

HiPer[®] Affinity Chromatography Teaching Kit

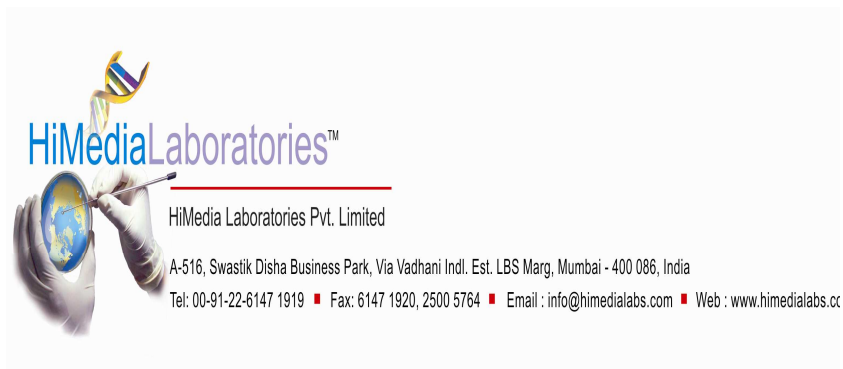
Product Code: HTC002

Number of experiments that can be performed: 5

Duration of Experiment:
Protocol: 3 hours (Approximately)

Storage Instructions:

- The kit is stable for 6 months from the date of receipt
 - Store Protein Standard at -20 °C
- Store Concanavalin A-Agarose Suspension, Crude Protein Sample, Elution Buffer, Sodium Acetate Buffer, Hydrogen Peroxide and ABTS at 2-8°C
- Other kit contents can be stored at room temperature (15-25°C)



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Aim:

To learn the technique of protein purification of Horse Radish Peroxidase (HRP) by affinity chromatography using Concanavalin A agarose and estimation of the activity as well as the protein concentration of the purified protein.

Introduction:

Chromatography is the process through which biomolecules are separated and analyzed from a complex mixture. This separation process consists of two phases: a stationary phase and a mobile phase. The mobile phase consists of the mixture to be separated which percolates through the stationary phase. Affinity chromatography is a chromatographic process through which proteins are separated on the basis of their reversible interaction with a specific ligand coupled to a chromatographic matrix. The bound protein of interest is eluted by a solution containing free ligand.

Principle:

Affinity chromatography is a very effective molecular technique for purification of protein on the basis of its biological function. Through this chromatography the desired protein is isolated from a mixed solution depending upon the protein's specific binding affinity to ligands mounted in a gel matrix through which the protein mixture is allowed to run. This chromatographic technique can be used for the purification of proteins from denatured or functionally different forms and for the removal of specific contaminants. Affinity chromatography relies on isolating the desired protein from a mixed solution through the protein's specific binding affinity to ligands mounted in a gel matrix through which the protein mixture is allowed to run. The principle of this chromatographic method is as described in figure 1. When a sample is loaded on the affinity chromatography column only the substances with affinity for the ligand are retained in the column. Rest of the substances with no affinity for the ligand is washed out from the column. Finally, the substances retained in the column are eluted from the column by changing pH or salt or organic solvent concentration of the eluent.

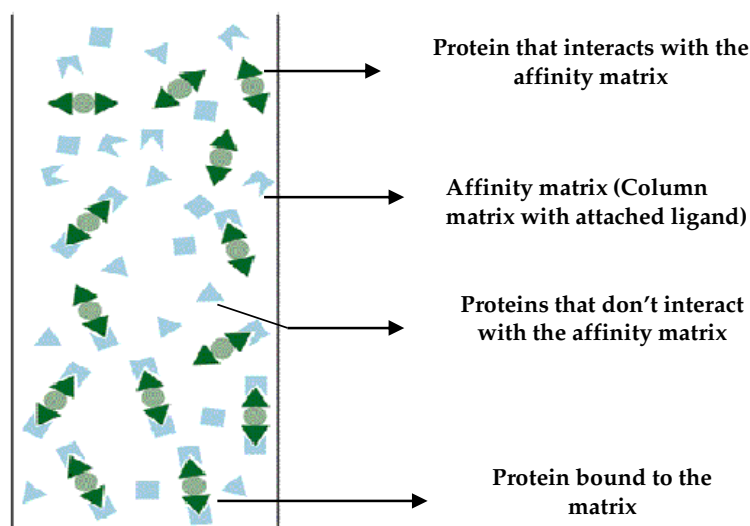


Fig 1: In affinity chromatography proteins are separated on the basis of their affinity for a column matrix

The materials required for affinity chromatography are as follows:

- 1) **Column matrix:** The column matrix is an inert support to which a ligand can be directly/indirectly coupled and should be chemically and physically inert. Sepharose, a bead-form of agarose, is a commonly used column matrix.
- 2) **Ligand:** The ligand should be covalently attached to the chromatographic matrix with the retention of its specific binding affinity for the target protein. Moreover, binding between the ligand and target protein should be reversible.
- 3) **Binding Buffer:** Binding buffer should be optimized for the effective interaction of ligand and target molecules.
- 4) **Wash Buffer:** Wash buffer should wash all unbound substances from the column without eluting the target molecules. It should also re-equilibrate the column back to the starting conditions. In affinity chromatography binding and wash buffer are the same.
- 5) **Elution Buffer:** Elution buffer elutes the bound substrate from its ligand by reversing the interaction between target molecules and ligand.

Affinity chromatography has applications for several purification procedures as follows:

Immobilized metal ion affinity chromatography (IMAC): During this chromatographic procedure proteins are purified on the basis of formation of a coordinate covalent bond of amino acids with metal ions, e.g. cobalt, nickel, copper. In this chromatography metal ions are immobilized on the column matrix. The bound proteins are eluted out by changing the pH or by adding a competitive molecule like imidazole.

Immunoaffinity chromatography: In immunoaffinity chromatography procedure antibodies are immobilized on a matrix through which biological samples are passed and the antigen which is specific for the immobilized antibody is captured. For this reason this procedure is used to purify or enrich antibodies.

Lectin chromatography: In this chromatography lectins are used to fractionate protein samples from a mixture. Lectins, e.g. [Concanavalin A](#), are sugar-binding plant proteins which specifically bind [carbohydrate](#) molecules. Lectins have major application for the isolation and separation of glycoproteins. Substances bound to lectin are eluted out by using a competitive binding substance.

Kit Contents:

This kit can be used to purify Horse Raddish Peroxidase (from crude extract) using Concanavalin A agarose followed by measurement of enzymatic activity of HRP (Horse Raddish Peroxidase).

Table 1: Enlists the materials provided in this kit with their quantity and recommended storage

Sr. No.	Product Code	Materials Provided	Quantity	Storage
			5 expts	
1	TKC270	Concanavalin A-Agarose Suspension	2.5 ml	2-8°C
2	TKC230	Standard Protein (1 mg/ml)	4 ml	-20°C
3	TKC271	Crude Protein Sample	5 X 1 ml	2-8°C
4	TKC272	Elution Buffer	6 ml	2-8°C
5	TKC273	Sodium Acetate Buffer	60 ml	2-8°C
6	TKC274	Hydrogen Peroxide	0.1 ml	2-8°C
7	TKC275	ABTS	50 ml	2-8°C
8	TKC231	Folin's Reagent (2N)	15 ml	RT
9	TKC232	Solution A	180 ml	RT
10	TKC233	Solution B	2 ml	RT
11	TKC329	Solution C	2 ml	RT
12	TKC276	Column	1 No.	RT

Materials Required But Not Provided:

Glass wares: Test tubes, Measuring cylinder

Reagents: Distilled water

Other requirements: Spectrophotometer, Column stand, Glass cuvette, Micropipette, Tips

Storage:

HiPer® Affinity Chromatography Teaching Kit is stable for 6 months from the date of receipt without showing any reduction in performance. On receipt, store standard protein (1mg/ml) at -20°C and Concanavalin A-Agarose Suspension, Crude Protein Sample, Elution Buffer, Sodium Acetate Buffer, Hydrogen Peroxide, ABTS at 2-8°C. Other kit contents can be stored at room temperature.

Important Instructions:

1. Before starting the experiment the entire procedure has to be read carefully.
2. Column should be washed thoroughly before each use.
3. Concanavalin A agarose column cannot be reused.
4. Mix gently the Concanavalin A-Agarose suspension before pipetting.
5. Prepare substrate solution for determining HRP activity just before use.
6. **Preparation of 1N Folin's Reagent (5ml):** Dilute Folin's reagent (2N) with equal amount of distilled water on the day of use. (2.5 ml of Folin's reagent + 2.5 ml of Distilled water)
7. **Preparation of Alkaline Copper Reagent (30 ml):** Mix 29.4 ml of Solution A with 0.3 ml of Solution B and 0.3 ml of Solution C on the day of use.

Procedure:

Purification of HRP from the crude extract using Concanavalin A agarose:

1. Take 0.5 ml of Concanavalin A-Agarose suspension into the empty column and allow the resin to settle.
2. Add 5 ml of sodium acetate buffer for the equilibration of the Con A-Agarose column. The buffer should be completely drained out.
3. Take out 0.1 ml of the crude sample and save it for the determination of enzyme activity and protein concentration.
4. The remaining 0.9 ml of the crude sample should be loaded onto the column and allow it to drain off completely.
5. Add 1 ml of sodium acetate buffer for washing the column. For washing out any unbound sample another 4 ml of the buffer has to be added.
6. Add 1 ml of elution buffer to elute out the bound HRP. Label this collected sample as "eluate". The enzymatic activity and protein concentration of this eluate has to be determined by the following methods:

Estimation of HRP activity:

1. Substrate solution for the enzymatic assay was prepared by adding 2 µl of H₂O₂ to 10 ml of ABTS solution.

2. Use this substrate solution as blank for the absorbance reading at 725 nm.
3. Add 10 µl of “eluate” to 2990 µl of substrate solution and mix properly and exactly after 20 seconds of mixing the reagents note down the change in absorbance at 725 nm ($\Delta A_{725\text{nm}}$).
4. Take 10 µl of crude sample and add 190 µl of distilled water for diluting the crude sample 1:20.
5. Add 10 µl of 1:20 diluted crude sample to 2990 µl of substrate solution. Mix the reagents properly and note down the change in absorbance at 725 nm exactly after 20 seconds ($\Delta A_{725\text{nm}}$).
6. Determine the enzymatic activity of crude and eluted sample as follows:

$$\text{Activity of enzyme} = \frac{3 \times \Delta A_{725\text{nm}} \times \text{RV} \times \text{DF}}{19 \times \text{SV}} \quad \text{Units/ml}$$

Where

Extinction coefficient of ABTS at a concentration of 1 µmol/µl = 19

$\Delta A_{725\text{nm}}$: Change in absorbance at 725 nm after 20 seconds

RV: Reaction Volume (in ml)

SV: Sample volume taken for assay (in ml)

DF: Dilution factor (for eluate: 1, for crude sample: 20)

7. Determine the total enzyme activity as follows:

Total enzyme activity of crude sample = activity x volume loaded onto the column

Total enzyme activity of eluate = activity x eluted volume from the column

Folin–Ciocalteu (Lowry) Assay for Protein Estimation:

1. Make dilutions of Protein (BSA) standards with concentrations of 200, 160, 120, 80, 40, 20, 10 µg/200 µl by transferring respective amount of BSA from the standard protein solution (1 mg/ml) and adjusting it to a total volume of 200 µl by adding distilled water as mentioned in table 2.
2. Add 3 ml of Alkaline Copper reagent to each test tube. Mix well.
3. Keep at room temperature for 10 minutes.
4. Add 0.5 ml of 1N Folin’s reagent to each test tube. Vortex the tubes and keep in Boiling Water Bath for 10 minutes
5. Switch on the Spectrophotometer, select the wavelength at 750 nm and let it warm before taking the absorbance (OD). First take the OD of Blank and make it zero.
6. Remove Blank tube and take the OD of all the tubes and record it. Wash the cuvette after taking OD of each sample.

Table 2:

Tube No.	Blank	1	2	3	4	5	6	7	8	9
Conc. Of BSA (µg)	0.0	10	20	40	80	120	160	200	Eluate	Crude sample (1:20)
Amt of Stock (µl)	0.0	10	20	40	80	120	160	200	200 µl	200 µl
Amt of diluent (µl)	200	190	180	160	120	80	40	0.0		
Alkaline Copper Reagent	3	3	3	3	3	3	3	3	3	3
Keep at Room temperature for 10 minutes										
Amt of Folin's Reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vortex and keep in Boiling Water Bath for 10 minutes										
Absorbance at 750 nm										

- Plot a Standard Curve of absorbance at 750 nm on "Y" axis versus concentration of protein µg/200 µl on "X" axis.
- Record the value "x" of Unknown [Eluate and crude sample (1:20)] from graph corresponding to the optical density reading of the samples.

Protein concentration can be calculated using following formula:

$$\text{Protein concentration} = \frac{\text{"x"} \times \text{DF}}{\text{V}} \quad \text{mg/ml}$$

Where

DF: Dilution factor (for eluate: 1, for crude sample: 20)

V: Volume of sample

x: Unknown (sample)

Estimation of Specific Activity of HRP:

Determine the specific activity of HRP for crude and eluted sample using the following formula:

$$\text{Specific activity} = \frac{\text{Enzyme activity}}{\text{Protein concentration}} \quad \text{U/mg}$$

Estimation of yield and recovery of HRP:

Calculate the percentage of recovered enzyme activity on loading the crude sample from the following formula:

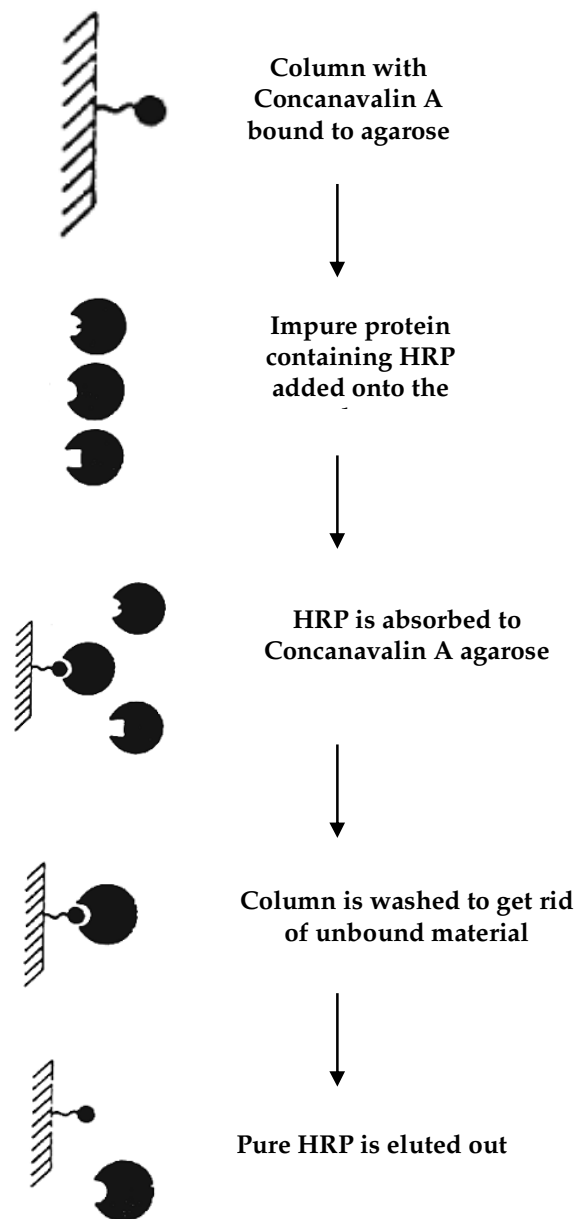
$$\text{Recovery} = \frac{\text{Total activity in eluate}}{\text{Total activity in crude sample}} \times 100$$

Yield = Total activity of eluate (U/ml) X Total volume of eluate (ml)

Estimation of fold purification:

Fold purification = $\frac{\text{Specific activity of eluate}}{\text{Specific activity of crude sample}}$

Flowchart:



Observation and Result:

Look for the colour change upon addition of HRP to the substrate solution. Measure the absorbance and determine the enzyme activity as mentioned in the procedure. Determine the protein concentration following the Lowry assay and record all the results as follows:

	Crude Sample	Eluate
Enzyme activity (U/ml)		
Protein concentration (mg/ml)		
Specific activity (U/mg)		

From all these data determine yield and fold purification of HRP as mentioned in the procedure.

Interpretation:

The column used in this chromatography teaching kit is Concanavalin A (ConA) agarose where ConA is a lectin. This lectin ligand is bound to the agarose matrix. Lectins, sugar-binding plant proteins, are mainly used for the purification of glycoproteins. Horse Raddish Peroxidase (HRP) is a glycoprotein and has a high affinity for ConA. When the crude protein extract is loaded onto the ConA agarose column, HRP binds to it and rest of the unbound proteins are washed off. HRP bound to ConA is eluted out by using a free sugar that competes with glycoprotein for the binding.

Troubleshooting Guide:

Sr.No.	Problem	Possible Cause	Solution
1	Poor flow through the column	Air bubble is trapped within the column	Allow the ConA-agarose to settle down before loading the crude sample
2	Eluate does not show any enzyme activity	Purification was not done properly	Follow the entire purification procedure properly

Technical Assistance:

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance mail at mb@himedialabs.com