Affinity Chromatography of Glucose Binding Protein

Storage: See Page 3 for specific storage instructions

EXPERIMENT OBJECTIVE:
Students will learn the principle of affinity chromatography by isolating a carbohydrate-binding lectin protein from an extract of jack bean meal.
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Safety Data Sheets can be found on our website:

Affinity Chromatography of Glucose Binding Protein

Experiment

Experiment Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Affinity Gel</td>
<td>Refrigerator</td>
</tr>
<tr>
<td>B Jack bean meal</td>
<td>Room temperature</td>
</tr>
<tr>
<td>C NaCl</td>
<td>Room temperature</td>
</tr>
<tr>
<td>D Dextrose</td>
<td>Room temperature</td>
</tr>
<tr>
<td>E Con A Control</td>
<td>Refrigerator</td>
</tr>
<tr>
<td>F Horseradish peroxidase</td>
<td>Refrigerator</td>
</tr>
<tr>
<td>G ABTS Substrate</td>
<td>Refrigerator</td>
</tr>
<tr>
<td>H Standard dilution buffer</td>
<td>Refrigerator</td>
</tr>
</tbody>
</table>

The following components can be stored at room temperature:
- Membrane
- Columns (syringe) and column tips
- Cheesecloth for column
- 50 ml conical tubes
- Transfer pipets
- Petri dishes

Requirements

- Shaking platform or vortex
- Rotating or rocking platform
- Clinical centrifuge
- Ring stands with clamps for columns
- Microtest tubes or small glass test tubes for collecting fractions
- Test tubes (15 ml) to collect elutant
- 50 ml, 100 ml beakers or flasks
- Beaker or flask
- Graduated cylinders: 10 ml, 100 ml, and 250 ml
- 10 ml pipets
- Forceps
- Distilled water
- Filter paper

This experiment is designed for 10 groups.

Store components A, E-H in the refrigerator. The remaining components can be stored at room temperature.

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Affinity Chromatography of Glucose Binding Protein

Protein purification by affinity chromatography takes advantage of the biological activity of the protein to be purified. Typically the protein to be purified has a binding site with an affinity for a specific ligand. The ligand-protein interaction can be: substrate-enzyme, antigen-antibody, hormone-hormone receptor, saccharide-saccharide binding protein, and so on. An affinity column is made by covalently coupling the ligand on a support that is usually a chromatography bead such as Sephadex or Sepharose bead matrix. A crude extract containing the protein is passed through the affinity column where the protein-ligand binding interaction occurs. Proteins and other biological macromolecules that do not bind to the ligand are eluted from (pass through) the column in a fraction called the effluent. After the sample is charged on the affinity column, it is washed with a suitable buffer to remove proteins from the extract that do not bind to the column.

The bound protein is eluted from the affinity column by one of the following procedures:

a. In most cases, a solution of purified ligand is used to elute the bound protein. The concentration of the ligand in the eluting buffer is significantly high so that the binding equilibrium for the ligand-protein pair shifts from the column-bound ligand to the ligand in the eluting solution. This results in the elution of the affinity column-bound protein. Subsequent dialysis against buffer that contains high salt breaks the complex, removes the ligand and makes it possible to recover the purified protein.

b. In cases where it is prohibitive to use high concentrations of purified ligand to elute the affinity column, the affinity bound-protein can be eluted by disrupting the ligand-protein interaction with an acidic pH buffer. This non-specifically disrupts the protein-ligand interaction and the protein elutes from the column in a low pH environment. Rapid neutralization of fractions that contain the eluted protein will help maintain the biological activity of the bound protein.

In this experiment, the saccharide binding protein from jack bean meal, Concanavalin A (Con A), binds to the glucose-based saccharide (dextran) of Sephadex. Thus no ligand needs to be chemically coupled to Sephadex as the glucose molecules making up the dextran polymer of Sephadex will serve as the ligands. Con A is a mannose-binding protein in jack bean meal. Mannose and glucose are similar in structure and Con A binds to glucose but not as strongly as it does to mannose or mannose-containing oligosaccharides. A one molar (1M) solution of dextrose (glucose) is used to elute the Con A from the Sephadex column.

The biological activity exhibited by Con A in this experiment is its ability to bind to horseradish peroxidase, a mannose containing glycoprotein with enzymatic activity. Horseradish peroxidase has an oligosaccharide core that contains mannose. Con A binds to the mannose of the enzyme without affecting the peroxidase activity.
Affinity Chromatography of Glucose Binding Protein

Con A eluted in fractions from jack bean meal is adsorbed to a nylon membrane. The ligand binding activity is maintained by Con A while bound to the nylon membrane. The binding is determined by incubating the adsorbed membranes in a solution of horseradish peroxidase. The enzyme will bind to the membrane bound Con A and the bound horseradish peroxidase will convert the substrate to a color product.
Experiment Overview

**EXPERIMENT OBJECTIVE:**

In this experiment, students will learn the principle of affinity chromatography by isolating a carbohydrate-binding lectin protein from an extract of jack bean meal.

**LABORATORY SAFETY**

1. Gloves and safety goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment which is used in conjunction with the heating and/or melting.
3. Do not mouth pipet reagents - use pipet pumps or bulbs.
4. Always wash hands thoroughly with soap and water after handling contaminated materials.
Experiment 277

Affinity Chromatography of Glucose Binding Protein

Student Experimental Procedures

1. In the 50 ml capped tube, suspend the 1.5 grams of jack bean meal (B) in 12 ml of 1M NaCl.

2. Extract the Con A from the Jack Bean meal at room temperature for thirty minutes with frequent, vigorous mixing (jack bean meal should be kept in suspension) or by placing on a shaking platform or vortex with the tube placed horizontally.

Prepare the Column

- Remove the plunger from the syringe.
- Fold the cheesecloth in half twice and then again to fit into the barrel of the syringe.
- Use the plunger or a pencil to push the cheesecloth to the bottom of the syringe (remove the plunger).

See Useful Hint at right.

During the extraction, proceed to steps 3 through 6.

3. Resuspend slurry of affinity gel by inverting tube several times.

4. Pour the 10 ml of slurry into affinity column and allow the gel to settle.

5. Start the elution of the affinity column and let liquid flow from column. If the gel leaks from the column, save it in a beaker. Remove and repack the cheesecloth at the bottom of the column and repack the column.

See Useful Hint at right.

6. When affinity gel surface in the column is moist and no liquid is visible;
   - Gently fill the rest of the column with a solution of 1 M NaCl.
   - Let the wash flow through; when the last of the wash enters the column, stop the flow.
   - The surface of the gel should be moist, not dry, and a very small amount of sodium chloride solution should remain on the surface of the gel.

7. Pour the extract into a clean conical centrifuge tube and centrifuge at 2000 rpm or at high speed for 15 minutes. Transfer the supernatant to a clean tube and spin again if there are pieces of undissolved jack bean meal remaining. Discard the pellets.

8. Save 0.5 ml of the volume of the extract and label it as “Jack bean meal extract - Sample #2”. There should be no precipitate.

Steps 1 and 2:

The jack bean meal should be swirled vigorously in the extraction fluid to ensure even dispersal.

Step 5:

Collect this wash in a 50ml beaker. If a considerable amount of affinity gel comes out of the column, then add one more column volume of wash solution and collect all of the wash in the beaker. Remove the packing at the bottom of the column and repack the column. Pass all of the wash through the column. The gel should be retained in the column. If buffer is leaking from the capped column, dab a small amount of petroleum jelly or stopcock grease on the column end piece. Be careful not to plug the column opening with the jelly or grease.
Student Experimental Procedures

9. Charge the column by gently pouring the remaining extract into the affinity column.

10. Start the column and collect the flow through (effluent) in bulk in a clean 50 ml beaker.
   • As the last of the extract enters the column, stop the flow.
   • The surface of the gel should be moist, not dry and no extract should be present on the surface of the gel.

11. Wash the affinity column with four volumes:
   • Fill the column with 1M NaCl.
   • Elute the column and discard the effluent into a 50 ml beaker.
   • Repeat these steps three more times. When the last (fourth) elution wash enters the column, collect a few drops and stop flow. Label this tube “Effluent - Sample #3”.
   • The surface of the gel should be moist, not dry and no wash should be present on the surface of the gel.

12. Pour 5 ml of elutant, 1M NaCl/1M Dextrose, into the column.

   **See Useful Hint at left.**

13. Allow the column to flow and collect the first 0.5 ml fraction. This is “Eluate fraction 1 - Sample #4”.

14. Stop the column flow and let the column set for 10 minutes.

15. Allow flow to continue and collect 0.5 ml fractions. Collect fractions until all the column is eluted.
   • Label the initial fraction collected as “Eluate fraction 5 - Sample #5”

16. Elute the final fraction as “Last eluate fraction collected - Sample #6”.

   **See Useful Hint at left.**

17. Assay the following fractions for enzyme binding activity:
   1: Con A Control
   2: Jack bean meal extract (Step 8)
   3: Effluent (Step 11)
   4: Eluate fraction 1 (Step 13)
   5: Eluate fraction 5 (Step 15)
   6: Last eluate fraction collected (Step 16)
APPLICATION OF SAMPLES TO MEMBRANE

In all steps, use gloves and forceps to handle the membrane.

Sample Application

18. Place a piece of membrane on a paper towel.

19. Using a micropipet, apply 10 microliters of each sample to the mem-
brane. Each membrane should have the pattern as shown.

Sample Numbers:
1. Con A Control, 1 mg/ml
2. Jack bean meal extract
3. Effluent
4. Eluate fraction 1
5. Eluate fraction 5
6. Last eluate fraction collected

See Useful Hint right.

20. Allow the membrane to dry completely for 15 minutes at room tem-
perature or 10 minutes in a 37°C incubation oven.

Optional Stopping Point:

The experiment can be stopped after step 20 and resumed
during the next lab period.
Student Experimental Procedures

DETECTION OF CON A PROTEIN

1. Obtain three 60 mm diameter Petri dishes and a 50 ml beaker which will be used for steps 2 through 9.

2. Label a different Petri dish for each of the following:
   - Standard dilution buffer (SDB)
   - Horseradish peroxidase (HRP)
   - Substrate (SUB).

3. Pour 40-45 ml distilled water into a beaker. Label this beaker as “Wash”.

4. Pipet 10 ml of Standard Dilution Buffer into Petri dish labeled "SDB".

5. Pipet 5 ml of diluted horseradish peroxidase into Petri dish labeled "HRP".

6. Dip the dried membrane in the Petri dish with the Standard Dilution Buffer (SDB) and transfer the wet membrane to "HRP" dish. Incubate the membrane in "HRP" dish at room temperature for 10 minutes with frequent mixing or place on a shaking or rotating platform.

7. Lift the membrane from the enzyme (HRP dish). Drain excess liquid from the membrane. Dip the membrane in the Standard Dilution Buffer (SDB dish) and drain excess SDB from the membrane.

8. Place the membrane in the dish labeled "SUB". Carefully pipet 2 ml of the ABTS substrate overtop of the center of the membrane (the substrate should spread evenly over the entire membrane). Avoid disturbing the dish and causing the substrate to spill off of the membrane. Allow the membrane to soak undisturbed for 30-90 seconds or until the color is sufficiently developed. Do not allow the membrane to become too dark.

9. Lift the membrane from the substrate solution and immediately immerse the membrane in the wash beaker of distilled water. Wash the membrane in the water by successive dipping for one minute.

10. Lift the membrane from the water and drain excess water from the membrane.

11. Place the membrane on a piece of filter paper and allow it to dry at room temperature for 30 min. Alternatively, place the membrane in a 37°C incubation oven for 10 min. After the membrane is dry, record the precipitated substrate in Samples 1 - 6. Initially, precipitin dots will appear very dark but will become lighter after the membrane dries.

12. Store the membrane in a plastic bag.
Affinity Chromatography of Glucose Binding Protein

Experiment Results and Study Questions

LABORATORY NOTEBOOK RECORDINGS:

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the experiment:
• Write a hypothesis that reflects the experiment.
• Predict experimental outcomes.

During the Experiment:
• Record (draw) your observations, or photograph the results.

Following the Experiment:
• Formulate an explanation from the results.
• Determine what could be changed in the experiment if the experiment were repeated.
• Write a hypothesis that would reflect this change.

STUDY QUESTIONS

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. What pattern of enzyme (HRP*) binding activity would you expect if you assayed the 0.5 ml effluent fractions?

2. Dextrose is used to elute the bound Con A from the affinity gel column, yet the bound dextrose was not removed from the Con A-containing eluate fractions before adsorbing the fractions to the membrane. Why does Con A bind to HRP* if the dextrose is still present in the Con A binding site?

3. Con A adsorbs strongly to the membrane, yet the HRP* protein binds only slightly. If HRP bound as strongly to the membrane as Con A, then the assay may not be possible unless an intermediate step was done. What would that intermediate step be?

* HRP is the abbreviation from horseradish peroxidase.
Instructor's Guide

NOTES TO THE INSTRUCTOR

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances.

If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK® web site. In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).
PreLab Preparations

The solutions required for this experiment should be prepared as follows:

- **Jack Bean Meal**
  Weigh out 1.5 grams of jack bean meal (component B) and place in a 50 ml conical tube for each group.

- **Affinity Gel**
  Mix the Affinity Gel to obtain an even suspension and dispense 10 ml into tubes or beakers for each group.

- **1 M NaCl**
  Dissolve the NaCl powder (component C) in 550 ml distilled or deionized water. Each group will require 45 ml. Save 60 ml for preparation of 1 M NaCl/1 M Dextrose (below).

- **1M NaCl/1 M Dextrose**
  To 60 ml 1M NaCl (saved from previous step), add all of Dextrose (component D), and stir to dissolve. Dispense 5 ml of 1M NaCl/1 M Dextrose for each group.

- **Con A Control**
  Dispense 15 µl of Con A Control (component E) for each group.

- **Cheesecloth for column “frit”**
  Completely unfold the cheesecloth and cut into 10 x 1.5” pieces. Each group will require one piece. Refer to student instructions for placement of cheesecloth and column packing.

- **Membranes**
  Cut membrane strips into 3.5 x 3.5 cm pieces to fit into 60 mm Petri dishes. Distribute one piece of membrane per group. Wear gloves when handling the membranes.

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**EACH GROUP WILL RECEIVE:**

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jack bean meal</td>
<td>1.5 grams</td>
</tr>
<tr>
<td>Affinity Gel</td>
<td>10 ml</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>45 ml</td>
</tr>
<tr>
<td>1 M NaCl/1 M Dextrose</td>
<td>5 ml</td>
</tr>
<tr>
<td>Con A Control</td>
<td>15 µl</td>
</tr>
<tr>
<td>cheesecloth (2 x 1.5”)</td>
<td>1 piece</td>
</tr>
<tr>
<td>Membrane strip (3.5 x 3.5 cm)</td>
<td>1 piece</td>
</tr>
<tr>
<td>SDB</td>
<td>10 ml</td>
</tr>
<tr>
<td>Horseradish Peroxidase</td>
<td>5 ml</td>
</tr>
<tr>
<td>ABTS Substrate</td>
<td>2 ml</td>
</tr>
<tr>
<td>Filter Paper (4 x 4 cm)</td>
<td>1 piece</td>
</tr>
</tbody>
</table>

- **Preparing Standard Dilution Buffer for Students**
  In a beaker or flask, dilute 10x Standard Dilution Buffer (component H) by mixing 20 ml of 10X SDB with 180 ml of distilled or deionized water. This is 1x SDB. Each group will require 10 ml of 1x SDB. Reserve 50 ml 1x SDB for preparation of Horseradish peroxidase.

- **Preparing Dilution of Horseradish Peroxidase**
  Dilute Horseradish peroxidase stock (component F) with Standard Dilution Buffer by mixing the contents of horseradish peroxidase stock with 50 ml of 1x Standard Dilution Buffer. Each group will require 5 ml of diluted enzyme.

- **ABTS Substrate**
  Dispense 2 ml of ABTS for each group. Keep refrigerated (in dark) until ready to use.

- **Filter Paper**
  Cut filter paper into 4 x 4 cm pieces. Distribute one piece of filter paper per group.
Anticipated Results

Dots of precipitated product will appear immediately upon immersing the membrane in the peroxidase substrate solution. Their intensity on the membrane is optimal after 30-90 seconds.

1. The Con A control at 1 mg/ml will show a light amount of precipitate. This is a control to indicate that the Con A - HRP assay is working.

2. The jack bean meal extract will give a spot of variable intensity from no precipitate to very light precipitate. This is dependent upon the concentration of the Con A in the extract plus the concentration of other non-specific jack bean meal proteins that bind to the membrane.

3. There should be no precipitate in the effluent if the column is thoroughly washed. There may be a very light spot of precipitate due to a slight amount of endogenous peroxidase activity in jack bean meal.

4. There will be no precipitate for Eluate fraction 1 since the elutant has not passed through the column.

5. The eluate fraction 5 will have an intense amount of precipitate, and the last eluate fraction will have a less intense spot of precipitate. Eluate fraction 5 contains the greatest amount of pure Con A. Depending on how much Con A bound to the column will determine how many subsequent fractions will contain Con A.

6. The last fraction will vary in intensity depending on how much Con A is in this last eluate fraction.

1: Con A Control
2: Jack bean meal extract
3: Effluent
4: Eluate fraction 1
5: Eluate fraction 5
6: Last eluate fraction collected
Please refer to the kit insert for the Answers to Study Questions