

Structural Studies as a Screening Tool in Development of Protein Purification Processes



Prathima Acharya
Analytical & Formulation Development
Diosynth Biotechnology, RTP, NC

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Biopharmaceutical Manufacturing

- Market Drivers (Time and Money)
 - Anyone can execute 100 studies with 100 people
 - How do we get more with less?
 - material
 - people
 - time
 - cost

- Conventional timelines 
- What can we do? 

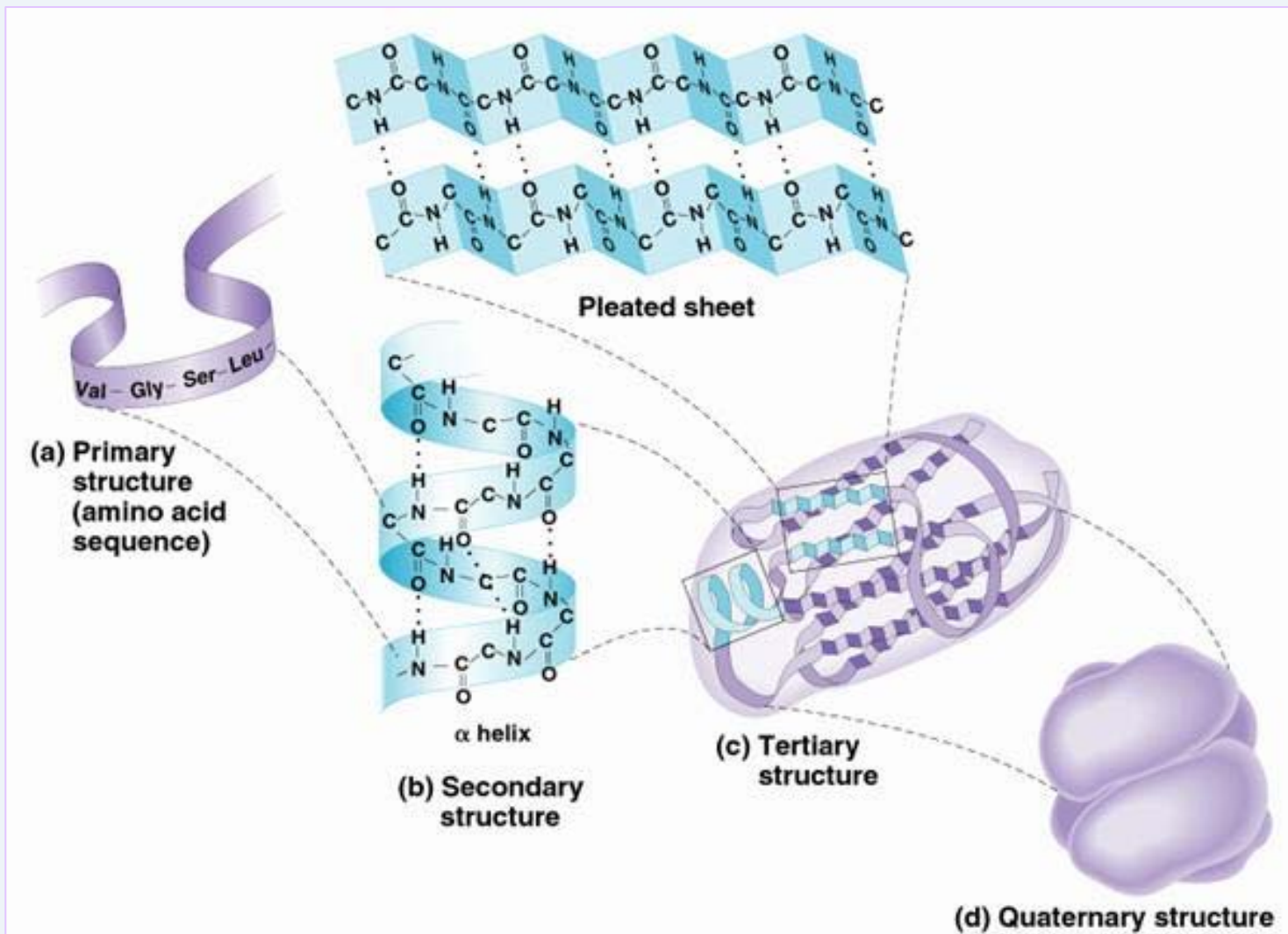
- Downstream processing is the most costly part of biopharmaceutical production and there is considerable demand to reduce costs

- Speed, understanding and robustness are needed for development of downstream process

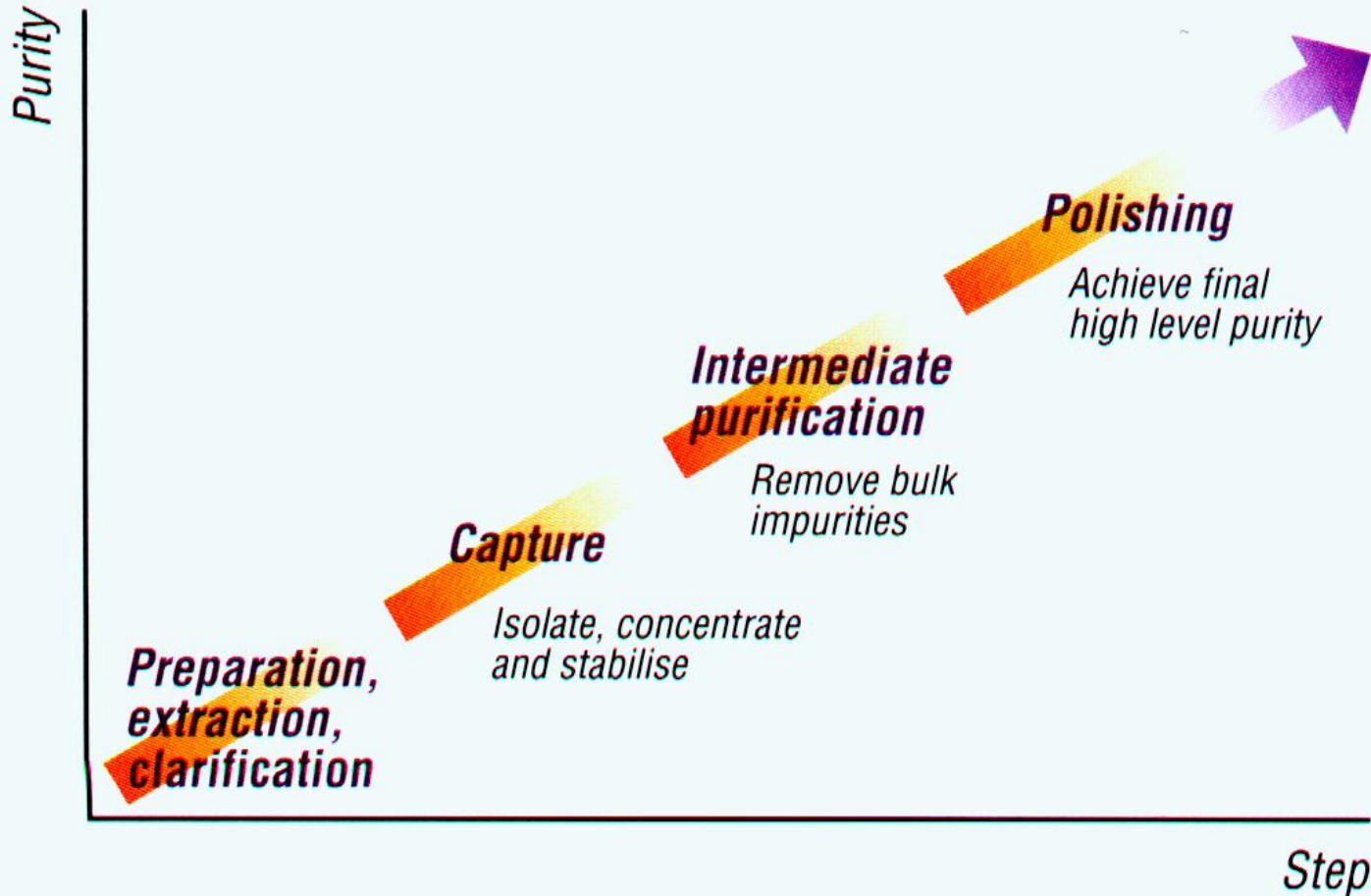
From Expression to Patient

- Must understand the effect of process and handling steps on the stability of proteins
 - Upstream processing (Expression and Harvest)
 - Downstream processing (Purification, Chemical Modifications)
 - Hold steps
 - UF/DF Exchange, Formulation
 - Short and Long Term Storage Conditions (Freeze/Thaw Cycles)
 - Fill/Finish Procedures (Filtration, Sterilization, Vialing, Lyophilization)
 - Delivery (Device Compatibility, Reconstitution)

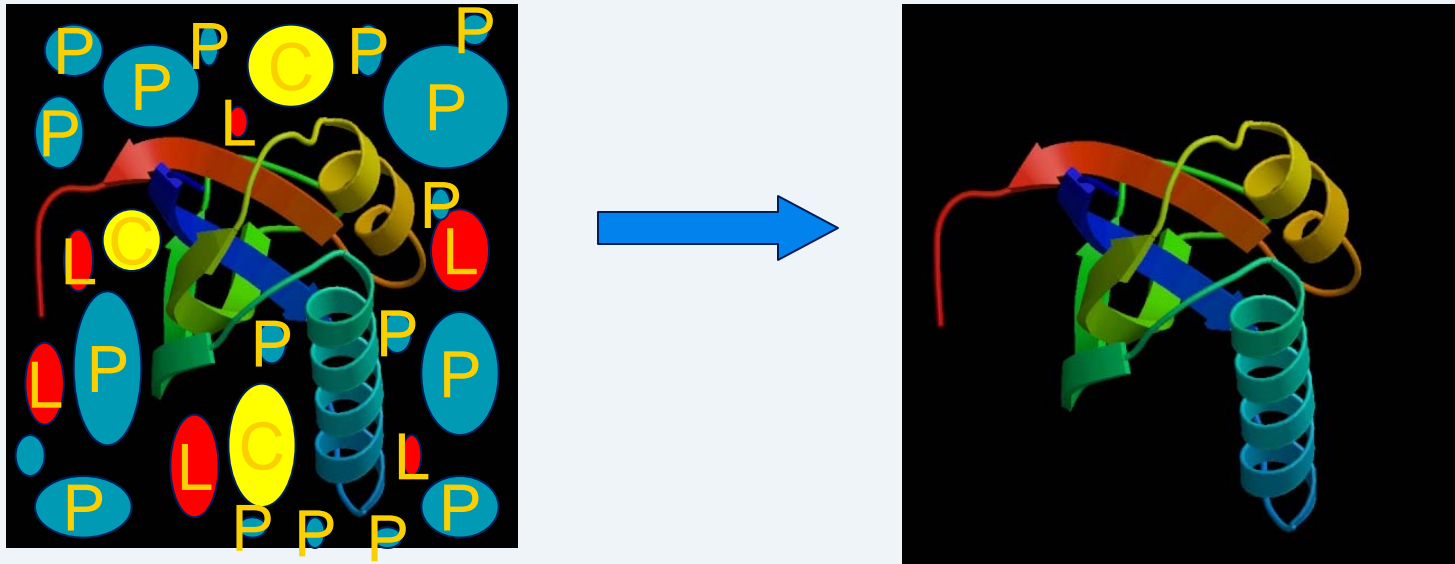
Protein Structure



Processing Steps



Protein Purification



Intracellular stabilizing scenario

- Purification process removes it ever further from the natural environment in which the protein is most stable
- A highly purified protein is rendered more sensitive to processes such as shear, agitation, enzymatic and chemical degradation and aggregation
- Very important to understand the native state and what destabilizes it

Protein Purification

- Potential denaturing forces include
 - Chemical stress from factors used in purification (pH, ionic strength, detergents)
 - Physical stress during UF/DF where surface adsorption and shear contribute to unwinding of tertiary structure
- The tertiary structure that must be stabilized against various disruptive forces during processing and handling
 - Freezing-Induced stresses
 - Decoupling production steps with freezing is essential for contract manufacturing operational efficiency to obtain
 - Greater scheduling flexibility
 - Secure shipping between manufacturing facilities

Instability of Protein in an Aqueous Environment

- Conformational changes
- Unfolding or denaturation
- Chemical modifications
- Physical degradation

Chemical Instability

- ✓ Deamidation
- ✓ Racemization
- ✓ Hydrolysis
- ✓ Beta-elimination
- ✓ Disulfide exchange
- ✓ Oxidation

Physical Instability

- ✓ Denaturation
- ✓ Aggregation
- ✓ Precipitation
- ✓ Adsorption



Safety and efficacy (activity)

Understanding Proteins

The set of activities related to overcoming the inherent instability of a protein molecule is called
Preformulation Development

- It is important to understand the critical properties of a protein
- Preformulation studies are designed to learn about the protein's susceptibility to a variety of relevant stresses
- Helps in deciding what features need to be in control and what features are non-critical

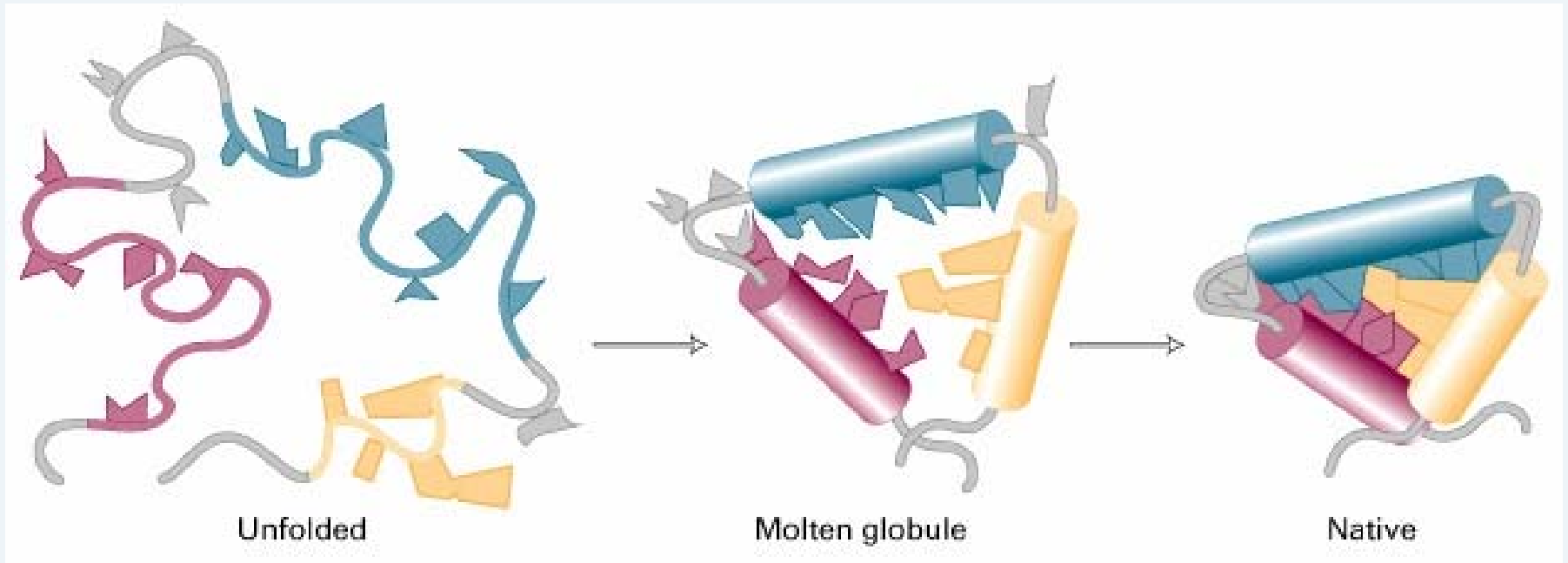
Ref: "Rational Design of Stable Protein Formulations – Theory & Practice", Eds., Carpenter J & Manning C

Protein Stability is a Balancing Act

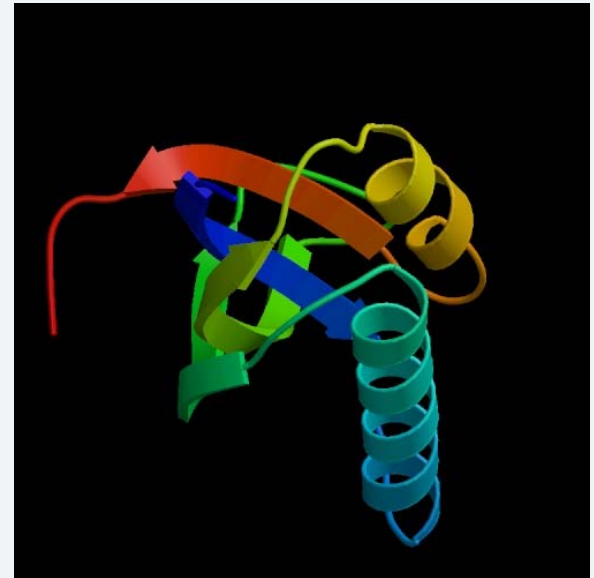
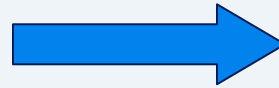
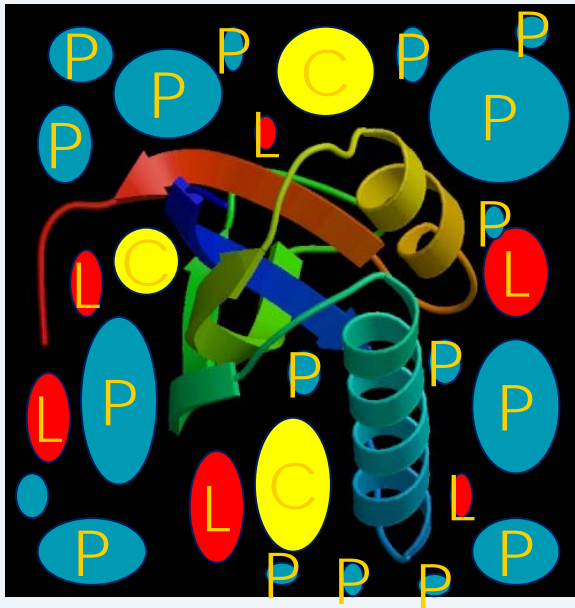
Formulation Variables	Desired attributes	Examples
<ul style="list-style-type: none"> pH 	<ul style="list-style-type: none"> Provides good physical properties of protein, minimize degradations 	
<ul style="list-style-type: none"> Stabilizer 	<ul style="list-style-type: none"> Inhibit degradations, effective at low concentrations 	<ul style="list-style-type: none"> Surfactants, sugars, salts, antioxidants
<ul style="list-style-type: none"> Solubilizer 	<ul style="list-style-type: none"> Improve the solubility, effective at low concentrations 	<ul style="list-style-type: none"> Salts, amino acids, surfactants
<ul style="list-style-type: none"> Buffer 	<ul style="list-style-type: none"> Good buffering capacity, stable to temperature change, stable to freezing, good safety record 	<ul style="list-style-type: none"> Phosphate, acetate, histidine, glutamate
<ul style="list-style-type: none"> Tonicity Modifier; Bulking Agent 	<ul style="list-style-type: none"> Inert, good safety record 	<ul style="list-style-type: none"> NaCl, Sorbitol, Mannitol, Glycine

Ref: "Rational Design of Stable Protein Formulations – Theory & Practice", Eds., Carpenter J & Manning C

How can we Stabilize Proteins?



Protein Purification- Formulation Development



**Formulation - stabilizing scenario
using compendial excipients**

Creating a Stability “Insurance Policy”

- Gain knowledge of a protein’s inherent instability triggers
 - Tailor steps throughout the manufacturing process
 - *minimize* stressors and *maximize* the level of stability of the finished drug product
 - Increased stability = more robust production processes
 - Saves *time and money*
 - Typical Preformulation screening studies
 - pH and buffer screens
 - Ionic strength, excipients
 - Stress studies
 - Analytical toolbox
 - Chromatography
 - Potency assays
 - Electrophoresis
 - ***Biophysical – Protein structural characterization***
- Helps us work smarter not harder***

Rational Approach to Purification Development

- Anyone can explain principles of IEX, PrA, etc., and go by theory for choice of load and elution buffers
- However, applying SPECIFIC molecule information results in quickly tailored process steps (effect of pH, buffer type, ionic strength etc.)
- Provides more reliable information than random trials and errors
- Helps define a working space
- Working space for product may drive decisions on selection and sequence of purification steps

CEX

**Protein
A**

AEX

**Non-Protein A
(Eg. MEP)**

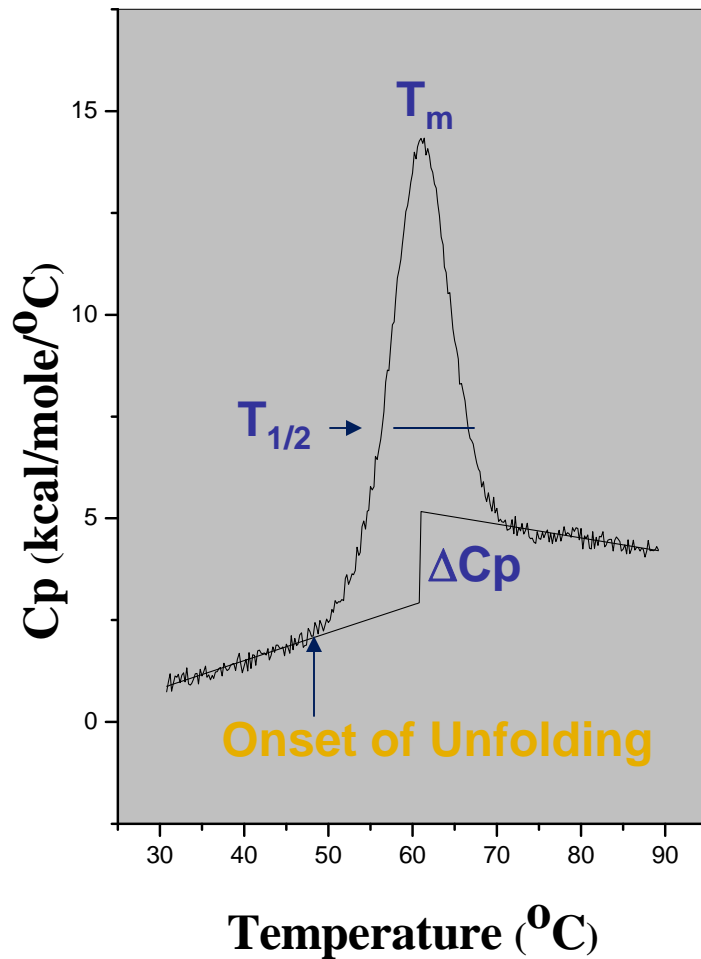
HIC

**Hold
Steps**

UF/DF

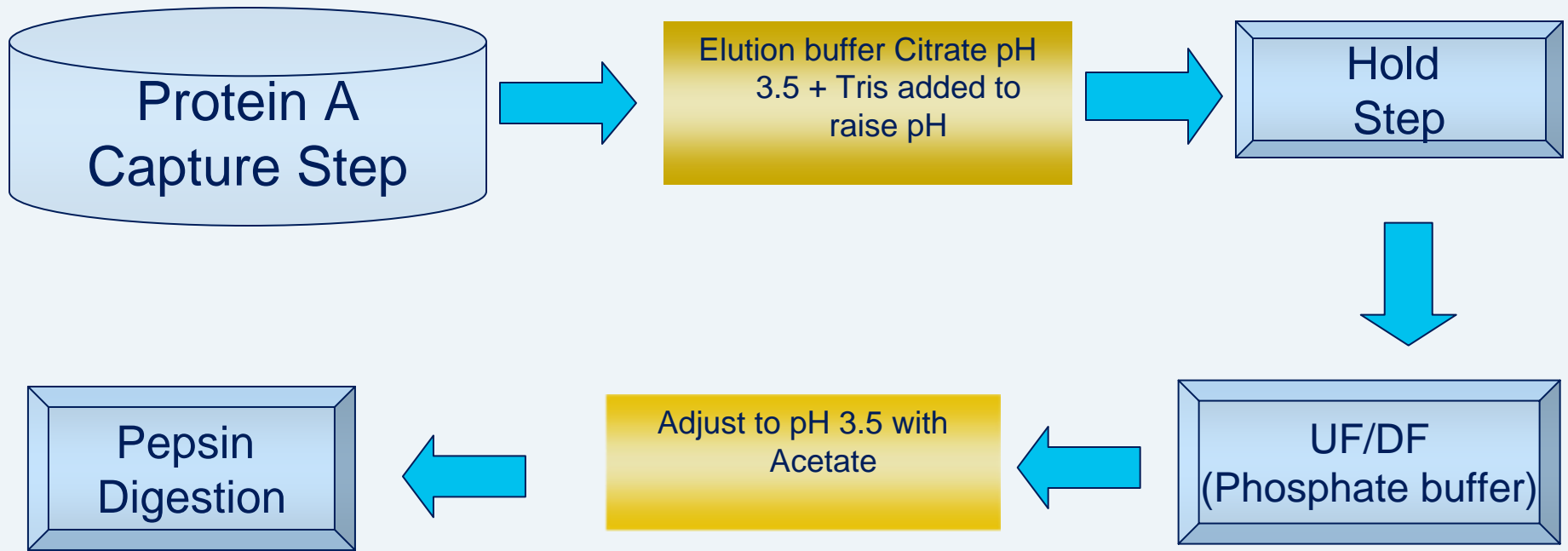
HCIC

Differential Scanning Calorimetry



- T_m - Midpoint of transition
- $T_{1/2}$ - Cooperativity of unfolding (compactness of the molecule)
- ΔH - Enthalpy of unfolding (area under the curve)
- C_p - Heat Capacity
- ΔC_p - Measure of change in exposure of polar and non-polar amino acid residues to the solvent
- Onset of unfolding

Case Study # 1 (Protein A Capture Step Development)



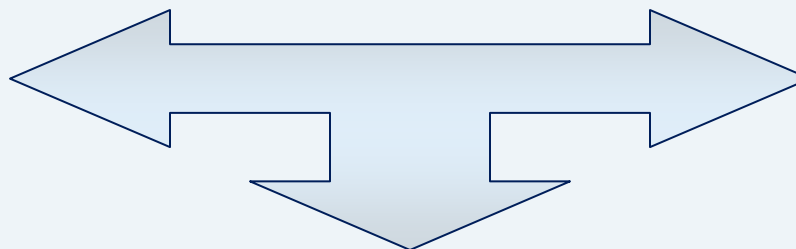
Key Issues with Process

- Most proteins are unstable at low pHs
- Precipitation of the protein at low pH is a major obstacle for
 - development of Protein A as a capture step
 - almost all alternatives to Protein A chromatography require elution at low pH
- Loading capacity of the column is compromised due to precipitation issues in the eluate at high protein concentrations
- Therefore the protein has to be stabilized in the elution buffer

How Can We Make the Process Economically Viable?

Stabilizing the protein in the elution buffer (low pH) will increase the capacity of the column

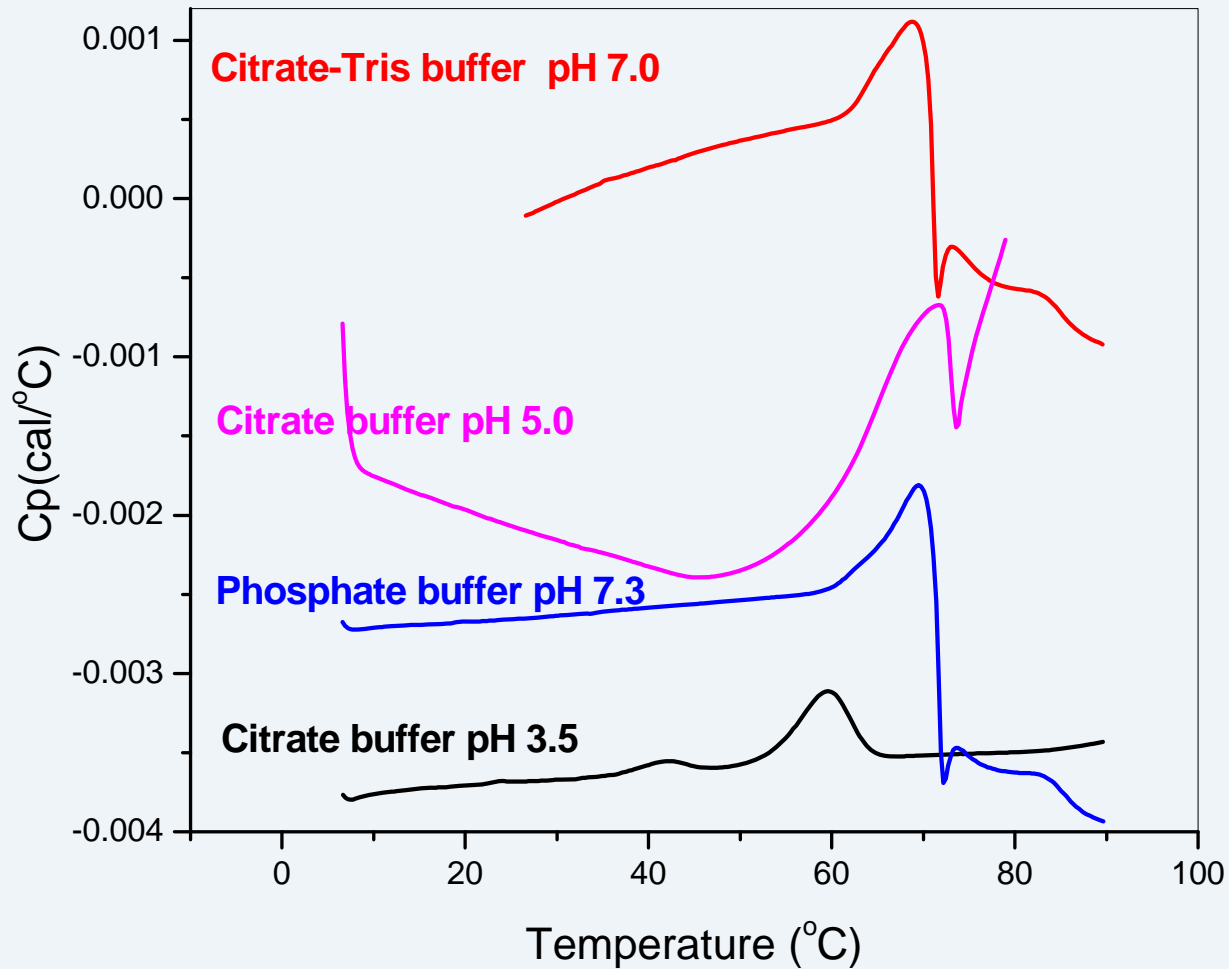
Protein A column can be made economically Feasible



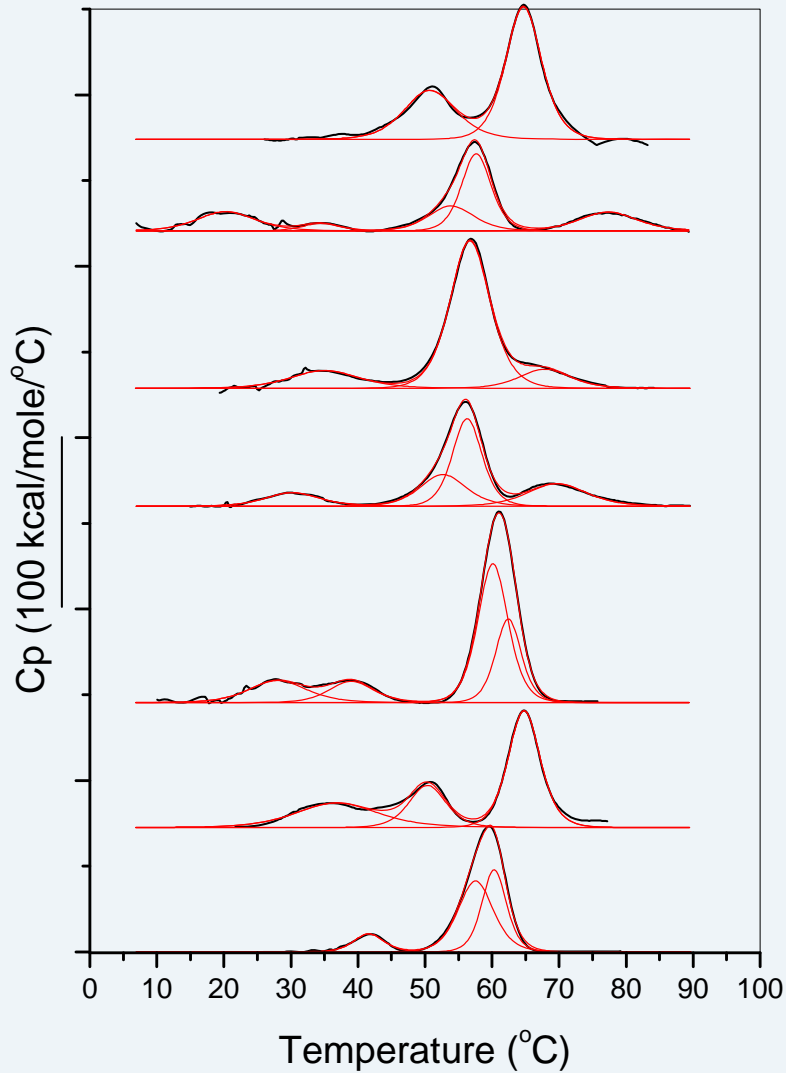
Increasing capacity 10 fold will result in savings of thousands of dollars

Eluting the protein at a higher concentration from Protein A will eliminate a UF/DF step (cost savings)

Differential Scanning Calorimetry



Differential Scanning Calorimetry



Citrate + Mannitol pH 3.5

Glycine + Arg pH 3.0

Glycine + Ile pH 3.0

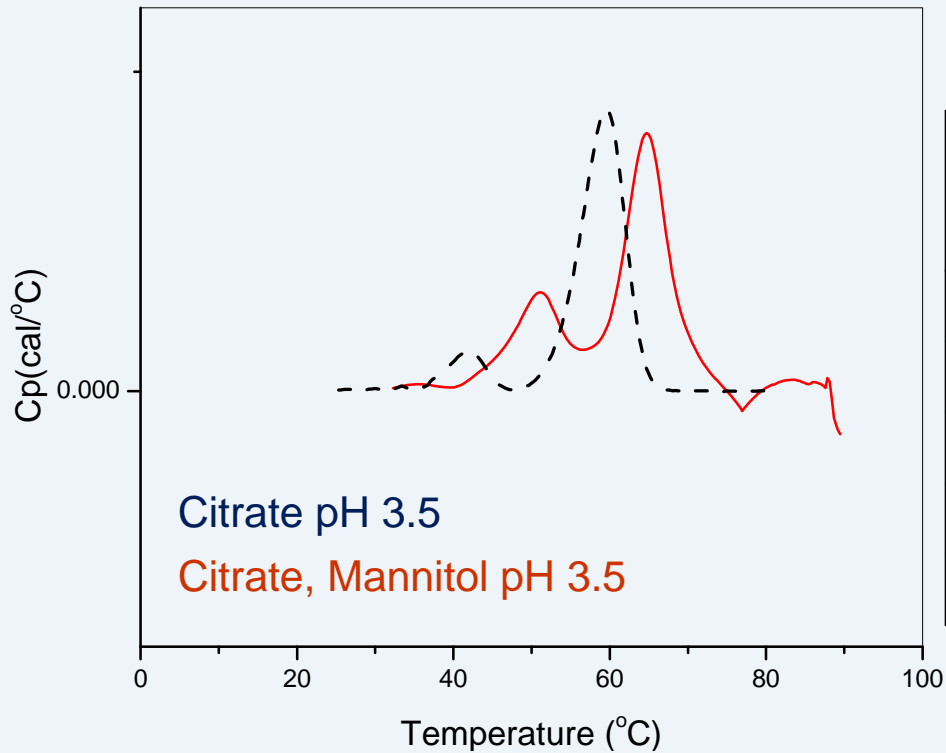
200 mM Glycine pH 3.0

50mM Glycine pH 3.0

Glycine + Leu pH 3.0

Citrate pH 3.5

Differential Scanning Calorimetry

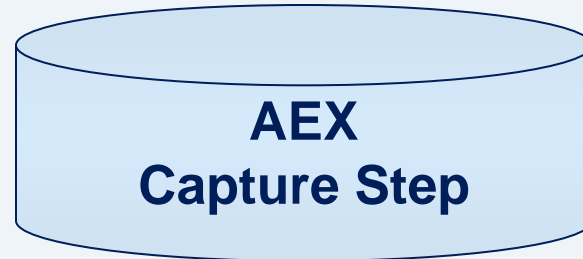


	Tm of major transition (°C)	Onset of Unfolding (°C)
Citrate-Tris buffer pH 7.0	68.7	60.1
PBS pH 7.3	69.5	58.5
Citrate pH 5.0	71.5	48.2
Citrate pH 3.5	59.3	34.1
Citrate, Mannitol pH 3.5	64.7	41.0

Summary of Development Efforts

- Achieved good stabilization of the protein at low pH
- Obtained increased loading capacity on the Protein A column (~ 7.5 fold up from 2 mg/ml to 15 mg/ml)
- Cost/Benefits realized during GMP runs (raw materials):
 - Initial process from client used a 19.5 L column - ~ cost \$175,000
 - Current redeveloped process used 2.6 L column that cost ~23,400
 - At 100 L scale, assuming titer of 0.5g/L, 4 cycles per run on PrA column, cost reduction would be \$38,000 on the resin alone
- At 1500 L cell culture scale savings would be ~570,000 for the resin

Case Study #2 - (CEX Capture Step Development)



- Low capacity
- EDTA competes for binding sites



- Only column removing impurities
- Purity ~ 90%
- Low yield ~ 50%

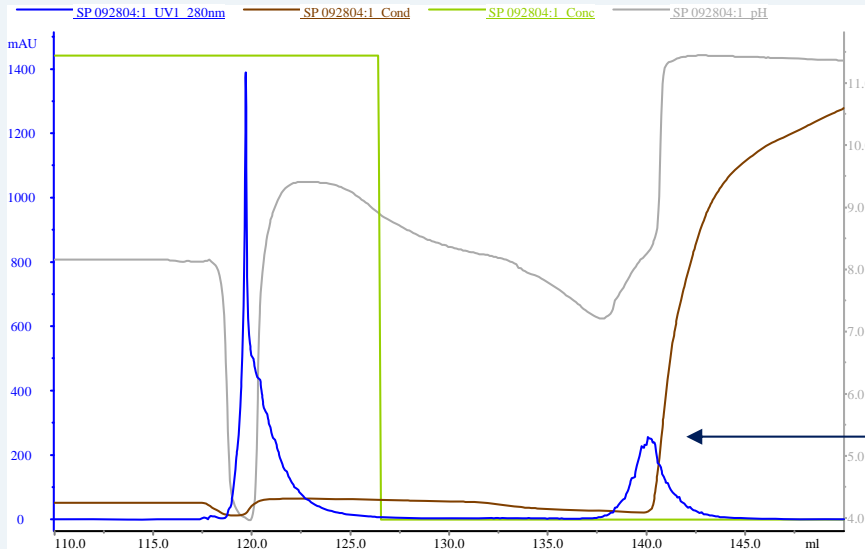


Situation Requires a Better Capture Step

Cation Exchange Chromatography Development

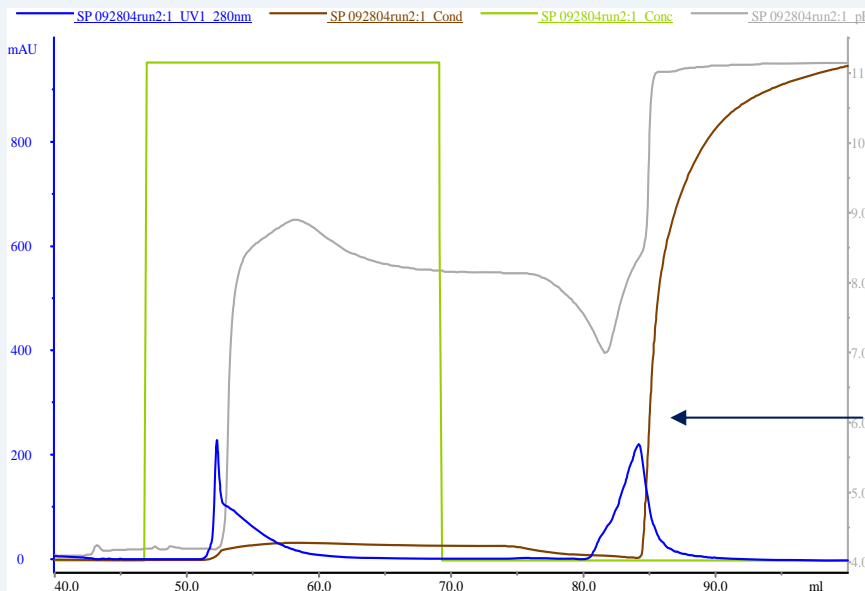
- Purpose:
 - High capacity capture step
 - Capable of handling large amount of clarified extract
 - High flow rate means less processing time
 - Remove proteases
 - Prevent degradation of protein
 - Removal of EDTA downstream
 - Problems Encountered
 - Protein found to be poorly soluble at low pH (pI is ~5.6)
 - Addition of salt resulted in precipitation at low pH, therefore elution had to be performed by pH shift, not ionic strength increase
 - Protein at pH 4.0 bound tighter to the CEX column than at pH 4.5

CEX Chromatography



Load Buffer:
25 mM Acetate, pH 4.5
Elution Buffer:
200 mM Tris, pH 9.0

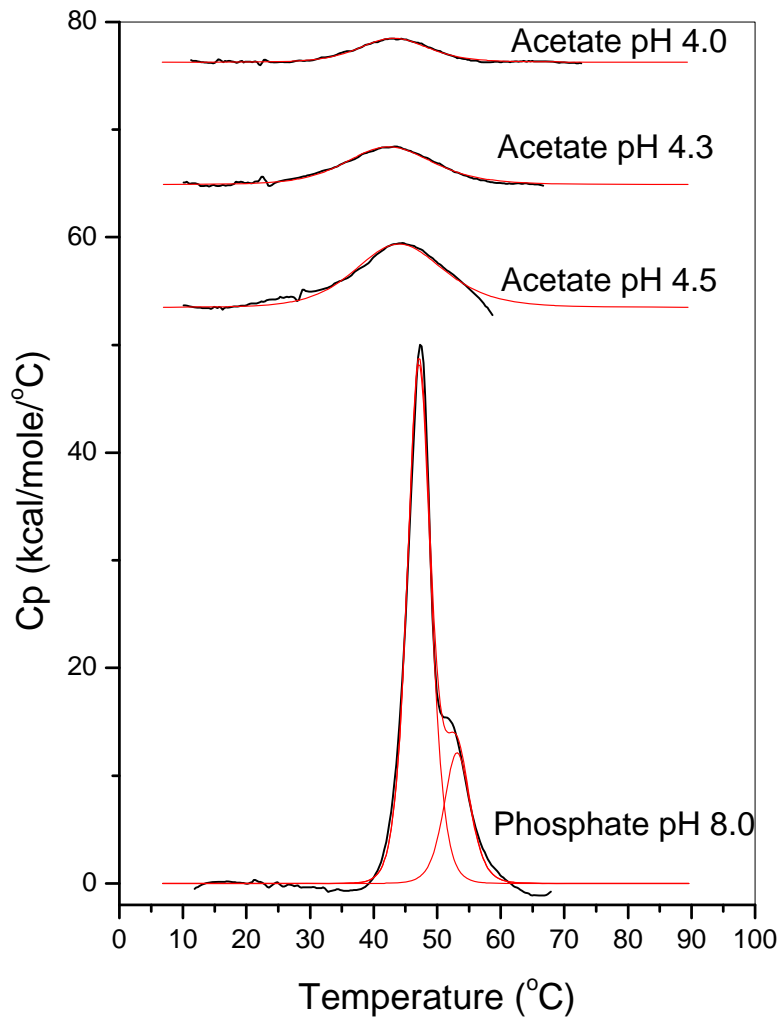
Strip with 0.5M
NaOH



Load Buffer:
25 mM Acetate, pH 4.0
Elution Buffer:
200 mM Tris, pH 9.0

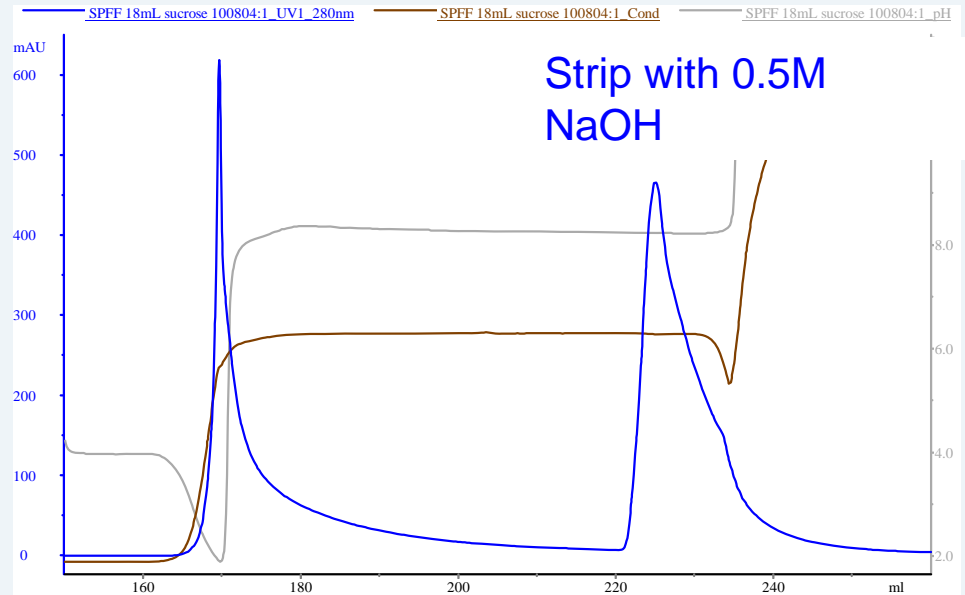
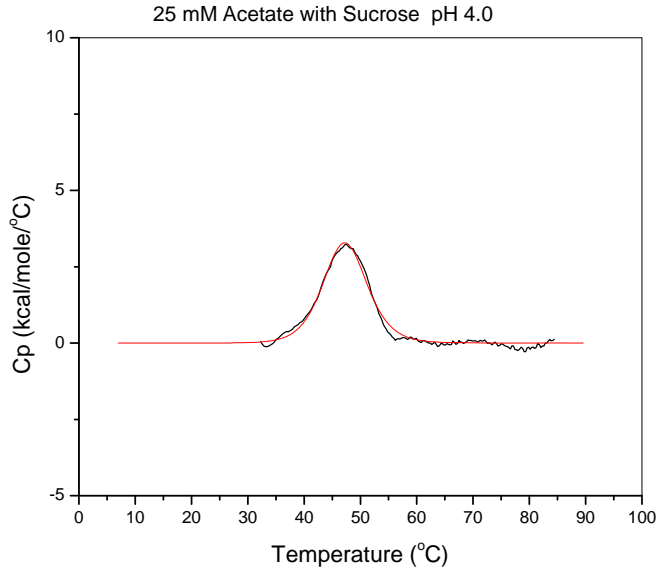
Strip with 0.5M
NaOH

Differential Scanning Calorimetry

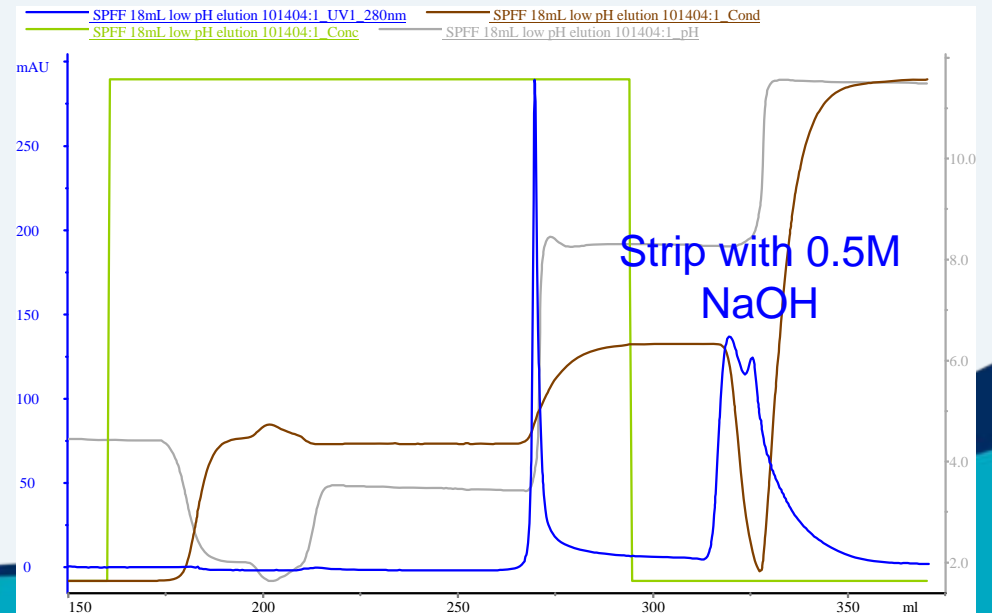
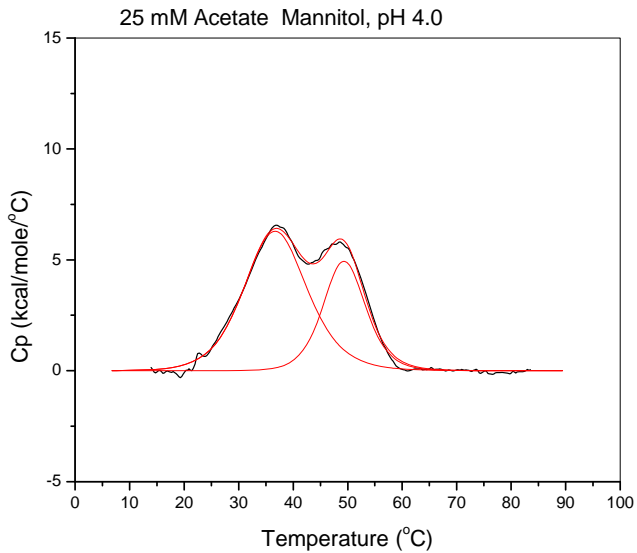


Samples	T_m (°C)	DH (kcal/mole/°C)	$T_{1/2}$ (°C)
Acetate pH 4.0	42.9	30.9	13.1
Acetate pH 4.3	43.4	62.2	16.3
Acetate pH 4.5	44.5	105.2	16.3
Phosphate pH 8.0	47.3	297.2	4.3

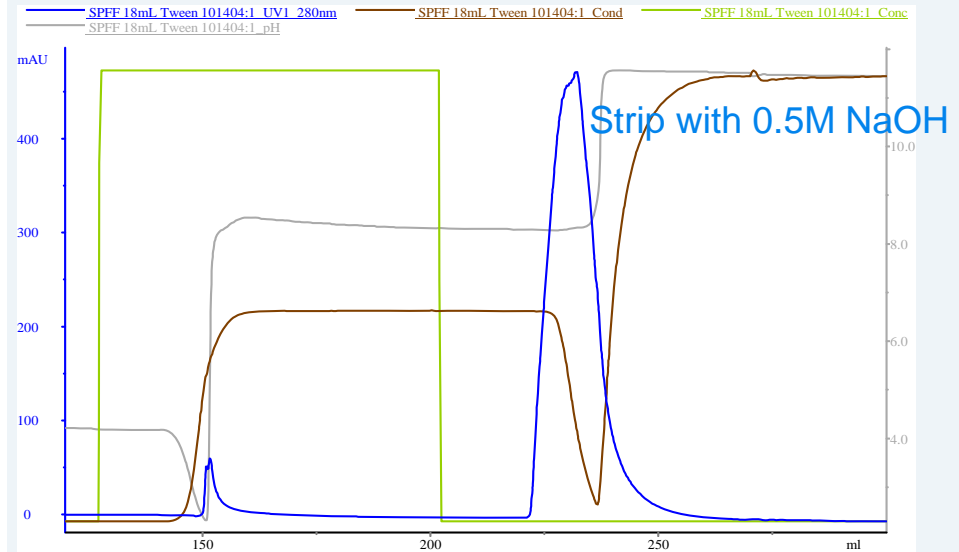
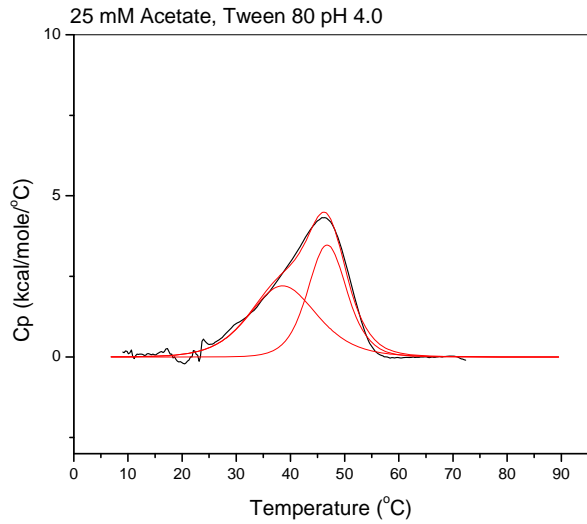
CEX Chromatography Load buffer – (Acetate + Sucrose, pH 4.0)



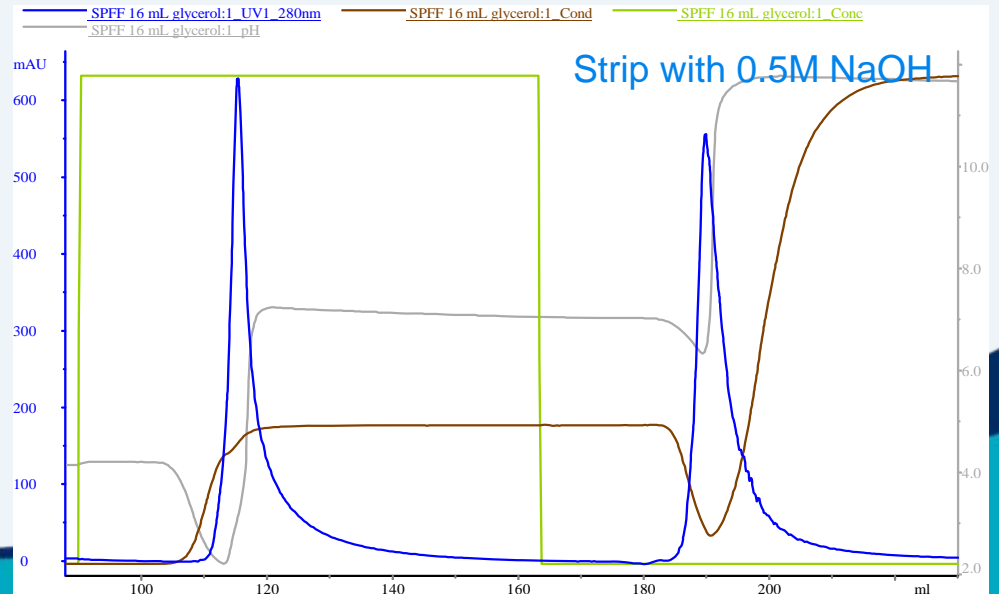
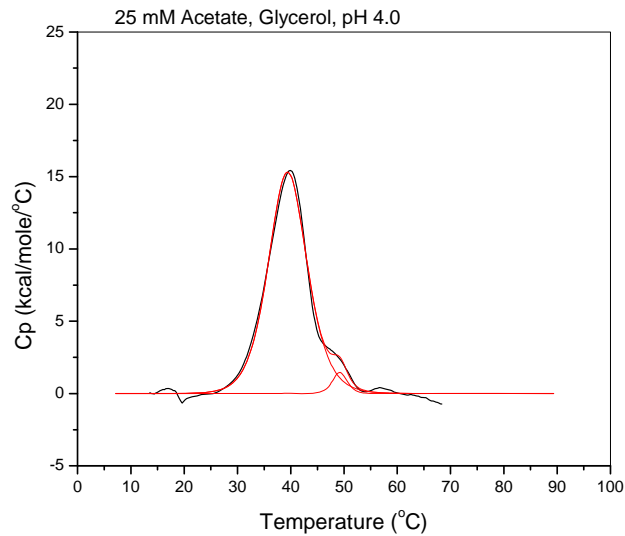
CEX Chromatography Load buffer – (Acetate + Mannitol, pH 4.0)



CEX Chromatography Load buffer – (Acetate + polysorbate 80, pH 4.0)



CEX Chromatography Load buffer – (Acetate + Glycerol, pH 4.0)

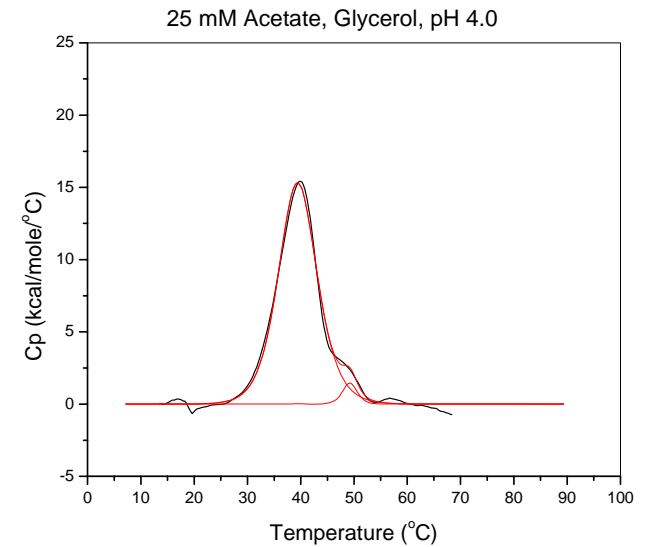
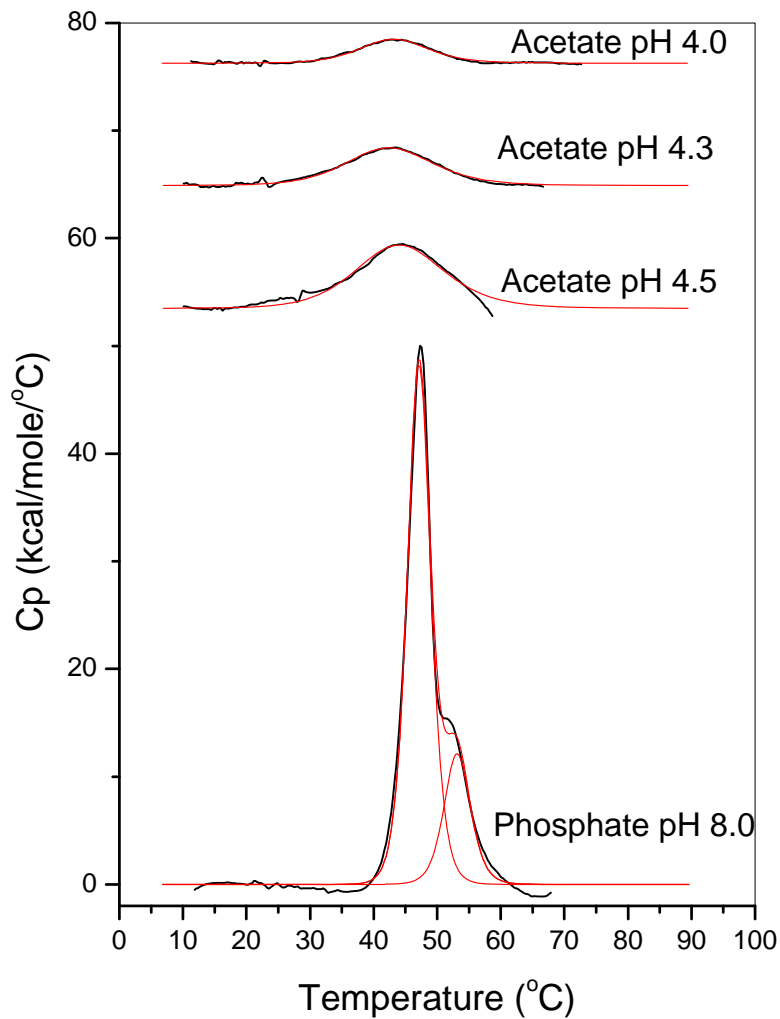


Capture Step Development

Alternative Method - Hydrophobic charge induction (HCIC)

- HCIC is based on the pH-dependent behavior of dual-mode ionizable hydrophobic ligands
- The pKa of 4-MEP is 4.8 and at physiological pH the aromatic pyridine ring is uncharged.
- Elution is accomplished by reducing the pH
 - ligand takes on a positive charge
 - bound proteins take on net positive charge
 - electrostatic charge-repulsion prompts desorption
 - pI of the target protein and its hydrophobicity will determine the pH at which it is eluted
- High capacity, selectivity, chemical and mechanical stability

Differential Scanning Calorimetry

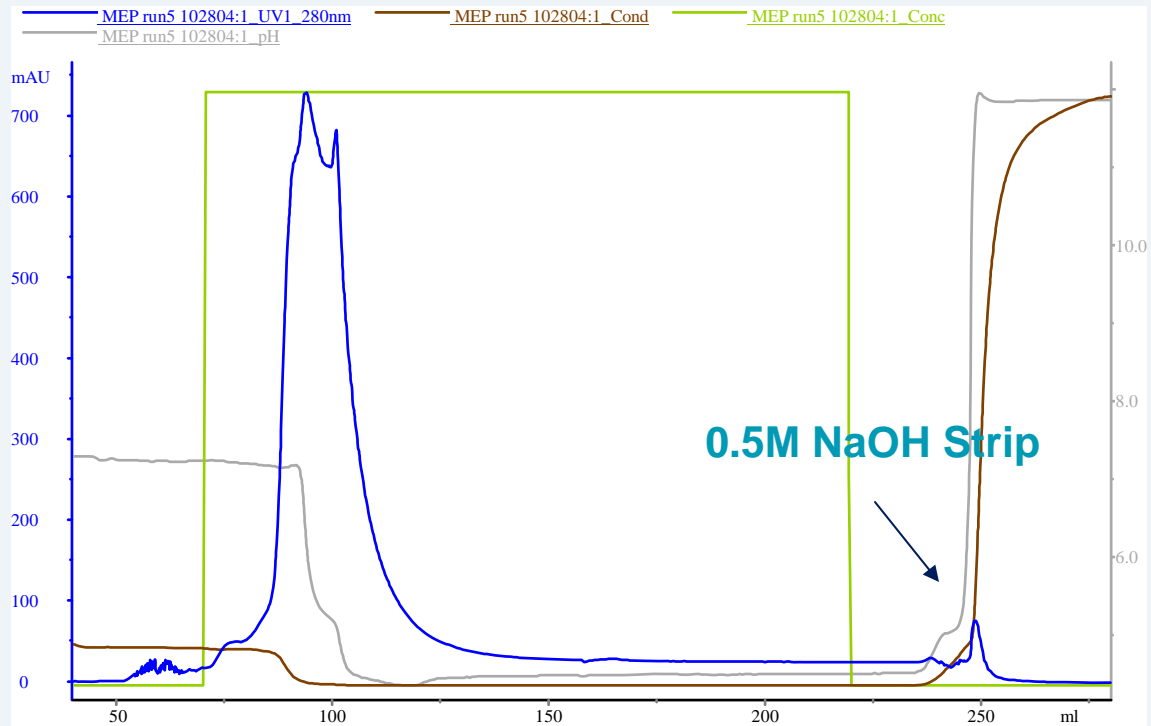


Capture Step Development

Hydrophobic Charge Induction Chromatography

Load Buffer: Phosphate, 0.1M NaCl, pH 7.25

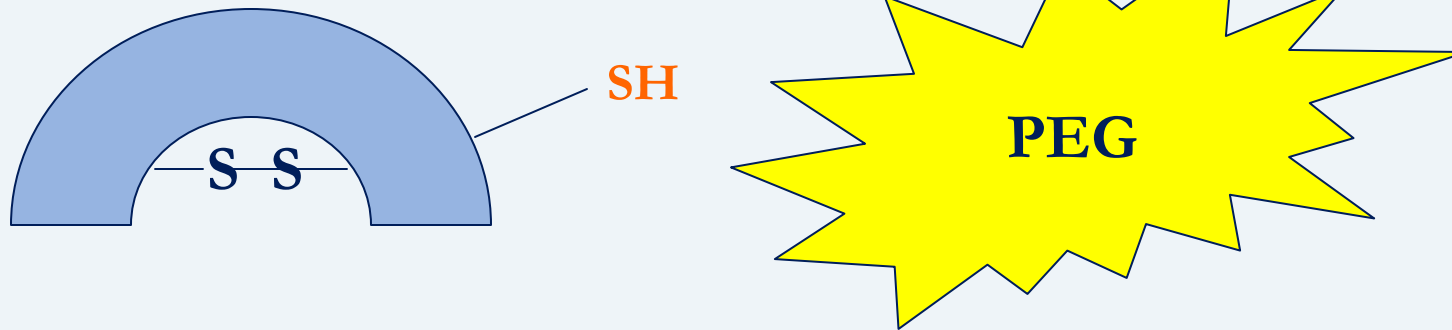
Elution Buffer: Acetate + Glycerol, pH 4.4



Summary of Development Efforts

- Protein is incompatible with traditional cation exchange methods
- Between pHs 4.0 and 6.0, protein is in an unstable, partially unfolded state
- The folded conformation can be forced at low pH to a certain degree with the addition of glycerol
- The pH requirements for HCIC and charge characteristics of the protein may make it suitable for purification by HCIC

Case Study #3 (Development of a Robust PEGylation Process)



- Protein with internal S-S bonds with 1 surface engineered Cys for PEGylation
- Instability of protein at high concentration at higher pHs required for PEGylation
- Large processing volumes at low protein concentration – possible issues with scale-up
- UF/DF to concentrate the protein + exchange into PEGylation buffer containing a reducing agent
- A 2nd DF step to dialyze out the reducing agent; following which
- The process calls for “rapid” PEGylation to avoid:
 - instability of high concentration of protein at high pH
 - reformation of S-S bonds involving the surface Cys

This scenario cannot translate into a robust process

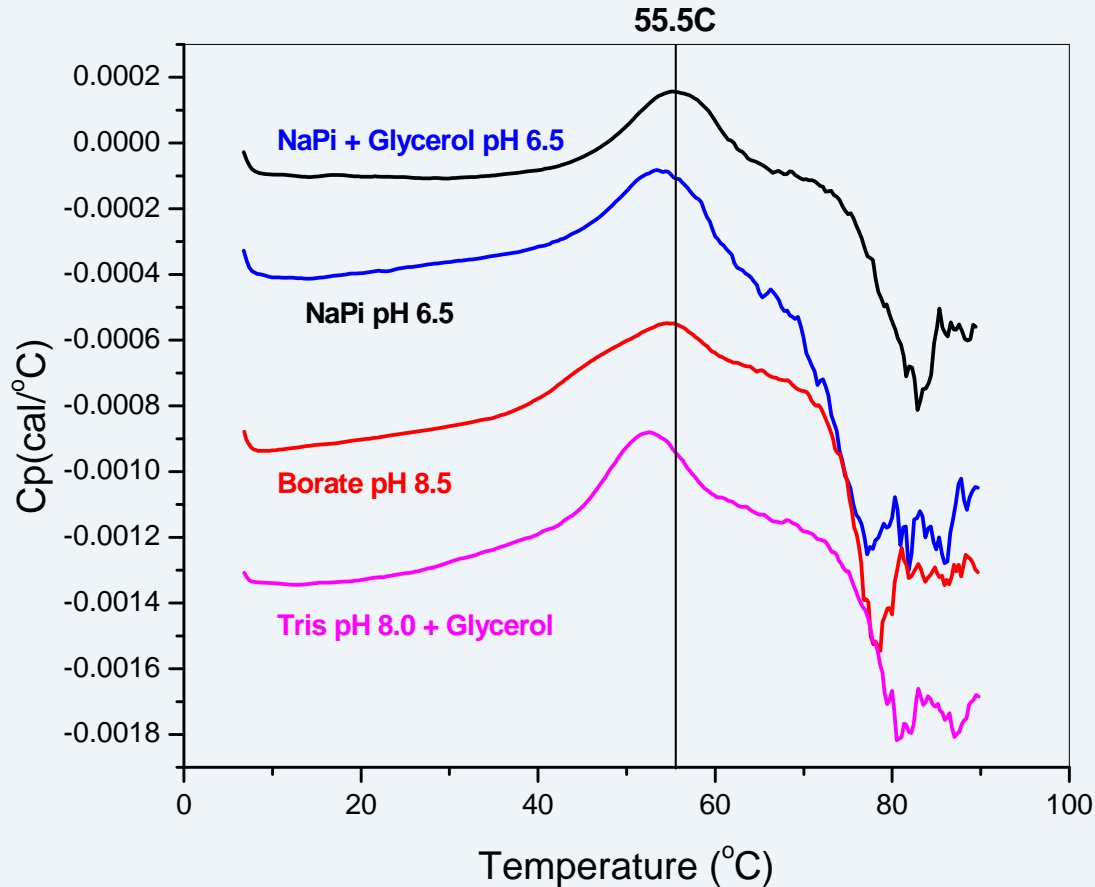
Can We Develop a Robust PEGylation Process?

Some of the questions we needed to address:

- Can we stabilize the protein at high pH required for PEGylation to allow for lower processing volumes?
- Can we PEGylate in the presence of a reducing agent to arrive at a robust processing step?
- In doing so, can we eliminate 1 UF/DF step and the 2nd DF step?

- Can we use Biophysical techniques to address these questions?
 - Fluorescence
 - DSC

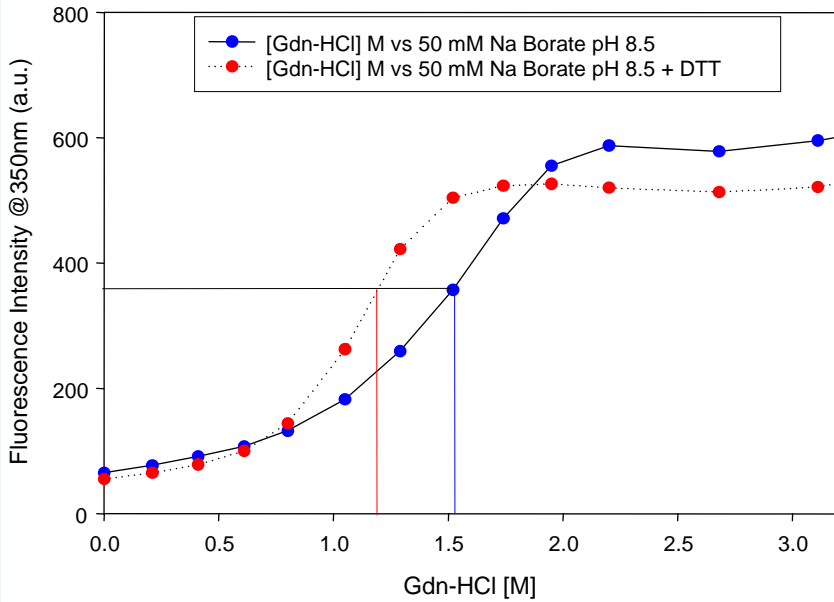
Differential Scanning Calorimetry



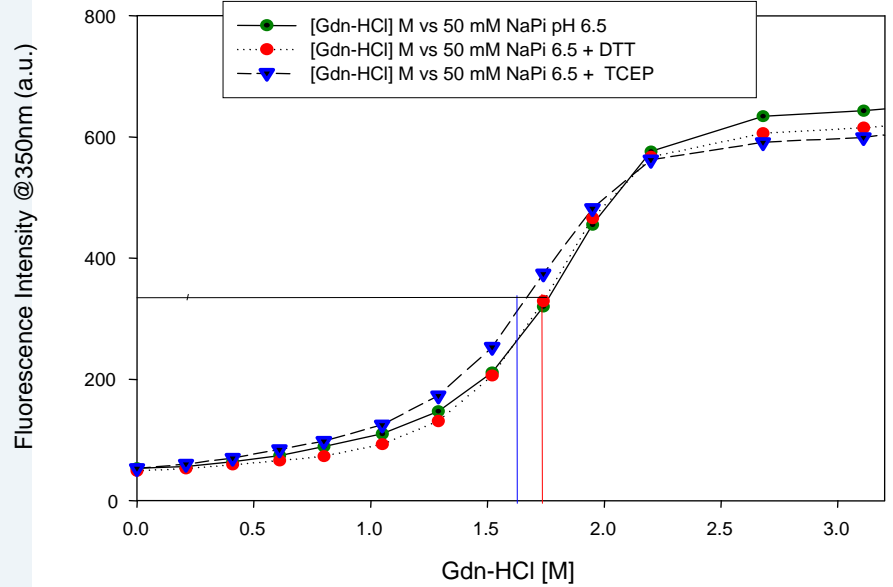
- Loosely folded structure at high pH which could result in
 - (a) instability of protein
 - (b) access of reducing agent to internal S-S bonds
- Glycerol is stabilizing and helps in formation of compact structures

Fluorescence Data

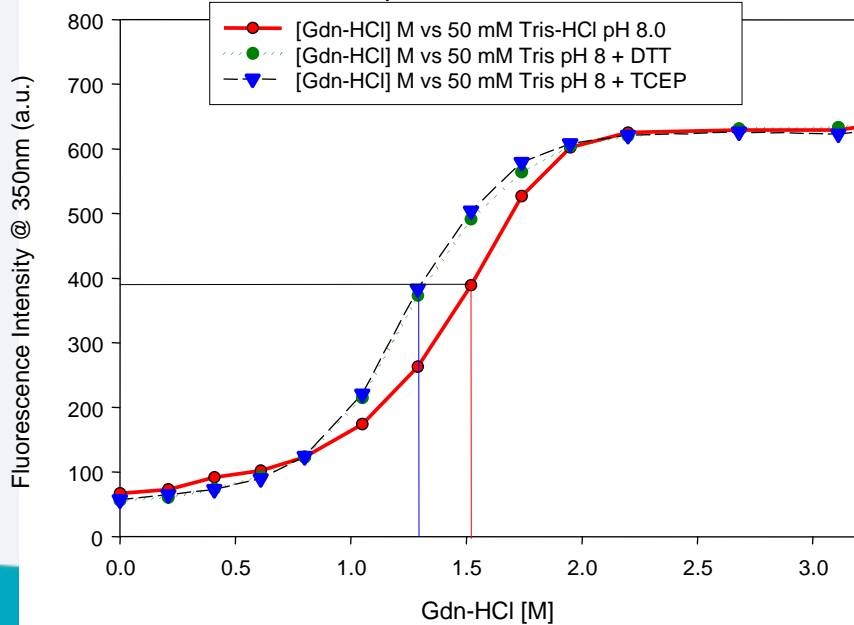
Borate buffer pH 8.5 +/- DTT



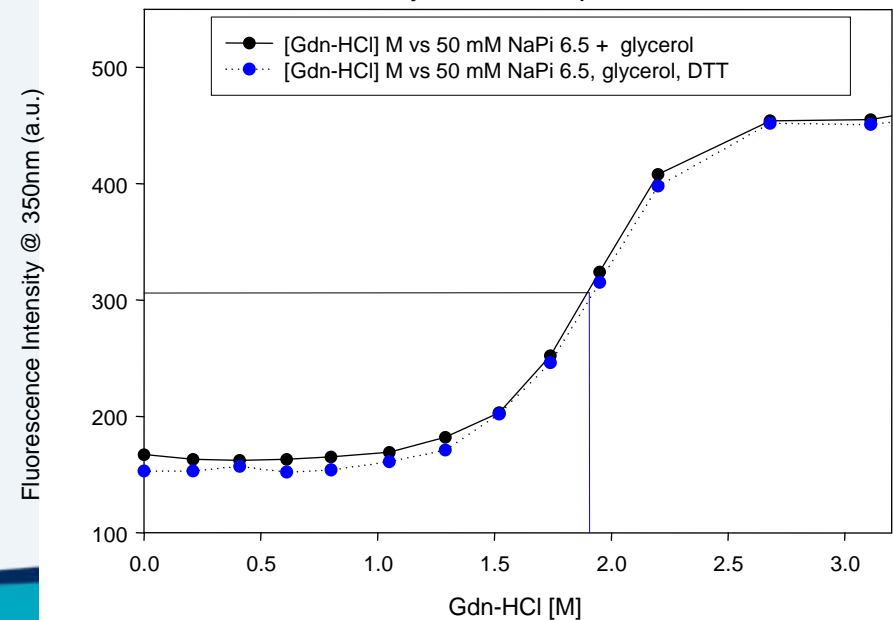
NaPi buffer pH 6.5 +/- DTT and TCEP



Tris Buffer pH 8.0 +/- DTT and TCEP



NaPi + Glycerol buffer pH 6.5 +/- DTT



Summary of Development Efforts

- Achieved good stabilization of the protein at higher pHs
- Obtained compact structures at higher pHs which would allow for PEGylation in the presence of a reducing agent
- Cost/Benefit projections:
 - Elimination of 1 UF/DF step and a 2nd DF step
 - Robust process with lower processing volumes

Summary

- A paradigm shift in approach to purification process development
 - Beyond final product structure to maintaining structure throughout
- Helps design an adaptive, customized process based on molecule specific info and not on general principles of chromatography
- Helps define a working space even for early clinical products
- Ensures adequate stability of molecule all through process steps and a stable baseline for future work (scale-up etc.)

FDA's Process Initiative

- Regulatory process tailored to recognize the level of scientific knowledge supporting product application, process validation and process capability
- Risk based regulatory scrutiny relate to
 - level of scientific understanding of how formulation and manufacturing process factors affect product quality and performance
 - the capability of process control strategies to prevent or mitigate risk of producing a poor quality product

Conclusions

- Biophysical assays, such as DSC, Fluorescence, and CD, are useful in efficiently detecting the effect of solvent and physical conditions on the stability of protein molecules
- Pre-formulation screening can be a powerful tool for tailoring production processes and final protein formulations to *maximize* the stability of the end product

Acknowledgements

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-