Affinity Chromatography of Glucose Binding Protein

Storage:
Store the entire experiment in the refrigerator.

EXPERIMENT OBJECTIVES:
Students will learn the principle of affinity chromatography by isolating a carbohydrate-binding lectin protein from an extract of jack bean meal.

All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.
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EDVO-Kit # 277  Affinity Chromatography of Glucose Binding Protein

Experiment Components

A  Affinity Gel
B  Jack bean meal
C  NaCl
D  1 M NaCl/1 M Dextrose
E  Con A Control
F  Horseradish peroxidase
G  ABTS Substrate
H  Standard dilution buffer

• 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 eluant
• 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

All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

This experiment is designed for 10 groups.
Store entire experiment in the refrigerator.
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.

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.
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 left.

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 left.

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.

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

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)

Step 12:
Most of the NaCl solution is absorbed by the dry meal the final volume of extract should be about 7-9 ml.

Step 15:
The total number of fractions collected from the column elution may vary, but each group should get 7 - 9 fractions.
Student Experimental Procedures

APPLICATION OF SAMPLES TO MEMBRANE

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

Sample Application

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

2. Using a micropipet, apply 10 microliters of each sample to the membrane. 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 at left.

3. Allow the membrane to dry completely for 15 minutes at room temperature or 10 minutes in a 37°C incubation oven.

Optional Stopping Point:
The experiment can be stopped after step 19 and resumed during the next lab period.
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.
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.
**Material Safety Data Sheet**

May be used to comply with OSHA’s Hazard Communication Standard. 29 CFR 1910.1200. Standard must be consulted for specific requirements.

### IDENTIFY (As Used on Label and List)

**Con A (Concanavalin A)**

Note: Blank spaces are not permitted. If any item is not applicable, or no information is available, the space must be marked to indicate that.

### Section I

**Manufacturer’s Name**

EDVOTEK, Inc.

**Address (Number, Street, City, State, Zip Code)**

14676 Rothgeb Drive

Rockville, MD 20850

**Emergency Telephone Number**

(301) 251-5990

**Date Prepared**

09-16-2002

**Signature of Preparer (optional)**

### Section II - Hazardous Ingredients/Identify Information

Hazardous Components [Specific Chemical Identity, Common Name(s)] OSHA PEL ACGIH TLV Other Limits Recommended % (Optional)

Concanavalin A

### Section III - Physical/Chemical Characteristics

**Appearance and Odor**

White powder

### Section IV - Physical/Chemical Characteristics

**Boiling Point**

No data

**Vapor Density (AIR = 1)**

No data

**Evaporation Rate**

(Butyl Acetate = 1)

No data

**Specific Gravity (H2O = 1)**

No data

**Flammable Limits**

LEL UEL

No data

**Melting Point**

No data

**Solubility in Water**

Soluble-cold

**Extinguishing Media**

Water spray, carbon dioxide, dry chemical powder or foam

**Special Fire Fighting Procedures**

Wear SCBA and protective clothing to prevent contact with skin

**Unusual Fire and Explosion Hazards**

Emits toxic fumes under fire conditions.

### Section V - Reactivity Data

**Stability**

Unstable

**Conditions to Avoid**

X

**Incompatibility**

Strong oxidizing agents

**Hazardous Decomposition or Byproducts**

Toxic fumes of carbon monoxide, carbon dioxide

### Section VI - Health Hazard Data

**Route(s) of Entry:**

Inhalation? Yes

Skin? Yes

Ingestion? Yes

**Health Hazards (Acute and Chronic)**

Harmful if swallowed, inhaled or absorbed through skin. May cause allergic reaction. Possible teratogens

**Carcinogenicity:**

NTF? Yes

IARC Monographs? Yes

OSHA Regulation?

### Section VII - Precautions for Safe Handling and Use

**Waste Disposal Method**

Normal solid waste disposal.

**Other Protective Clothing or Equipment**

Wear protective gear to avoid skin/eye contact

**Other Precautions**

None

### Section VIII - Control Measures

**Respiratory Protection (Specify Type)**

NIOSH/MSHA approved respirator

**Protective Gloves**

Yes

Eye Protection Safety goggles

**Other Protective Clothing or Equipment**

None

**Work/Hygienic Practices**

Do not breathe dust. Avoid eye, skin contact.

### Section V - Reactivity Data

**Stability**

Unstable

**Conditions to Avoid**

X

**Incompatibility**

None

**Hazardous Decomposition or Byproducts**

No data

**Polymerization**

Will Not Occur

### Section VI - Health Hazard Data

**Route(s) of Entry:**

Inhalation? Yes

Skin? Yes

Ingestion? Yes

**Health Hazards (Acute and Chronic)**

No data available

**Carcinogenicity:**

NTF? None

IARC Monographs? None

OSHA Regulation?

### Section VII - Precautions for Safe Handling and Use

**Waste Disposal Method**

Place in suitable container for waste disposal

**Other Protective Clothing or Equipment**

None

**Work/Hygienic Practices**

Treat symptomatically and supportively

### Section VIII - Control Measures

**Respiratory Protection (Specify Type)**

Any dust and mist respirator

**Protective Gloves**

Not required

Eye Protection Not required

**Other Protective Clothing or Equipment**

Not required

**Work/Hygienic Practices**

None

### Section V - Reactivity Data

**Stability**

Unstable

**Conditions to Avoid**

X

**Incompatibility**

None

**Hazardous Decomposition or Byproducts**

No data

**Polymerization**

Will Not Occur

### Section VI - Health Hazard Data

**Route(s) of Entry:**

Inhalation? Yes

Skin? Yes

Ingestion? Yes

**Health Hazards (Acute and Chronic)**

No data available

**Carcinogenicity:**

NTF? None

IARC Monographs? None

OSHA Regulation?

### Section VII - Precautions for Safe Handling and Use

**Waste Disposal Method**

Place in suitable container for waste disposal

**Other Protective Clothing or Equipment**

None

**Work/Hygienic Practices**

Treat symptomatically and supportively

### Section VIII - Control Measures

**Respiratory Protection (Specify Type)**

Any dust and mist respirator

**Protective Gloves**

Not required

Eye Protection Not required

**Other Protective Clothing or Equipment**

Not required

**Work/Hygienic Practices**

None
### Material Safety Data Sheet

**IDENTITY** (As Used on Label and List)
- Sodium Chloride

**Section I**

<table>
<thead>
<tr>
<th>Manufacturer's Name</th>
<th>EDVOTEK, Inc.</th>
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<tbody>
<tr>
<td>Address</td>
<td>14676 Rothgeb Drive, Rockville, MD 20850</td>
</tr>
<tr>
<td>Telephone Number</td>
<td>(301) 251-5990</td>
</tr>
<tr>
<td>Date Prepared</td>
<td>09-19-2002</td>
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**Section II - Hazardous Ingredients/Identification Information**

| CAS # | 7647-14-5 |

**Section III - Physical/Chemical Characteristics**

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<th>Property</th>
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<td>Appearance and Odor</td>
<td>Clear liquid</td>
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**Section IV - Physical/Chemical Characteristics**

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<th>Property</th>
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<td>Flash Point (Method Used)</td>
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<td>Evaporation Rate</td>
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**Section V - Reactivity Data**

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<td>Flammable Limits</td>
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<td>Incompatible</td>
<td>Strong oxidizing agents, strong acids</td>
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**Section VI - Health Hazard Data**

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<th>Route(s) of Entry:</th>
<th>Inhalation?</th>
<th>Skin?</th>
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<tr>
<td>Inhaled</td>
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<td>Ingestion</td>
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<td>Yes</td>
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**Section VII - Precautions for Safe Handling and Use**

- Avoid contact. Wear self-contained breathing apparatus, rubber boots and rubber gloves.
- Observe federal, state, and local laws.
- Use suitable protective clothing and equipment.
- Do not ingest. Avoid contact with skin, eyes and clothing.
- Do not store near incompatible materials.

---

### Material Safety Data Sheet

**IDENTITY** (As Used on Label and List)
- ABTS Substrate

**Section I**

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**Section II - Hazardous Ingredients/Identification Information**

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**Section III - Physical/Chemical Characteristics**

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<td>Odor</td>
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**Section IV - Physical/Chemical Characteristics**

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**Section V - Reactivity Data**

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**Section VI - Health Hazard Data**

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<th>Inhalation?</th>
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</tr>
<tr>
<td>Ingestion</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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**Section VII - Precautions for Safe Handling and Use**

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- Do not store near incompatible materials.