Shelf life of protein-based pharmaceuticals

Definition:

- Shelf life is the time period during which a drug product is expected to remain within the approved shelf life specification, provided that it is stored under the conditions defined on the container label. (according to ICH).

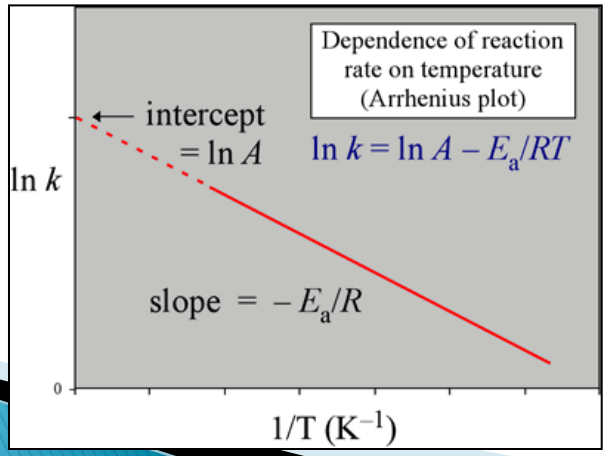
Proteins can be stored

- (1) as an aqueous solution,
- (2) in freeze-dried form, and
- (3) in dried form in a compacted state (tablet).

- Protein aggregation presents one of the key challenges in the development of protein biotherapeutics.
- Due to the **complexity of the aggregation process** and **temperature-dependent conformational stability**,

temperature-induced protein aggregation is often **non-Arrhenius** over even relatively small temperature windows relevant for product development,

and this makes **low-temperature extrapolation difficult** based simply on accelerated stability studies at high temperatures.



Does not work very well with protein therapeutics!!

Shelf life of protein drugs

- Protein therapeutics shelf life should be derived from representative **real time** / **real conditions** stability studies

- While accelerated studies can be:
  - only supportive to establish shelf life
  - generate help to elucidate the degradation profile

Protein stability

- Although freshly isolated proteins may be folded in to a distinct three-dimensional structure, this fold structure is not necessarily retained indefinitely in aqueous solution.

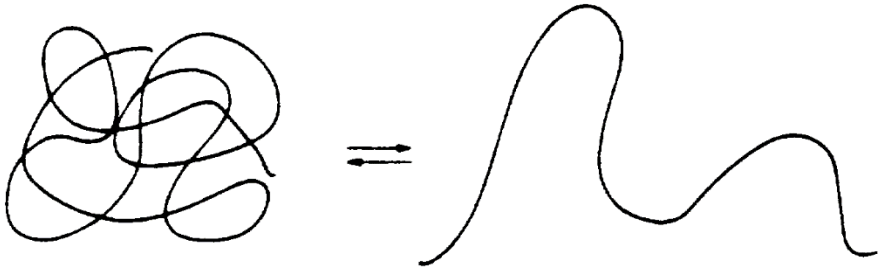


Figure 9-3 Two-state model of protein folding–unfolding.

- The reason is that proteins are neither chemically nor physically stable.
- The protein surface is chemically highly **heterogeneous** and contains **reactive groups**. Long-term exposure of these groups to **environmental stresses** causes various chemical alterations.

Common reactions affecting the stability of proteins

	Physical property effected	Method of analysis
Oxidation	Hydrophobicity size	RP-HPLC, SDS-PAGE, size-exclusion chromatography, and mass spectrometry
Cys	Hydrophobicity	
Disulfide		
Intrachain		
Interchain		
Met, Trp, Tyr		
Peptide bond hydrolysis	Size	Size-exclusion chromatography SDS-PAGE
N to O migration	Hydrophobicity	RP-HPLC inactive in Edman reaction
Ser, Thr	Chemistry	
α-carboxy to β-carboxy migration	Hydrophobicity	RP-HPLC inactive in Edman reaction
Asp, Asn	Chemistry	
Deamidation	Charge	Ion-exchange chromatography
Asn, Gln		
Acylation	Charge	Ion-exchange chromatography Mass spectrometry
α-amino group, ε-amino group		
Esterification/carboxylation	Charge	Ion-exchange chromatography Mass spectrometry
Glu, Asp, C-terminal		
Secondary structure changes	Hydrophobicity	RP-HPLC
	Size	Size-exclusion chromatography
	Sec/tert structure	CD
	Sec/tert structure	FTIR
	Aggregation	Light scattering
	Sec/tert structure, Aggregation	Analytical ultracentrifugation

Oxidative reactions

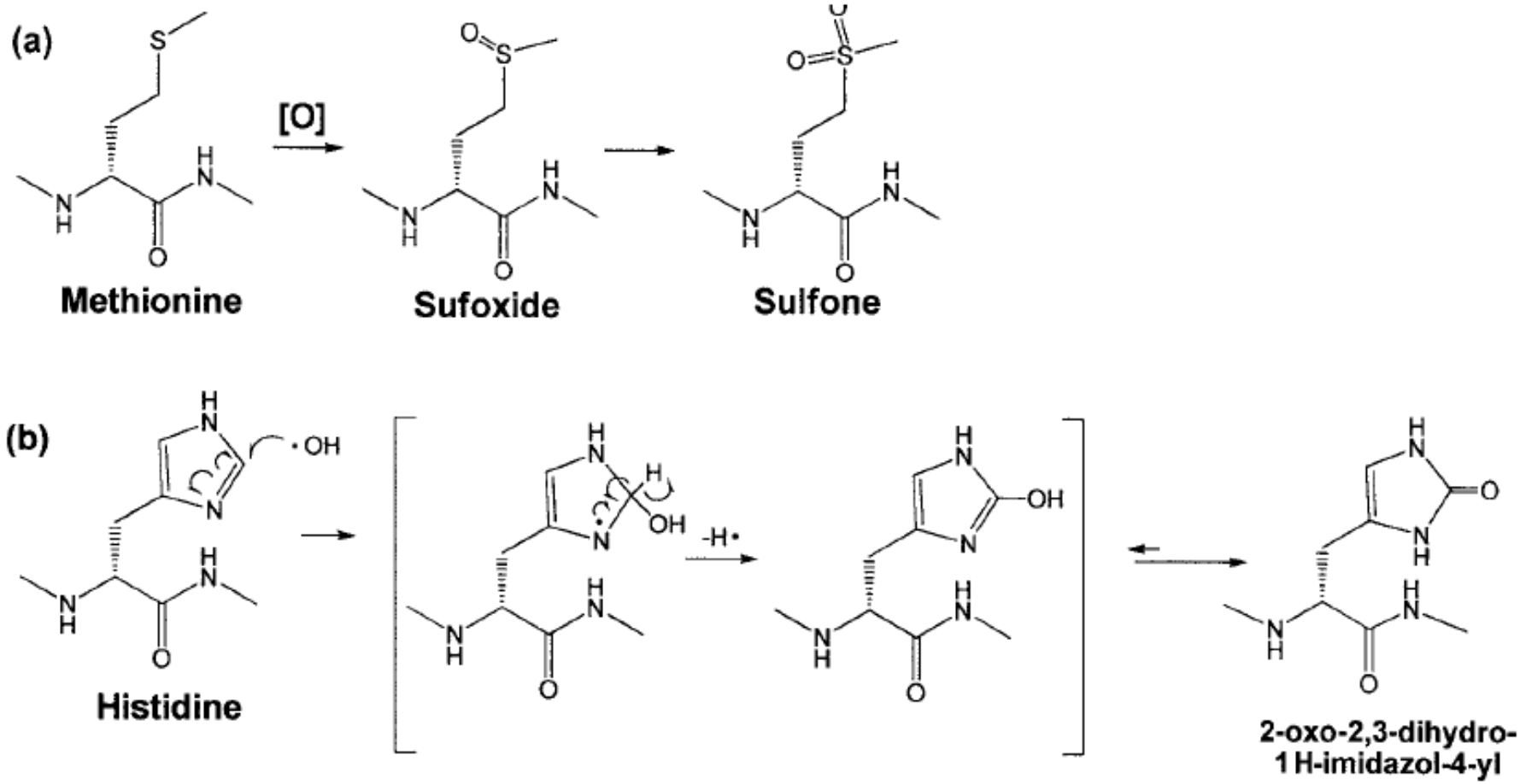


Fig. 21–30. (*a*) The oxidation of methionine to form sulfoxide and sulfone. (*b*) Oxidation of a histidine residue by hydroxyl radicals to form oxo-imidazole.

Source: Martin's physical pharmacy, 6th edition

Disulfide bond exchange

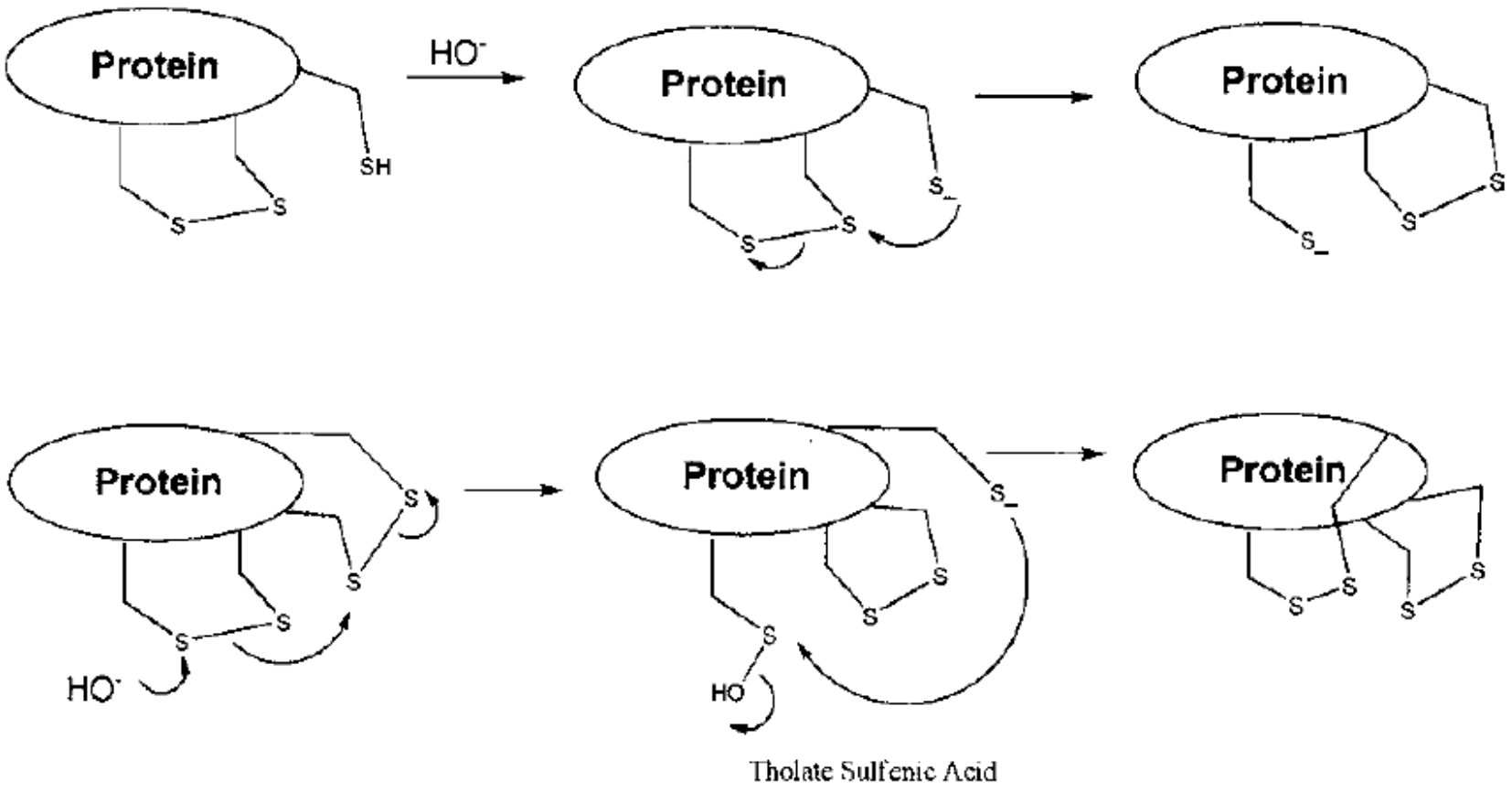


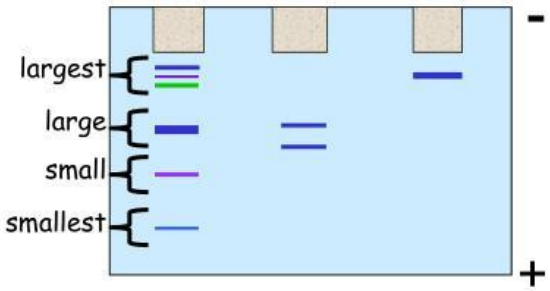
Fig. 21–29. Disulfide bond exchange in proteins under basic conditions.

Source: Martin's physical pharmacy, 6th edition

SDS-PAGE analysis

How does SDS-PAGE work?

- Proteins (negatively charged due to SDS) move to positive electrode
- Proteins separate by size
- Smaller proteins move faster



Source: https://www.slideserve.com/lynna/electrophoresis-sds-page

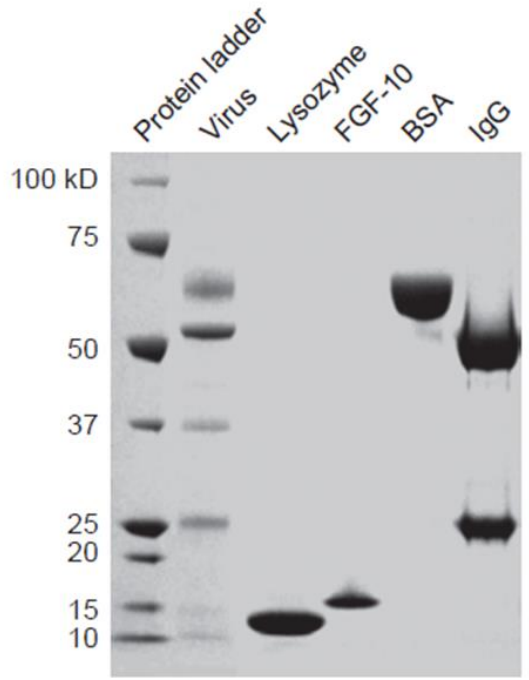
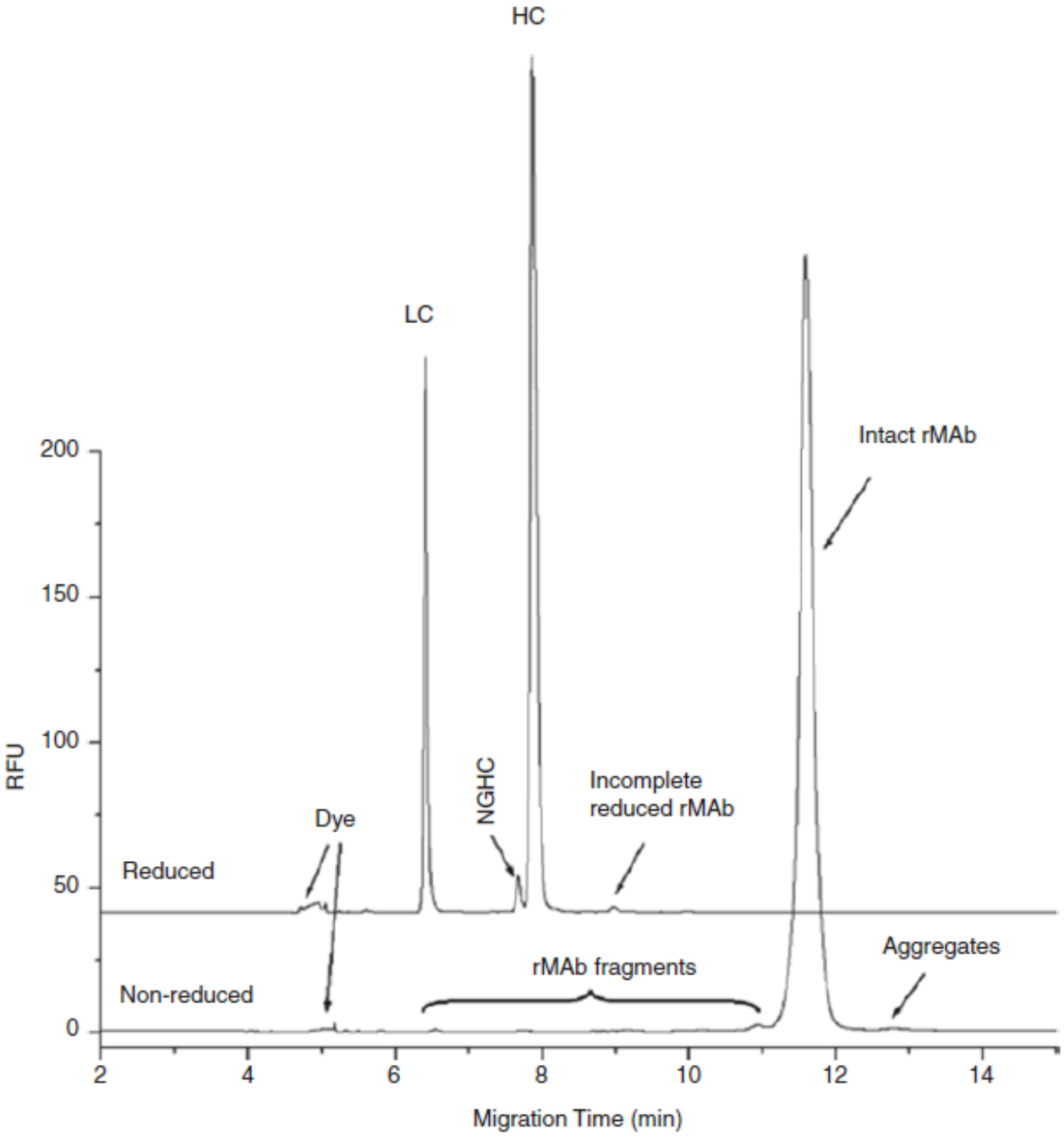


Fig. 21–7. SDS-PAGE of representative biopharmaceuticals under reducing conditions employing a gradient gel. As expected, the virus produces multiple protein components and the IgG, two bands corresponding to the heavy and light chains. Molecular weights can be estimated by comparison to the known standards present in the left-most lane.

Source: Martin's physical pharmacy, 6th edition

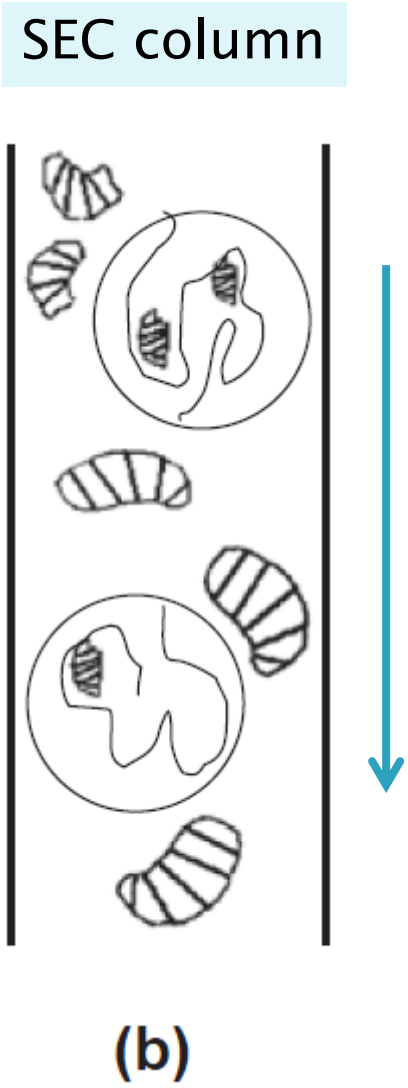
CE-SDS

- CE-SDS: A Modern Alternative to SDS-PAGE for Protein Analysis
  - Traditional slab gel SDS-PAGE is being replaced by CE-SDS due to its advantages:
  - Convenience and automation: CE-SDS is faster and easier to automate than SDS-PAGE.
  - Superior separation and reproducibility: CE-SDS provides better separation and more consistent results.
- Key differences between CE-SDS and SDS-PAGE:
  - Sieving matrix: CE-SDS uses a wider variety of flexible polymers, while SDS-PAGE uses only cross-linked polyacrylamide.
  - Detection: CE-SDS uses online UV or fluorescence detection, eliminating the need for staining and destaining steps in SDS-PAGE.
  - Analysis speed and automation: CE-SDS offers faster analysis and is more amenable to automation due to its online detection and instrument design.


HC
LC
Intact rMAb
200
150
100
50
0
RFU
Dye
NGHC
Incomplete
reduced rMAb
Reduced
Aggregates
rMAb fragments
Non-reduced
2
4
6
8
10
12
14
Migration Time (min)

Size exclusion chromatography

- (b) In size exclusion chromatography (SEC), no ligand is used but **smaller** molecules are able to diffuse into particle support interiors **slowing** their progress through the column resulting in **separations based on size.**



Source: Martin's physical pharmacy, 6th edition

N,O-Acyl Migration in Ser or Thr Residues

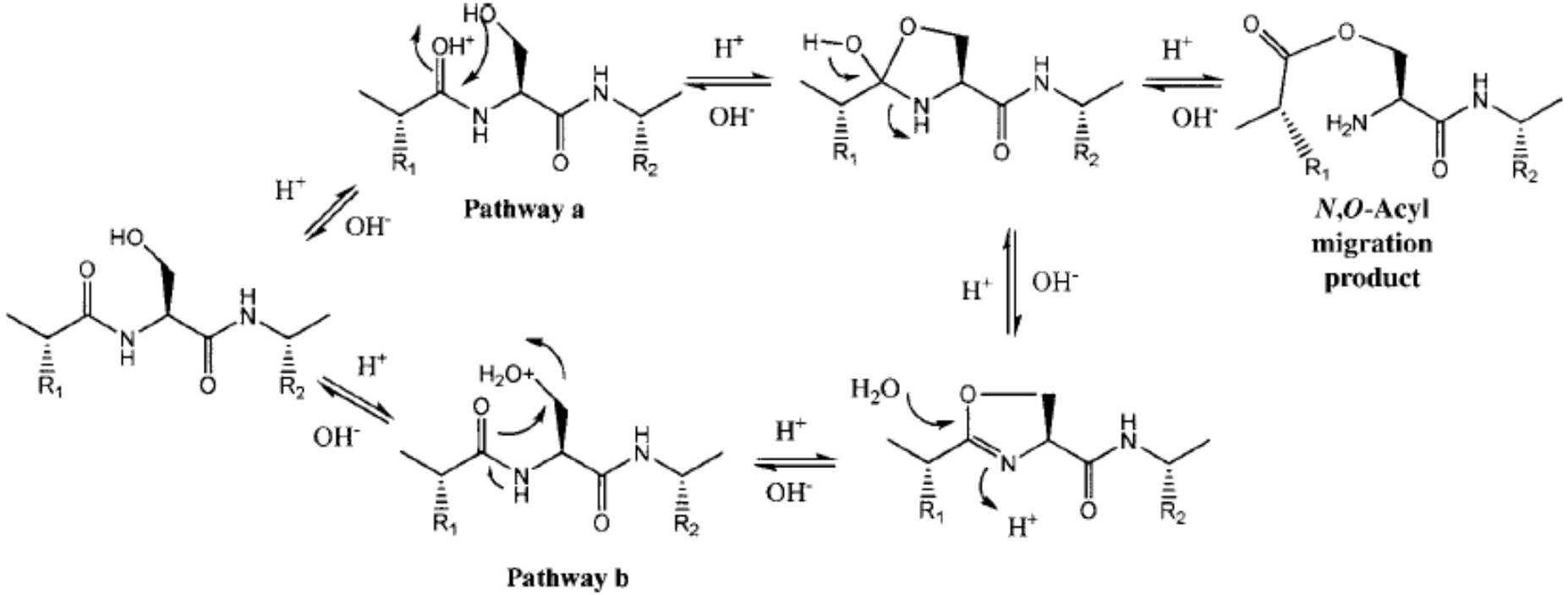


Fig. 21–27. The *N,O*-acyl migration reaction occurs at Ser and Thr residues. This reaction produces a rearrangement of the peptide backbone at the N-terminus of the side chain of the Ser or Thr residue to make an ester bond.

Source: Martin's physical pharmacy, 6th edition

Thermodynamics of protein unfolding and aggregation

- Physical stability of a protein is expressed as the difference in free energy, ΔGU, between the native and denatured states.

- Native state $\overset{\Delta G_U}{\Longleftrightarrow}$ Denatured state $\overset{k}{\Longrightarrow}$ Aggregated state

- As long as this unfolding is reversible and **ΔG_U is positive**, protein remains in native state.

$$\Delta G_U = \Delta H - T\Delta S$$

- ΔG_U is decreased (becomes negative) by heating. Why?
- Most proteins denature upon heating and subsequent aggregation of the denatured molecules results in irreversible denaturation.

Continued…

- Also, any stress that decreases ΔG_U and increases *k* will cause the accumulation of irreversibly inactivated forms of the protein.
- Such stresses may include:
  - chemical modifications (see previous table)
  - physical parameters,
    - such as pH,
    - ionic strength,
    - protein concentration,
    - in addition to temperature.

- Development of a suitable formulation that prolongs the shelf life of a recombinant protein is essential when it is to be used as a human therapeutic.

Approaches to enhance storage stability of proteins

A. **Use of protein stabilizing agents**. These compounds affect protein stability by increasing ΔG_U. These compounds, however, may also increase *k* and hence their net effect on long-term storage of proteins may vary among proteins, as well as on the storage conditions.

B. **Minimizing the irreversible step** should increase the stability, and often, this may be attained by the addition of **mild detergents**

C. **Lyophilization** can minimize the aggregation step during storage, since both chemical modification and aggregation are reduced in the **absence of water**.

Note: The effects of a lyophilization process itself on ΔG_U and *k* are **not fully understood** and hence such a process must be optimized for each protein therapeutic

- Stability of protein solutions strongly depends on factors such as **pH**, **ionic strength**, **temperature**, and the **presence of stabilizers**.

For example, Fig. 4.5 shows the pH dependence of α 1 -antitrypsin and clearly demonstrates the critical importance of pH for the shelf life of proteins.

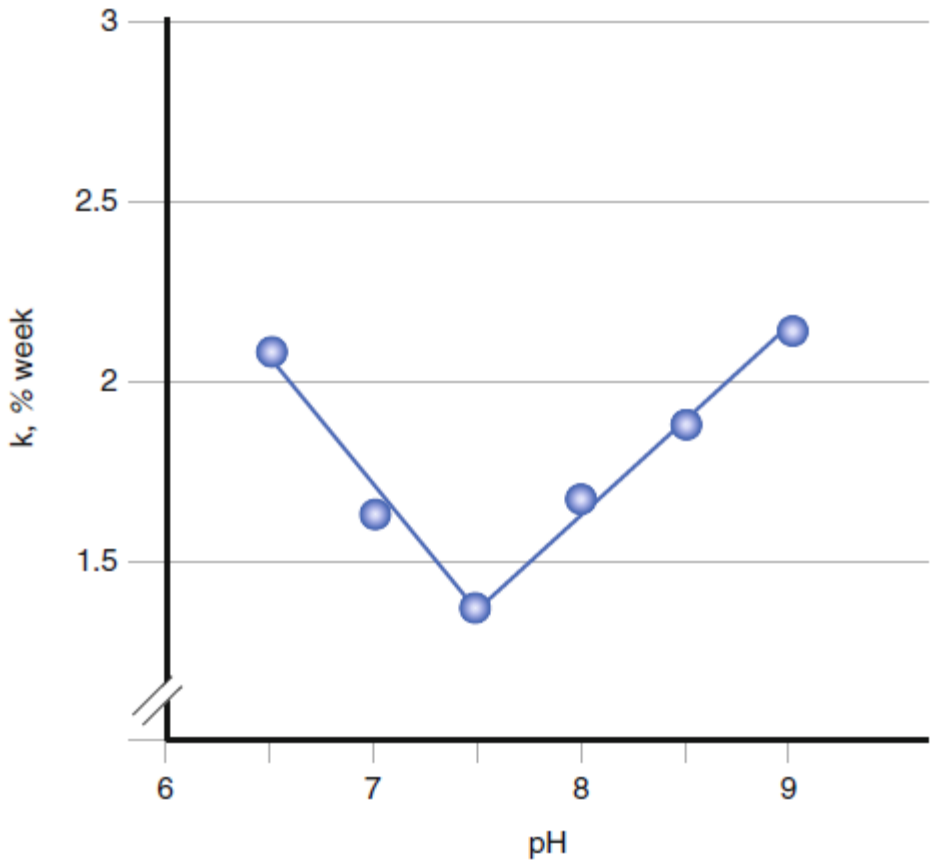


Figure 4.5 ■ pH stability profile (at 25 °C) of monomeric recombinant α_1-antitrypsin (rAAT) by size exclusion-HPLC assay, *k* degradation rate constant. Monomeric rAAT decreased rapidly in concentration both under acidic and basic conditions. Optimal stability occurred at pH 7.5 (Adjusted from Vemuri et al. (1993)).

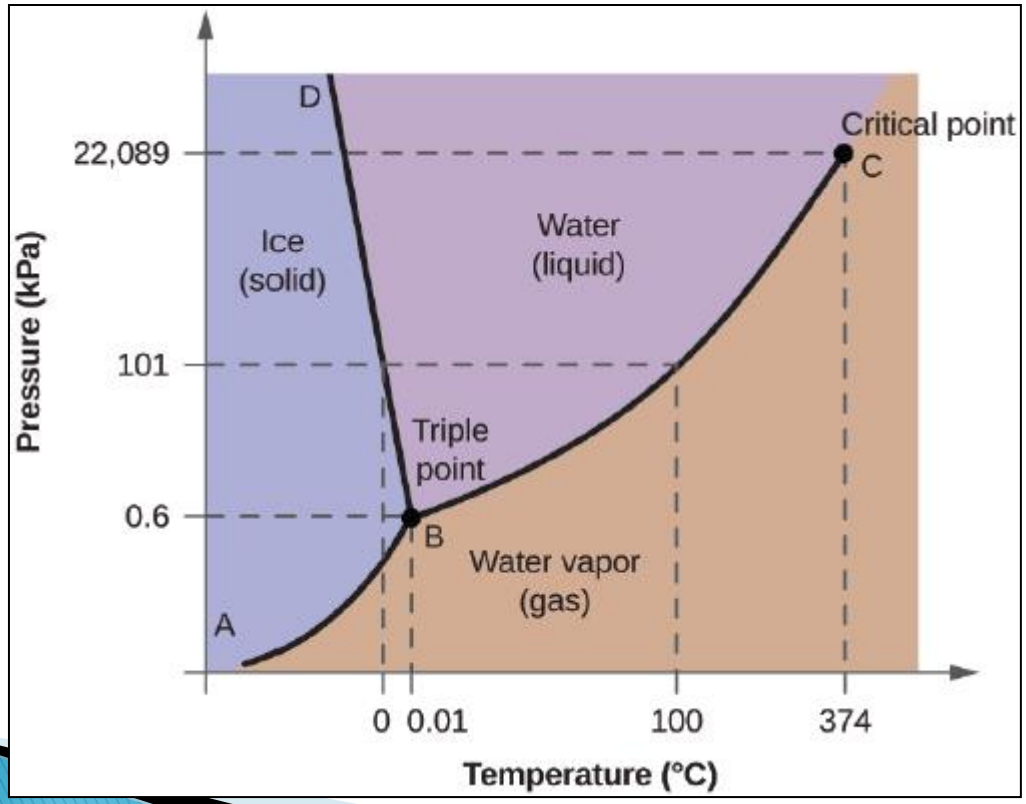
Freeze-Drying of Proteins

Freeze-drying: also known as lyophilization, is a **dehydration process** typically used to preserve a perishable material or make the material more convenient for transport. Freeze-drying works by **freezing** the material and then reducing the surrounding **pressure** to allow the frozen water in the material to **sublime directly** from the solid phase to the gas phase.

- Proteins in solution often do not meet the preferred stability requirements for industrially pharmaceutical products (>2 years), even when kept permanently under refrigerator conditions (cold chain).
- The abundant presence of **water promotes chemical and physical degradation** processes.

Lab-scale freeze-drier

- Freeze-drying may provide the desired stability by extending shelf life. During freeze-drying water is removed via sublimation and not by evaporation.
- It works by freezing the material, then reducing the pressure and adding heat to allow the frozen water in the material to sublimate.



Phase diagram for water

Source: http://www.chegg.com/homework-help/questions-and-answers/phase-diagram-water-figure

- Steps in Freeze-Drying: three stages can be discerned in the freeze-drying process:
  1. freezing step
  2. primary drying step
  3. secondary drying step.

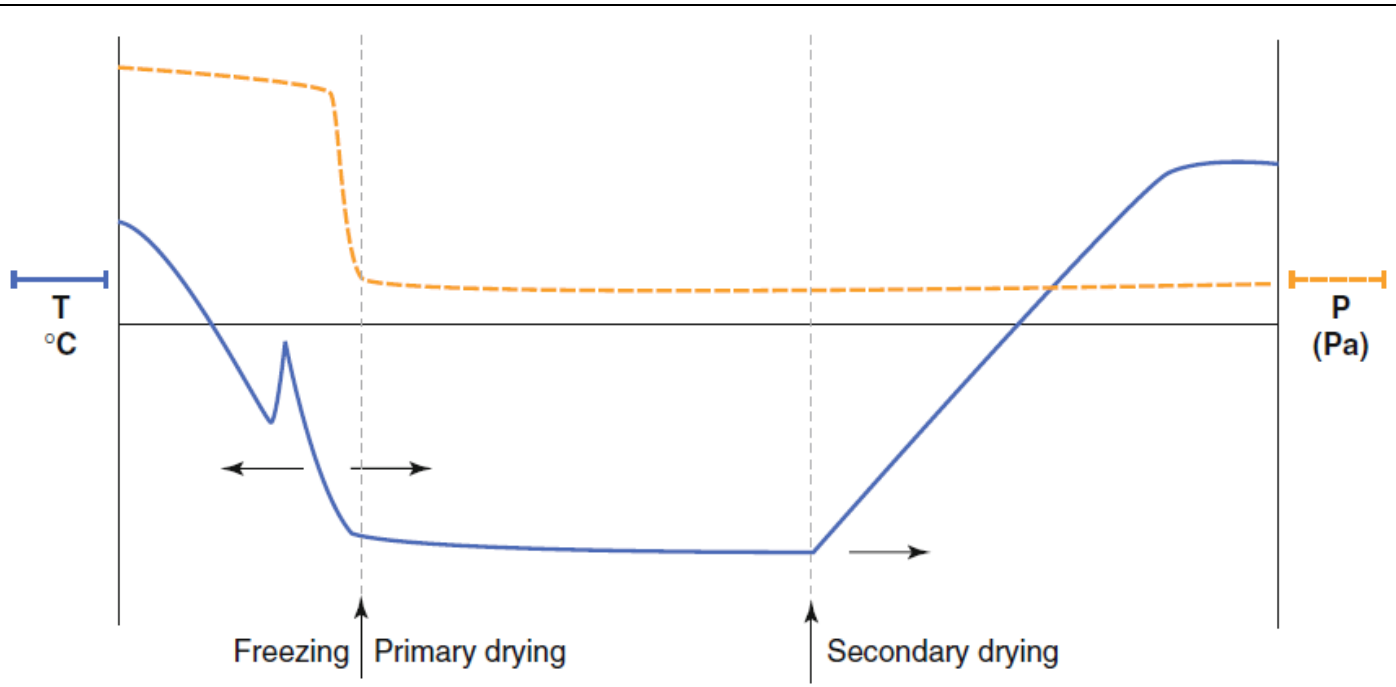


Figure 4.6 Example of freeze-drying protocol for systems with crystallizing water. *T* temperature, *P* pressure.

1- Freezing

The temperature of the product is reduced from ambient temperature to a **temperature below the eutectic temperature (Te), or below the glass transition temperature (Tg)** of the system. A Tg is encountered if amorphous phases are present.

2- Primary drying

Crystallized and **water not bound** to protein/excipients is removed by sublimation. The temperature is below the Te or Tg; the temperature is for example -40°C and reduced pressures are used.

3- Secondary drying

Removal of **water interacting with the** protein and excipients. The temperature in the chamber is kept below Tg and rises gradually, e.g., from -40°C to 20°C.

Typical excipients in a freeze-dried protein formulation

1- Bulking agents: examples are mannitol/ glycine

- Reason: elegance/ blowout prevention

Note: **Blowout** is the loss of material taken away by the water vapor that leaves the vial. It occurs when little solid material is present in the vial.

2- Collapse temperature modifier: dextran, albumin/ gelatine

- Reason: prevent increase collapse temperature.

3- Lyoprotectant: sugars, albumin

- Reason: protection of the physical structure of the protein.
- Mechanism of action of lyoprotectants is not fully understood. Factors that might play a role are:

1) **Lyoprotectants** replace water as stabilizing agent (water replacement theory),
2) Lyoprotectants increase the Tg of the cake/ frozen system- prevents collapse
3) Lyoprotectants will absorb moisture from the stoppers
4) Lyoprotectants slow down the secondary drying process and minimize the chances for overdrying of the protein.

* Overdrying might occur when residual water levels after secondary drying become too low.

Note: The freeze-drying of a protein solution without the proper excipients causes, as a rule, irreversible damage to the protein.

Vials of freeze-dried product

The product in the "Poor" vials has become soft and dense during freeze-drying, because it has become warmer than its "Critical Temperature"

Source:https://www.slideshare.net/Sophie_Koenig/use-of-freeze-drying-microscopy-to-determine-critical-parameters

Final note

- During the primary drying stage the vial content should never reach or exceed the eutectic temperature (Tc) or glass transition temperature (Tg) range. Typically a safety margin of 2–5°C is used; otherwise, the cake will **collapse.**
- Collapse causes a strong reduction in sublimation rate and poor cake formation.

Homework!

- Reducing lyoprotectants such as glucose and lactose should be avoided in protein formulation, why?

Thank you