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Refolding of Recombinant Protein

Introduction

The vast majority of protein purification is now done with cloned, **recombinant proteins expressed** in a suitable host.

Expression of cloned gene in bacteria is widely used in both **industry**, for the production of pharmaceutical protein, and in **research**, for structural and\or biochemical studies.

Introduction

When proteins are expressed at high levels in *E. coli* and other expression hosts, it is common to find that most of expressed protein are found in an **insoluble** form called an **inclusion body**.

Inclusion body

Inclusion body are very dense partial containing the most of recombinant protein in incorrect stereo structure without the desired biological activity.

Strategies to Increase Proportion of Soluble Protein

1. Induce overproduction at lower temperatures

- Temperatures of 20–25 ºC are commonly used (below the normal growth temperature of 37 ºC)

2. Addition sucrose to the culture medium

- High osmolyte concentration inducing the osmotic shock response, thus providing conditions more favorable for refolding.

3. Fuse to easily refolded protein

- Fusion with a hydrophilic protein can enhance solubility. ‘‘solubility tag’’ such as maltose-binding protein (MBP) and glutathione-s-tranferase (GST).

Strategies to Increase Proportion of Soluble Protein

4. Co-expression of molecular chaperones

- Since molecular chaperones are known to suppress misfolding and aggregation in the cell, one can increase the levels of these proteins by introducing a separate plasmid that contains inducible genes for these chaperones.

Inclusion Body Advantages

Expression of recombinant proteins as inclusion bodies can be advantageous

1. high levels of enriched protein produced;
2. Easy separated from soluble impurities;
3. protection of the protein product from proteolytic degradation;
4. If the recombinant protein is toxic or lethal to the host cell, inclusion body may be the best available production method.

The General Strategy used to Recover Active Protein from Inclusion Bodies

The general strategy used to recover active protein from inclusion bodies involves three steps:

1. **Inclusion body isolation and washing;**
2. **Solubilization of the aggregated protein;**
   - ➢ causes denaturation
3. **Refolding of the solubilized protein.**
   - ➢ folding yields may be limited by the production of inactive misfolded species as well as aggregates.

General Refolding Consideration

- The literature includes many examples in which recombinant proteins have been produced by refolding from inclusion bodies. In spite of it, there is **No universal method for refolding of proteins**.

- However, solution conditions can have significant effects on the folding pathway of a given protein.

- The best general strategy in refolding is “to refold at the lowest protein concentration that is feasible”.

1. Inclusion Body Isolation and Washing

Releasing inclusion body from cells

Cells are typically disrupted by high-pressure homogenization, sonication or by combination of lysozyme treatment and sonication.

Isolation of inclusion body from cell homogenate

The stranded method is differential centrifugation to remove the soluble fraction.

Washing of inclusion body

- **Low concentrations of chaotropic agents,** such as urea or guanidinium chloride (GdmCl)
- **Detergents**, such as Triton X-100 and sodium deoxycholate.

✓ This washing step is designed to **remove contaminants, especially proteins**, that may have adsorbed onto the hydrophobic inclusion bodies during processing, and **could affect protein refolding yield**.

2. Inclusion Body Solubilization

Inclusion bodies are solubilized using **strong denaturants** and a **reducing agent**.

- **Strong denaturants**
  - such as urea, guanidinium chloride (GdmCl), or thiocyanate salts,
- **Detergents**
  - such as sodium dodecyl sulfate (SDS), n-cetyl trimethylammonium chloride (CTAB), or sarkosyl
- **Reducing agent (If recombinant protein contains cysteine residues)**
  - such as β-mercaptoethanol, or dithiotreitol (DTT).

- Temperatures above 30ºC are typically used to facilitate the solubilization process.

- A chelating agent, such as EDTA or EGTA, can be included in the solubilization buffer to scavenge metal ions, which could cause unwanted oxidation reactions.

- Solubilized inclusion body proteins can be contaminated with varying levels of host proteins, nucleic acids, and cell membrane components. It is thought that the presence of these microbial contaminants may induce aggregation during refolding, thus reducing overall yields.

- Thus, some inclusion body processes include a purification step prior to refolding. Typically this step may be ion exchange, size exclusion, metal affinity, or reverse phase chromatography. A common feature of these chromatographic steps is that they all operate with buffers that keep the protein in the denatured reduced state

Refolding of Solubilized Protein

There are two problems during refolding process

1. Aggregation

- The best general strategy in refolding is to refold at the lowest protein concentration that is feasible.

2. Misfolding

3. Refolding of Solubilized Protein

Protein refolding is initiated by the **removal of excess denaturants and reducing agents** by either **dilution** or **buffer-exchange step**, such as dialysis and gel filtration chromatography.

Dialysis

The denatured protein solution is dialyzed against renaturation buffer.

➢ **Disadvantage**

- Dialysis may be too slow to be used in commercial scale production of proteins.
- Exposure of the protein to intermediate concentration of denaturants for a prolonged period of time may cause aggregation.

➢ **Membrane-based alternative, "Diafiltration"**

Diafiltration is a faster, as rate of denaturant removal is not diffusion limited, the driving force being pressure difference.

https://www.youtube.com/watch?v=l9JaMbdCqrk

3. Refolding of Solubilized Protein

Direct Dilution

Dilution of the denatured solution directly into renaturation buffer is the easiest process and the most commonly used method.

Gel filtration chromatography

- This method of buffer-exchange is much faster than dialysis.
- Gel filtration column is equilibrated with the final refolding buffer. Unfolded protein sample in denaturant is applied to the column and run through it the refolding buffer.
- Potential complications may arise if folding of the protein is inhibited by binding to the solid support, which could be prevented by using fusion proteins

➢ **Advantages**

In addition to buffer exchange, column chromatography allows for some degree of purification of the desired product.

Disadvantage

however, problems in flow through the column may arise due to protein aggregation upon buffer exchange.

Other Refolding Procedures

On-column refolding

- In chromatographic refolding, the **solid medium** acts as a kind of **chaperone or assistant to help the protein refolding** occur in a correct way, which minimizes misfolding and aggregation.
- The solution containing **denatured protein** and **denaturant** is loaded into the column packed with porous microspheres.
- **Renaturation buffers** are introduced to elute the denatured protein to move through the column, during this process, **simultaneous refolding** take place.
- The solid phase helps the correct folding of the protein and the protein may exit in the correct form.

Use of folding catalysts such as protein and chemical chaperones

- Another nice approach is to **covalently attach various enzymes** and **chaperones** to a gel filtration column resin and then **refold in column**.

- The enzymes that have been immobilized, either individually or perhaps better in combinations are:
  - PDI (Protein disulfide-isomerase), DsbA (thiol disulfide oxidoreductase) and DsbC (disulfide bond isomerase) to catalyze disulfide formation,
  - Chaperones GroEL/GroES to aid in refolding.

High-Pressure Refolding

- Solubilization of inclusion bodies by exposure to high hydrostatic pressure within a certain range will disaggregate the aggregated protein.

- The process of pressurizing, holding at high pressure, and slowly decreasing the pressure in a variety of buffers result in high recovery of soluble and active enzymes.

- This method has been commercialized by Barofold (Boulder, CO) using a specialized pressure cells.

Reoxidation to form correct disulfide bonds

- In case of disulfide bonded proteins, renaturation buffer must promote disulfide formation (oxidation).

- **There most common methods used to promote oxidation during refolding are:**

1. Air oxidation

- Expose to air, without reducing agent for several days

2. Redox buffer

- Addition of a mixture of reduced and oxidized forms of low molecular weight thiol reagent usually provides the appropriate redox potential to allow formation and reshuffling of disulfides.
- Such as reduced/oxidized glutathione, or dithiothreitol.

3. Protein disulfide isomerase

- An enzyme that catalyzes disulfide shuffling

References

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