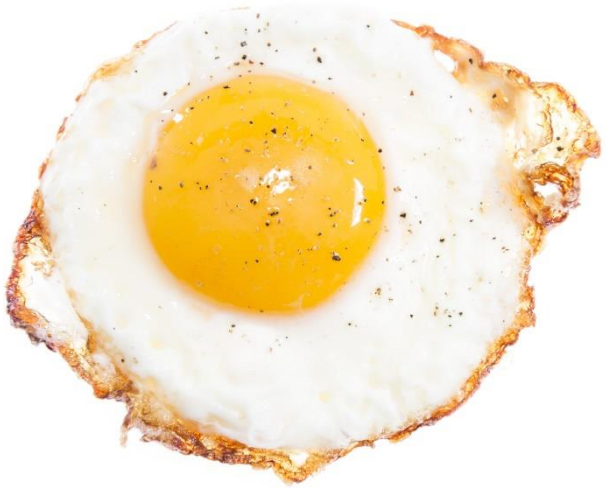
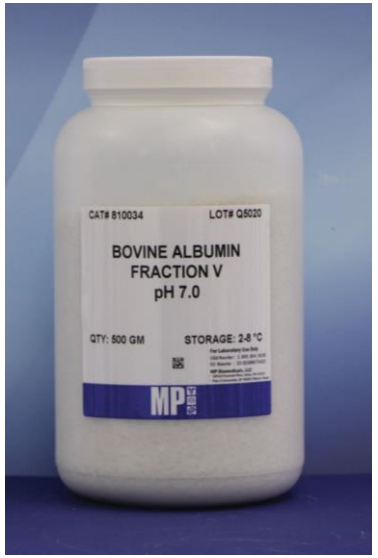
What do these items have in common?





Their preparation/production involves

the **precipitation of proteins**

Module ChE 311 Biochemical Engineering

Downstream processing

Lecture 4A - Protein precipitation and crystallization

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3.5 Preliminary observations (1/2)

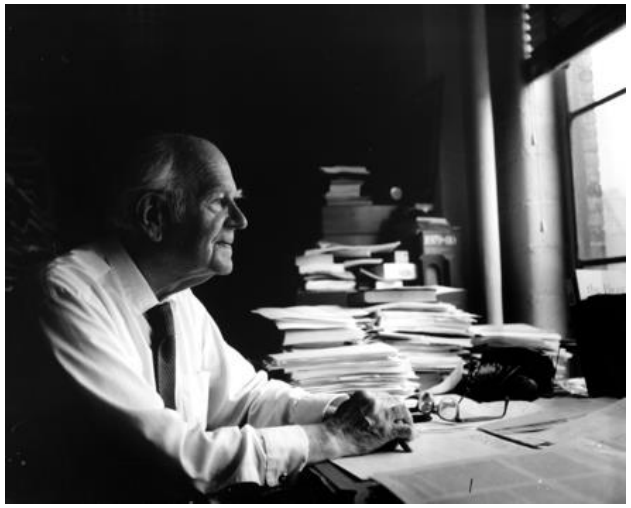
- Precipitation is a widely used technique for the isolation of proteins (as well as antibiotics, biopolymers)
- It is practiced from lab-scale to industrial-size operations
- It is very simple to set-up and requires low-cost equipments and (most often) reagents
- It is mostly used in the Capture phase of the purification process and usually permits dramatic reductions of the volumes to be handled
- The selectivity of the method is extremely variable
- As usual, the selection of this method must take into account the impact of the treatment on the target molecule

3.5 Preliminary observations (2/2)

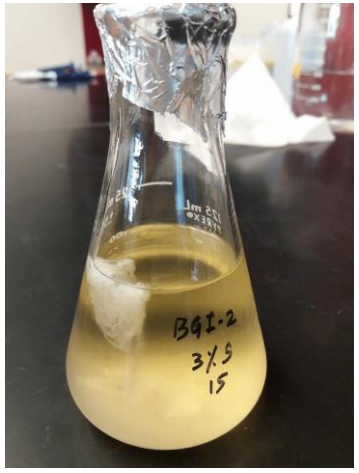
- Precipitation is obtained by a modification of the protein interactions with its environment, i.e. the solvent that surrounds it. It results from a change in the complex interplay between water molecules and hydrophilic/hydrophobic patches on the protein surface which are caused by the precipitating agent
- Protein precipitates differentiate themselves from chemical precipitates since they are usually far from pure and may contain other proteins, water, or precipitating agents
- The solubility behaviour of some proteins is peculiar and resembles more a partition between a protein-depleted solution and a protein-rich phase
- Precipitation can be reversible (salting-out), partially reversible (solvents) or irreversible (heat). Reversibility has to be carefully assessed at an early stage

3.5 The most common precipitating agents

- Heat
- pH change
- Salting-out with electrolytes
- Organic solvents
- Soluble polymers
- Polyelectrolytes
- Affinity precipitation



John T. Edsall
(1903-2002)

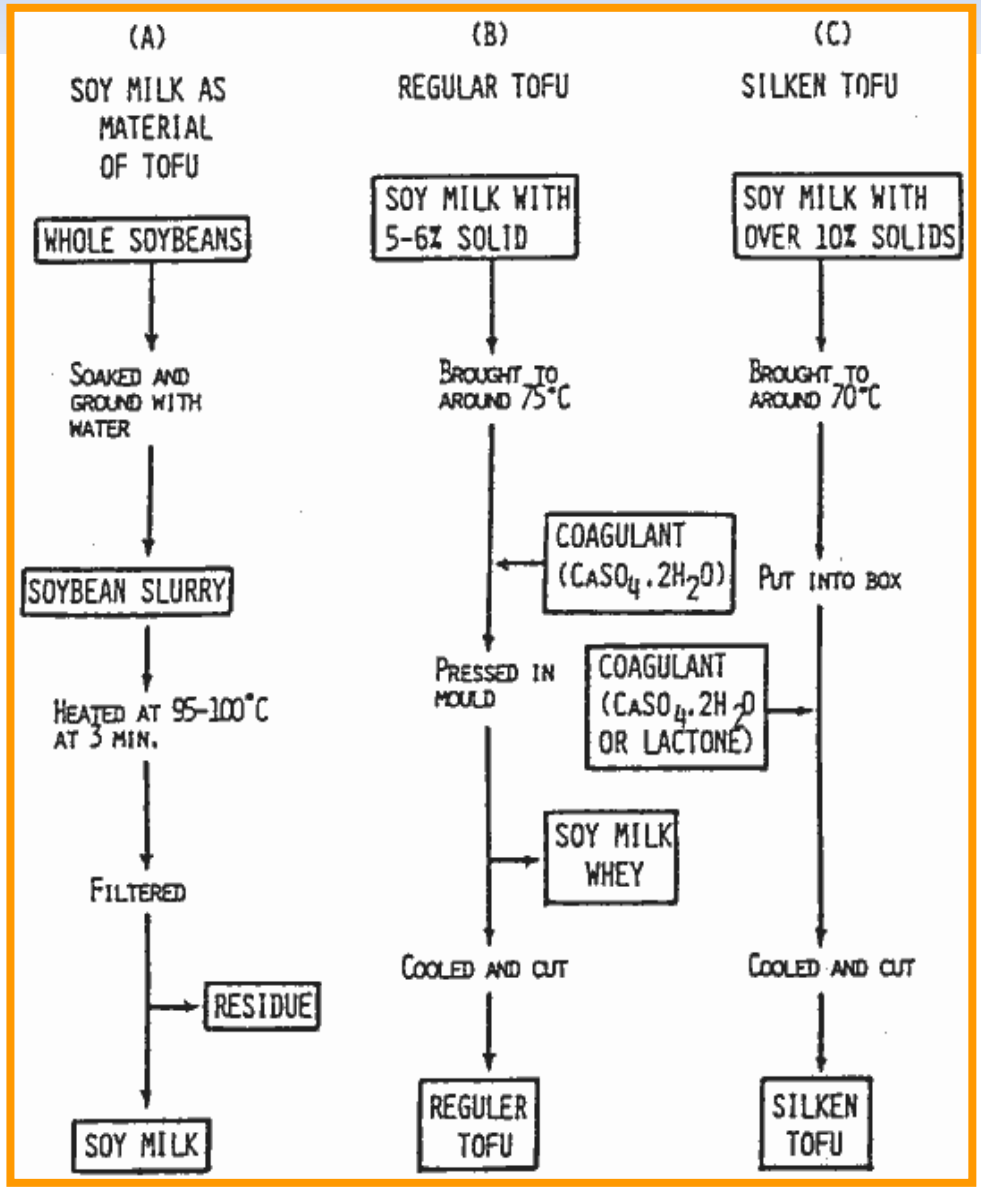


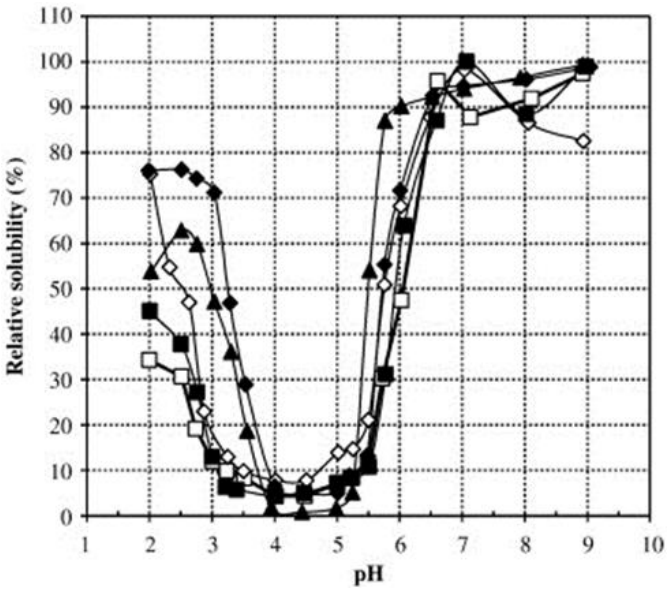
Edwin J. Cohn
(1892-1953)

Affinity chromatography is a powerful, highly selective technique. However, it only found a limited number of applications.

1. R. Freitag et al. (2007) Affinity precipitation, an option for early capture in bioprocessing. Biotechnol. J., 2, 685-690
2. L. Janoschek et al. (2014) Protein A affinity precipitation of human immunoglobulin G. J. Chromatogr. B, 965, 72-78
3. S. Raghava et al. (2008) Strategy for purifying maltose binding protein fusion proteins by affinity precipitation. J. Chromatogr. A, 1194, 90–95

pH-induced precipitation: tofu production





Solubility of soy proteins vs. pH

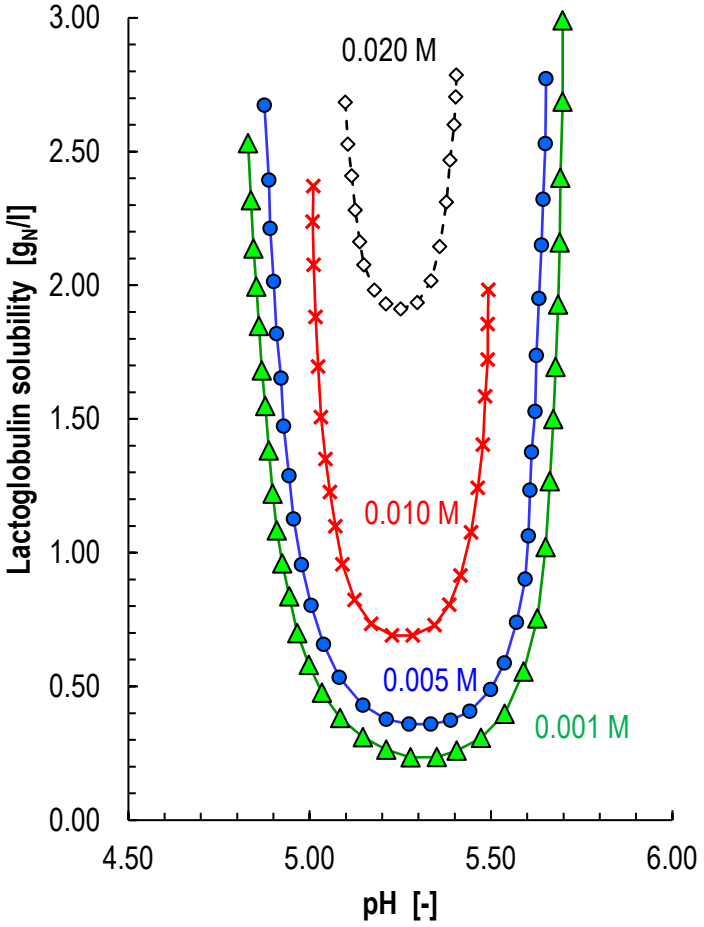
There are several ways to precipitate soy proteins in the manufacture of tofu, e.g. coagulation induced by a calcium salt or acidification using acetic acid or delta-glucono-lactone

Source: Sarah's vegan kitchen

Electrolyte-induced precipitation (salting-out)

- Most widely used technique, involves hydrophobic characteristics of the protein
- The exact nature and mechanism of the process is still uncompletely characterized and understood
- Numerous factors come into play, including a reduction of the hydration layer, the solvent molecules being trapped by the electrolyte
- Other contributions:
  - *Charge of hte protein being masked by the ionic atmosphere*
  - *Osmotic-type of interaction due to reduction of salt concentration between two approaching protein molecules*
  - *Specific ionic effects linked to the structure of water around the protein*

Protein solubility is minimal at the isoelectric point



Solubility of β-lactoglobulin as a function of pH at different NaCl concentrations (taken from Fox S. & Foster J.S., Introduction to protein chemistry, p.242, Wiley (1957))

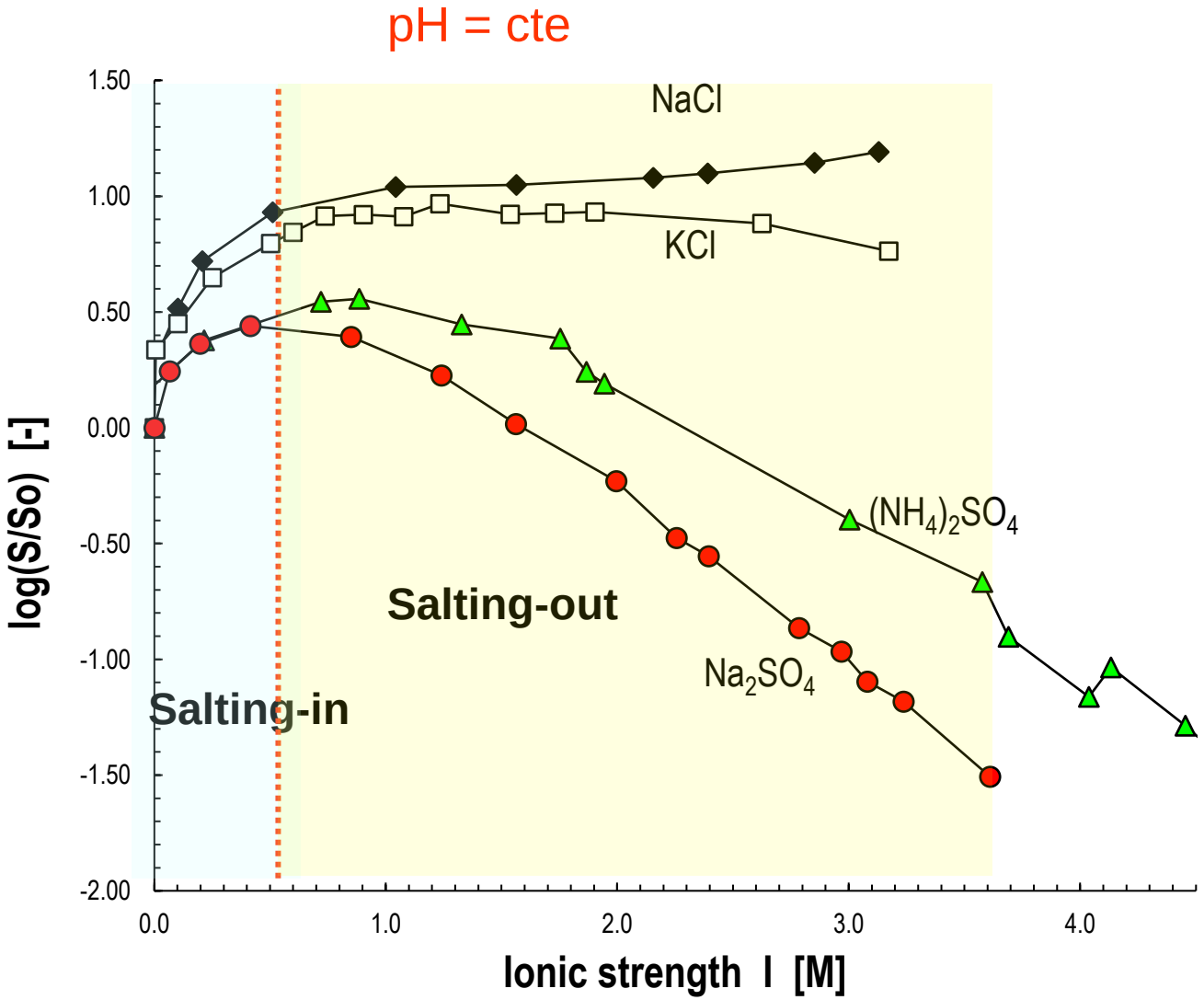
The solubility of a protein is generally lowest at its isoelectric point

At the pI, the net charge of the protein is zero, and the intensity of the repulsive forces between protein molecules is then at its lowest

For a given pH value, protein solubility first increases with electrolyte concentration up to a certain point, and then drops due to precipitation. This first increase is known as the salting-in phenomenon

Salting in at low concentrations is explained by the Debye–Huckel theory. Proteins are surrounded by the salt counterions (ions of opposite net charge) and this screening results in decreasing electrostatic free energy of the protein and increasing the activity of the solvent, which in turn, leads to increasing solubility. This theory predicts that the logarithm of solubility is proportional to the square root of the ionic strength.

3.5 Salting-in and salting-out



Solubility of hemoglobin-CO in the presence of different salts at a fixed pH value

Addition of a small quantity of salt leads to increased solubility in the salting-in range

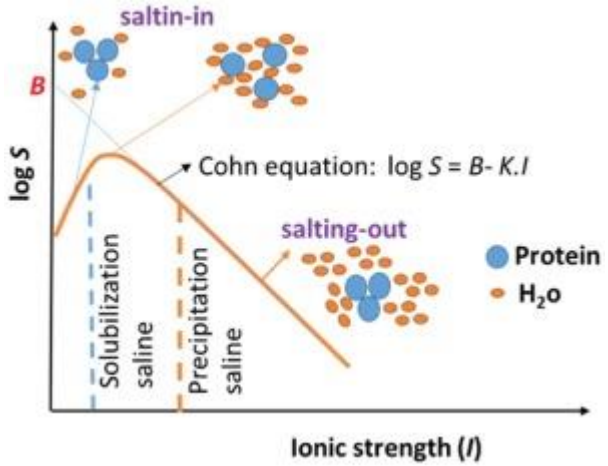
At higher salt concentrations, certain salts (not all of them) lead to a drastic decrease of protein solubility and to its precipitation (salting-out)

3.5 The Cohn (& Edsall) equation

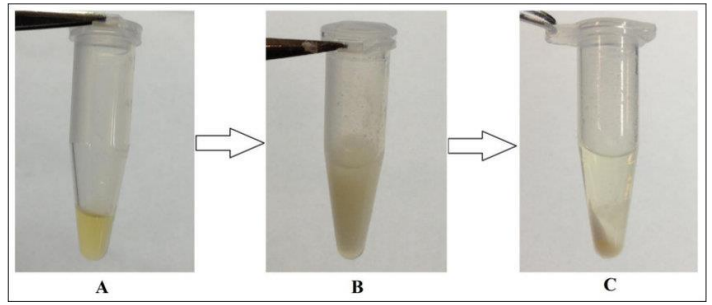
- The empirical equation of Cohn & Edsall is often used to describe the change of solubility S as a function of electrolyte concentration C (or ionic strength I)

$$\log S = \beta - K_s \cdot I$$

where $I = \frac{1}{2} \cdot \sum_i c_i \cdot Z_i^2$

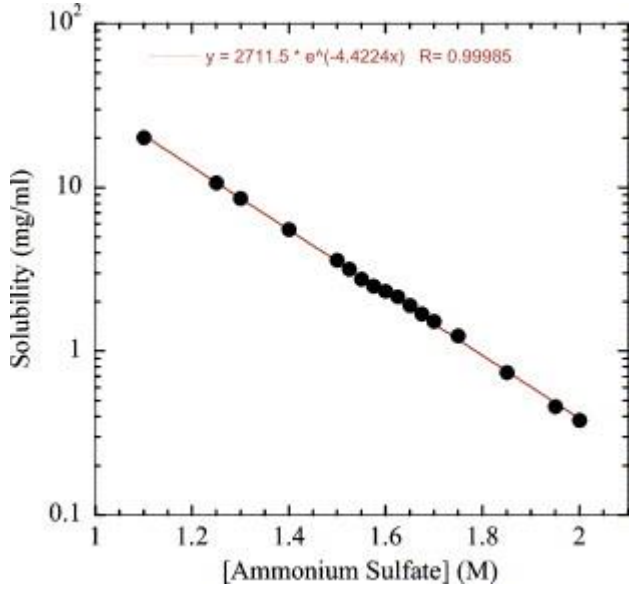


  - β: Debye constant; value extrapolated from Cohn's linearized equation (hence does not correspond to the solubility in pure water). β depends on pH and T, and reaches a minimum at the protein pI
  - K_s : salting-out constant; does not depend on pH or T, but is a function of the electrolyte-protein pair under investigation.

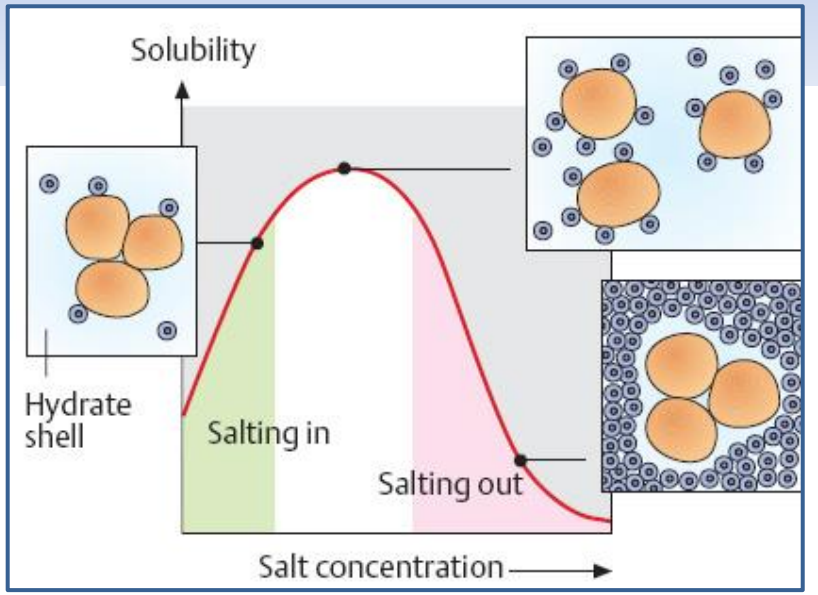


- Although completely empirical, the Cohn equation was «validated » in 1977 by two authors who took a thermodynamic approach to protein salting-out (Melander W. & Horvath C., Arch. Biochem. Biophys. 183, 200-215, 1977)

Protein salting-out illustrated



RNase Sa solubility as a function of ammonium sulfate concentration in 50 mM sodium acetate buffer (pH 4.25). S. R. Trevino et al., J. Mol. Biol. 366, 449-460 (2007)



Source: Wikipedia

- NB: The form of the Cohn equation is also applicable to other precipitation agents, e.g. organic solvents, neutral polymers, polyelectrolytes

- The so called Cohn process, developed during WWII, is still widely used today for blood plasma fractionation (fractionated precipitation of plasma proteins with ethanol at low temperature)

The Hofmeister series

- Increase protein stability
- Less denaturing
- Salting out (aggregates)
- Kosmotropic

- Decrease protein stability
- More denaturing
- Salting in (solubilizes)
- Chaotropic


Franz Hofmeister
1850 - 1922

$$F^- > SO_4^{2-} > HPO_4^{2-} > Acetate^- > Citrate^- > Cl^- > NO_3^- > Br^- > I^- > ClO_4^- > SCN^-$$

HOFMEISTER SERIES

$$N(Me)_4^+ > NH_4^+ > K^+ > Na^+ > Cs^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+} > Gdm^+$$

Source: https://water.lsbu.ac.uk/water/hofmeister_series.html

In 1888, Franz Hofmeister first reported that the salts to precipitating proteins and macromolecules out of aqueous solution generally follow a specific ions series, known as the HS, lyotropic sequences, or ion specificity.

The series include two different groups: strongly hydrated ions and weakly hydrated ions. Anions on the left of Cl– are well hydrated, classified as kosmotrope ions (water structure maker), and tend to stabilize the native fold structure of proteins, leading to salting-out behavior.

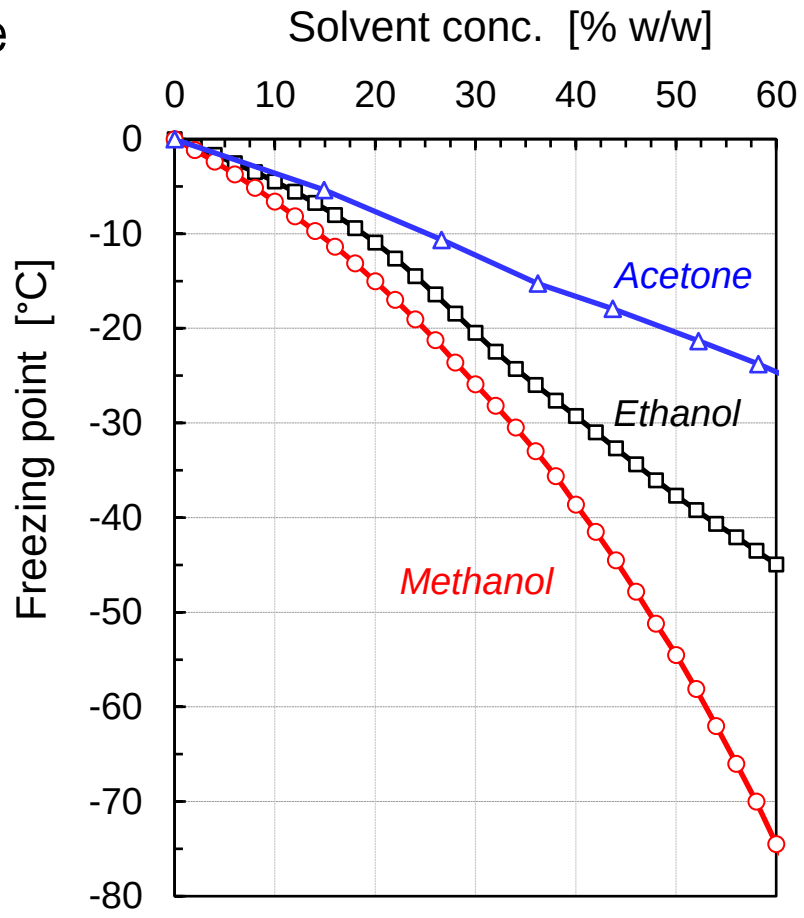
Anions on the right of Cl– are poorly hydrated, called chaotropes (water structure breaker), and tend to facilitate protein denaturation and unfolding, showing salting-in behavior.

Similar observations can be made on the left- and right-hand sides of Na^+ for the cations

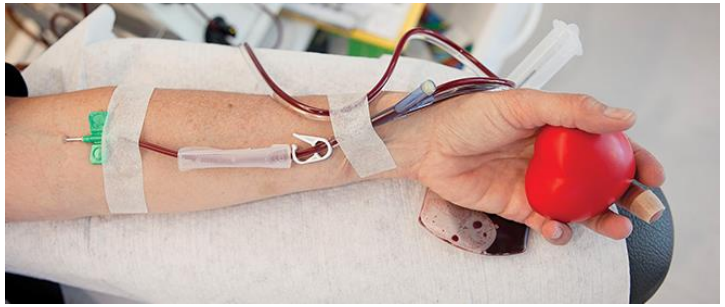
B. Kang et al., ACS Omega 2020, 5, 12, 6229–6239

4.2.4 Precipitation using organic solvents

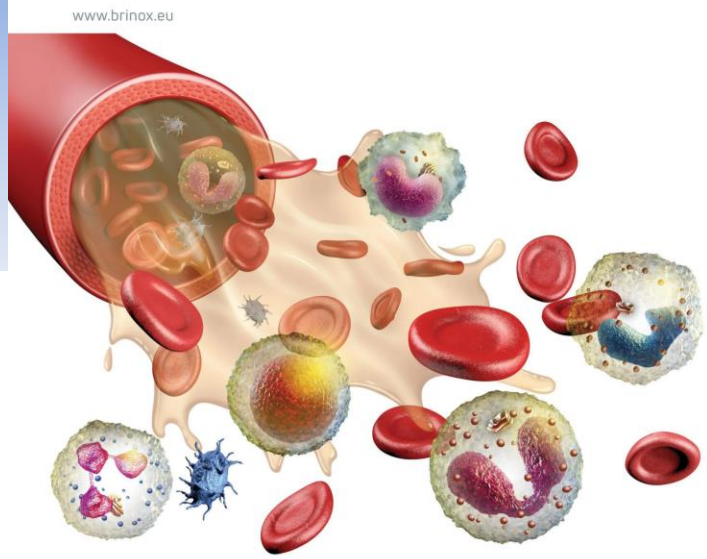
- Adding a miscible organic solvent to a protein solution will result in a reduction of the dielectric constant, which increases interactions of the charge-dipole and dipole-dipole types between protein molecules, thus promoting precipitation
- As for electrolytes, the technique is popular because it is simple, moderately selective (playing with pH and ionic strength), easy to scale-up, efficient, and involves low-cost reagents.
- Organic solvents also have a strong sterilization effect and allow working at low temperatures.
- Water-solvent mixtures also have a lower density than water, which facilitates the centrifugal recovery of the precipitate
- However, there are safety and environmental issues linked to the use of solvents.
- In addition, solvents often have denaturing effects on proteins.



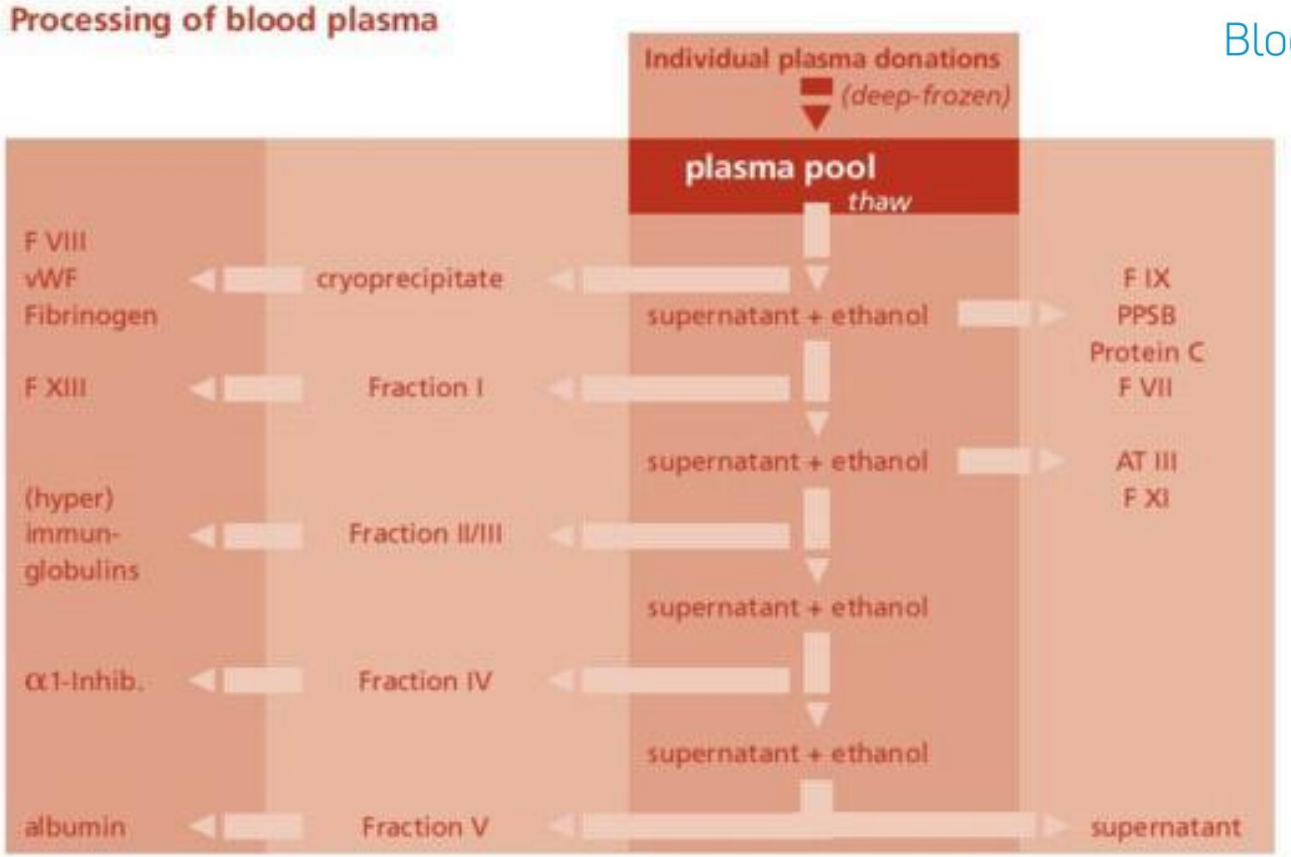
The Cohn blood plasma fractionation process

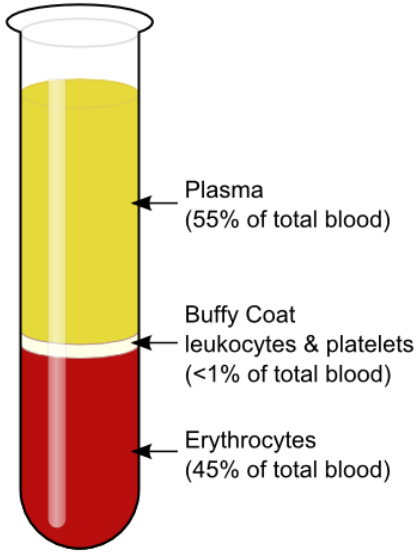


Source: www.usinenouvelle.com



Blood plasma fractionation

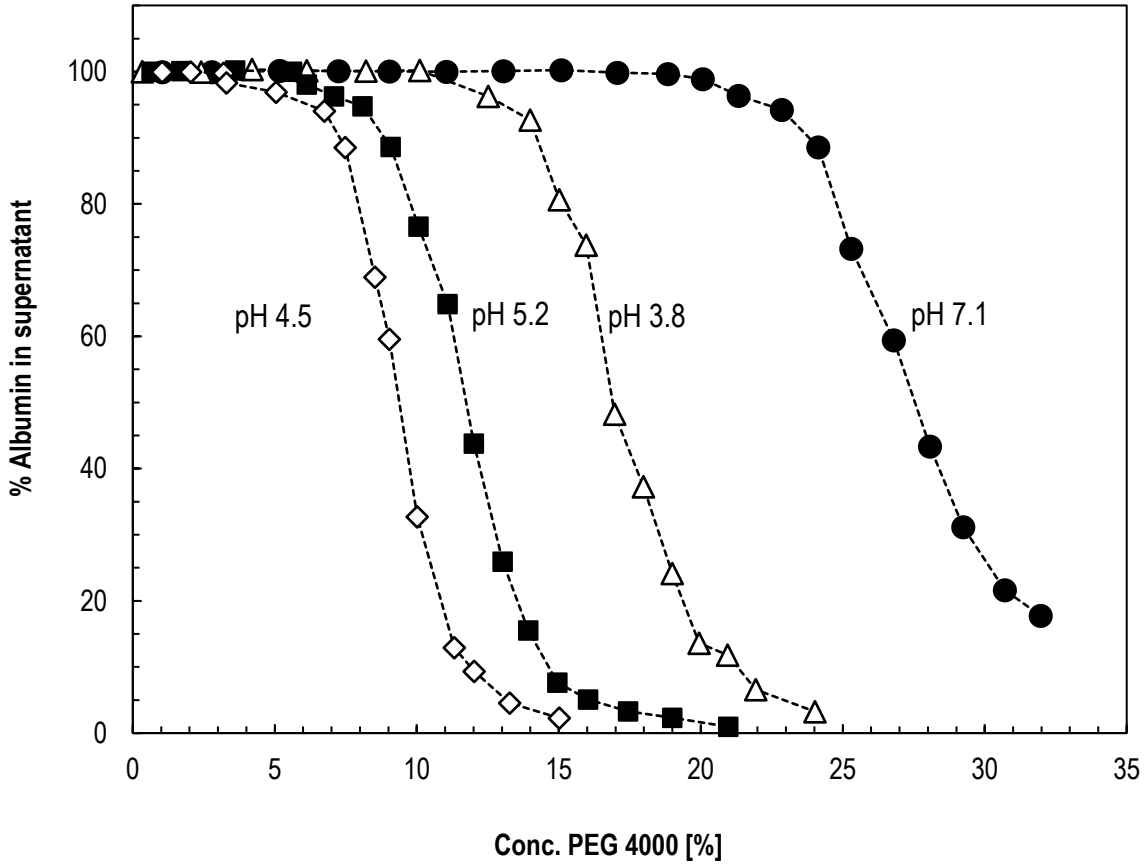




HSA precipitation by PEG at T_{amb}

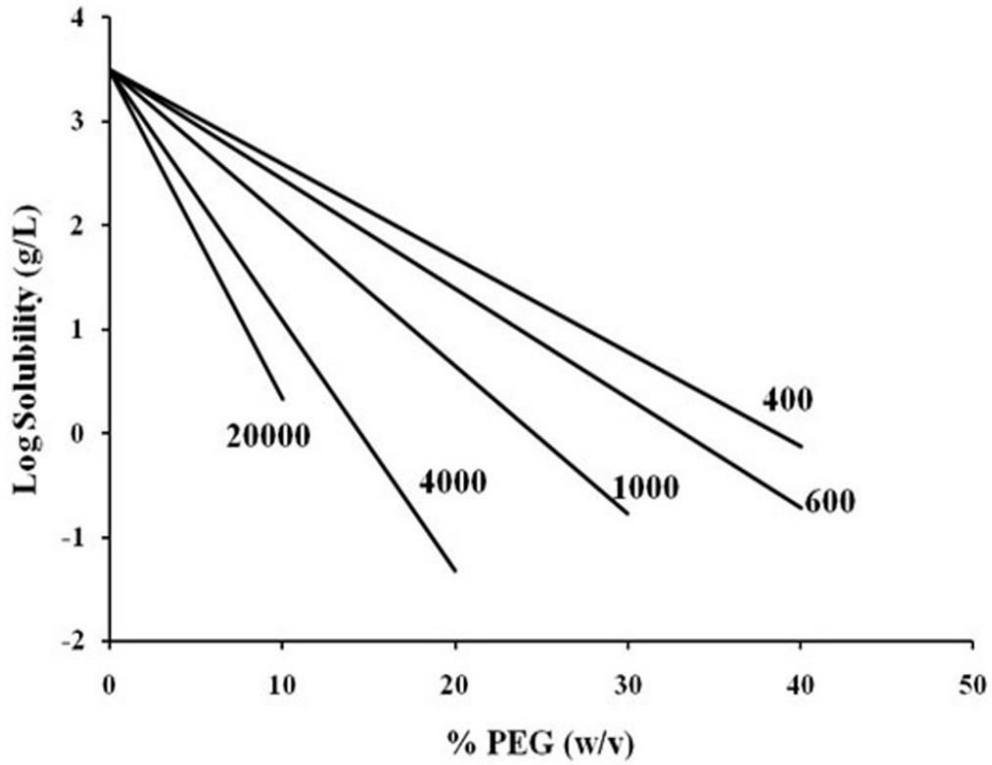
HSA = Human Serum Albumin
PEG = Polyethylene glycol

K. C. Ingham, Arch. Biochem. Biophys. 186, 106 (1978).



Effect of pH on the precipitation of human serum albumin by PEG 4000 at room temperature. All solutions contained 0.1 M KCL and initial albumin concentration was always 20 mg/ml

D. H. Atha, K. C. Ingham, J. Biol. Chem. 256, 12108 (1981)

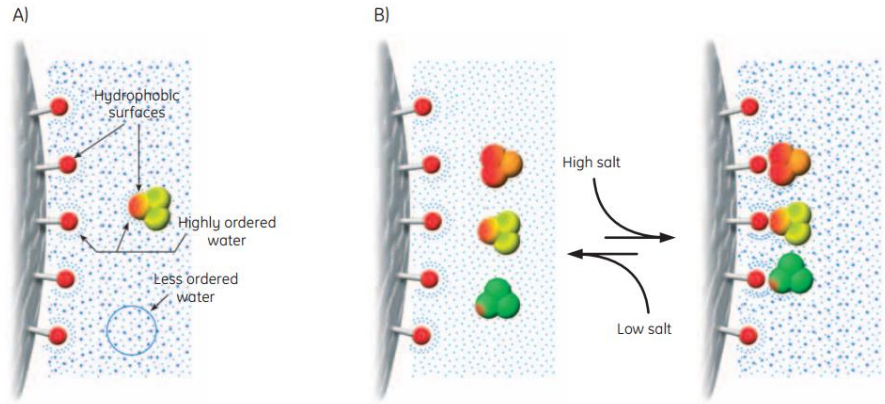


Effect of PEG molecular weight on the solubility of HSA. The initial concentration of protein was 20 mg/ml in a pH 4.5 buffer containing 0.1 M KCl.

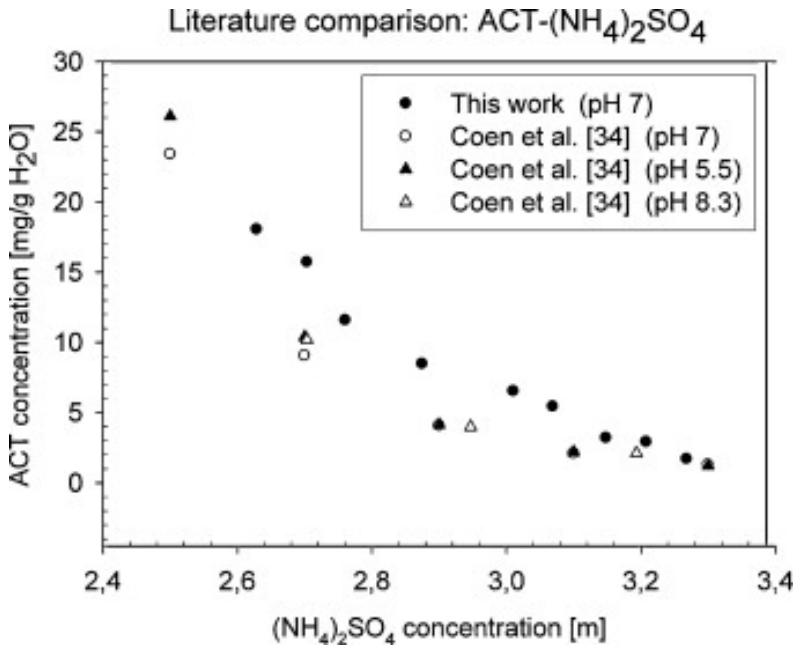
Some more data on salt-induced precipitation of proteins

B. K. Nfor et al., 2011: High-throughput protein precipitation and hydrophobic interaction chromatography ***: Salt effects and thermodynamic interrelation. Journal of Chromatography A 1218 (49), 8958–8973

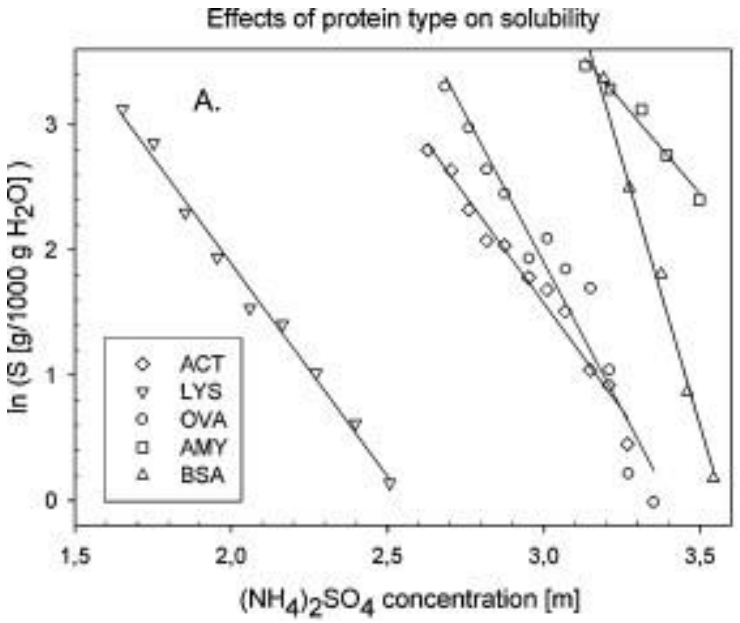
*** yes, there is a strong connection between salt-induced protein precipitation and hydrophobic interaction chromatography



A) Highly ordered water shells surround the hydrophobic surfaces of ligands and proteins. Hydrophobic substances are forced to merge to minimize the total area of such shells (maximize entropy). Salts enhance the hydrophobic interaction. B) The equilibrium of the hydrophobic interaction is controlled predominantly by the salt concentration.

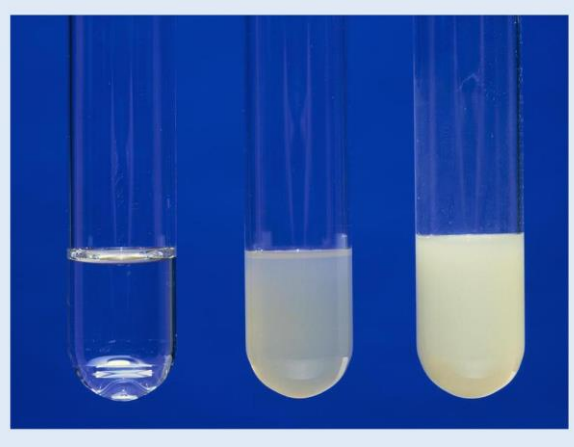


ACT : α-chymotrypsin
LYS: lysozyme
OVA: ovalbumin
AMY: α-amylase
BSA: bovine serum albumin



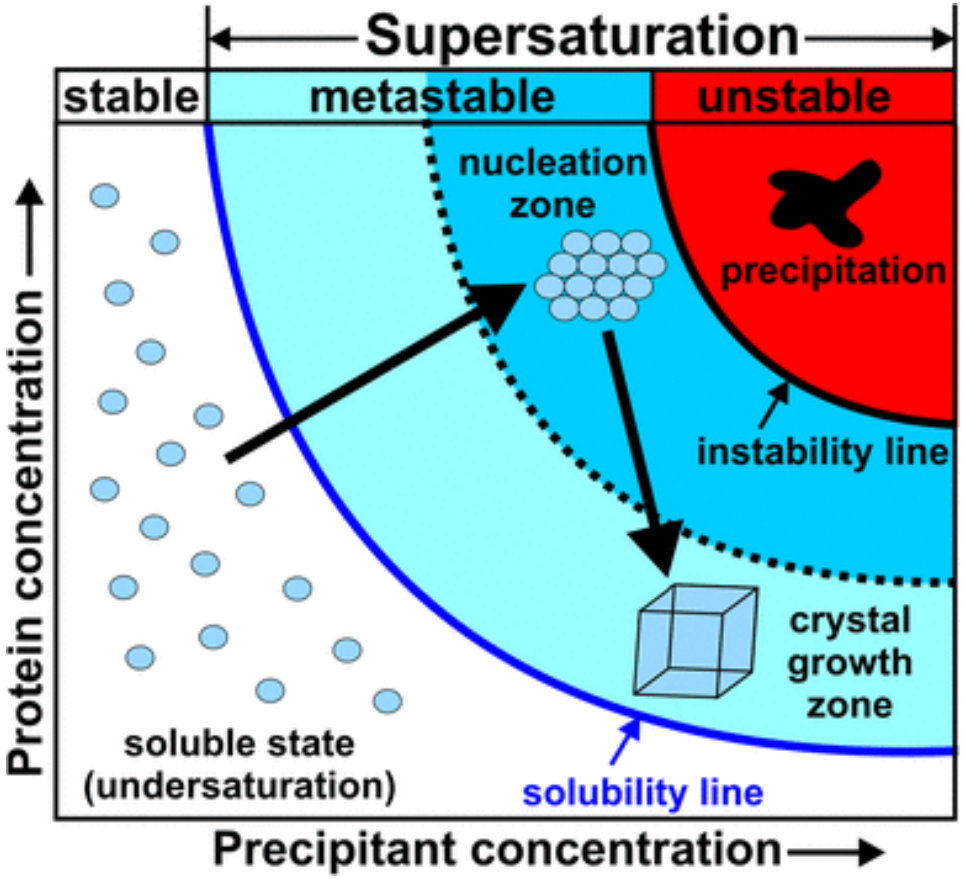
The revival of protein precipitation?

- DSP of monoclonal antibodies has reached certain limits in terms of purification costs and capacity, described in the press as «DSP bottleneck»
- Alternatives to the classical approach involving protein A chromatography are hence actively sought
- Investigations aiming at investigating the potential of precipitation, due to the simplicity and low cost:
  - Kuczewski M. et al. G.: PEG Precipitation: A Powerful Tool for Monoclonal Antibody Purification. Biopharm International Supplements, March 2, 2010
  - Wang J. M. et al.: Precipitation of Process-Derived Impurities in Non-Protein A Purification Schemes for Antibodies. BioPharm International Supplements, Oct. 2, 2009
  - Glynn J.: Process-Scale Precipitation of Impurities in Mammalian Cell Culture Broth. BioPharm International Supplements, March 2, 2008

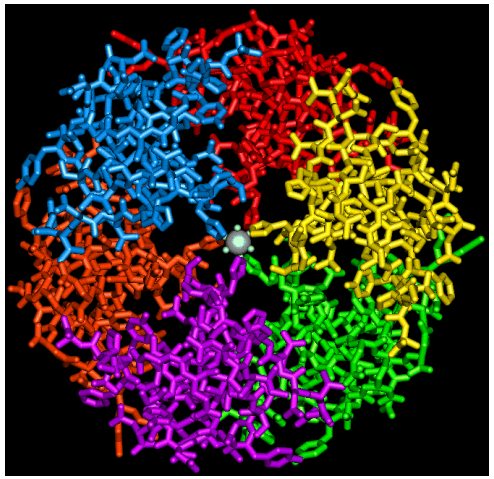


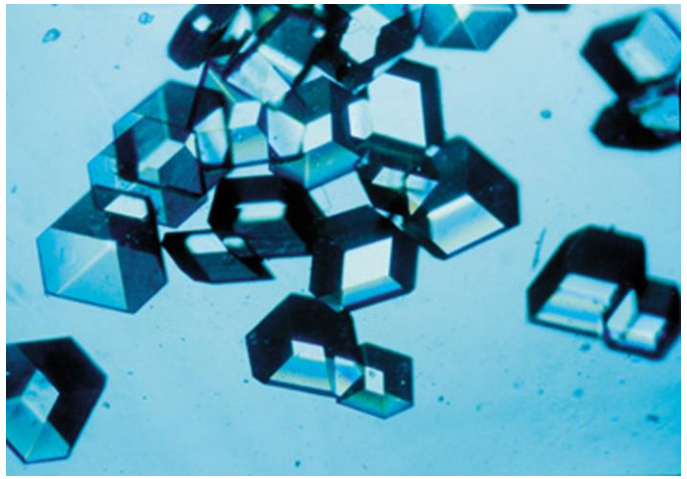
Nickerson J. L. & Doucette A. A.: Rapid and Quantitative Protein Precipitation for Proteome Analysis by Mass Spectrometry. J. Proteome Res. 2020, 19, 5, 2035–2042 (2020) https://doi.org/10.1021/acs.jproteome.9b00867

Crystallization of proteins

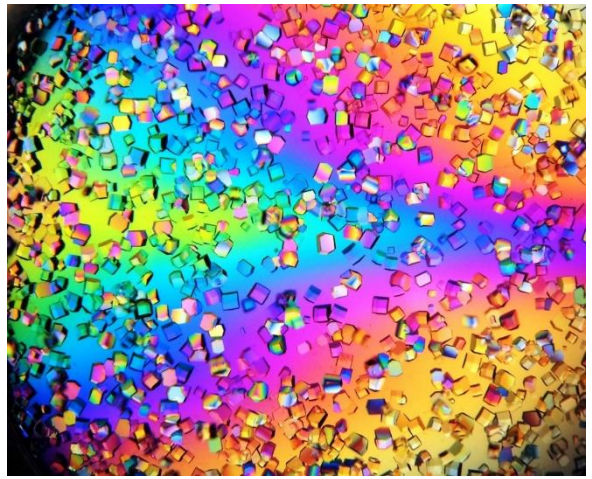


Source: A. Bijelic & A. Rompel, ChemTexts (2018) 4:10
https://doi.org/10.1007/s40828-018-0064-1





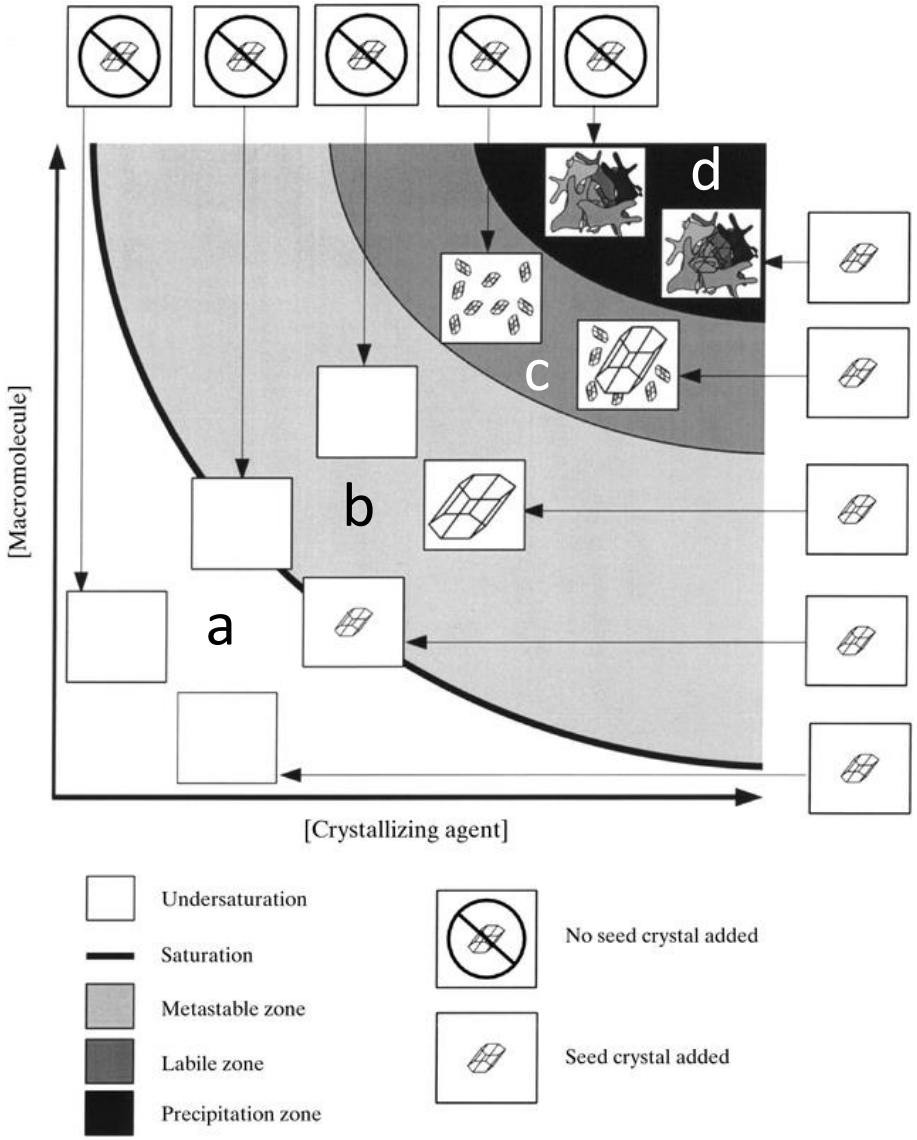
Hexameric insulin crystals with coordination Zn^{2+} ion Source: Wikipedia



Source: Wikiwand.com

Crystallization is obtained from a solution of protein using the same agents as seen for precipitation (but under strictly controlled conditions)

Formation of aggregates and crystal growth

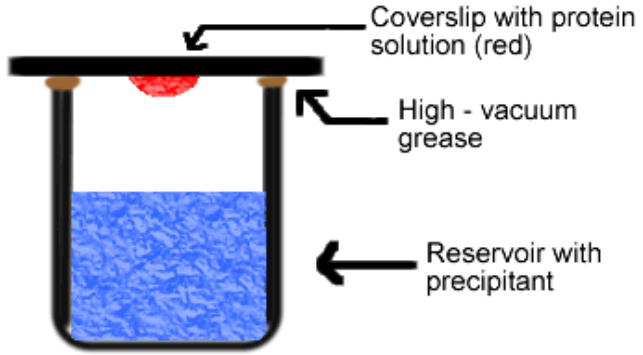


The diagram shows zones with varying degrees of supersaturation and what happens there (be it psontaneously or after addition of seed crystals):

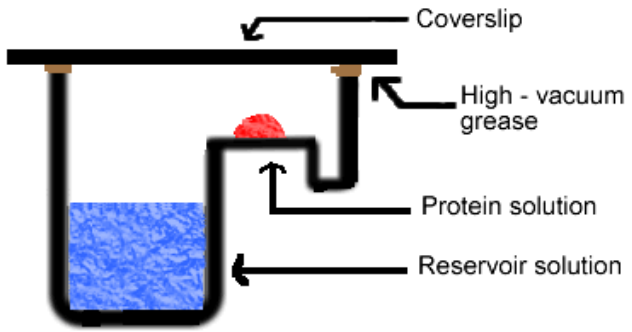
a) No crystal formation
b) Growth of seed crystals
c) Formation and growth of crystals
d) Precipitation

Protein crystallization: hanging drop and sitting drop techniques

Hanging drop



Sitting drop



http://www.bio.davidson.edu/courses/MolBio/MolStudents/spring2003/Kogoy/protein.html

Crystallization of a therapeutic antibody at Novartis

ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Fast and Scalable Purification of a Therapeutic Full-Length Antibody Based on Process Crystallization

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[2]Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts

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ABSTRACT: The potential of process crystallization for purification of a therapeutic monoclonal IgG1 antibody was studied. The purified antibody was crystallized in nonagitated micro-batch experiments for the first time. A direct crystallization from clarified CHO cell culture harvest was inhibited by high salt concentrations. The salt concentration of the harvest was reduced by a simple pretreatment step. The crystallization process from pretreated harvest was successfully transferred to stirred tanks and scaled-up from the mLscale to the 1 L-scale for the first time. The crystallization yield after 24 h was 88–90%. A high purity of 98.5% was reached after a single recrystallization step. A 17-fold host cell protein reduction was achieved, and DNA content was reduced below the detection limit. High biological activity of the therapeutic antibody was maintained during the crystallization, dissolving, and recrystallization steps. Crystallization was also performed with impure solutions from intermediate steps of a standard monoclonal antibody purification process. It was shown that process crystallization has a strong potential to replace Protein A chromatography …

B. Smejkal et al., *Biotechnol. & Bioeng.* 110 (9), 2452-2461, 2013