

Use of Microcalorimetry in Early Development of Pharmaceutical Proteins in Solution

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Outline

- **Protein Instability in Solutions**
- **Protein Aggregation**
- **Considerations for Purified Bulk**
- **Strategy to Determine Sensitivity of Proteins to Stress Conditions**
- **Case Studies**
 - **Physical and Chemical Stability in Early Development**
 - **Alternate Technique: Microcalorimetry**

Potential Protein Instability in Solutions

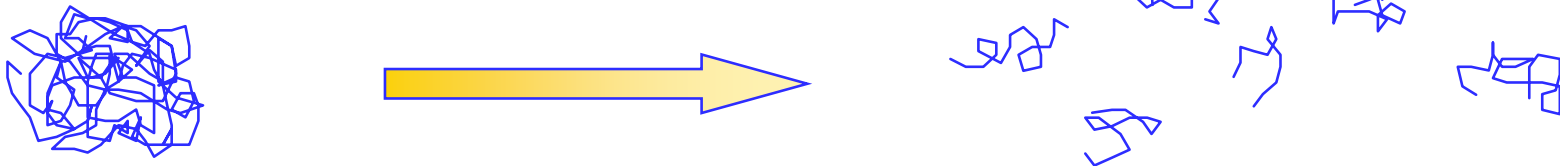
1) Denaturation



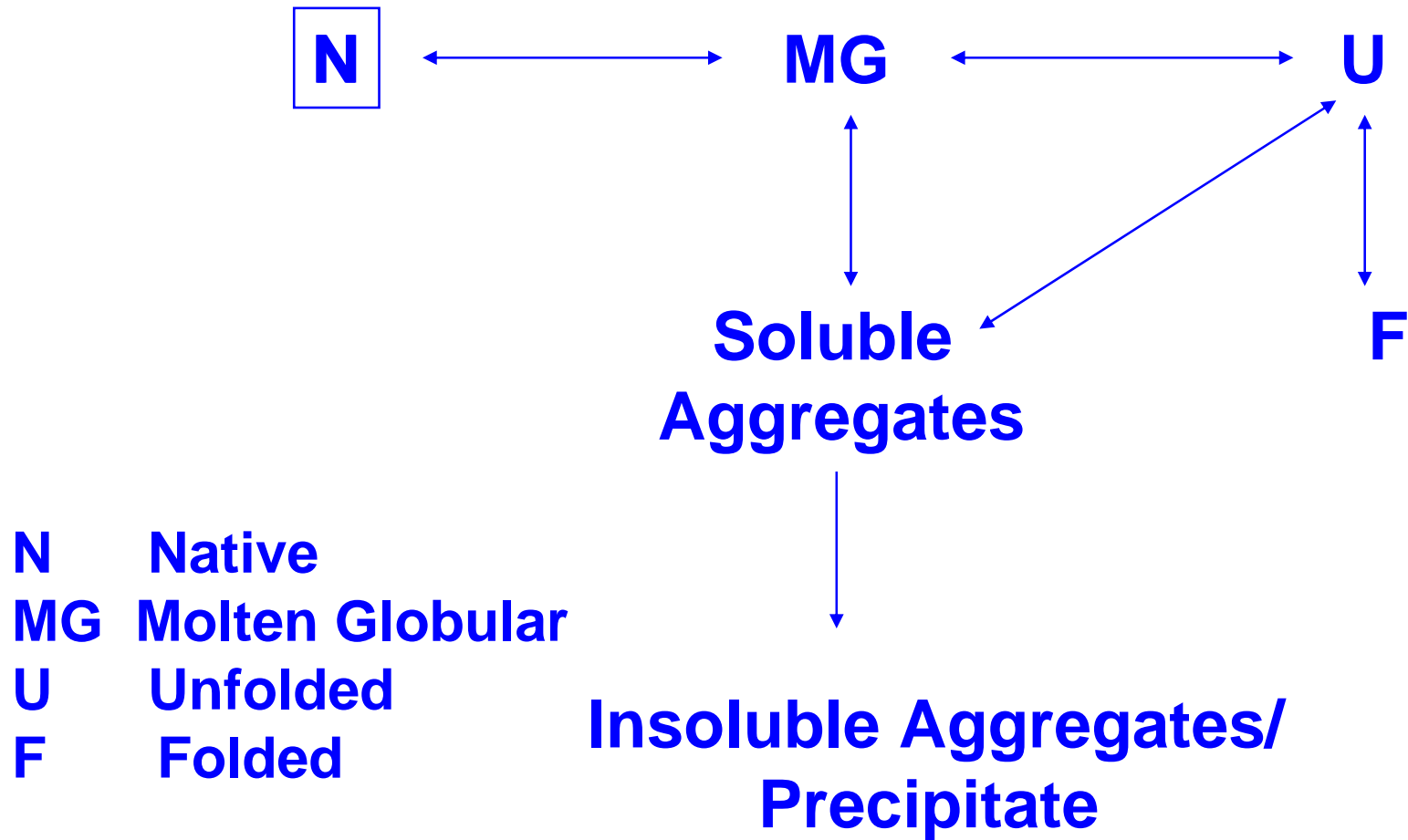
2) Aggregation



3) Degradation



Unfolding, Aggregation and Precipitation



Protein Aggregation

- **Types of Protein Aggregates**
- **Mechanisms of Protein Aggregation**
- **Causes of Protein Aggregation During Development**
- **Factors Affecting Physical and Chemical Stability of Proteins**

Types of Protein Aggregates

- **Non-covalent, rapidly reversible, small oligomers (dimer, trimer, tetramer etc)**
- **Irreversible, noncovalent oligomers**
- **Covalent oligomers (fusion proteins with disulfide linkage)**
- **Large (>10mers) and very large (50nm – 3um) soluble aggregates. Could be reversible if noncovalent**
- **Visible particulates (probably irreversible)**

Mechanisms of Protein Aggregation

Protein Stabilizing Forces

- Electrostatic interaction
- Van der Waals forces
- Hydrogen Bonding
- Cation – Π interactions
- α -helical content

Protein Destabilizing Forces

- Extended Hydrophobic regions
- β -sheet content

Causes of Protein Aggregation During Development

- **Incorrect folding during**

Protein expression

- **Denaturation during**

Protein Purification

Formulation

Mixing

Filtration

Filling operation

Lyophilization

Container/closure

Transportation

Storage

Characterization studies

- Solubility
- pH
- Temperature
- Buffer concentration
- Buffer components
- Sodium chloride concentration
- Protein concentration
- Excipients
- Multiple freeze/thaw
- Agitation (transportation)
- Shear effect (manufacturing)
- Adsorption
- Compatibility studies with Packaging/Mfg components
- Stability under selected processing conditions

Considerations for Purified Bulk

- **Buffer / Salt Composition**
- **Protein Concentration**
- **Storage Conditions**
- **Drug Product Requirements**

Considerations for Buffer Composition of Purified Bulk

■ Stability Considerations

- Real-time data necessary
- Stress-stability vs. Accelerated Development
 - Arrhenius Relationship?
- Always utilize several assays including binding assays

Strategy to Determine Sensitivity of Proteins to Stress Conditions

- Small quantity of bulk
- Size exclusion chromatography
 - Effect of pH
 - Effect of temperature



Aggregation?
Degradation?

Physical and Chemical Stability Studies in Early Development

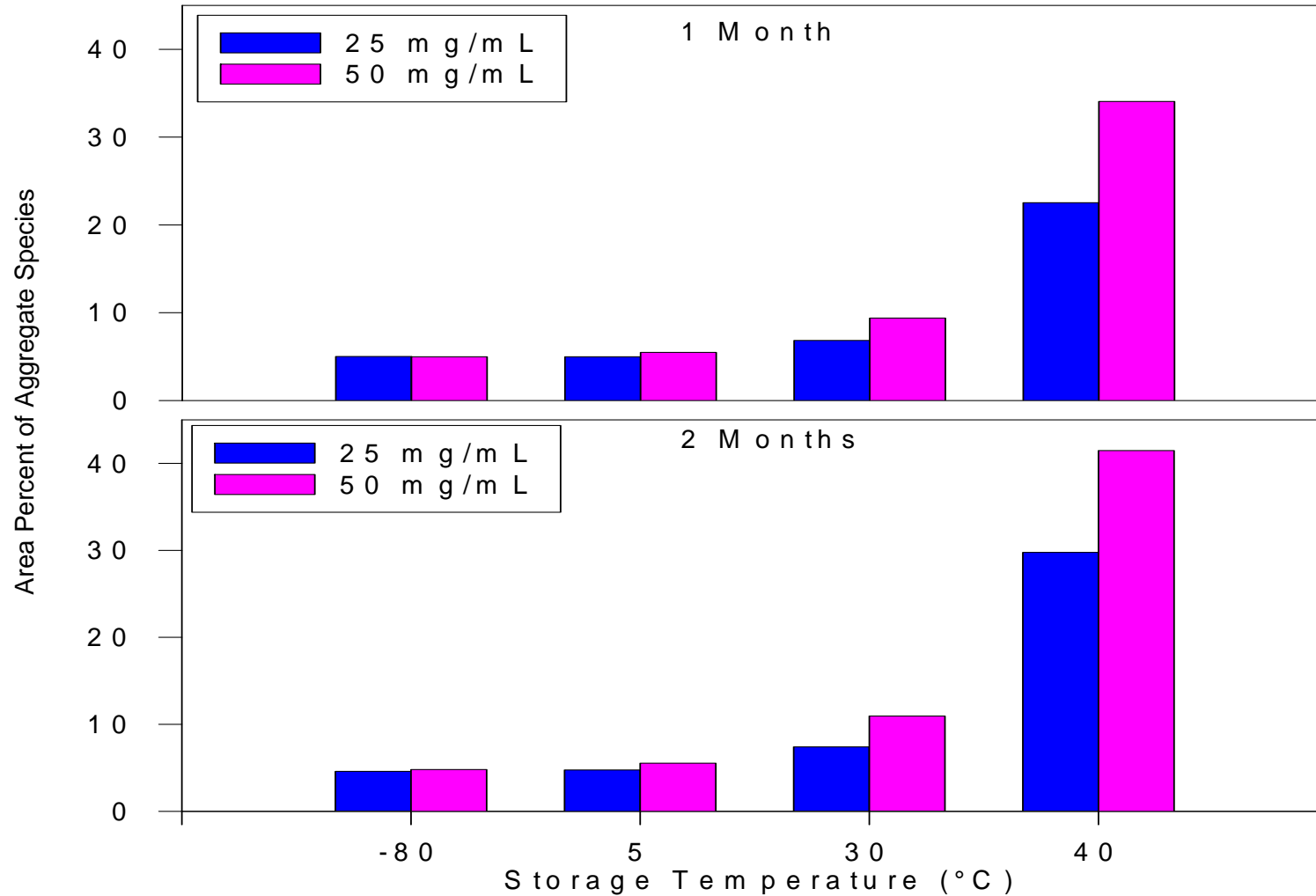
Detailed studies are undertaken to evaluate chemical and physical stability of various lots during process development.

- Integration of clinical requirements, e.g., dose & route of administration

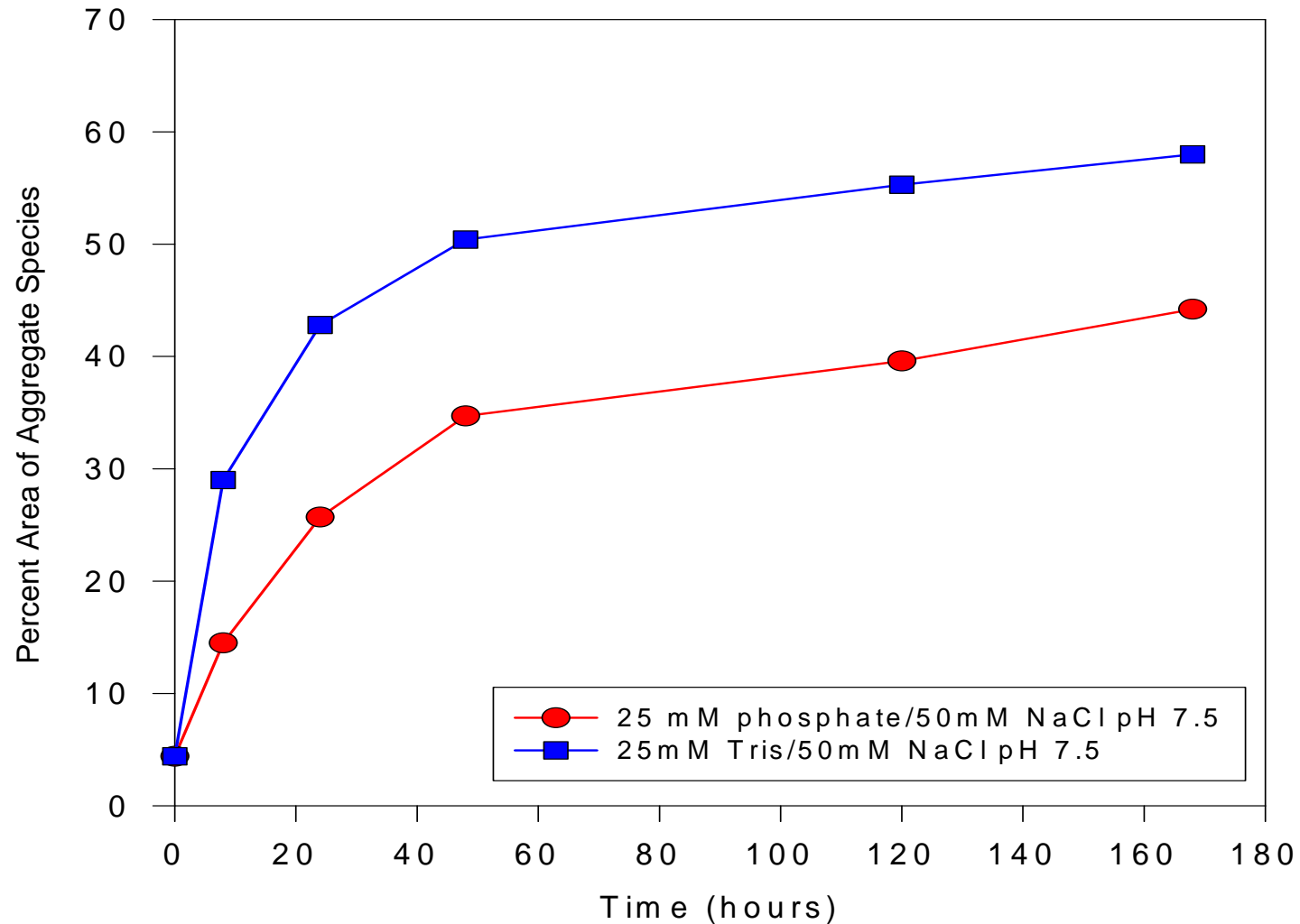
Evaluation of parameters:

- pH
- Temperature
- Buffer/salt
- Protein concentration
- Agitation/vortexing
- Multiple freeze/thaw

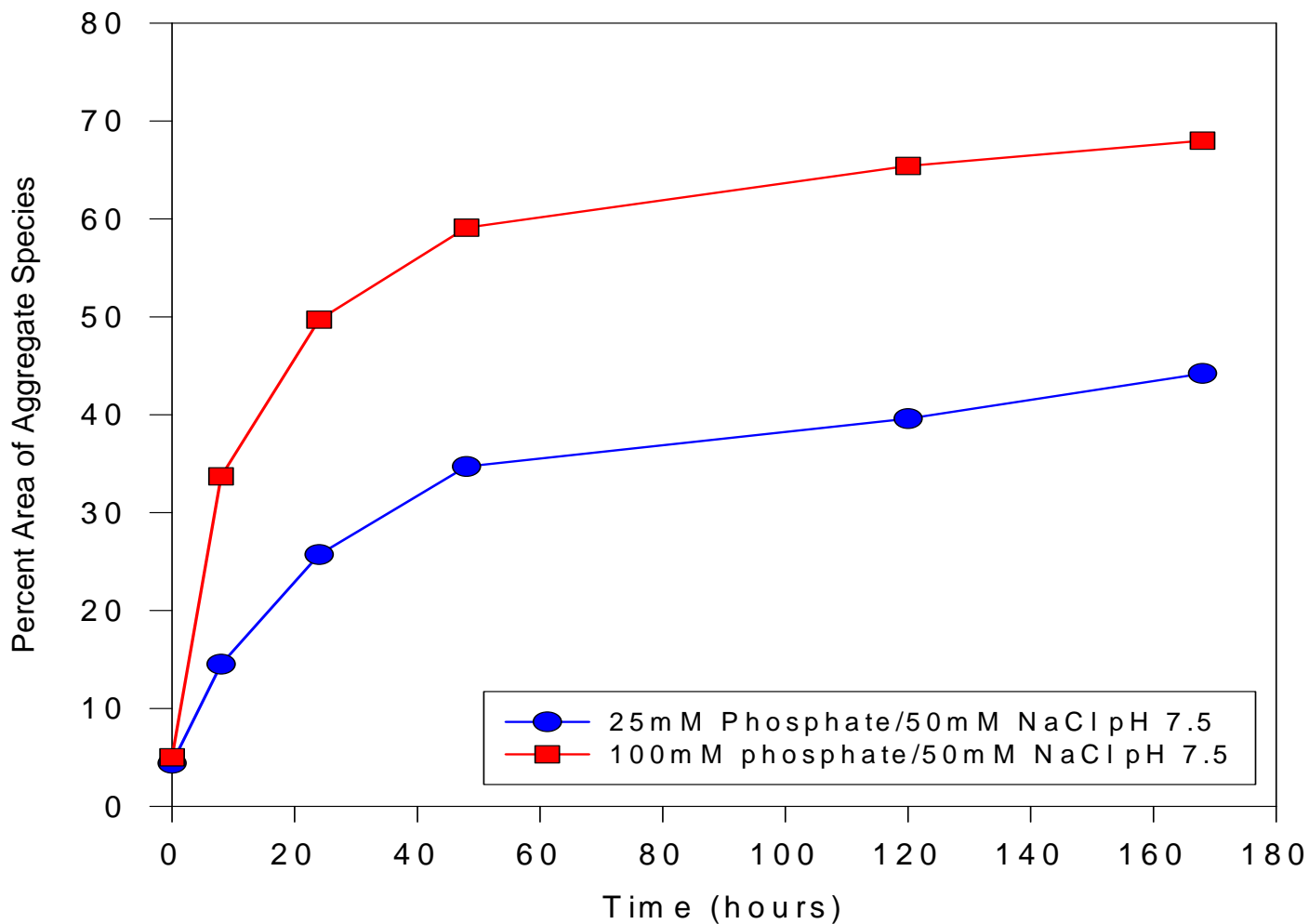
Effect of Temperature on Solution Stability of Protein I



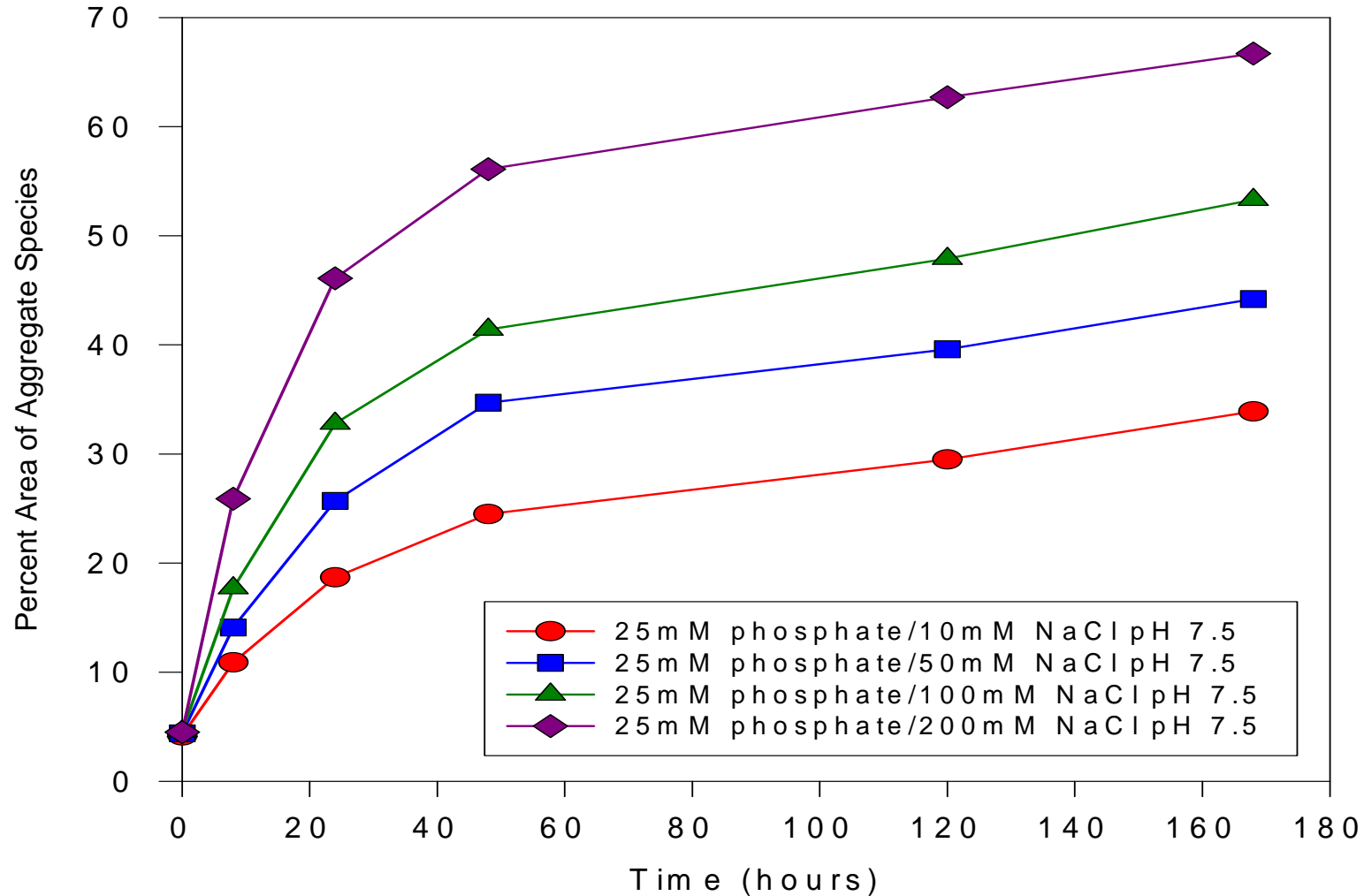
Effect of Buffer Type on Solution Stability (5 mg/mL) of Protein I at 50°C



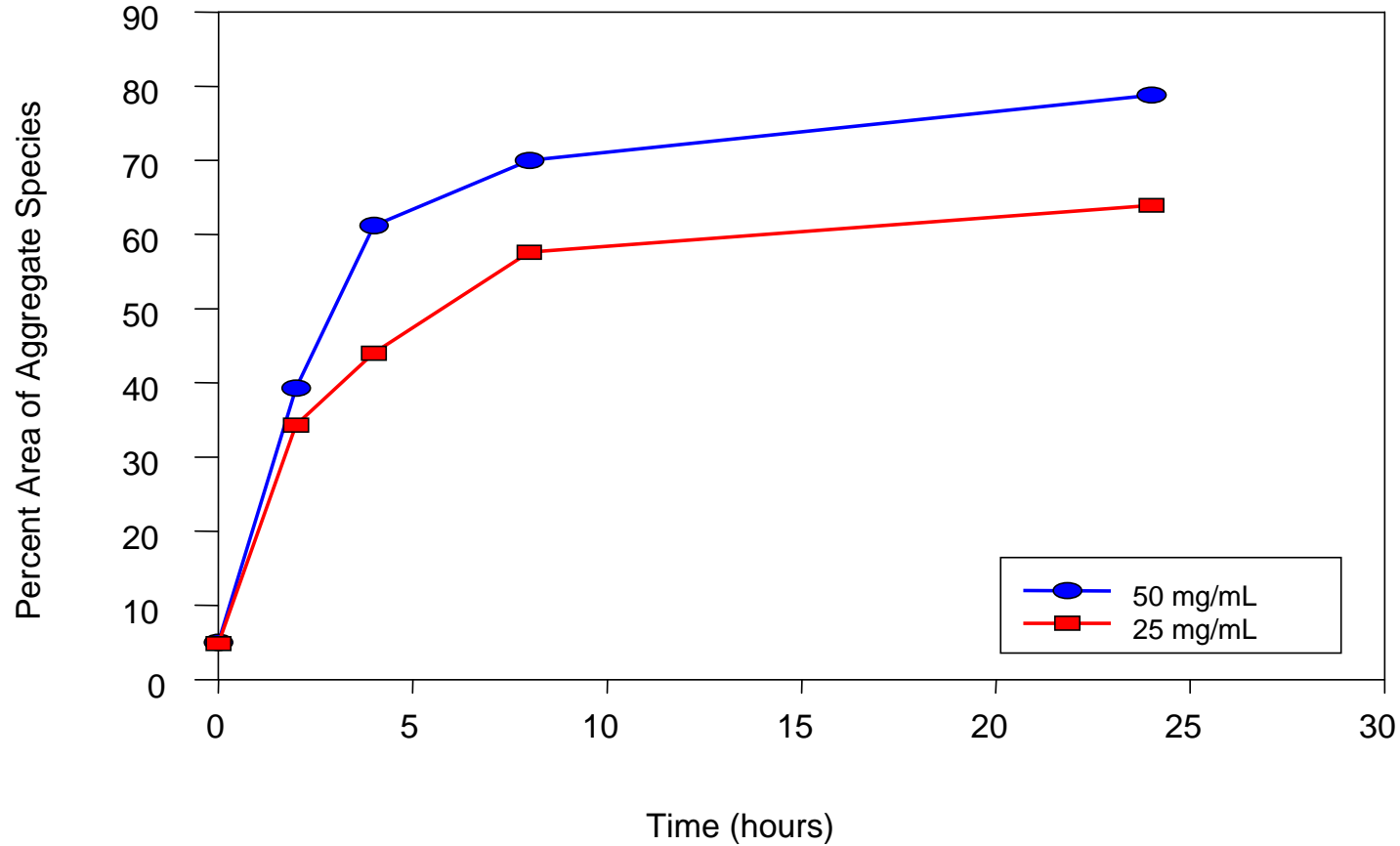
Effect of Buffer Concentration on Solution Stability (5 mg/mL) of Protein I at 50°C



Effect of Sodium Chloride Concentration on Solution Stability (5 mg/mL) of Protein I at 50°C



Effect of Protein Concentration on Solution Stability of Protein I at 50°C



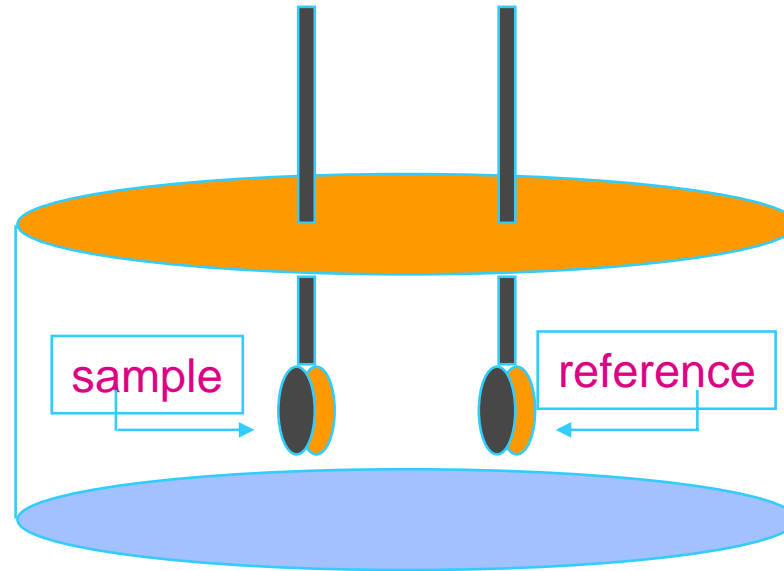
Effect of Agitation and Vortexing on Solution Stability (6.6 mg/mL) of Protein I

<i>Conditions</i>	<i>Percent Remaining</i>	<i>Total Aggregates Percent Area</i>
<i>Control</i>	<i>100</i>	<i>4.6</i>
<i>100 RPM/22°C for 24 hours</i>	<i>100.4</i>	<i>4.8</i>
<i>Vortex for 30 sec</i>	<i>97.4</i>	<i>4.8</i>
<i>Vortex for 60 sec</i>	<i>100.6</i>	<i>5.3</i>

Findings of Stability Studies

- *Based on SEC, Protein I is most stable in the pH range of 7 to 8*
- *Aggregation is the predominant pathway at elevated temperature and is highly pH dependent*
- *Increase in sodium chloride, sodium phosphate, or protein concentration promotes aggregation.*
- *Agitation, vortexing or multiple freeze-thaw cycling of bulk Protein I does not appear to induce any aggregation or degradation.*
- *The recommended buffer for handling and storage of Protein I is 25 mM sodium phosphate/10 mM sodium chloride pH 7.5.*

Alternate Technique: Microcalorimetry



- Measures energy required to maintain sample cell at the same temperature as reference cell.
- Sample energy changes are associated with thermal transitions such as protein denaturation, aggregation, and precipitation.
- Measurements require approximately 0.5-1 mg sample.

Solution Stability Studies

Conditions investigated:

- 1) pH (range 4-9)
- 2) Temperature (SEC studies) (5-50°C, -70°C control)
- 3) Excipients (Protein I)*
 - Sucrose
 - Maltose
 - L-Arginine HCl

*Buffer composition for Protein I excipient screening studies was 25 mM sodium phosphate buffer with 10 mM sodium chloride, pH 7.5. Protein-to-excipient ratio was 1:2.

Analysis:

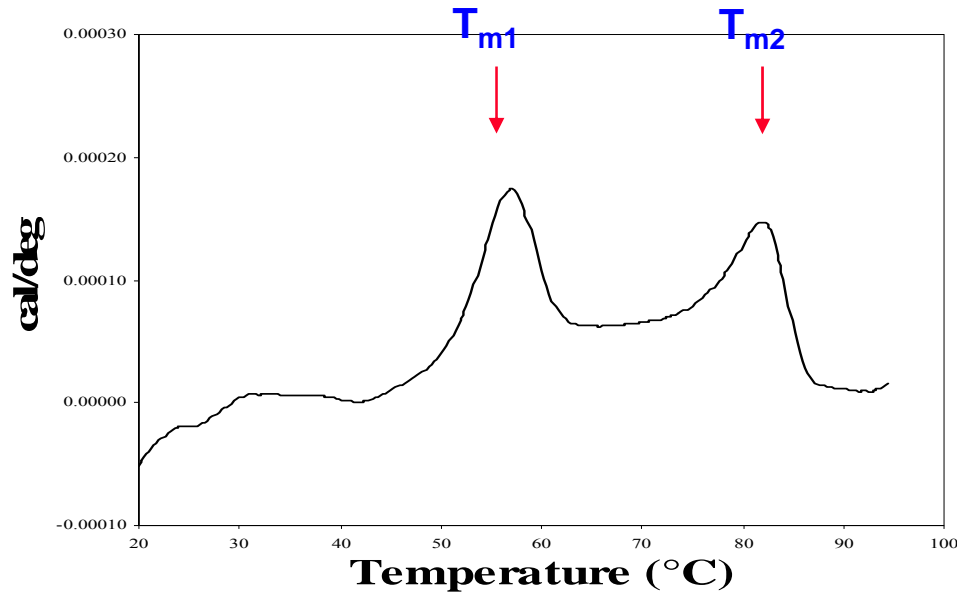
1. Microcalorimetry

Microcal VP-DSC microcalorimeter
Temperature Range: 25-90°C
Scan Rate: 1°C/min
Sample Volume: 0.5 mL

2. SEC

Column: TSK Gel G3000 SWXL (30 cm x 7.8 mm i.d., 5 µm)
Mobile Phase: 0.2 M KH₂PO₄ w/ 0.9% NaCl, pH 6.8
Flow Rate: 1 mL/min
Injection Volume: 10 µL
UV Detection: 280 nm

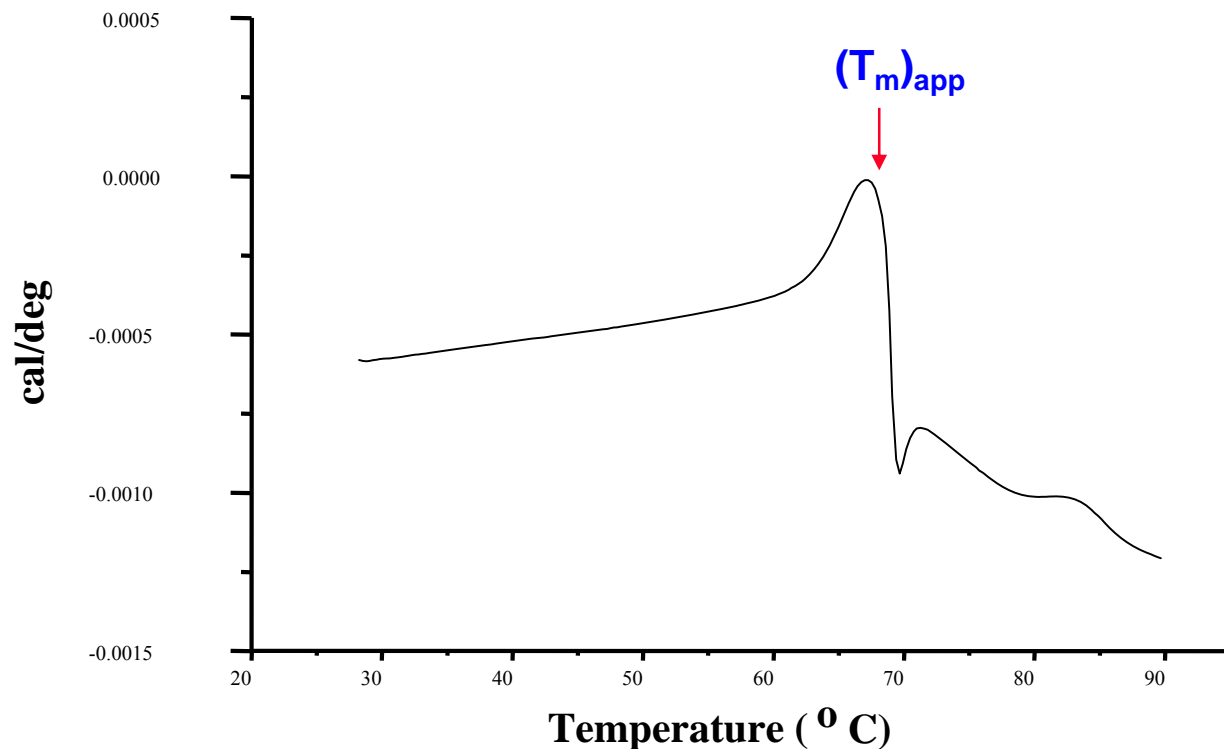
Typical Microcal DSC Thermogram for Protein I



Scans for Protein I showed two endothermic transitions, T_{m1} and T_{m2} . Based on denaturing SEC and non-reducing SDS-PAGE, T_{m1} has been identified as the transition of Protein I from its native, monomeric conformation to a non-covalent dimer. T_{m2} has not yet been characterized.

Protein stabilization is indicated by a shift of T_{m1} to higher temperatures.

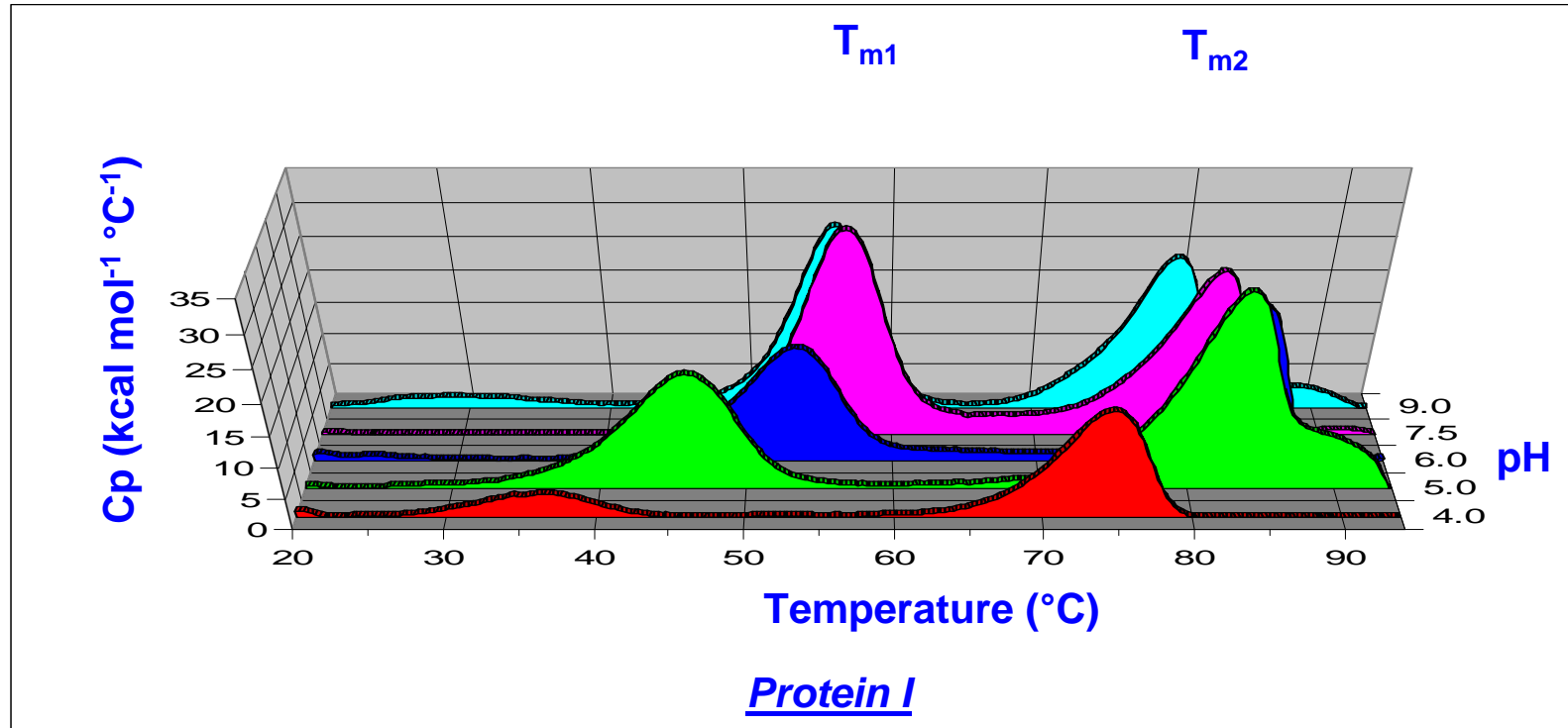
Typical Microcal DSC Thermogram of Protein II



Scans for Protein II showed a single endothermic transition $(T_m)_{app}$, likely indicating protein denaturation, followed by aggregation and precipitation from solution.

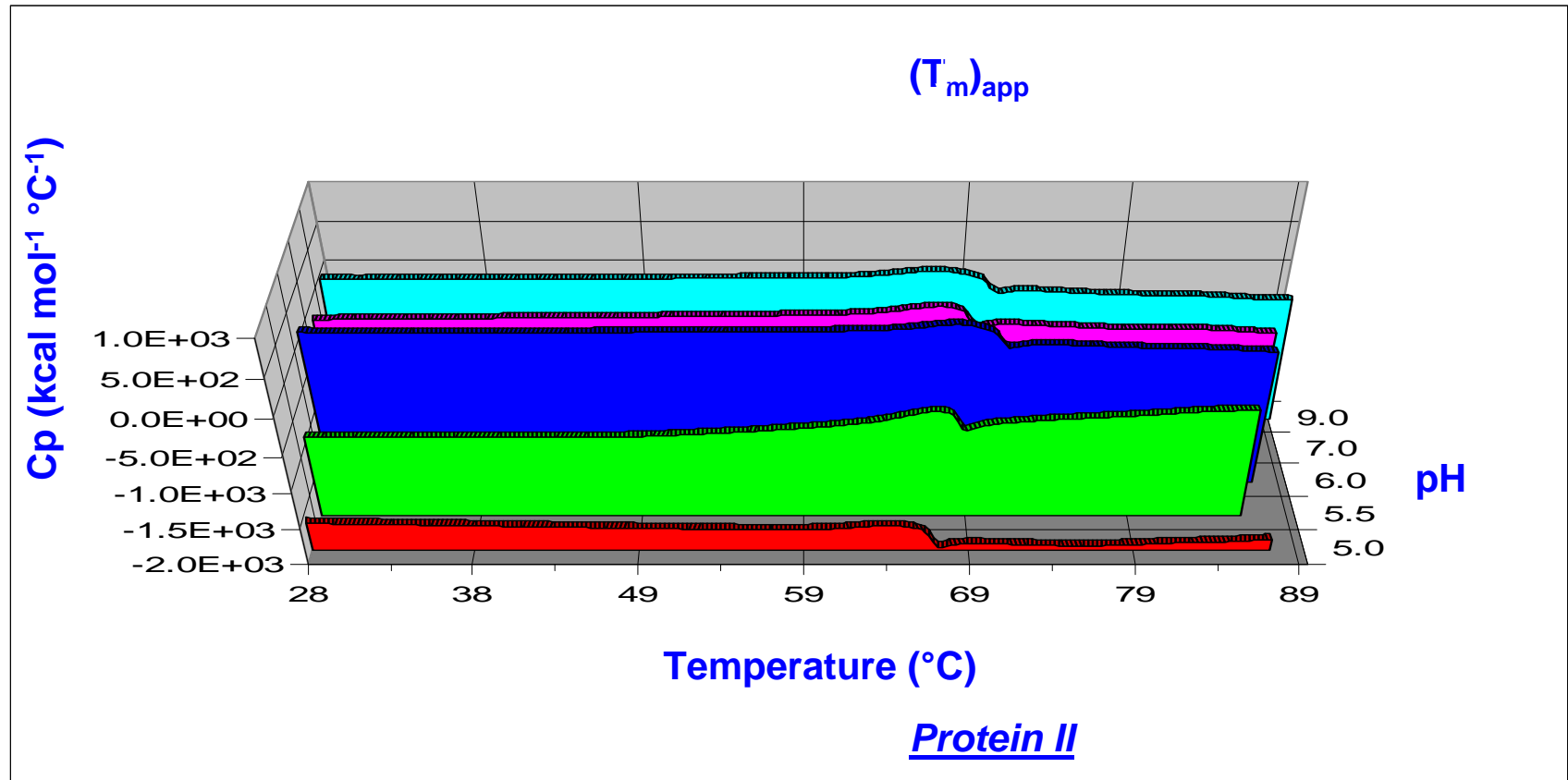
Protein stabilization is reflected by a shift of the $(T_m)_{app}$ value to higher temperatures.

Effect of Solution pH on Physical Stability of Protein I by Microcalorimetry



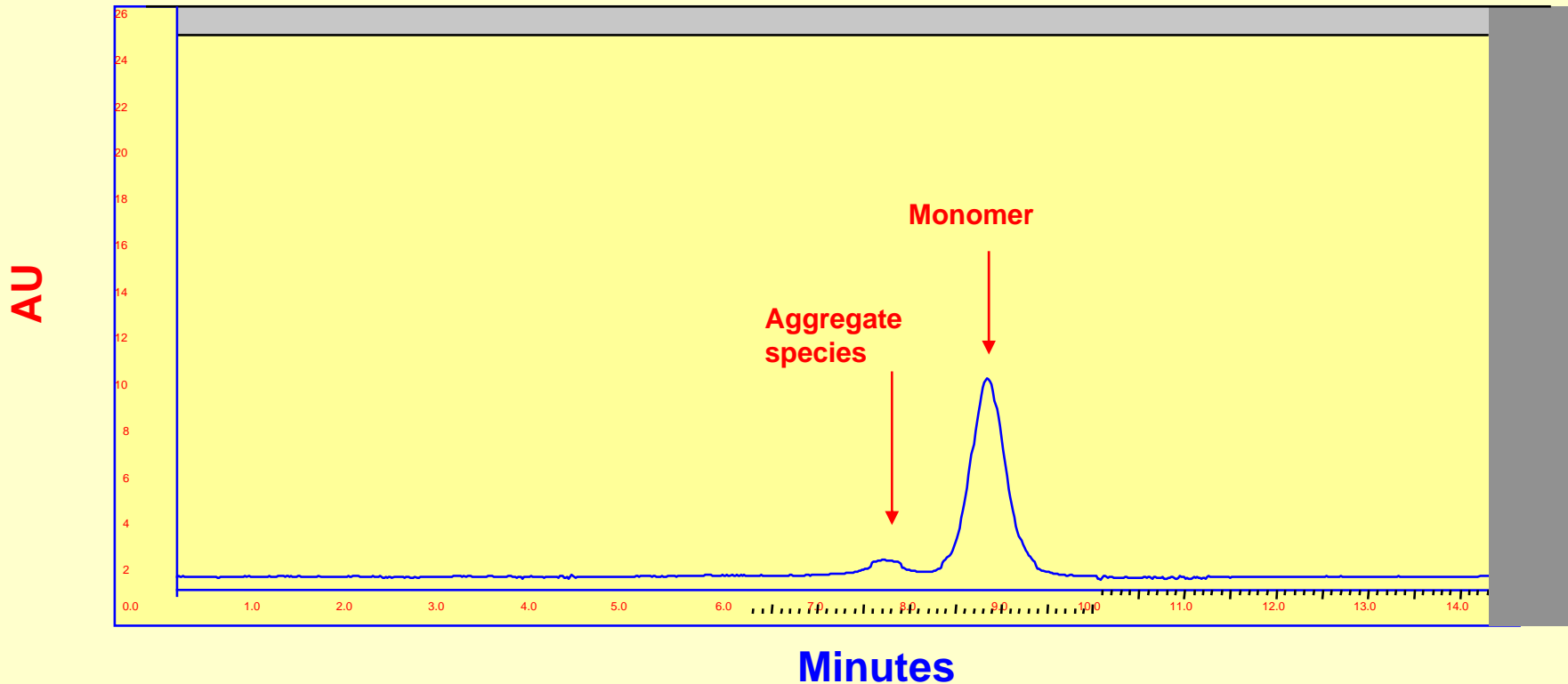
The value of T_{m1} was pH-dependent, and maximum solution stability for Protein I was obtained at approximately pH 7.5.

Effect of Solution pH on Physical Stability of Protein II by Microcalorimetry



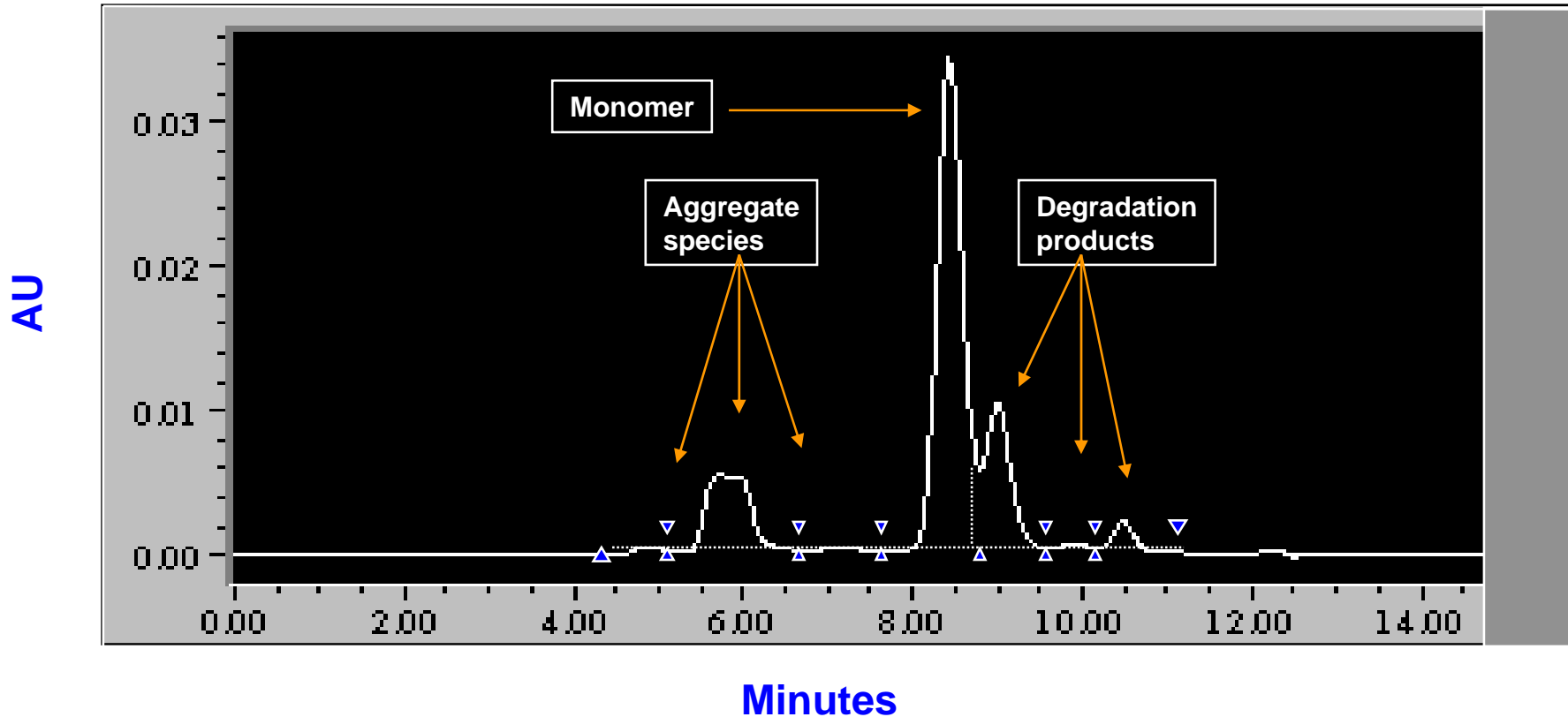
The value of T_m was pH-dependent, and maximum solution stability for Protein II is obtained at approximately pH 6.

Typical SEC Chromatogram for Protein I Solution Stored at 50°C, pH 8, for 2 Days



The major route of degradation of Protein I at elevated temperature is aggregation. Aggregate formation increases with decreasing solution pH and is maximal below pH 5. Small amounts of low molecular weight degradation products resulting from peptide backbone cleavage are observed at pH values above 6.

Typical SEC Chromatogram for Protein II Solution Stored at 50°C, pH 9, for 2 Weeks

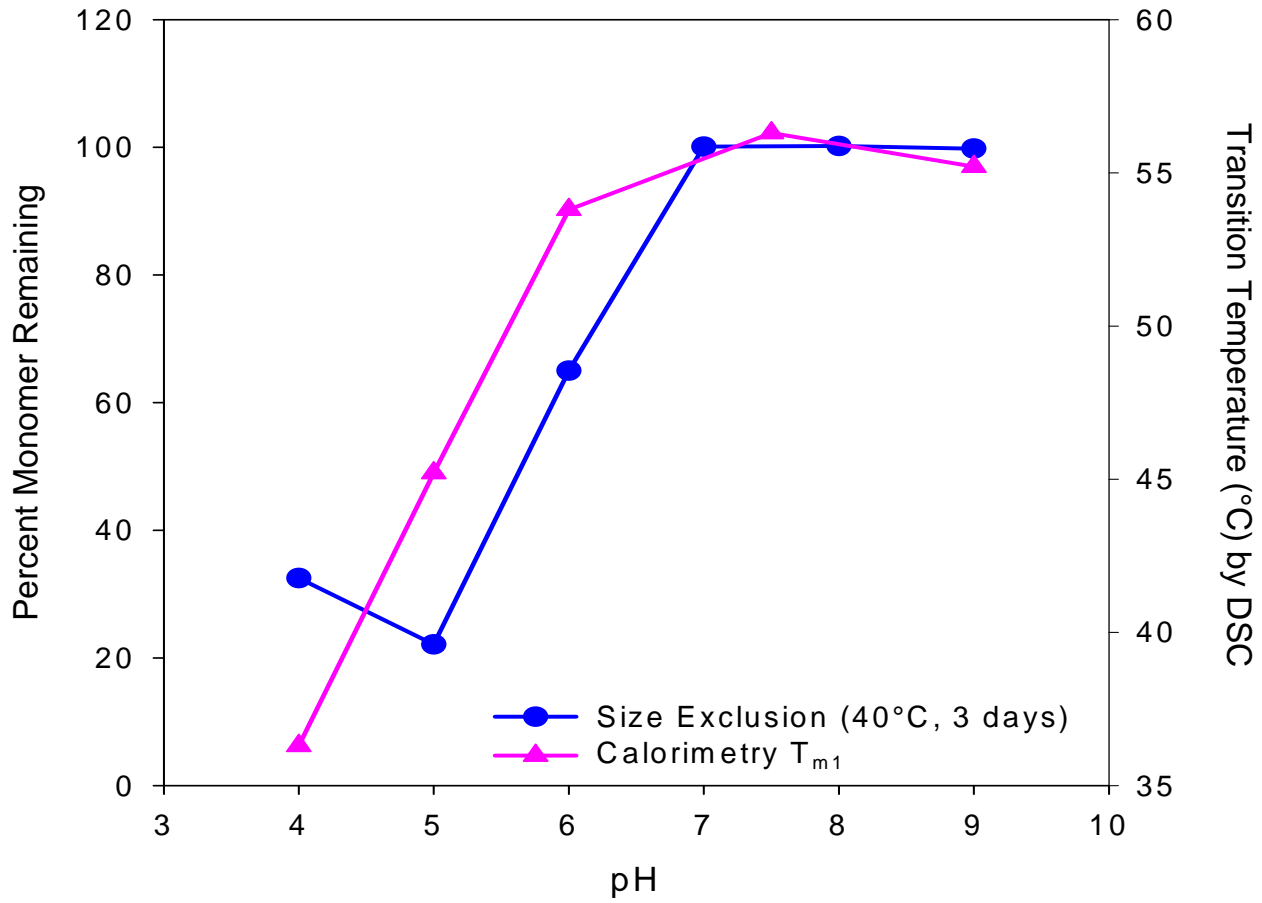


Both aggregates and degradation products are observed by SEC at pH below 5 and above 8.

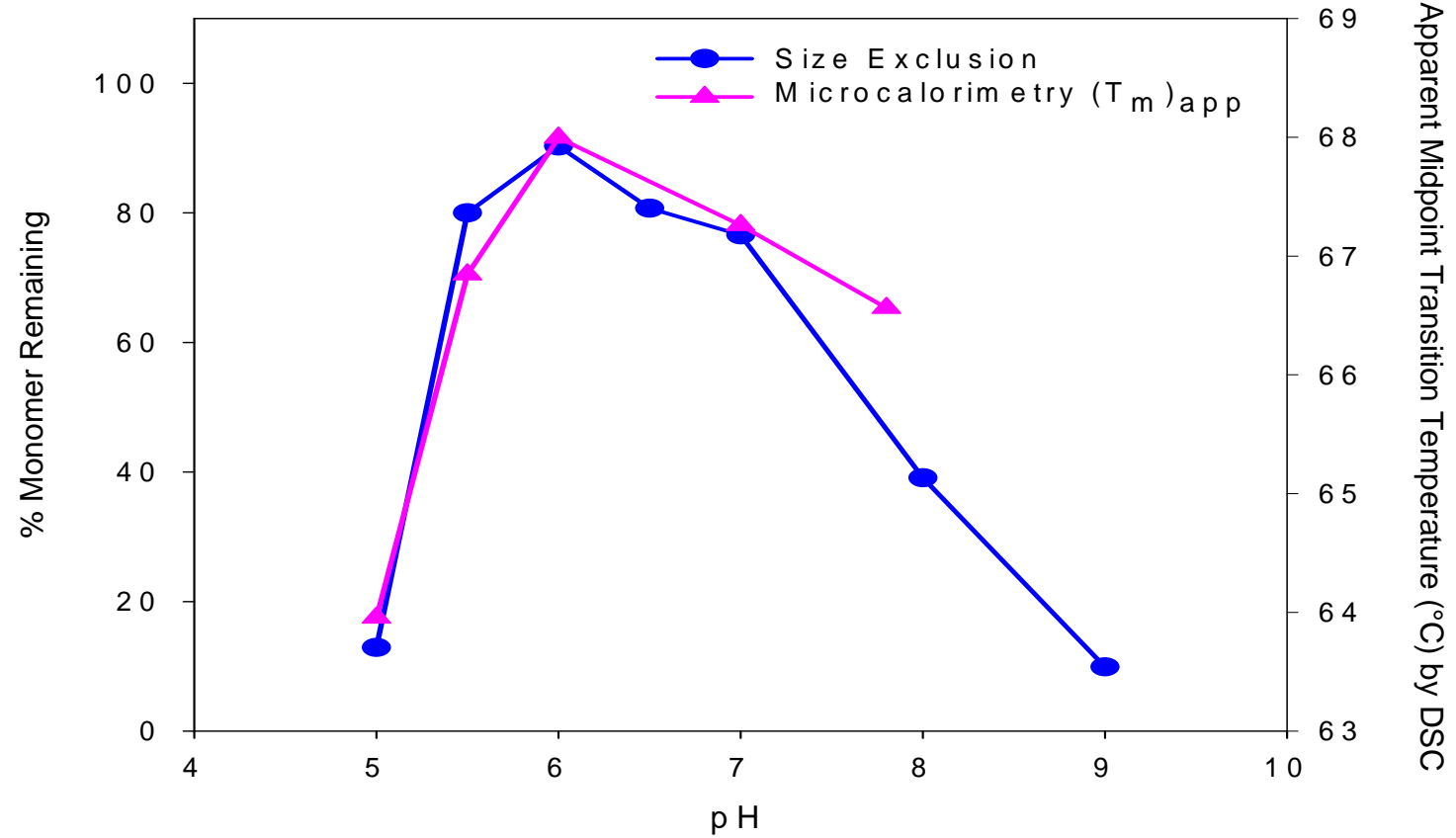
Formation of aggregate species of varying molecular weight increases with decreasing solution pH and is maximal below pH 5.

Degradation to low molecular weight fragments becomes significant above pH 8.

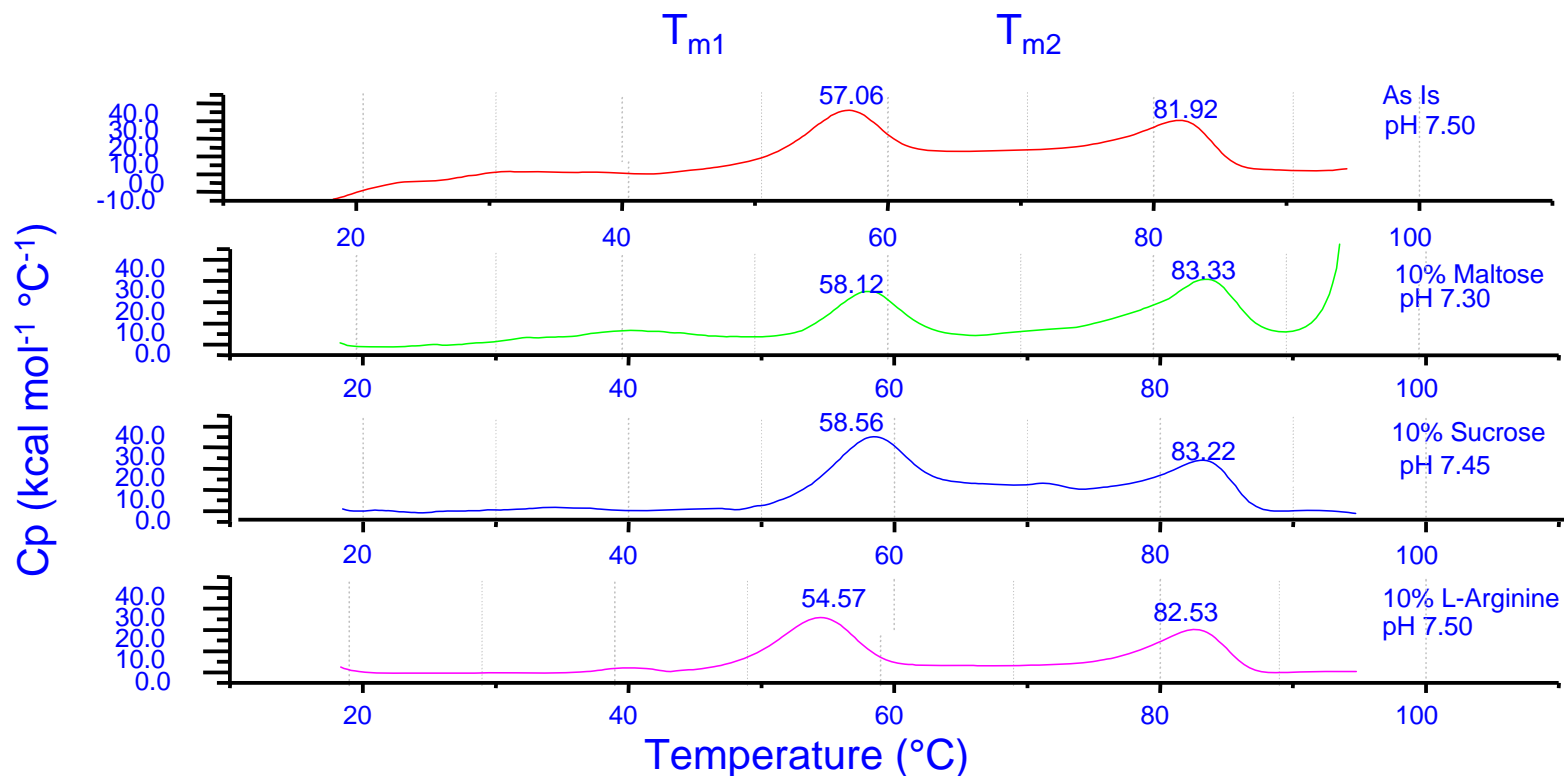
Comparison of Microcalorimetry to SEC Protein I



Comparison of Microcalorimetry to SEC Protein II



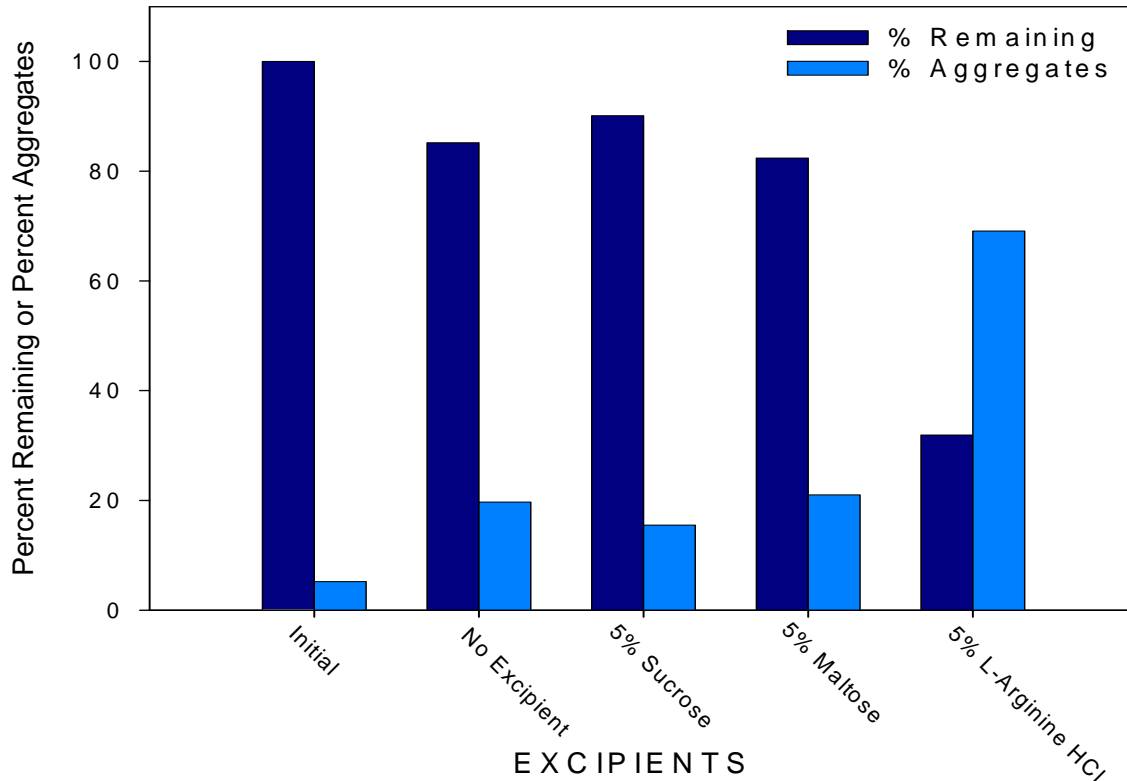
Effect of Excipients on Solution Stability of Protein I by Microcalorimetry



Solution composition: 1 mg/mL Protein I in 25 mM sodium phosphate buffer, pH 7.5, with 10 mM NaCl.

Similar to SEC results, microcalorimetry data indicated that sucrose increased the stability of Protein I, while L-Arginine HCl destabilized the Protein Is indicated by shifts in the position of T_{m1} .

Effect of Excipients on Solution Stability of Protein I by Size Exclusion Chromatography



Solutions stored for two weeks at 40°C under the following conditions:

- 25 mM sodium phosphate buffer, pH 7.5, with 10 mM NaCl
- 25 mg/mL Protein I

- Based on SEC data, sucrose showed greatest ability to stabilize Protein I in solution under stress conditions while L-arginine HCl showed a destabilizing effect as indicated by significant increase in aggregate levels.

Optimal Buffer Compositions for Protein I and Protein II

Based on SEC and microcalorimetry results, along with additional data collected to investigate the effects of buffer type, buffer concentration, and salt concentration the optimal buffer composition for storage and handling of Protein I and Protein II bulk solutions are:

- **Protein I:** 25 mM sodium phosphate buffer, pH 7.5, with 10 mM NaCl
- **Protein II:** 5 mM sodium citrate buffer, pH 6.0, with 25 mM NaCl

Conclusions

- The pH of maximum stability predicted by microcalorimetry corresponded well to that predicted by longer-term SEC studies for both proteins.
- Both microcalorimetry and SEC techniques indicated that sucrose stabilized Protein I in solution, while L-Arginine destabilized the protein.
- Results of these studies suggest that microcalorimetry can be a useful tool for rapid screening of protein stability in solution, particularly where aggregation is the predominant pathway for degradation.
- Use of microcalorimetry to conduct early solution stability investigations may allow for more efficient design of longer term stability studies for biomolecules.

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