorbance.

ii. To detect the presence of acid phosphatase, perform the enzyme assay of alternate fraction.

iii. For the fractions that need to be pooled, carry out Lowry's estimation method to determine the amount of protein.

iv. Calculate the percentage recovery and fold purification for the fractions.

C.5. Observations

5.1 Observation Table:

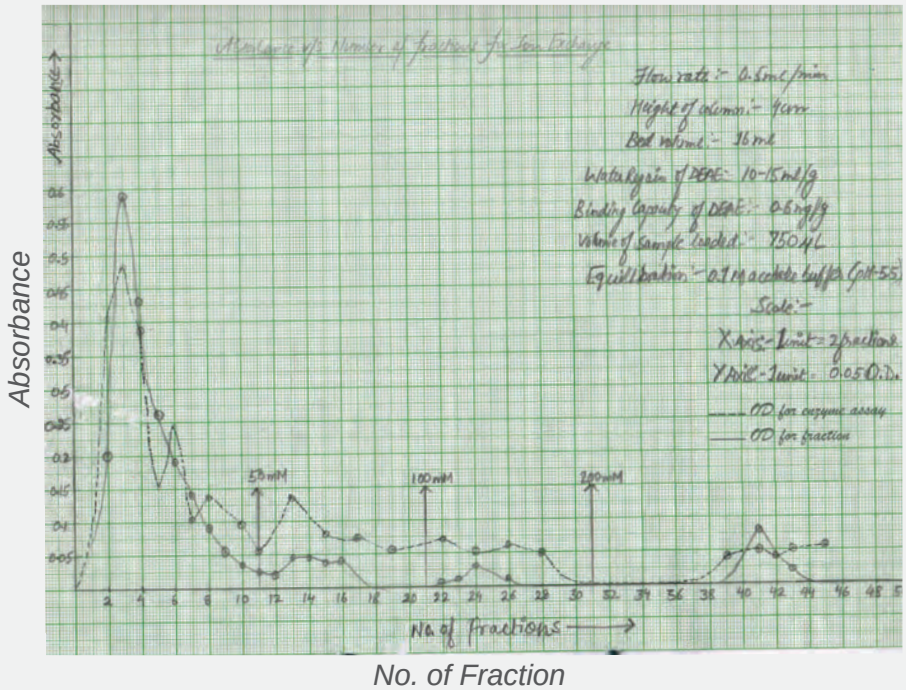
Fraction No.	Absorbance at (280 nm)	Fraction No.	Absorbance at (280 nm)
Unbound (1-10) fractions		100 mM (1-10) fractions	
50mM(1- 10)			

5.2 Enzyme Assay of Fractions:

Sample No	Absorbance	Optical Density
i. Unbound		
ii. 50 mM NaCl		
iii. 100 mM NaCl		
iv. 200 mM NaCl		

5.3 Estimation of Activity, Specific Activity, percentage recovery

Sample	Total Volume	Activity	Total Activity	Protein $mgmL^{-1}$	Total Protein	Specific Activity	% yield	% Recovery	Fold purification
Crude									
50 mM pool									
Unbound pool									
Unbound fraction									



No. of Fraction

Fig. 7: Graph for purification of acid phosphatase by ion exchange chromatography

C.6. Result

i. The fold purification of P_2 after Ammonium Sulphate Fractionation was found to be _____.

ii. The fold purification of the three samples is as follow.

50 mM pool = _____.

Unbound pool = _____.

Unbound fraction = _____.

C.7 Precautions

i. Packing of the column should be done with accuracy and precision. No air bubble should be present in the column.

ii. Selling and precycling should be done properly.

iii. Pipetting for enzyme assay and protein estimation should be done accurately.

iv. The filter of the spectrophotometer should be set in accordance with the required wavelength and calibrate the spectrophotometer with the suitable buffer solution (Blank).

v. Keep adding the required buffer until all the fraction are collected otherwise column will get dried and gel bed may be broken.

Suggested Reading(s)

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ii. Richard, J. Simpson. *Purifying Proteins for Proteomics.* CSHL Press.
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iv. Wilson, K., & Walker, J. (2008). *Biochemistry and Molecular Biology.* (6th ed.). Cambridge University Press.
v. Rodney Boyer (2009). *Biochemistry Laboratory: Modern theory and techniques.* (International Edition) Benjamin Cummings.

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