Experiment 8: Cell lysis and protein purification

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Purpose

• To lyse cells and purify GFP

Materials

• Column
• Ni-NTA beads (BioPLUS, 30210)
• Equilibration buffer: 50 mM Tris pH= 8.0, 500 mM NaCl, 5 mM imidazole
• Lysis buffer: 50 mM Tris pH= 8.0, 500 mM NaCl, 5 mM imidazole, 1 mM PMSF (Phenylmethanesulfonyl Fluoride)
• Wash buffer: 50 mM Tris pH=8.0, 500 mM NaCl, 50 mM imidazole
• Elution buffer: 50 mM Tris pH=8.0, 500mM NaCl, 250 mM imidazole
• 2X gel loading buffer: 4 % SDS, 100 mM Tris pH=6.8, 20 % v/v glycerol, 0.2 % w/v bromophenol blue + 10% BME
• 1X PAGE running buffer: 250 mM Tris, 2.5 M glycine, 0.5 % SDS
• SDS-PAGE gel staining buffer: 3 mM brilliant blue R, 50 % v/v EtOH, 10 % v/v AcOH

Machines to use

• Microfludizer (Unitronics. LM10 Microfludizer)
• SDS-PAGE power supply (Cytiva, EPS-301)
• high-speed centrifuge
• Microwave

Principles

1. Methods of cell lysis

Cell membrane and walls must be disrupted to extract target proteins. In physical disruption methods, the cell membrane is physically broken down by shear or external forces to release cellular components (Figure 1). Depending on the stability of the target proteins, one of the following methods can be used: sonication, manual grinding, homogenization and freeze-thaw. Lysing a wide range of cells with high efficiency is important, but protein denaturation and aggregation can occur due to localized heating. In solution-based cell lysis,

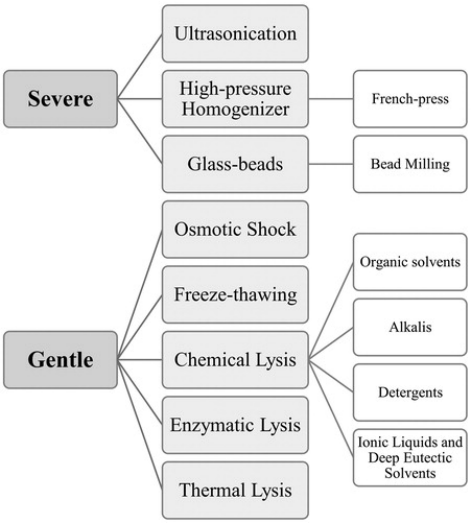

Figure 1 Cell lysis methods

detergents break the lipid barrier surrounding cells by solubilizing proteins and disrupting lipid-lipid, protein-protein, and protein-lipid interactions.

2. Protein purification

Proteins can be purified based on different properties as follows:

- Separation based on size: Size exclusion chromatography
- Separation based on charge or hydrophobicity: Ion exchange chromatography
- Separation by affinity difference: Affinity chromatography (Figure 2)

Ligand (stationary phase)	Target protein
Substrate analogue	Enzymes
Antibody	Antigen tagged proteins (FLAG, Myc, HA, NE)
Lectin	Glycoproteins
Hormone	Receptors
Avidin	Biotinylated proteins
Glutathione	GST fusion proteins
Amylose	MBP fusion proteins
Protein A & G	Immunoglobulins
Metal ions	His-tagged proteins

In our experiment, affinity chromatography using histidine tag and IMAC beads will be utilized (Figure 2). Histidine tag (or His tag) contains multiple (5-10), continuous histidine residues that can bind to metal ions. IMAC (Immobilized Metal ion Affinity Chromatography) beads fix metal ions (nickel or cobalt) on agarose resin (Figure 3). NTA (nitrilotriacetic acid) or IDA (iminodiacetic acid) resin are commonly used.

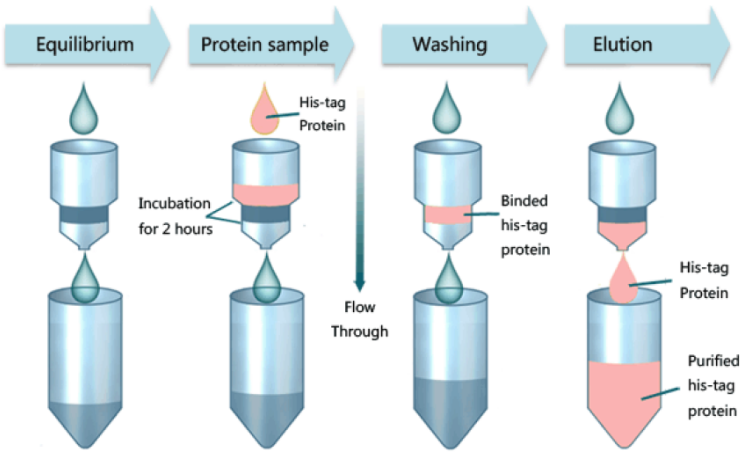

Figure 2 Principle of Affinity chromatography

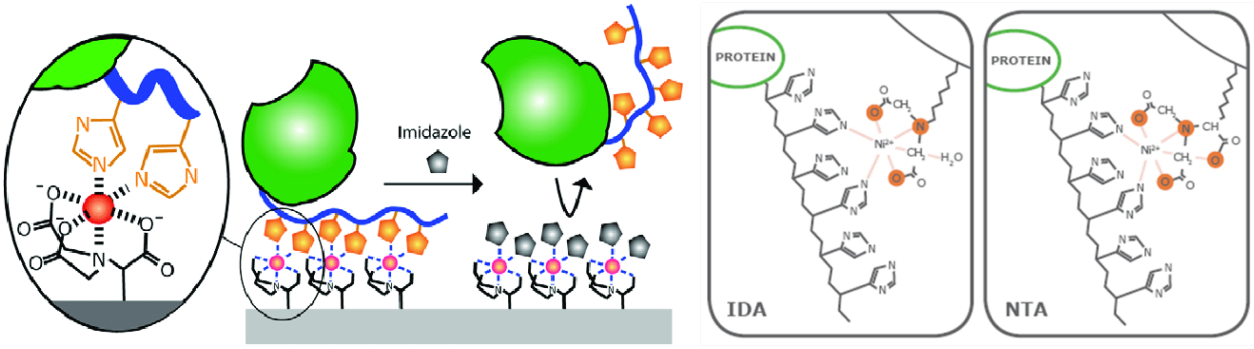

Figure 3 Principles of IMAC beads

Procedure

1. Thaw the frozen cell pellet in water at room temperature.

2. Resuspend the cell pellet with lysis buffer.

3. Pour the cell in the reservoir of the microfludizer. Break the cell with 15000 psi by two times.
   - ❖ Before adding the cell, about 50 ml of equilibration buffer should be applied with 10000 psi pressure and the elution loop should be cooled down by ice

4. Centrifuge the lysate at 15000 rpm for 25 min at 4°C.

5. To 500 uL of Ni-NTA resin in a 1.5-ml EP tube, add 1-ml of Equilibration buffer and mix well by flipping over multiple times. Spin down the tube briefly and remove the supernatant, trying not to remove any resin.

6. Repeat the step five times to equilibrate the resin. Then, transfer the resin to a 50-ml falcon tube using ~ 1 ml of equilibration buffer.

7. Transfer the supernatant of the centrifuged lysate onto the equilibrated Ni-NTA resin in a tube, close the lid tightly and let it rotate at 4°C for 1 hour.
   - ❖ Before transferring the supernatant, save 10 ul of the supernatant for SDS-PAGE analysis

8. Load the protein-beads mixture to the column and collect flow-through.

9. Wash the resin with 2.5 ml of wash buffer, and elute the protein with 1 ml of elution buffer. Collect the elution 3 times, separately.

10. Prepare the SDS-PAGE samples by adding 10 ul of 2X gel loading buffer to 10 ul of samples – supernatant (5-times diluted), flow-through (5-times diluted), wash, elution 1, elution 2, elution 3, left-over resin.

11. Boil the samples in a heat block at 95 ºC for 5 min, spin them down briefly, and load 5 ul of samples to an SDS-PAGE gel well with a protein marker. Run the gel at 200 V for 50 minutes.

12. When the run is done, carefully separate the gel from the glass plates,

Result

1. Describe your experiment experience by adding details to the procedure above and your observations.

2. How does the color of the Ni-NTA beads is changed during the experiment?

3. Add the image of SDS-PAGE gel to the report

Analysis

1. What does the color of Ni-NTA beads indicate in each step of the experiment – flow-through, wash, elution.

2. In the SDS-PAGE gel, which is the band of the protein of interest (GFP, here)?

3. From the SDS-PAGE gel image, can you say that you have recovered most of the over-expressed GFP with the resin? How can you know that?

4. From the SDS-PAGE gel, can you see if the purification is well-performed? Explain your answer regarding your gel image.

Discussion

1. Write down the differences between NTA and IDA (coordination number, specificity, yield, etc..)

2. List three more affinity chromatography methods in pairs (for example, MBP-tag: amylose resin)

3. Describe the function of each component used the in the protein purification
    - PMSF
    - Imidazole

4. Why is the pH of Tris buffer used in stacking gel and resolving gel different?

5. Calculate the amount of each stock buffer volume to add to make the Buffers in the table below:

Stock solution	1M Tris-HCl pH=8.0	5M NaCl	2M imidazole pH=8.0	Total volume (mL)
Equilibration buffer				10
Wash buffer				5
Elution buffer				5