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A Geno Technology, Inc. (USA) brand name

Protein Purification from Tissue

Teacher's Guidebook

(Cat. # BE-418)

MATERIALS INCLUDED

This kit has enough materials and reagents for 24 students (six groups of four students).

- 6 vials Protein: Tissue Extract & Pestle
- 1 vial Precipitant-Mes
- 1 bottle Salt Solution (Amm. Sulfate)
- 6 Column: Size Exclusion Chromatography
- 1 bottle Gel Filtration Buffer
- 6 Column: Hydrophobic Columns
- 1 bottle HP Loading Buffer (2X)
- 1 bottle HP Elution Buffer
- 1 bottle Enzyme Substrate (starch)
- 1 bottle Enzyme Color Dye-I
- 1 vial Enzyme Color Dye-II
- 250 Centrifuge Tubes (1.5ml)

SPECIAL HANDLING INSTRUCTIONS

- Store Columns and Tissue Extract at 4°C.
- All other reagents can be stored at room temperature.

The majority of reagents and components supplied in the *BioScience Excellence*™ kits are non toxic and are safe to handle, however good laboratory procedures should be used at all times. This includes wearing lab coats, gloves and safety goggles.

For further details on reagents please review the Material Safety Data Sheets (MSDS).

The following items need to be used with particular caution.

Part #	Name	Hazard
C111	Column: Hydrophobic Column	Flammable
E141	Enzyme Color Dye-I	Corrosive

ADDITIONAL EQUIPMENT REQUIRED

- Low Speed Centrifuge for 1.5-2ml tubes
- Waterbath or Beaker and thermometer

TIME REQUIRED

- 3-4 hours

OBJECTIVES

- Learn protein purification strategies from biological samples /tissues.
- Learn step by step approaches to protein purification.
- Perform salt fractionation, size exclusion, and hydrophobic chromatography to purify target proteins in a single purification task.

BACKGROUND

A key step in proteomics, the study of proteins function and structure, is the purification of proteins. The ability to isolate and purify specific proteins is an essential feature of modern biochemistry as it allows scientist to study proteins in isolation from other proteins, which greatly aids the understanding of a particular protein's function.

Unfortunately there is no single ideal protein purification procedure and often the purification of a protein involves several techniques. The main idea behind protein purification is to select the best techniques to isolate a protein of interest, based on differences in its physical properties from other "unwanted proteins. The "Protein Purification" kits aim to cover many of the common techniques used in protein purification.

The first stage in purifying proteins from complex biological samples is to use protein fractionation. The main purpose of protein fractionation is to simplify the complex starting material into less complicated fractions. Protein fractionation uses protein properties that are affected by changing the pH and/or the ionic strength of the protein solutions. The use of acid and/or salt fractionations concentrates and enriches proteins into defined fractions, depending on their precipitation at differing pH and salt concentrations. Students understand the importance of enrichment of target proteins in the scheme of purification of protein molecules.

Adjusting the pH has been used as a simple and efficient way to precipitate proteins. Proteins have their lowest solubility at their isoelectric point. When the pH is gradually changed the pH of the solution passes through the isoelectric point of some proteins, causing some proteins to precipitate. Isoelectric precipitation is often used to precipitate unwanted proteins rather than to precipitate the protein of interest.

Precipitation of proteins by increasing the ionic strength of the solution is also knows as protein salting out. Salting out is dependent on the hydrophobicity of the surface of the protein. Hydrophobic regions are enriched in the hydrophobic amino acids, phenylalanine, tyrosine, tryptophan, leucine, isoleucine, methionine and valine. Proteins with more hydrophobic regions will aggregate and precipitate before those with smaller and fewer hydrophobic regions, thus resulting in fractionation. Salting out is usually performed at 4°C to decrease the risk of inactivation.

Salting out proteins from a crude extract using ammonium sulfate is a convenient purification step. Salts affect the electrostatic and non-polar properties of proteins in a reversible manner. At concentrations above 0.2M, salts not only neutralize the electrostatic forces on the protein surface but also affect the three dimensional structure of proteins, making them less soluble.

Salts, such as ammonium sulfate, have the tendency to disrupt the water structure, increase the water surface tension and increase the hydrophobic effect in the solution (i.e. decrease the solubility of non-polar molecules) and promote protein aggregation by association of hydrophobic surface. Ammonium sulfate is the most frequently used salt for salting-out experiments and can precipitate ~70% of proteins in a complex sample.

Once the complex starting material has been broken down into simpler fractions the fractions can be assayed for the protein of interest, often using an enzymatic assay. This allows scientist to locate the correct fraction, which can then be further purified.

Further purification normally utilizes conditions related to the specific protein. One set of techniques routinely used is chromatographic purification. Numerous chromatography techniques exist that can purify proteins based on their ionic strength, size, hydrophobicity or their affinity for specific ligands, such as an enzyme for its substrate or an antibody for its antigens.

This protein purification kit is a comprehensive kit designed to teach students strategies for the purification of a novel protein from a complex biological sample, such as a tissue extract. Students explore a multi-step sequential approach to purification and incremental increases in purification and enrichment of target proteins.

Using this kit students will purify proteins from a mammalian tissue extract and will use various fractionation and chromatography techniques to purify two target proteins. The purification procedure will teach students how to closely monitored purification of target proteins using target protein specific assays. Students will then be able to assign specific characteristics to the isolated proteins, based on the purification techniques.

This kit specifically teaches a sequential use of protein fractionation, size exclusion, and hydrophobic chromatography to achieve partial purification for the target proteins, assays of purification fractions and identification of purified proteins.

TEACHER'S PRE EXPERIMENT SET UP

1. Transfer 1ml 2X HP Loading Buffer to a 1.5ml tube; this will be aliquoted according to the next section.

2. Add 25ml HP Elution Buffer to the bottle containing 2X HP Loading Buffer. Mix the content by inverting 5-6 times to prepare 1X HP Loading Buffer.

3. Prepare the Enzyme Detection Dye: Take 1ml Enzyme Color Dye-I solution and add to the vial of Enzyme Color Dye-II. Mix the contents by inverting the tube until the tube contents have dissolved. This may take up to 10 minutes to completely dissolve. Transfer the dissolved Enzyme Color Dye-II into the remaining Enzyme Color Dye-I solution and briefly mix the solution. This is the Enzyme Detection Dye.

4. Aliquot reagents to each student group according to the amount needed in the next section.

MATERIALS FOR EACH GROUP

Supply each group with the following components. Several components will be shared by the whole class and should be kept on a communal table.

- 1 vial Tissue Extract & Pestle
- 100µl Precipitant-Mes
- 0.8ml Salt Solution (Amm. Sulfate)
- 4ml Enzyme Substrate
- 4ml Enzyme Detection Dye
- 1 Size Exclusion Chromatography Column
- 4ml Gel Filtration Buffer
- 1 Hydrophobic Column
- 100µl 2X HP Loading Buffer
- 8ml 1X HP Loading Buffer
- 5ml HP Elution Buffer
- 41 Centrifuge Tubes (1.5ml)

PROCEDURE

Always wear gloves and protective clothing through the whole experiment.

I. Protein Extraction

1. Add 150µl Gel Filtration Buffer to the Tissue Extract vial. Incubate at room temperature for 10 minutes. Use the Pestle to grind the tissue extract for 2 minutes. Centrifuge the tube for 5 minutes at maximum speed in a microcentrifuge.

2. Transfer 100µl supernatant, without disturbing the pellet, to a 1.5ml tube. Save the rest of the extract tube to be used as the enzyme assay control.

If necessary, the samples can be placed in a freezer until required to continue the experiment.

II. Protein Fractionation

1. Add 100µl Precipitant-Mes to the supernatant extracted from section I (step 2) and mix the content by inverting the tube a few times or vortexing briefly.

2. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 1 – Acid precipitation.

3. Carefully transfer the supernatant to a new tube. Add 100µl Salt Solution (Amm. Sulfate) and mix the content by inverting the tube a few times or vortexing briefly.

4. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 2 [Represents ~33% Ammonium Sulfate solution].

5. Carefully transfer the supernatant to a new tube. Add 200µl Salt Solution (Amm. Sulfate) and mix the content by inverting the tube a few times or vortexing briefly.

6. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 3 [represents ~60% Ammonium Sulfate solution].

7. Carefully transfer the supernatant to a new tube. Add 500µl Salt Solution (Amm. Sulfate) and mix the content by inverting the tube a few times or vortexing briefly.

8. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 4 [represents ~80% Ammonium Sulfate solution].

9. Carefully transfer the supernatant to a new tube. This is your Fraction 5.

10. Examine the fractions. The fraction with the most concentrated red color is enriched in Hemoglobin.

11. Add 50µl Gel Filtration Buffer to each fraction pellet and vortex briefly to dissolve the pellets.

If necessary, the samples can be placed in a freezer until required for sections III, IV and V. Samples can be frozen again to allow each section to be completed at different times.

12. Follow the protocol in section III to find out the fraction containing the target proteins/enzyme using 5µl of each fraction for each assay. Setup a positive control tube using the crude.

III. Target Enzyme (Amylase) Detection Assay

1. Label one tube for each fraction to be assayed and a tube for a positive control.

2. Add 200µl Enzyme Substrate (starch) to each tube.

3. Transfer 5µl of each fraction to the appropriate tube and add 5µl crude tissue extract saved in section I step 2 to the positive control.

4. Close the tubes and invert 5-6 times to mix the content.

5. Incubate the tubes for 20 minutes at room temperature.

6. Add 200µl Enzyme Detection Dye to each tube. Mix the contents by inverting the tubes 5-6 times.

7. Place the tubes in a warm water bath or incubator (50-65°C) for a maximum of 30 minutes.

    ***NOTE****: Depending on the temperature of the bath or incubator the reaction may proceed too rapidly making it difficult to see a difference between the tubes. Check the tubes every 3-5 minutes and note the color differences.*

8. Observe the tubes. The tubes with the most intense brown color contain the highest concentration of the target protein/enzyme.

IV. Size Exclusion Chromatography to Purify Hemoglobin from the Enriched Fraction

1. Clamp the Size Exclusion Column in an upright position to the stand.

2. Open the top cap first and then the bottom cap of the column to prevent air entering the resin. Allow the buffer to drain out of the column, under gravity, to a waste container.

3. *Equilibrate the column*: Apply 100µl Gel Filtration Buffer to the top of the column and allow it to drain out freely. Repeat this step 9 more times, so that a total of 1ml Gel Filtration Buffer has been added.

4. Apply 50µl hemoglobin enriched fraction from Section II to the column without disturbing the column surface. Allow the sample to enter the column.

5. *Elute the sample*: Apply 100µl Gel Filtration Buffer to the top of the column and allow it to drain freely into a waste container.

6. Repeat step 5 until sample begins to elute from the column. When the red hemoglobin starts to elute from the column, collect the fractions in a 1.5ml tube.

V. Use Hydrophobic Chromatography to purify the target enzyme from the Enriched Fraction

1. Clamp the Hydrophobic Column in an upright position on to a stand.

2. Open the top cap first and then the bottom cap of the column to prevent air bubbles entering the column. Let the buffer drain out of the column under gravity to a waste container.

3. *Equilibrate the column*: Apply 2 bed volumes (4ml) of 1X HP Loading Buffer. Add 0.5ml 1X HP Loading Buffer and let the buffer drain out freely in to a waste container. Repeat until all 4ml has been applied.

4. Add 50µl 2X HP Loading Buffer to the fraction containing the higher concentration of the target protein/enzyme (in Section-III). Save 10µl of this fraction for use as a control in the enzyme assay. Carefully load the remainder of this fraction to the column

5. Wash the column 3 times with 0.5ml 1X HP Loading Buffer: Apply 0.5ml 1X HP Loading Buffer to the column and let the buffer drain into a 1.5ml tube. Collect all 3 wash fractions separately.

6. Elute the proteins with 10x0.5ml elution steps: Apply 0.5ml HP Elution Buffer to the column and let the buffer drain into a 1.5ml tube. Collect all 10 fractions separately.

7. Follow the protocol in Section III to determine the fraction containing the target proteins.

If necessary, the samples can be placed in a freezer and the assay in Section III can be performed at a different time.

RESULTS, ANALYSIS & ASSESSMENT

Q. Briefly describe the reason behind the use of a multi-step procedure instead of a single chromatography procedure.

A. Single step will not offer the degree of purity. A multi-step procedure is used to remove unwanted proteins, in subsequent steps, from the sample, and at each step the purity of the target proteins are increased.

Q. What other purification techniques could you apply to purify target proteins?

A. Ion Exchange and affinity are two additional widely used protein purification techniques that could be applied to this sample.

Q. What information is needed before you could use affinity chromatography for the purification of one the target proteins in this lab activity?

A. *Information about properties of the target proteins and potential ligand specific for each target proteins.*

Q. What information is needed before you could use Ion Exchange chromatography for purification of one the target proteins in this lab activity?

A. *Information about properties of the target proteins and what the charge of the protein is. This will determine the selection of the column.*

Q. What determines the scheme of purification for a target protein?

A. The complexity of the starting material, properties of the target proteins, and the level of purity desired.

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Salts, such as ammonium sulfate, have the tendency to disrupt the water structure, increase the water surface tension and increase the hydrophobic effect in the solution (i.e. decrease the solubility of non-polar molecules) and promote protein aggregation by association of hydrophobic surface. Ammonium sulfate is the most frequently used salt for salting-out experiments and can precipitate ~70% of proteins in a complex sample.

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4. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 2 [Represents ~33% Ammonium Sulfate solution].

5. Carefully transfer the supernatant to a new tube. Add 200µl Salt Solution (Amm. Sulfate) and mix the content by inverting the tube a few times or vortexing briefly.

6. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 3 [represents ~60% Ammonium Sulfate solution].

7. Carefully transfer the supernatant to a new tube. Add 500µl Salt Solution (Amm. Sulfate) and mix the content by inverting the tube a few times or vortexing briefly.

8. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 4 [represents ~80% Ammonium Sulfate solution].

9. Carefully transfer the supernatant to a new tube. This is your Fraction 5.

10. Examine the fractions. The fraction with the most concentrated red color is enriched in Hemoglobin.

11. Add 50µl Gel Filtration Buffer to each fraction pellet and vortex briefly to dissolve the pellets.

If necessary, the samples can be placed in a freezer until required for sections III, IV and V. Samples can be frozen again to allow each section to be completed at different times.

12. Follow the protocol in section III to find out the fraction containing the target proteins/enzyme using 5µl of each fraction for each assay. Setup a positive control tube using the crude.

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1. Label one tube for each fraction to be assayed and a tube for a positive control.

2. Add 200µl Enzyme Substrate (starch) to each tube.

3. Transfer 5µl of each fraction to the appropriate tube and add 5µl crude tissue extract saved in section I step 2 to the positive control.

4. Close the tubes and invert 5-6 times to mix the content.

5. Incubate the tubes for 20 minutes at room temperature.

6. Add 200µl Enzyme Detection Dye to each tube. Mix the contents by inverting the tubes 5-6 times.

7. Place the tubes in a warm water bath or incubator (50-65°C) for a maximum of 30 minutes.

   

   ***NOTE**: Depending on the temperature of the bath or incubator the reaction may proceed too rapidly making it difficult to see a difference between the tubes. Check the tubes every 3-5 minutes and note the color differences.*

8. Observe the tubes. The tubes with the most intense brown color contain the highest concentration of the target protein/enzyme.

IV. Size Exclusion Chromatography to Purify Hemoglobin from the Enriched Fraction

1. Clamp the Size Exclusion Column in an upright position to the stand.

2. Open the top cap first and then the bottom cap of the column to prevent air entering the resin. Allow the buffer to drain out of the column, under gravity, to a waste container.

3. *Equilibrate the column*: Apply 100µl Gel Filtration Buffer to the top of the column and allow it to drain out freely. Repeat this step 9 more times, so that a total of 1ml Gel Filtration Buffer has been added.

4. Apply 50µl hemoglobin enriched fraction from Section II to the column without disturbing the column surface. Allow the sample to enter the column.

5. *Elute the sample*: Apply 100µl Gel Filtration Buffer to the top of the column and allow it to drain freely into a waste container.

6. Repeat step 5 until sample begins to elute from the column. When the red hemoglobin starts to elute from the column, collect the fractions in a 1.5ml tube.

V. Use Hydrophobic Chromatography to purify the target enzyme from the Enriched Fraction

1. Clamp the Hydrophobic Column in an upright position on to a stand.

2. Open the top cap first and then the bottom cap of the column to prevent air bubbles entering the column. Let the buffer drain out of the column under gravity to a waste container.

3. *Equilibrate the column*: Apply 2 bed volumes (4ml) of 1X HP Loading Buffer. Add 0.5ml 1X HP Loading Buffer and let the buffer drain out freely in to a waste container. Repeat until all 4ml has been applied.

4. Add 50µl 2X HP Loading Buffer to the fraction containing the higher concentration of the target protein/enzyme (in Section-III). Save 10µl of this fraction for use as a control in the enzyme assay. Carefully load the remainder of this fraction to the column

5. Wash the column 3 times with 0.5ml 1X HP Loading Buffer: Apply 0.5ml 1X HP Loading Buffer to the column and let the buffer drain into a 1.5ml tube. Collect all 3 wash fractions separately.

6. Elute the proteins with 10x0.5ml elution steps: Apply 0.5ml HP Elution Buffer to the column and let the buffer drain into a 1.5ml tube. Collect all 10 fractions separately.

7. Follow the protocol in Section III to determine the fraction containing the target proteins.

If necessary, the samples can be placed in a freezer and the assay in Section III can be performed at a different time.

RESULTS, ANALYSIS & ASSESSMENT

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