Refolding

Advantages of Inclusion Bodies (IB) expression

Three step procedure

Parameters for refolding

Refolding of proteins with disulfide bridges: oxidative folding

Protein Refolding Methods

On Column Refolding, some examples

How to Check Protein Refolding Success

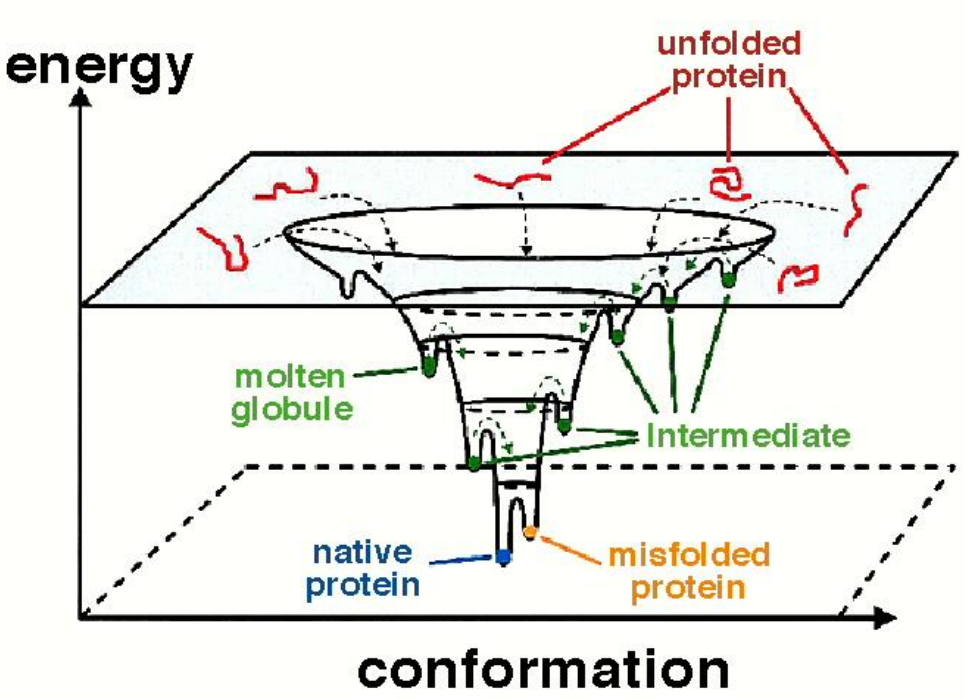
Screen Kit

Forces and interactions that drive folding

Protein Aggregation and Its Impact on Product Quality
Christopher J. Roberts - Curr Opin Biotechnol. 2014 December ; 0: 211–217.
doi:10.1016/j.copbio.2014.08.001.

- ✓ Van der Waals and hydrophobic attractions between side-chain and backbone atoms
- ✓ Maximizing hydrogen bonding
- ✓ Minimizing steric clashes and energetically unfavorable bond torsional angles
- ✓ Maximizing chain entropy
- ✓ Minimizing (maximizing) electrostatic repulsions (attractions)
- ✓ Minimizing unfavorable interactions between amino acids and the solvent (water) and its co-solutes

Refolding: A folding funnel for single protein



- During folding there is competition between correctly folded and misfolded protein.
- Inactive and denatured recombinant proteins sometime accumulate intracellular in insoluble aggregates called **inclusion bodies**.
- In addition, different oligomeric states (like dimers, multimers, etc) may be present: **soluble aggregates**

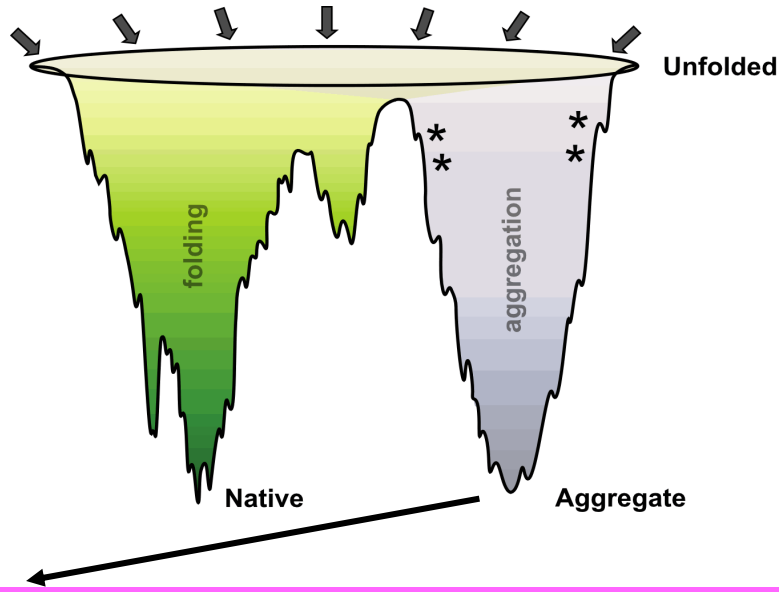
Advantages of IB expression

- The recombinant protein deposited in IB can be 50% or more of the total cellular protein.
- The IB often contain almost exclusively the over-expressed protein.
- Inside IB the protein is protected from proteolysis
- IB expression will protect the cell against the toxicity of the recombinant protein
- **The problem is that the protein must be refolded to get the correct structure**

Three step procedure

- Major problem: how to recover biologically active and/or soluble protein in high yield.
- To accomplish this the protein in the IB must by solubilized and refolded *in vitro*.
- This procedure is carried out in three steps:

1. Isolation of inclusion bodies
2. Solubilization of aggregated proteins
3. Refolding of the solubilized proteins



Is the aggregated protein completely unfolded? Or it is partially folded?

Isolation of inclusion bodies

According to In Vitro Denaturation and Refolding in the web-site of The Protein Expression and Purification Facility of The European Molecular Biology Laboratory: http://www.embl-heidelberg.de/ExternalInfo/geerlof/draft_frames/index.html

Cell Diruption

Cells are disrupted by high pressure homogenization (optionally following a lysosyme treatment). It is important that cell lysis is complete, because intact cells sediment together with the inclusion bodies, and contaminate the preparation.

Centrifugation

IB have a relatively high density and, therefore, can be pelleted by centrifugation.

Wash with low concentration of chaotropic agents or detergents

After centrifugation, the pellet is washed with buffer containing either low concentrations of chaotropic agents (*e.g.* 0.5-1 M guanidine-HCl or urea) or detergents (*e.g.* 1% Triton X-100 or 1 mg/ml sodium deoxycholate). This wash step is necessary to remove contaminants, especially proteins (proteases), that may have absorbed onto the hydrophobic inclusion bodies during processing. Addition of DNase (e.g., 10 to 20 µg/ml) will reduce the amount of contaminating DNA.

Protein solubilization with low concentration of chaotropic agents or detergents means that the protein is not soluble in simple buffers that and is not IB

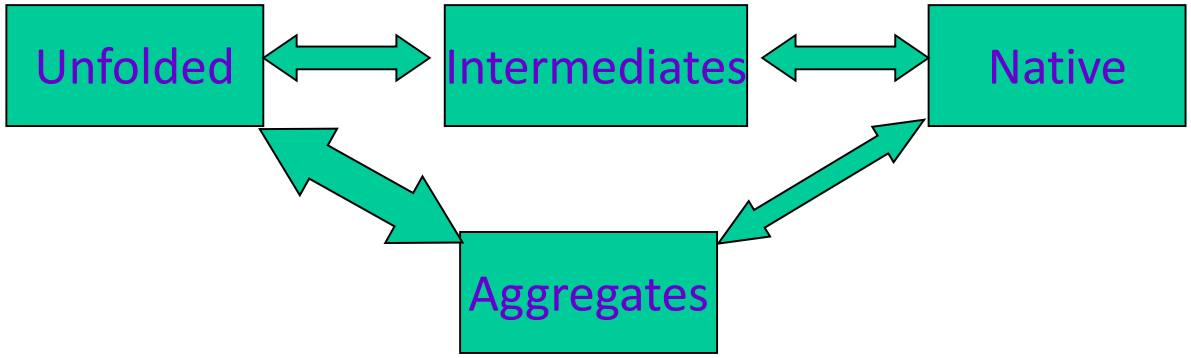
Solubilization of aggregated proteins

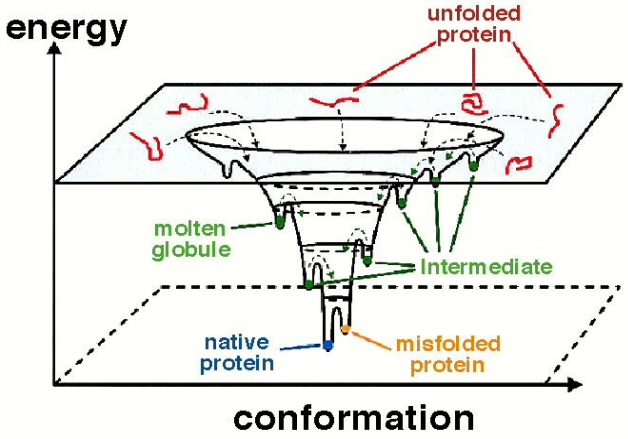
According to In Vitro Denaturation and Refolding in the web-site of The Protein Expression and Purification Facility of The European Molecular Biology Laboratory
http://www.embl-heidelberg.de/ExternalInfo/geerlof/draft_frames/index.html

- The washed inclusion bodies are resuspended and incubated in buffer containing a strong denaturant and a reducing agent (usually 20 mM DTT or b-mercaptoethanol).
- The addition of a reducing agent keeps all cysteines in the reduced state and cleaves disulfide bonds formed during the preparation.
- Incubation temperatures above 30°C are typically used to facilitate the solubilization process.
- Optimal conditions for solubilization are protein specific and have to be determined for each protein. This is done most effectively by carrying out small-scale experiments (1-2 ml) to screen the different variables
- After solubilization the solution should be centrifuged to remove remaining aggregates which could act as nuclei to trigger aggregation during refolding. The best results are obtained by ultracentrifugation (30 min at >100,000 g).

Variable	Good Starting Point
buffer composition (pH, ionic strength)	50 mM Tris-HCl, pH 7.5
incubation temperature	30°C
incubation time	60 min
concentration of solubilizing agent	6 M guanidine-HCl or 8 M Urea
total protein concentration	1-2 mg/ml
ratio of solubilizing agent to protein	

The big headache of the refolding process: Competing pathways and pitfalls





- ✓ How to promote native conformation and suppressed aggregation??
- ✓ There are not known general rules for how to achieve this in practice
- ✓ Suitable refolding conditions for a given protein have to be established empirically.

  What works for one protein not necessarily works for similar proteins
- ✓ Refolding success depend on the careful optimization of a number of parameters

Ideal environment during refolding step

Aggregation rate is decreased

The native folded protein is stabilized

Rather than undergoing a reverse transition to intermediate states

Rate of refolding is increased

Parameters for refolding

According to In Vitro Denaturation and Refolding in the web-site of The Protein Expression and Purification Facility of The European Molecular Biology Laboratory: http://www.embl-heidelberg.de/ExternalInfo/geerlof/draft_frames/index.html

- Refolding is initiated by the removal of the denaturant to allow non-covalent interactions
- The efficiency of refolding depends on the competition between correct folding and aggregation.
- Additives and conditions that reduce aggregation and or stabilize the protein
- Slow down aggregation process by refolding at **low protein concentrations** (10-100 µg/ml).
- Refolding conditions must be **optimized for each individual protein**.
- Performed under an appropriate **oxidizing environment** to form the correct S–S bonds
- **Important variables are**:

Buffer composition (pH, ionic strength)

Disulfide exchange reagents

Additives (alone or in combination)

Refolding Method

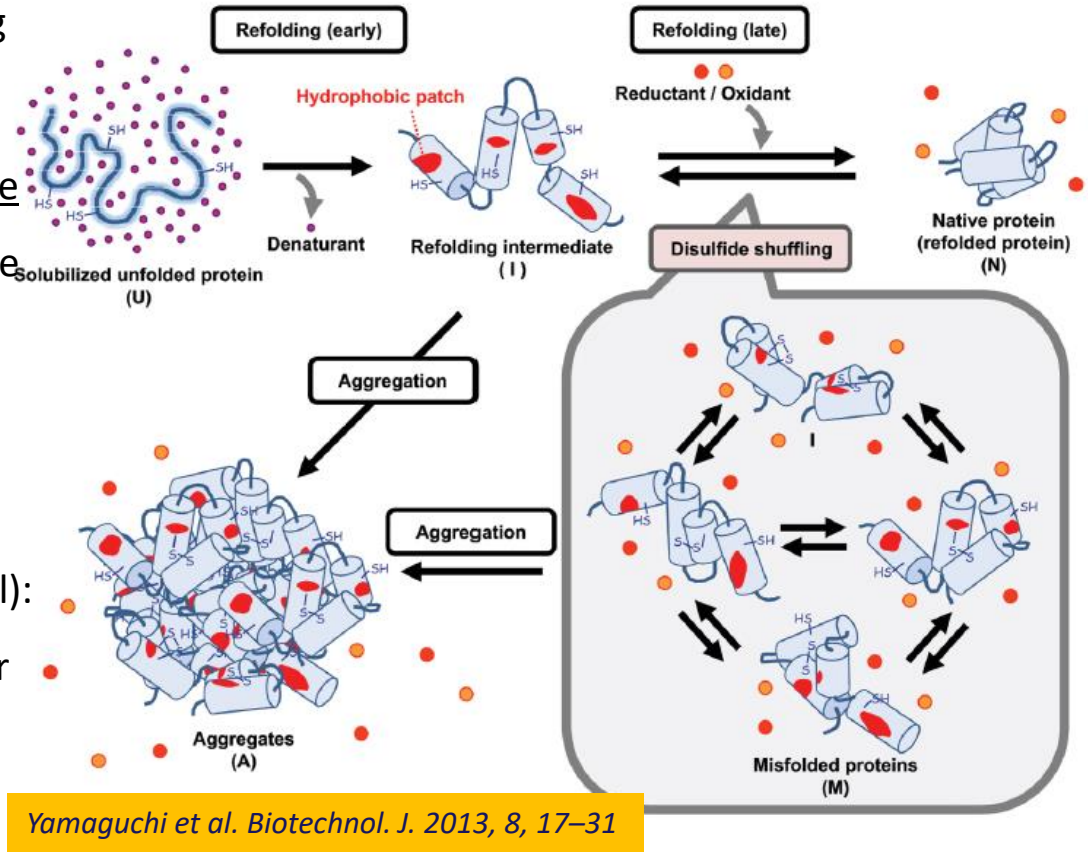
Time

Temperature

Refolding of proteins with disulfide bridges: oxidative folding

- Reduce completely disulfide bonds during solubilization with a reducing agent
- The reducing agent is replaced by disulfide exchange reagents (that provide a suitable redox potential) during refolding.
- Disulfide exchange reagents: mixture of reduced and oxidized forms

  (5:1 to 1:1 ratio of reduced/oxidixed thiol): 1-3 mM oxidized + reduced glutathione or cysteamine + cystamin
- The reagents form mixed disulfide intermediates with cysteine residues.

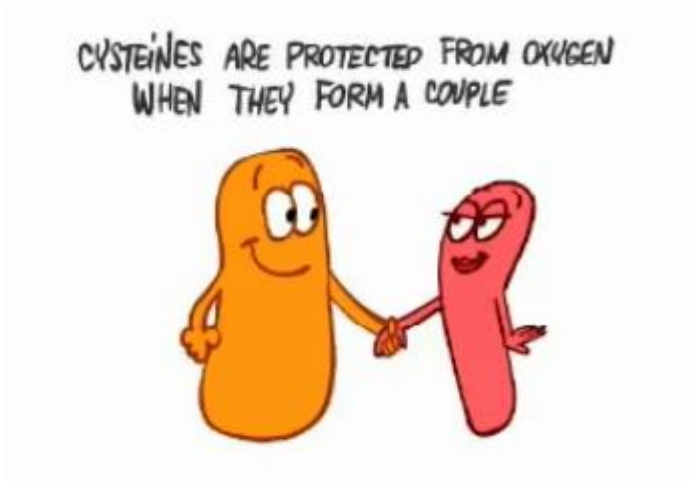


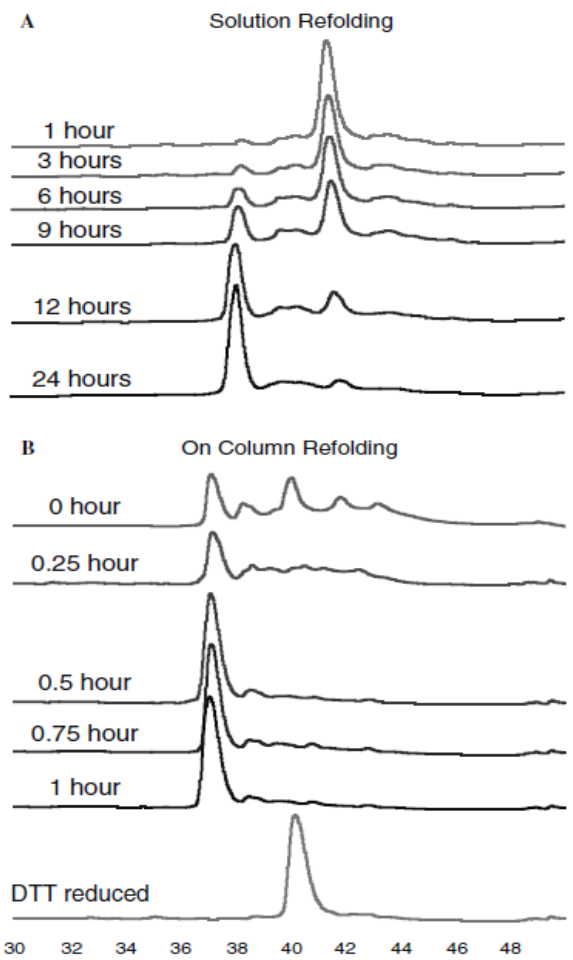
Yamaguchi et al. Biotechnol. J. 2013, 8, 17–31

- This reaction is reversible and the protein disulfide bonds are formed or broken until the most favorable protein disulfide bond formation has occurred.

Disulfide bond formation: RPC analysis

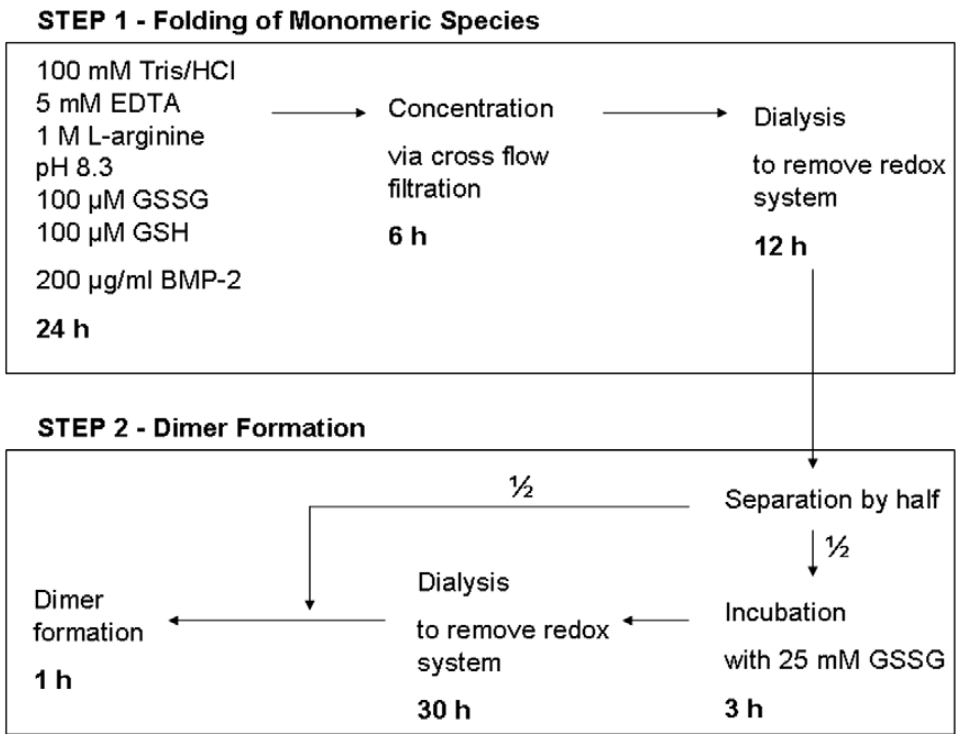
- The process is time dependent (days)
- Samples are collected at different time points during refolding
- Extent of folding determine by analytical HPLC
- As the disulfide bonds are formed, the retention time is reduced by ≈ 3.5 min.

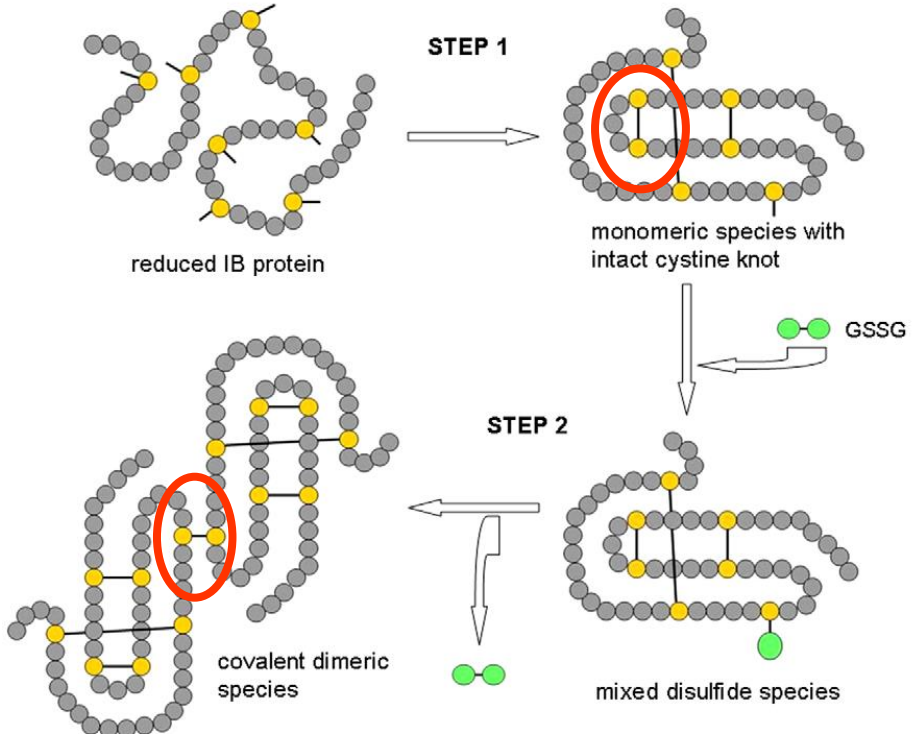




A novel TWO-STEP renaturation procedure for efficient production of biologically active homodimeric proteins with inter and intra-molecular disulfide bonds

*S. von Einem, E. Schwarz *, R. Rudolph - Protein Expression and Purification (September 2010), 73 (1): 65-69*





First step: formation of monomeric species with the correct intra-molecular disulfide bridges at low protein concentration

Second step: formation of the inter-molecular disulfide bridge at higher protein concentration

Efficient production of recombinant IL-21 proteins for pre-clinical studies by a two-step dilution refolding method

Chen Z, et al, Int Immunopharmacol (2013),http://dx.doi.org/10.1016/j.intimp.2013.02.017

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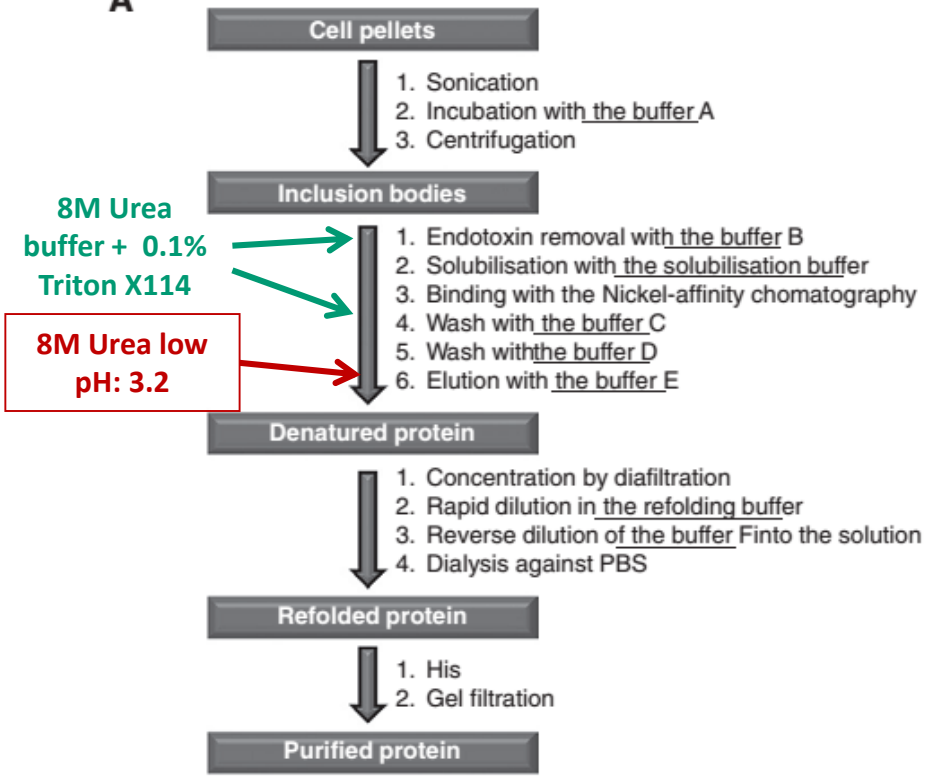

- ✓ First drop-wise dilution in the refolding buffer with 1 M Gdn-HCl + redox condition (GSSG and GSH) with additives (0.7M L-Arg).
- ✓ Has been shown to be highly efficient environment for the formation of native disulfide-bond within IL-21 (protein concentration < 10μM).
- ✓ Second dilution with buffer L-Cystine 1.6 mM (without Redox) was then added to stop disulfide shuffling.
- ✓ Finally dialysis to eliminate Arginine

B

Refolding Method	Refolding Efficiency	Time Expense (hours)	Reaction Volume (mL)
Step-wise dialysis [16]	~50%	36-72	5000
Dilution [15]	~20%	12-24	100
Dilution [17]	<10%	12-24	100
Two-step dilution	~50%	18-36	100

Two-step dilution strategy significantly reduce the time and reagent cost

Solubilization in partial denaturative conditions

Recovery of bioactive protein from bacterial inclusion bodies using trifluoroethanol as solubilization agent

Upadhyay et al. Microb Cell Fact (2016) 15:100 DOI 10.1186/s12934-016-0504-9

- High pH buffers, detergents and organic solvents like n-propanol have been successfully used as mild solubilization agents for high throughput recovery of bioactive protein from bacterial inclusion bodies.
- These mild solubilization agents preserve native-like secondary structures of proteins in inclusion body aggregates and result in improved recovery of bioactive protein as compared to conventional solubilization agents.
- Solubilization of human growth hormone inclusion body aggregates using 30 % trifluoroethanol in presence of 3 M urea and its refolding into bioactive form.
- Solubilized protein was refolded by dilution and purified by anion exchange and size exclusion chromatography.

- The effect of organic solvents on protein structure has long been studied.
- In general, alcohols like methanol, ethanol and trifluoroethanol (TFE) are known to stabilize secondary structure, mainly helical structure.
- The effect of organic solvents on the tertiary structure is more generalized and most of the solvents destabilize the tertiary structure.
- Organic solvents affect the secondary and tertiary structure of proteins by increasing the polypeptide intramolecular hydrogen bonding, disrupting the hydrophobic interactions or by interacting with water molecules and influencing their hydrogen bonding potential

Efficient renaturation of inclusion body proteins denatured by SDS

Chuan He, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.07.003

- Removal of SDS from protein solution by the addition of potassium salts on ice
- SDS is replaced by mild detergents
- Ceramic hydroxyapatite is successfully applied to remove SDS bound to proteins
- Some of the examples of non-denaturing solubilization agents include N-lauroyl sarcosine, dimethysulfoxide (DMSO) and low percentage (5 %) of n-propanol [Jevsevar S, Biotechnol Prog. 2005;21:632–9].

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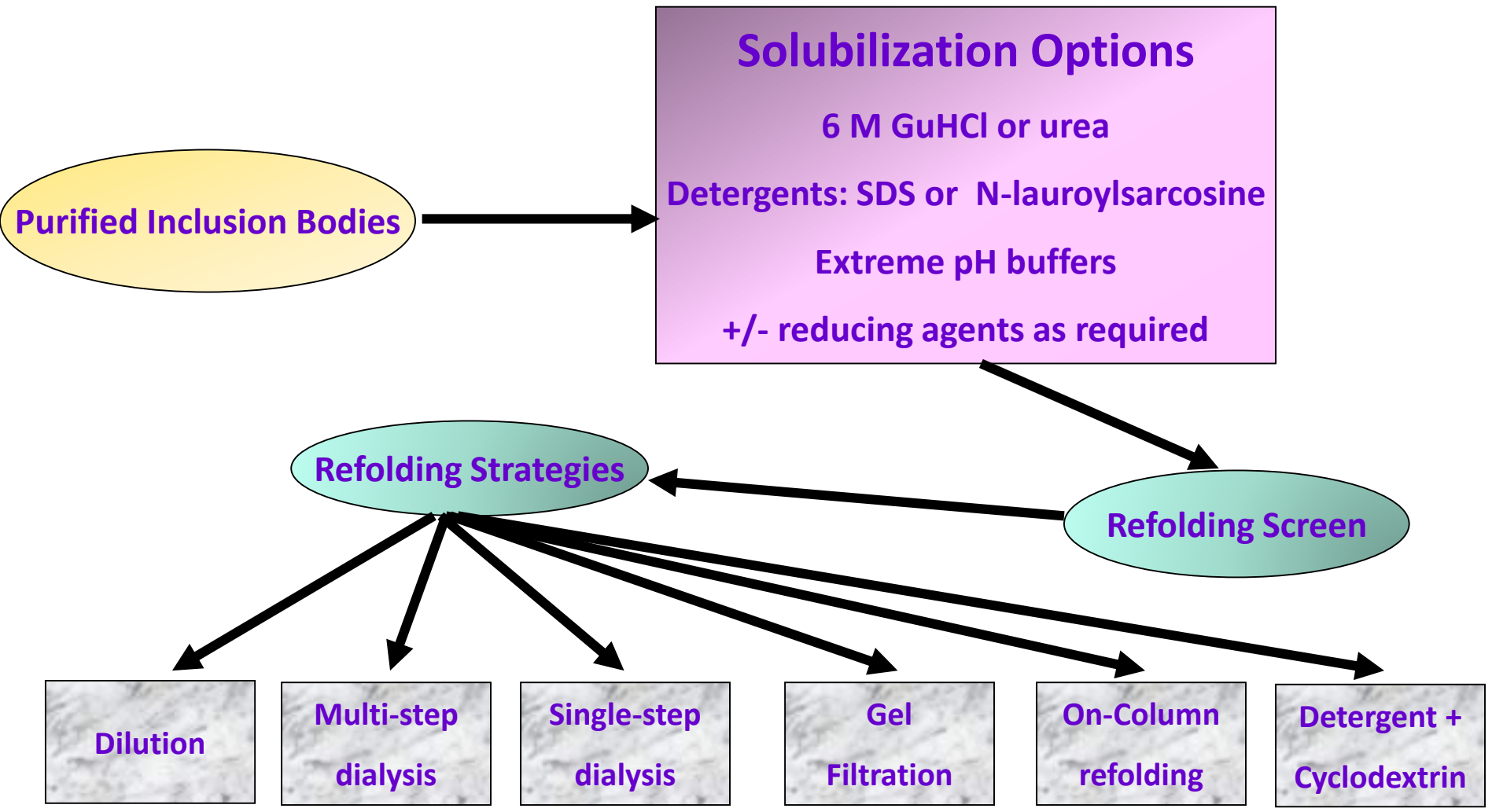
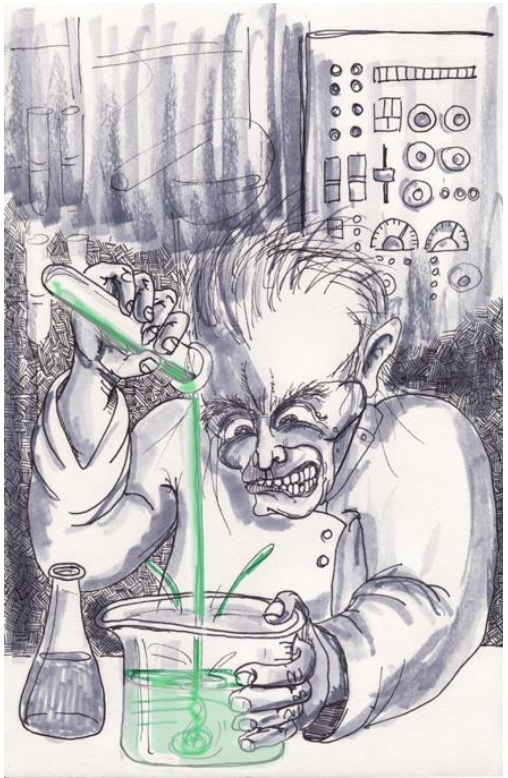


Refolding Strategies: Dilution

According to Ming Li et al. In vitro protein refolding by chromatographic procedures.
Protein Expr. & Purific. 33 (2004) 1-10

- Dilution of the solubilized protein directly into the renaturation buffer (~0.1mg/ml)
- Very simple
- Most popular for small-scale
- Very useful for screening
- Time-consuming
- Buffer- consuming
- Not optimal for large-scale production

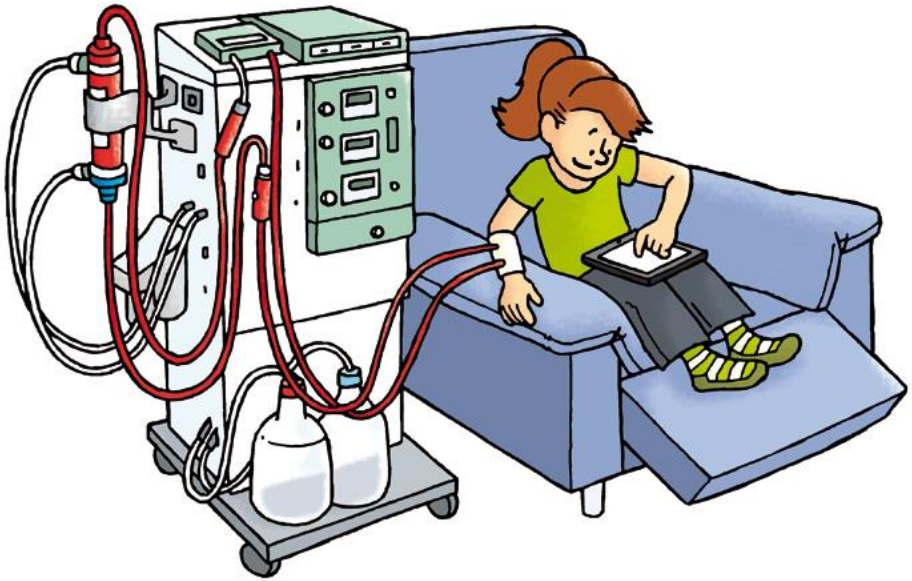
Method of Choice



Refolding Strategies: Solvent Exchange by Dialysis, Multi-step Dialysis or Diafiltration

According to Ming Li et al. In vitro protein refolding by chromatographic procedures.
Protein Expr. & Purific. 33 (2004) 1-10

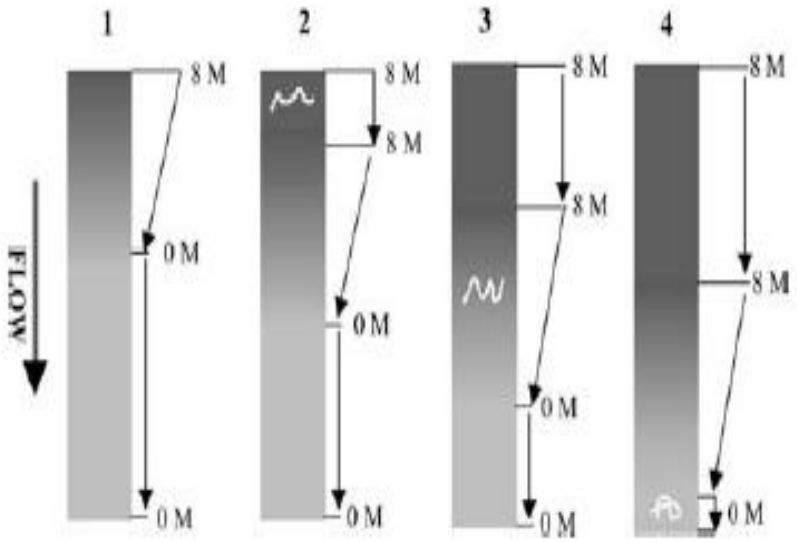
- Reduce high denaturant concentration by dialysis or diafiltration in one step or multiple step
- Very simple
- Very popular for small-scale
- Very useful for screening
- Time-consuming
- Buffer- consuming
- Aggregation deposits can impair refolding yields and clog the membranes
- Large scale: Tangential flow diafiltration / ultrafiltration, using hollow fiber or flat sheet membrane cassettes



Refolding Strategies: Solvent exchange -Size Exclusion Chromatography (SEC)

According to Ming Li et al. In vitro protein refolding by chromatographic procedures.
Protein Expr. & Purific. 33 (2004) 1-10

- Easily automated using commercially available preparative chromatography systems.
- Can be combined with simultaneous partial purification
- Facilitate the separation of correctly folded from aggregated species
- Refolding conditions must be known
- Not practical for screening conditions



Option of SEC refolding in a decreasing urea gradient. Provides a gentle and easily controllable environment for protein renaturation avoiding a quick change in urea concentration

Refolding Strategies: Adsorption Refolding - Solvent exchange – On Column Refolding

According to Ming Li et al. In vitro protein refolding by chromatographic procedures. *Protein Expr. & Purific. 33 (2004) 1-10*

- Reversible adsorption of the denatured protein to a solid support, separating the individual protein from each other and subsequent denaturant removal to promote refolding
- Molecules are spatially constrained during the refolding process
- Proteins can be adsorbed to *IEX, HIC or RPC* at suitable conditions & high urea concentration
- His-tags proteins can be bound to *chelating columns* in denaturating buffers
- Easily automated using FPLC systems. **Bonus:** partial purification
- Refolding conditions must be known
- Not practical for screening conditions

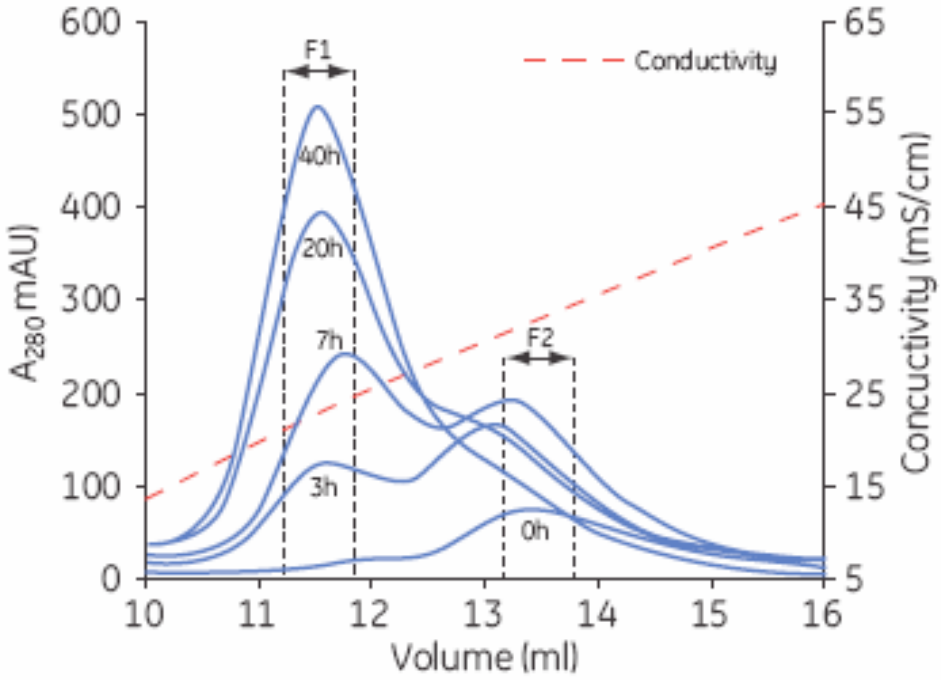
Method of choice for industrial or lab production once you know the refolding conditions

Refolding of albumin on-column using AEIX

Kinetics of Refolding: refolding buffer (Redox) was applied to the column and the flow stopped for 0 to 40 h before salt elution

GE Healthcare: Purifying Challenging Proteins. Principles and Methods

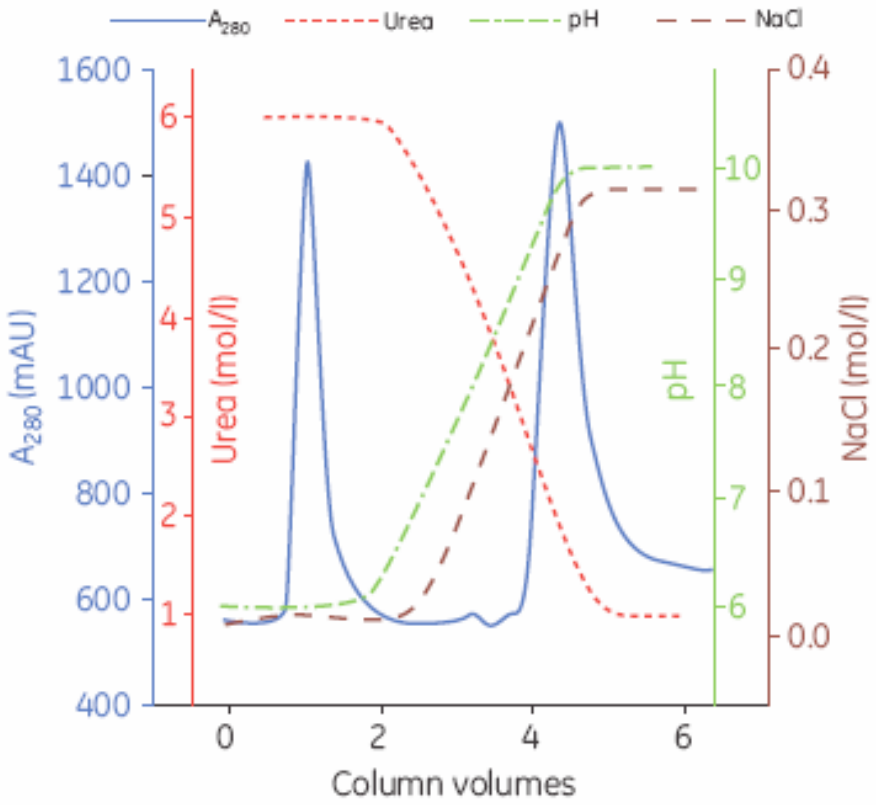
Column:	HiTrap Q FF, 1 ml
Sample:	1 ml BSA 0.5-20 mg/ml denatured-reduced (contains 17 disulfide bonds)
Start buffer:	50 mM Tris-HCl, 3 mM EDTA, 8 M urea, pH 8.5
Refolding Buffer:	50 mM Tris-HCl, 1 mM EDTA, 79 mM urea, 1.1 mM oxidized glutathione (GSSG), 2.2 mM reduced glutathione (GSH), pH 8.5
Incubation period:	0 to 40 h
Wash buffer:	50 mM Tris-HCl, 1 mM EDTA, pH 8.5
Elution buffer:	0 to 1 M NaCl in 50 mM Tris HCl, pH 8.5



Refolding of lysozyme on-column (CEIX)
Decreasing urea gradient

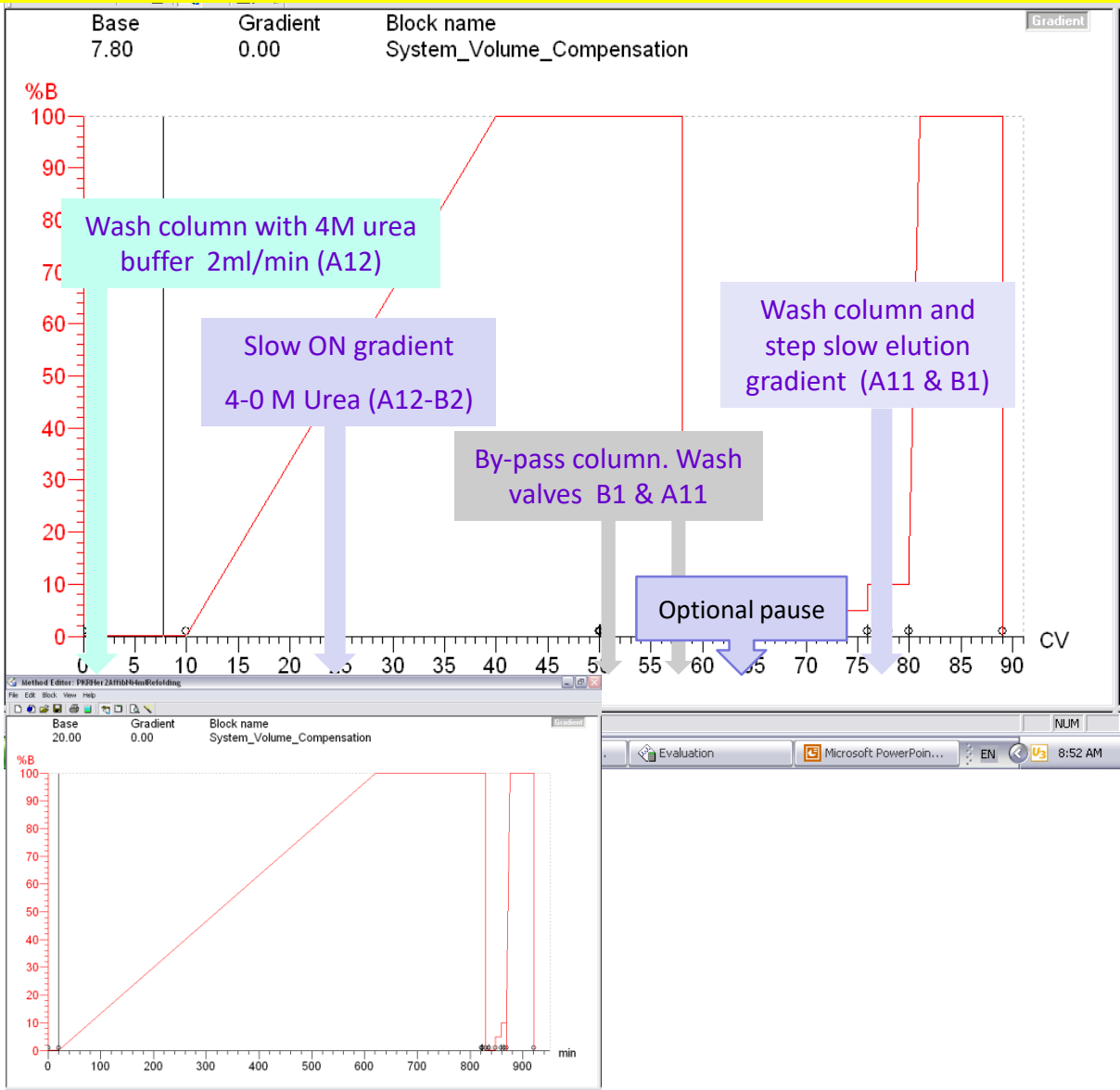
GE Healthcare: Purifying Challenging Proteins. Principles and Methods

Column:	SP Sepharose HP, 5 ml
Sample:	Lysozyme (8 mg)
Start buffer:	50 mM Tris HCl, 6 M urea, 3 mM reduced glutathione (GSH)/0.3 mM oxidized glutathione (GSSG), pH 6.2
Elution buffer:	50 mM Tris HCl, 1 M urea, 300 mM NaCl, 3 mM GSH/0.3 mM GSSG, pH 10
Flow rate:	0.4 ml/min
Instrument:	ÄKTApurifier



Our approach with His tag protein for on-column refolding

- IB resuspension in 6M Urea or GuHCl
- Batch binding to IMAC
- Wash resin till low OD 280nm
- Dilute beads with 20 cv of different refolding buffers and additives
- Mix and incubate ON 4°C
- Elute with high Imidazole in suitable buffers
- Check refolding yield (same day and after ON 4°C): turbidity, SDS PAGE, LS, others
- Scale up on FPLC

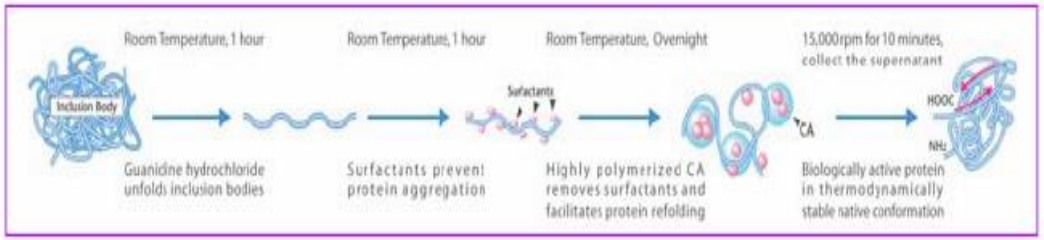


Refolding Strategies: Cycloamylose or Cyclodextrins together with Detergents as an Artificial Chaperone for Protein Refolding

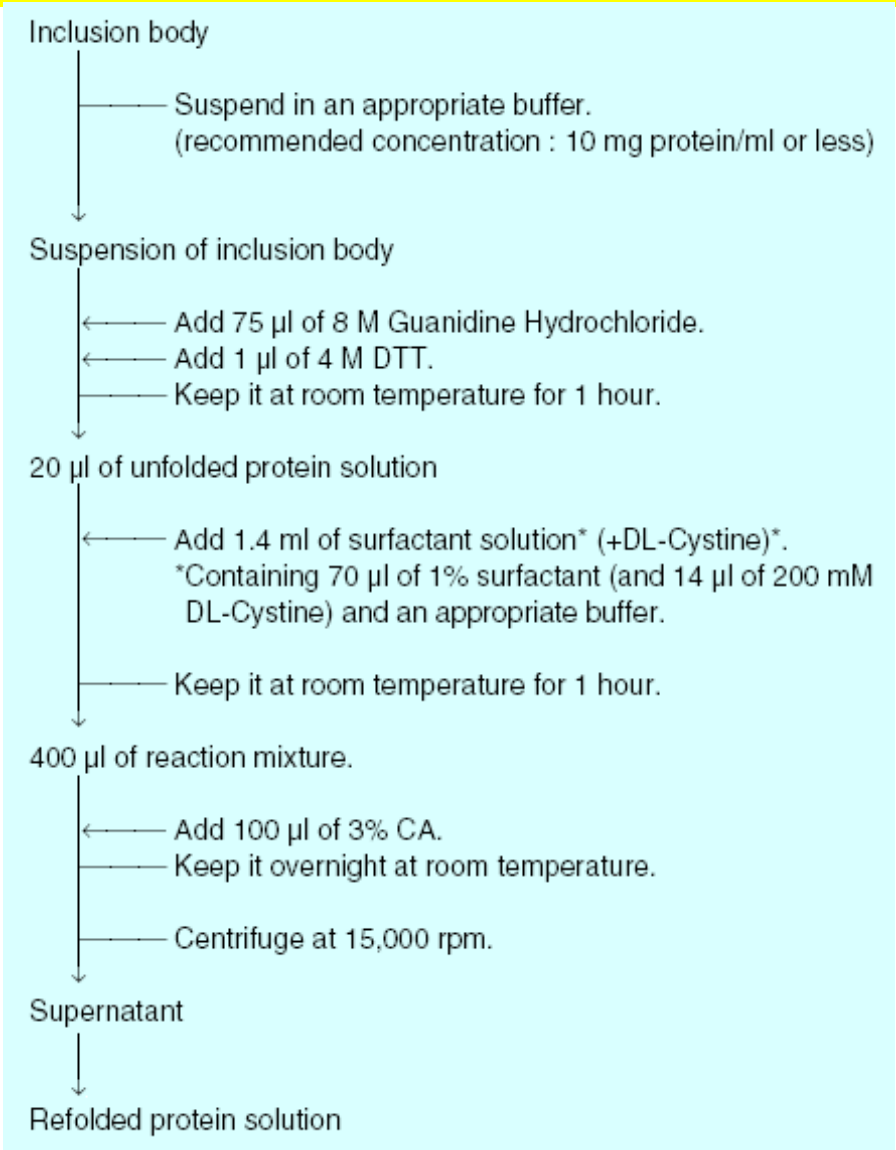
According to Ming Li et al. In vitro protein refolding by chromatographic procedures. *Protein Expr. & Purific. 33 (2004) 1-10*

- The artificial chaperone technique introduced the sequential use of two low MW agents to reconstitute the mimetic GroEL system in vitro
- In the first step the dissolved IB is diluted in buffer with detergent that prevent protein aggregation (presumably shields the hydrophobic regions of the non-native protein)
- In the second step, Cyclodextrins or Cycloamylose (polymers of α-1,4-glucan) strips the detergent from the protein-detergent complex, allowing proper folding.
- Very simple
- Very useful for small-scale screening
- Time-consuming
- Buffer- consuming
- Not optimal for large-scale production
- Not popular

Refolding Kit of TAKARA using this idea



Refolding Kit from TAKARA using Cycloamylose and different surfactants: CTAB, Sulfobetaine, Tween 40 and Tween 60 together with reducing agents



Other Refolding Strategies

According to Ming Li et al. In vitro protein refolding by chromatographic procedures. *Protein Expr. & Purific. 33 (2004) 1-10*

- **Immobilized folding catalysts** onto a chromatographic support causing the column to behave like a catalytic folding reactor. Catalysts as chaperones and foldases (GroEL, DsbA & peptydil-propyl-isomerase) immobilized to agarose; or antibodies. Can be reused a few times. Easily automated using commercially available preparative chromatography systems. Expensive
- **Immobilized liposome chromatography**. Lipid bilayers can selectively recognize the intermediate states and prevent them from forming intermolecular inactive aggregates (like chaperones).
- **Immobilized short synthetic peptides** that contain the self-recognition motif of the protein and engineered to destabilize the abnormal conformation and facilitate refolding

Refolding Strategies: PreEMT™ High Pressure Technology (HHP)

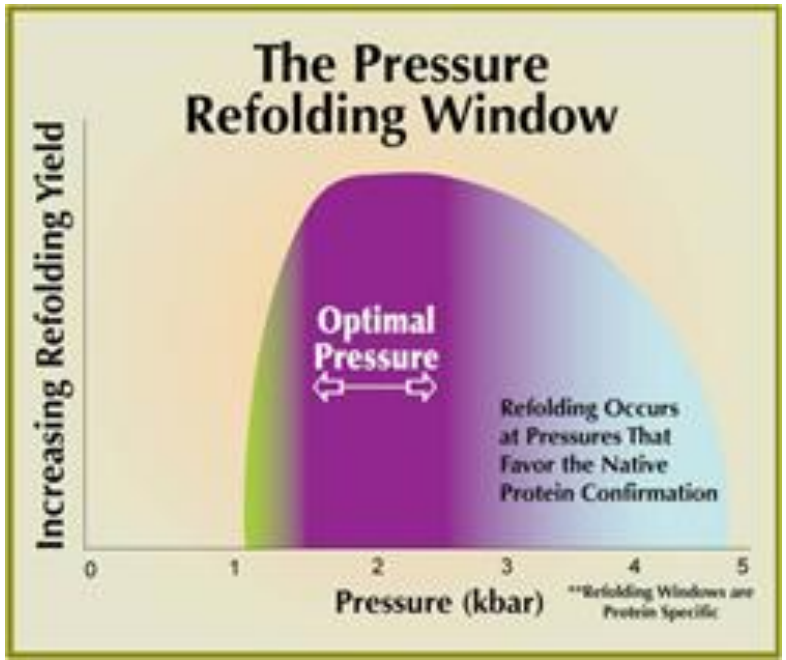
John Carpenter & Ted Randolph

IMPROVING PROTEIN THERAPEUTICS
IMPROVING PEOPLE'S LIVES

BAROFOLD

HHP disaggregates and refolds proteins from aggregates by disfavoring intermolecular **hydrophobic** and **electrostatic** interactions because hydration of hydrophobic and charged residues reduces system volume.

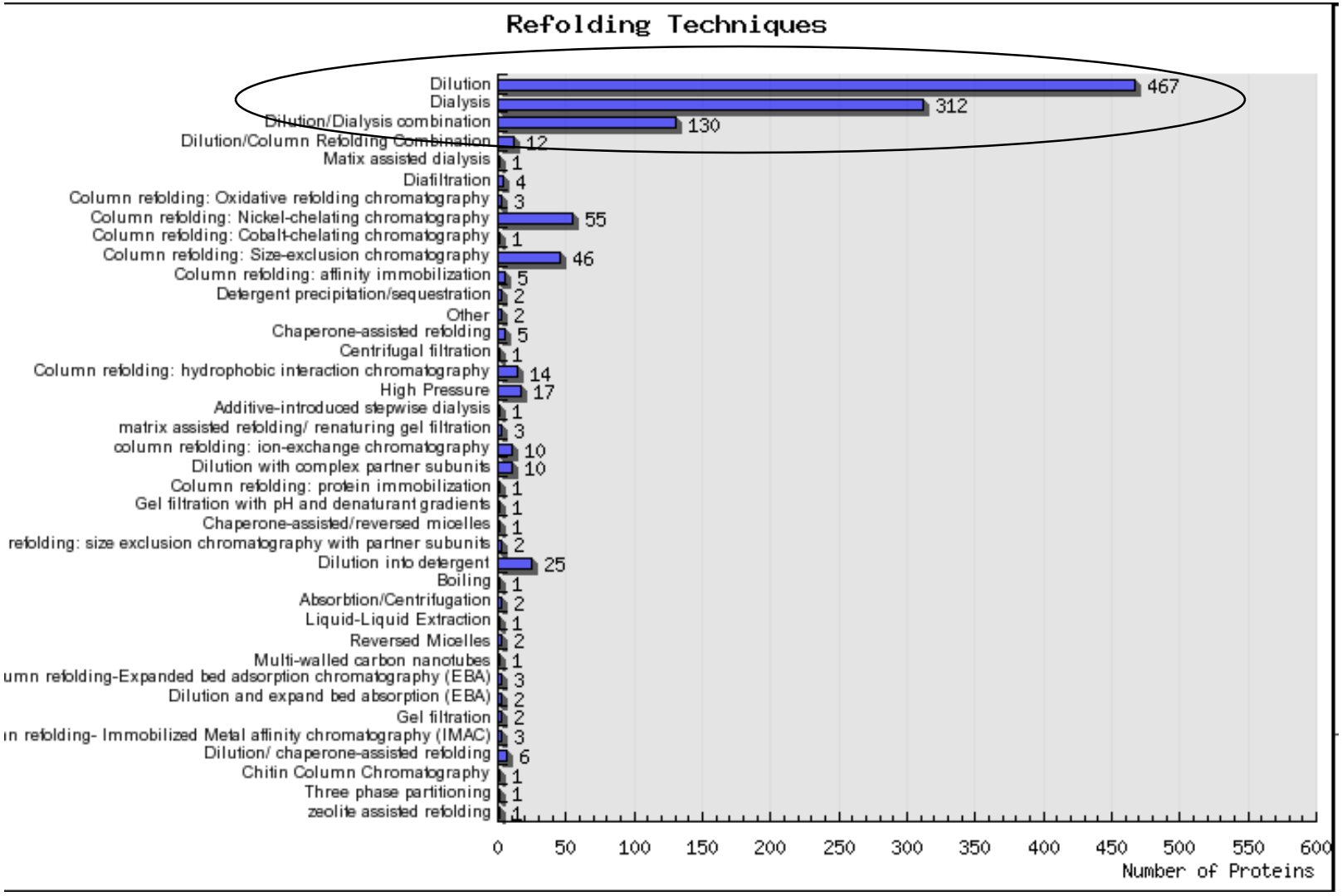
The high pressures forces the protein into its most compact form, usually the **native state**.



refold

http://refold.med.monash.edu.au/

Welcome to REFOLD! This website contains entries for refolding protocols for a wide range of proteins. **REFOLD is an excellent way of disseminating refolding protocols and techniques to the scientific community**. The data on this site is freely available to all.



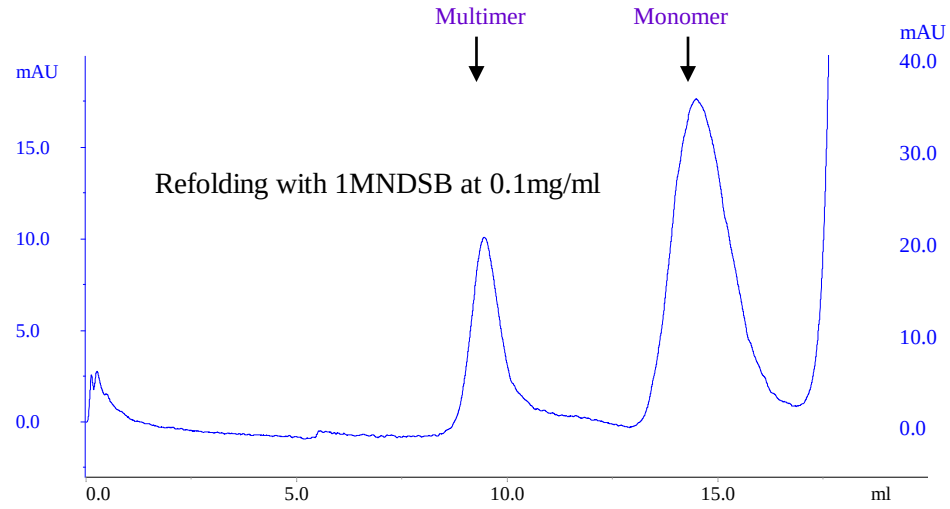
How to Check Protein Refolding Success

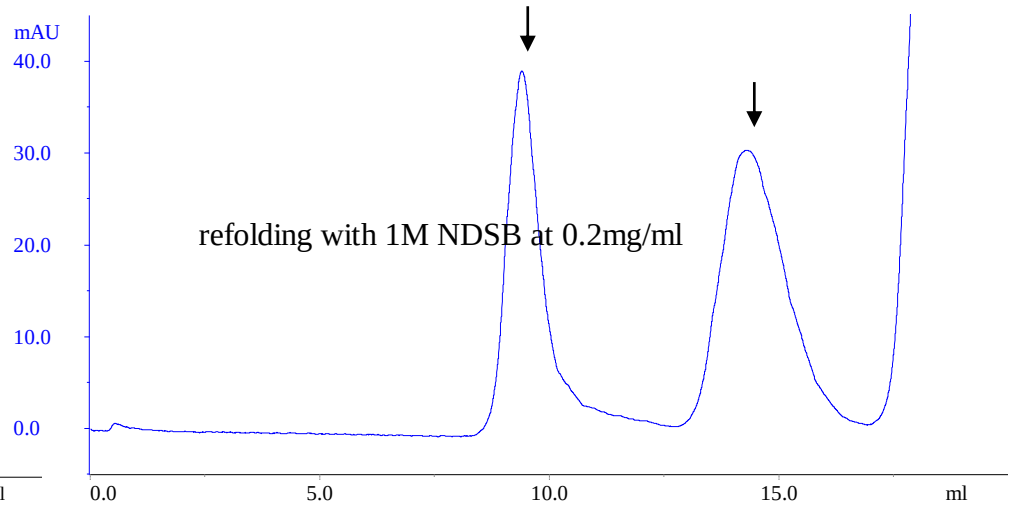
- **Bioactivity determination**: is a good option. *But* are all the molecules active?
- *And* many times an appropriate assay do not exist or is difficult to perform
- *So,* a good first measure could be solubility: eye turbidity, PAGE-SDS after spin, LS, etc
- *However* care has to be taken concerning soluble aggregates or stable misfolded species,
- *So,* in the absence /or regarding bioassay, the native state of the folded protein must be confirmed by structural analysis like:

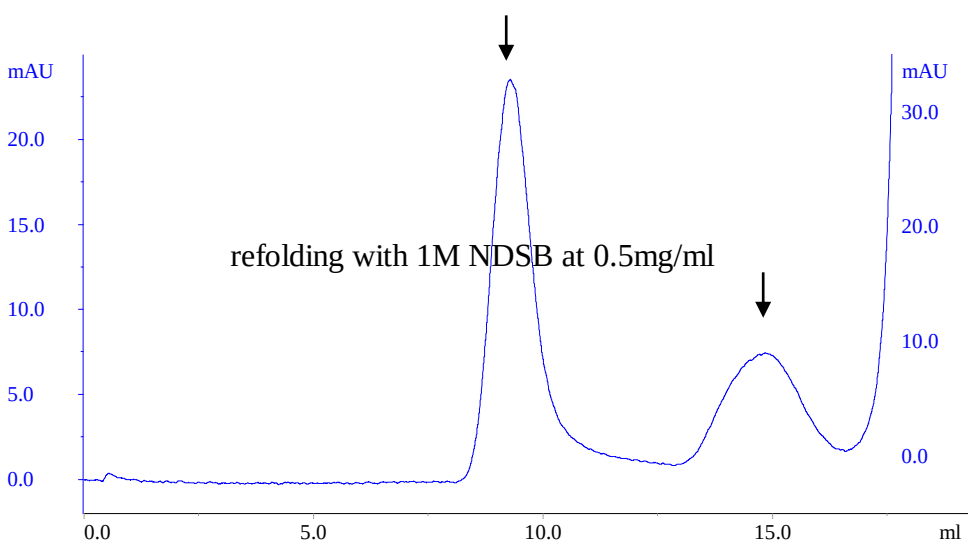
SEC / SEC-MALS	DLS
Ultracentrifugation	**Circular Dichroism**
Fluorescence / DFS	Native PAGE

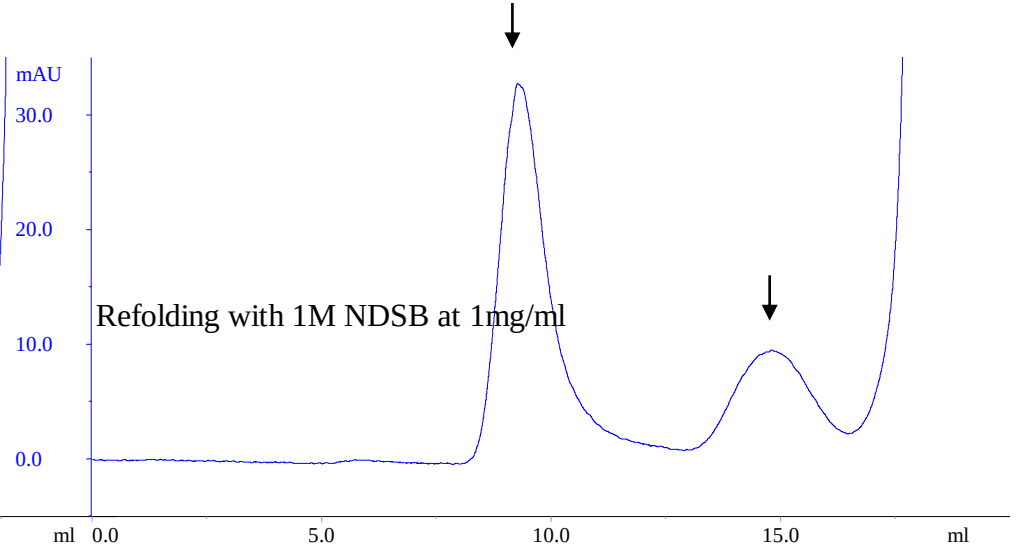
- *Nevertheless,* what percentage of the molecules are active?

Refolding by Dilution of Protein X with NDSB









Empirical Screen for Protein Renaturation

(Armstrong N. - Protein Science 1999 8:1475-1483)

Mode	1) Dilution 2) Dialysis
Protein Concentration	1) 0.1 mg/ml 2) 0.5 mg/ml
Polar Factor	L-Arginine 0.5M
Detergent	0.3mM Lauryl Maltoside
Ionic Strength	1) 10mM 2) 250mM
Prevent Aggregation	0.05% PEG (MW ~3550Da)

pH	1) 50mM TrisHCl pH8.5 2) 50mM MES pH 6.0
Redox	1) 1mM DTT 2) 1mM red.GSH/ 0.1mM ox. GSSG
Chaotropic Agent	0.5M Guanidine HCl
Divalent Cation	1) 1mM EDTA 2) 2mM $MgCl_2$ 3) 2mM $CaCl_2$
Others	0.4M Sucrose Ligand

Molecular Dimensions - Screen Kit

50 mM Tris-maleate pH 6.0, 9.6 mM NaCl, 0.4 mM KCI, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.75 M Guanidine HCI, 0.5% Triton X-100, 1 mM DTT

50 mM Tris-maleate pH 6.0, 9.6 mM NaCl, 0.4 mM KCI, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.05% polyethylene glycol 3550, 1 mM GSH, 0.1 mM GSS

50 mM Tris-maleate pH 6.0, 9.6 mM NaCl, 0.4 mM KCI, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.05% polyethylene glycol 3550, 1 mM GSH, 0.1 mM GSSH

50 mM Tris-maleate pH 6.0, 240 mM NaCl, 10 mM KCI, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.5% Triton X-100, 1 mM GSH, 0.1 mM GSSH

50 mM Tris-maleate pH 6.0, 240 mM NaCl, 10 mM KCI, 1 mM EDTA, 0.4 M sucrose, 0.75 M Guanidine HCI, 1 mM DTT

50 mM Tris-maleate pH 6.0, 240 mM NaCI, 10 mM KCI, 1 mM EDTA, 0.5 M arginine, 0.4 M sucrose, 0.5% Triton X-100, 0.05% polyethylene glycol 3550, 1 mM GSH, 0.1 mM GSSH

50mM Tris-maleate pH 6.0, 240 mM NaCI, 10 mM KCI, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.75 M Guanidine HCI, 0.05% polyethylene glycol 3550, 1 mM DTT

50 mM Tris-maleate pH 8.5, 9.6 mM NaCl, 0.4 mM KCI, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.4 M sucrose, 0.5% Triton X-100, 0.05% polyethylene glycol 3550, 1 mM GSH, 0.1 mM GSSH

50 mM Tris-maleate pH 8.5, 9.6 mM NaCl, 0.4 mM KCI, 1 mM EDTA, 0.5 M arginine, 0.75 M Guanidine HCI, 0.05% polyethylene glycol 3550, 1 mM DTT

50 mM Tris-maleate pH 8.5, 9.6 mM NaCl, 0.4 mM KCI, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.4 M sucrose, 0.75 M Guanidine HCI, 1 mM GSH, 0.1 mM GSSH

50 mM Tris-maleate pH 8.5, 9.6 mM NaCI, 0.4 mM KCI, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT

50 mM Tris-maleate pH 8.5, 240 mM NaCl, 10 mM KCI, 1 mM EDTA, 0.05% Polyethylene glycol 3550, 1 mM GSH, 0.1 mM GSSH

50 mM Tris-maleate pH 8.5, 240 mM NaCl, 10 mM KCI, 1 mM EDTA, 0.5 M arginine, 0.75 M Guanidine HCI, 0.5% Triton X-100, 1 mM DTT

50 mM Tris-maleate pH 8.5, 240 mM NaCl, 10 mM KCI, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.4 M sucrose, 0.75 M Guanidine HCI, 0.5% Triton X-100, 0.05% polyethylene glycol 3550, 1 mM GSH, 0.1 mM GSSH

50 mM Tris-maleate pH 8.5, 240 mM NaCI, 10 mM KCI, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.4 M sucrose, 1 mM DTT

iFOLD™ Protein Refolding System 1 from Novagen

Inclusion body preparation and a 96-well matrix of buffers and additives for convenient optimization of refolding conditions

Table 1. *Thirty-two condition fractional factorial screen*

	Buffer[a]	Detergent[b]	Reductant[c]	Salt[d]	PEG 3350[e]	GdnHCl[f]	Cation[g]	Sucrose[h]	Arginine[i]	Ligand[j]
1	MES 5.5	0	BMC	0	0	0	0	0	0	0
2	MES 6.5	DDM	BMC	1	1	0	0	1	1	1
3	BORATE 9.5	DDM	GSH:GSSG	1	0	0	1	1	0	0
4	MES 6.5	T80	BMC	1	0	1	1	0	1	1
5	TRIS 8.2	NDSB	DTT	1	1	0	1	0	0	1
6	TRIS 8.2	T80	BMC	1	1	0	0	1	1	0
7	MES 5.5	DDM	DTT	0	0	0	1	1	1	1
8	MES 5.5	T80	DTT	0	1	1	0	0	1	1
9	MES 5.5	NDSB	TCEP	1	0	0	0	0	1	0
10	TRIS 8.2	T80	TCEP	0	0	1	1	0	0	0
11	MES 6.5	NDSB	GSH:GSSG	0	1	0	1	0	1	0
12	MES 5.5	DDM	GSH:GSSG	1	1	1	0	0	0	1
13	TRIS 8.2	DDM	TCEP	0	1	0	0	1	0	0
14	MES 6.5	NDSB	DTT	1	0	1	0	1	0	0
15	BORATE 9.5	NDSB	TCEP	1	1	1	1	1	1	1
16	BORATE 9.5	T80	DTT	0	0	0	1	1	1	0
17	TRIS 8.2	0	DTT	1	0	1	0	1	0	1
18	TRIS 8.2	DDM	BMC	1	0	1	1	0	1	0
19	TRIS 8.2	0	GSH:GSSG	0	1	0	1	0	1	1
20	BORATE 9.5	0	BMC	0	1	1	1	1	0	1
21	TRIS 8.2	NDSB	GSH:GSSG	0	0	1	0	1	1	1
22	MES 6.5	0	DTT	1	1	0	1	0	0	0
23	MES 5.5	T80	GSH:GSSG	1	0	0	1	1	0	1
24	MES 6.5	DDM	TCEP	0	0	1	1	0	0	1
25	MES 5.5	NDSB	BMC	0	1	1	1	1	0	0
26	MES 6.5	0	GSH:GSSG	0	0	1	0	1	1	0
27	BORATE 9.5	0	TCEP	1	0	0	0	0	1	1
28	BORATE 9.5	T80	GSH:GSSG	1	1	1	0	0	0	0
29	BORATE 9.5	NDSB	BMC	0	0	0	0	0	0	1
30	MES 6.5	T80	TCEP	0	1	0	0	1	0	1
31	MES 5.5	0	TCEP	1	1	1	1	1	1	0
32	BORATE 9.5	DDM	DTT	0	1	1	0	0	1	0

Willis M. et.al.Protein Science (2005), 14:1818–1826

[a] 50 mM buffer.
[b] DDM, 0.3 mM; Tween 80, 0.5 mM; NDSB 201, 1 M.
[c] BMC, TCEP, and DTT, 5 mM; GSH:GSSG, 1 mM GSH:0.1 mM GSSG.
[d] 0 = 10.56 mM NaCl, 0.44 mM KCl; 1 = 264 mM NaCl, 11 mM KCl.
[e] 0 = no PEG 3350; 1 = 0.06% PEG 3350 w/v.
[f] 0 = no GdnHCl; 1 = 550 mM GdnHCl.
[g] 0 = 1.1 mM EDTA; 1 = 2 mM $MgCl_2$, 2 mM $CaCl_2$.
[h] 0 = no sucrose; 1 = 440 mM sucrose.
[i] 0 = no arginine; 1 = 550 mM arginine.
[j] 0 = no ligand; 1 = presence of ligand (target:ligand, kinases:100 μM AMP-PNP, phosphatases:100 μM *o*-phospho-L-tyrosine, proteases, RNase A, sRNase A, helicase:1–10 μM assay substrate, dehydrogenases:20 μM NADH or NADP, lysozyme:10 μg/mL *Micrococcus lysodeikticus*).

Refolding Literature

Challenges with osmolytes as inhibitors of protein aggregation: Can nucleic acid aptamers provide an answer? Kinjal A. Patel http://dx.doi.org/10.1016/j.ijbiomac.2016.05.014

Nucleic acid aptamers, on the other hand, are highly specific for their targets. When selected against monomeric, natively folded proteins, they bind to them with very high affinity. This binding inhibits the unfolding of the protein and/or monomer–monomer interaction which are the initial common steps of protein aggregation. Thus, by changing the approach to a protein-centric model, aptamers are able to function as universal stabilizers of proteins

A novel protein refolding system using lauroyl-L-glutamate as a solubilizing detergent and arginine as a folding assisting agent, M. Kudou et al., Protein Expr. Purif. (2010), doi:10.1016/j.pep.2010.08.011

Disulfide-bridging PEGylation during refolding for the more efficient production of modified proteins. Claire Ginn et al. Biotechnol. J. 2016, 11 DOI 10.1002/biot.201600035

Simultaneous refolding and disulfide bridging PEGylation of proteins could be a useful strategy in the development of affordable modified protein therapeutics.

Single pH buffer refolding screen for protein from inclusion bodies, B. Coutard Q1 et al., Protein Expr. Purif. (2012),

doi:10.1016/j.pep.2012.01.014

Found that proteins with an acidic isoelectric point (pI) refolded in buffers the average pH of which was alkaline and conversely. In addition, the number of refolding buffers wherein a protein remained soluble increased with the difference between its pI and the average pH of the buffers in which it refolded. new refolding screen made of a single buffer for acidic proteins and a single buffer for alkaline proteins.

Refolding Literature

✓**Efficient refolding of the bifunctional therapeutic fusion protein VAS-TRAIL by a triple agent solution**.

Jiying Fan et al. Protein Expression and Purification 125 (2016) 68–73

The solubilization of aggregated VAS-TRAIL was achieved by a triple agent solution, which consists of an alkaline solution (pH 11.5) containing 0.4 M L-arginine and 2 M urea. Stepwise dialysis: eliminates urea in the presence of Arg and neutral pH, and finally Arg

✓**Access to gram scale amounts of functional globular adiponectin from E.coli inclusion bodies by alkaline-shock solubilization**.

John T. Heiker Biochemical and Biophysical Research Communications 2010 10.1016/j.bbrc.2010.06.020

pH was increased to 12.5 using 1 M NaOH resulting in complete solubilization of the inclusion bodies within seconds

✓**Production of non-classical inclusion bodies from which correctly folded protein can be extracted**.

Jevsevar S, Biotechnol Prog 2005;21:632–9

IB obtained at low growth Temp might contain correctly folded recombinant protein and thus could be readily extracted with non-denaturing solvents

Purifying natively folded proteins from inclusion bodies using sarkosyl, Triton X-100, and CHAPS.

Hu Tao et al. BioTechniques48:61-64 (January 2010)

His6-MBP fusion proteins required the pellets to be incubated with 10% sarkosyl, others were soluble with 1% sarkosyl in the lysis buffer. Even though the His6-MBP fusion proteins could be purified with Ni2+ resin in the presence of 1% sarkosyl, the addition of Triton X-100 and CHAPS increased the binding

✓Solubilization of inclusion body proteins usingn-propanol and its refolding into bioactive form. Surinder M. Singh (India)

Protein Expression and Purification 81 (2012) 75–82

r-hGH solubilization with: 100 mM Tris buffer containing 6M n-propanol and 2M Urea. Refolding by dilution. Use of propanol along with 2 M urea improved r-hGH solubilization from IB without significantly disturbing the existing native-like secondary structure of the protein. It is easy to remove propanol from the protein mixture as it comes in the flow-through during ion-exchange chromatography

It has been reported that many miscible organic solvents promote the formation of secondary structure of protein both in native and non-native states. Alkyl alcohols are protein denaturants[31]and destabilize the tertiary structure of proteins with retention of secondary structure[25]

✓REVIEW: Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process

Singh et al. Microbial Cell Factories (2015) 14:41 DOI 10.1186/s12934-015-0222-8

Low concentrations of organic solvents like 5% npropanol and DMSO and detergents like 0.2% N-lauroyl sarcosine has been used as mild solubilization agents for solubilization of non-classical IBs

Mild solubilization processes using alkaline pH [19,61], high pressure [64], detergents [65,66], organic solvents [60] and low concentration of chaotropes [46,63] have been used for recovery of bioactive proteins from IB

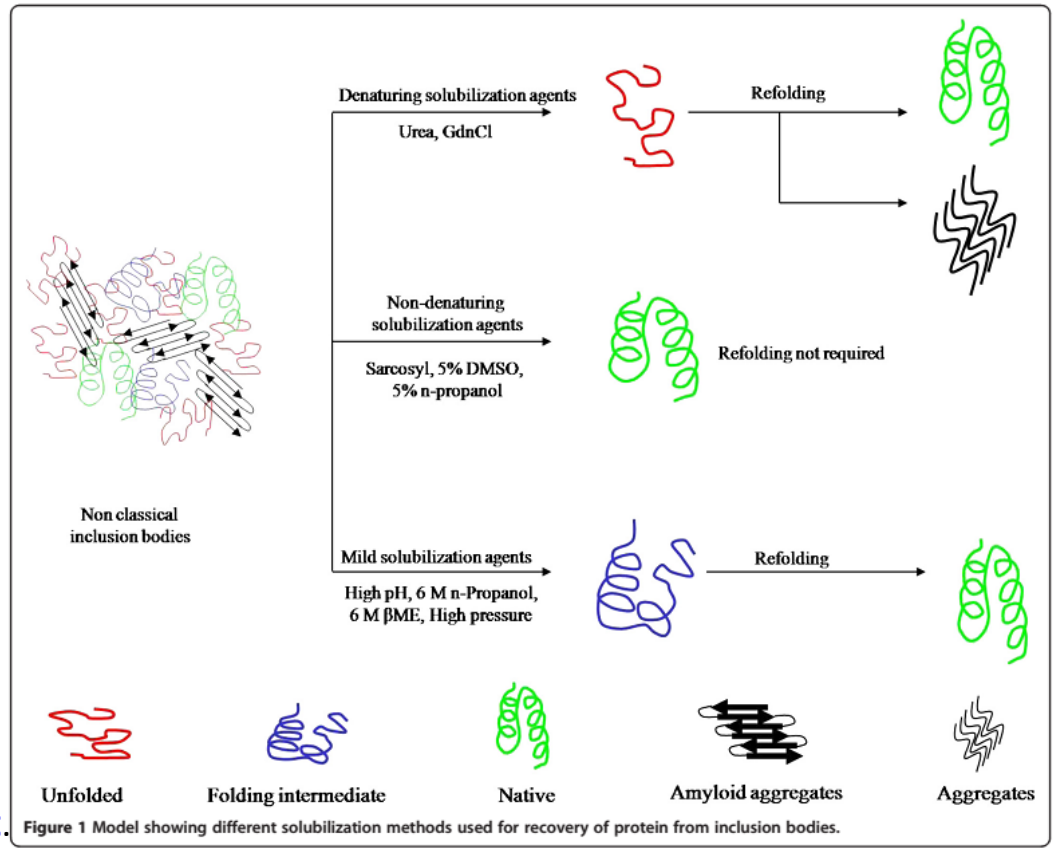


Figure 1 Model showing different solubilization methods used for recovery of protein from inclusion bodies.

✓Recovery of bioactive protein from bacterial IB using trifluoroethanol as solubilization agent.

Upadhyay et al. Microb Cell Fact (2016) 15:100

A rapid method for refolding cell surface receptors and ligands

Zhai L. et al. - *Sci Rep* 2016 May 24; 6:26482 –

Department of Biochemistry, Virginia Polytechnic Institute & State University, Blacksburg, VA 24061, USA

In this article, the authors compare on-column refolding of cell surface receptors and ligands against the popular and time-/reagent-consuming dialysis and dilution refolding methods. The on-column refolding method is not new, it has had many years in the field, but it is still not so widely used since it needs a previous buffer condition optimization in order to know which are the best refolding conditions.

The authors compare here several targets by three methods and show that, for these targets, the on-column refolding can be completed in a single day, compared to the classical more than one week required for the other two, with almost the same yield and protein quality. Moreover, the on-column method coupled to FPLC purification simplified the entire operation. In addition, the elution volumes of refolded targets can be easily controlled, avoiding an extra concentration step.

Instead of the classical overnight gradient refolding from 6 or 8M to 0M urea, the authors introduce here an interesting change, replacing the gradient with two quick washing steps: 3M and 1.5M urea buffers as key intermediate steps between the fully denatured state (8M urea) and fully folded state (0M urea). They claim that these steps are intended to stabilize intermediate folding or unfolded states of the proteins, which may in turn facilitate the refolding reactions to be more productive. Regarding the difficult disulfide bridges, the redox pair GSH:GSSG ratio of 10:1 (at a concentration of 1-5mM GSH) was selected because this condition was very efficient.

Anyone with experience in protein refolding knows that there is not a "magic solution", as the authors claim. It would be interesting to try this method for more difficult projects. Nevertheless, it looks to be a great advance