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Objective:
To learn protein purification by affinity chromatography which involves:
- Purification of Horse Radish Peroxidase (HRP) using Concanavalin A Agarose column (Con-A).
- Estimation of HRP activity.
- Estimation of Protein concentration by Lowry’s method.

Principle:
Affinity chromatography is a method of selectively and reversibly binding proteins to a solid support matrix based on the fact that biological affinities exist between molecules, e.g., antigen with antibody. One of the components, the ligand is immobilized onto a solid matrix, which is then used to selectively purify the target protein. Including a competing ligand in mobile phase or changing pH then elutes the target protein out.

The performance of affinity chromatography is determined by comparing the specific activity of protein before and after purification. Specific activity of protein (enzyme) is defined as its activity per mg of protein. In order to determine specific activity, enzyme activity and protein concentration are estimated.
Kit Description:

Using this kit, students will carry out purification of horse radish peroxidase (from crude extract) using a lectin column (Con A). Lectins are plant proteins that have high affinity for particular sugars such as those present in glycoproteins. HRP is a glycoprotein, which helps in its selective binding to the column. Pure protein will then be eluted out using a free sugar (e.g., fructose) that competes with the glycoproteins for the immobilized lectin.

Students will estimate the HRP activity and protein concentration of samples before & after purification to determine specific activity of HRP.

Enzyme activity of HRP will be determined using hydrogen peroxide as substrate and ABTS [2,2’-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid] as a chromogen. HRP acts on hydrogen peroxide to release nascent oxygen that oxidizes ABTS to give a coloured product. The intensity of the coloured product is measured using spectrophotometer at 725 nm or colorimeter using a suitable filter.

Enzyme HRP activity is expressed in ABTS units, which is defined as the amount of HRP that oxidizes 1 ìmol of ABTS in 1 minute to ABTS oxide.

Protein Estimation by Lowry’s method: This method is based on both Biuret reaction and Folin-Ciocalteau reaction. Here, the peptide bonds of protein react with copper under alkaline conditions producing Cu+, which reacts with Folin’s reagent to give blue colour due to the reduction of phosphomolybdotungstate to heteropoly molybdenum. The intensity of the colour is dependent on the tyrosine and tryptophan content of the proteins.
Affinity Chromatography

Materials Provided:
The list below provides information about the materials supplied in the kit. The products should be stored as suggested. Use the kit within 6 months of arrival.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Quantity</th>
<th>Store</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>50 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Column</td>
<td>1 No</td>
<td>4°C</td>
</tr>
<tr>
<td>Concanavalin A-Agarose suspension</td>
<td>2.5 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Crude sample</td>
<td>5 Nos.</td>
<td>4°C</td>
</tr>
<tr>
<td>Elution buffer</td>
<td>5 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>0.1 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Sodium acetate buffer</td>
<td>60 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>BSA (Protein standard)</td>
<td>5 x 1 mg</td>
<td>4°C</td>
</tr>
<tr>
<td>Solution I</td>
<td>1.5 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Solution II</td>
<td>100 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Solution III</td>
<td>10 ml</td>
<td>4°C</td>
</tr>
</tbody>
</table>

Materials Required:

- **Equipment:** Colorimeter or Spectrophotometer.
- **Glassware:** Measuring cylinder, Test tubes.
- **Reagent:** Distilled water.
- **Other Requirements:** Glass cuvette, Micropipette, Tips.

Procedure:

Purification of HRP using Con A:

1. Resuspend an aliquot of crude sample in 1 ml of sodium acetate buffer.
2. Gently pipette 0.5 ml of Concanavalin A-Agarose suspension into the column and allow the resin to settle.
3. Equilibrate Con A-Agarose column with 5 ml of sodium acetate buffer. Allow the buffer to drain out completely.
4. Save 0.1 ml of the crude sample for determination of enzyme activity and protein concentration.
5. Load the remaining 0.9 ml of the crude sample onto the column; allow it to drain off completely.
6. Wash the column with 1 ml of sodium acetate buffer. Add another 4 ml of the buffer to wash out any unbound sample.
7. Elute the bound HRP by using 1 ml of elution buffer. Collect the entire amount in a test tube. Label this sample as eluate.
Estimation of HRP activity:

1. Prepare substrate solution by adding 2 µl of H₂O₂ to 10 ml of ABTS solution.
2. Using the substrate solution, blank the reading to zero at 725 nm (spectrophotometer/colorimeter).
3. To 2990 µl of substrate solution, add 10 µl of eluate and mix. Note down the change in absorbance at 725 nm, exactly after 20 seconds of mixing the reagents (dA-725).
4. Dilute the crude sample 1:20 (10 µl of crude sample + 190 µl of distilled water).
5. To 2990 µl of substrate solution add 10 µl of 1:10 diluted crude sample and mix. Note down the change in absorbance at 725 nm exactly after 20 seconds of mixing the reagents (dA-725).
6. Estimate the activity of enzyme in both the crude and eluted samples, as follows:

   Extinction coefficient of ABTS at a concentration of 1 µmol/µl = 19.0

   Activity of enzyme in the reaction mixture
   \[ \frac{3 \times (dA-725)}{19} \times X \] µmol / min.

   Therefore, activity of enzyme in the sample
   \[ \frac{X \times rv \times df}{sv} \] U / ml

where: (dA-725) – change in absorbance at 725 nm after 20 seconds
U - ABTS units.
rv - reaction volume (in ml).
sv - sample volume taken for assay (in ml).
df - dilution factor.
df - 1 for eluate; 10 for crude sample.

7. Estimate the total enzyme activity in crude & elute samples as follows:
   Total enzyme activity in crude sample
   = activity/ml x volume loaded onto the column
   Total enzyme activity in eluate
   = activity/ml x eluted volume from the column

Estimation of Protein concentration by Lowry’s Method:

1. Dissolve 1 aliquot of standard BSA in 1 ml of distilled water to get stock concentration of 1 mg/ml.
2. Use 1:10 diluted crude sample for estimation of protein concentration. Do not dilute the eluate for protein estimation.
3. Pipette standard BSA solution and samples (crude and eluate) as given in table I and adjust the volume to 0.1 ml with distilled water.
4. Add 2 ml of complex forming reagent to each tube, mix and incubate at room temperature (RT) for 10 minutes.
5. Add 0.2 ml of Solution III, mix and incubate for 20 minutes at RT.
6. Read the optical density using spectrophotometer at 660 nm or a colorimeter using a suitable filter. Record your results as in table 1.
Table 1: OD readings - Lowry's method.

7. Construct a calibration curve by plotting optical density reading on 'Y' axis against standard protein concentration in µg on X axis.
8. Record the value ‘X’ from the graph corresponding to the optical density reading for eluted and crude samples.
9. Calculate the concentration of protein in these samples using the following formula:

Sample concentration = \( \frac{(X \times df)}{v} \) mg/ml \\

where: X - Value from graph in µg \\
      v - Volume of sample in µl \\
      df - dilution factor of sample if any \\
      (df = 1 for eluate; 10 for crude sample)

Estimation of Specific Activity of HRP:
Calculate the specific activity using the following formula for both eluted and crude samples.

Specific activity = \( \frac{\text{Enzyme activity}}{\text{Protein concn.}} \) \\

\[ \text{Specific activity} = \frac{\text{Enzyme activity}}{\text{Protein concn.}} = \text{______ µmol/min/mg} \]

Estimation of yield & recovery of HRP:
The following formula is used to estimate the percentage of enzyme activity recovered on loading the crude sample onto the column:

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

Yield = Total activity of eluate/ml x Total volume of eluate \\

Estimation of Fold Purification:
Fold purification = \( \frac{\text{Specific activity of eluate}}{\text{Specific activity of crude sample}} \) \\

This is a measure of efficiency of purification of the enzyme using affinity chromatography.
Result
Record your results as follows:

<table>
<thead>
<tr>
<th></th>
<th>Crude Sample</th>
<th>Eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activity (U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein concentration (mg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity (U/mg)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From the above data, calculate and report recovery, yield and fold purification of the enzyme HRP.

Ordering Information

<table>
<thead>
<tr>
<th>Product</th>
<th>Size</th>
<th>Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeNeiTm Affinity Chromatography</td>
<td>1 Pack</td>
<td>KT41</td>
</tr>
<tr>
<td>Teaching Kit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Consumables for 5 experiments)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Email:
Sales: geneisales@sanmargroup.com

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