Kód předmětu: C8980

MASARYKOVA UNIVERZITA

Protein expression and purification

IX. Protein quality

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Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

OP Vzdělávání pro konkurenceschopnost

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

Recombinant protein quality evaluation

Microbial Cell Factories

BioMed Central

Commentary

Open Access

Minimal information: an urgent need to assess the functional reliability of recombinant proteins used in biological experiments

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Published: 23 July 2008

Microbial Cell Factories 2008, **7**:20 doi:10.1186/1475-2859-7-20

Received: 24 June 2008

Accepted: 23 July 2008

MIPFE (Minimal Information for Protein Functionally Evaluation)

Table 2: Experimental information check list. MI for each experiment is in bold, optional information is in italics.

Experiment type	Analyses to perform for evaluating proteins before starting the experiments	Protocol references (from a list of accepted standard protocols for each experiment type that guarantee output congruency)	Equipments (define minimal requisites of the devices in terms of quality, maintenance, controls)
Protein pull-down	**SDS PAGE; AI;** *SEC; DLS; CD far UV; CD near UV; FT-IR; NMR;*	SDS: protocol 1 (or protocol 2,..) AI protocol 1	SDS: Hoefer minigel.... AI: Spectrofluorimeter Jasco ..... SEC: column xy and FPLC model z.....
Monoclonal Ab purification	**SDS PAGE; AI;** *SEC; DLS; CD far UV; CD near UV;*		
Protein purification	**SDS PAGE; AI; SEC;** *DLS;* **CD far UV**; *CD near UV; FT-IR; NMR;*		
SPR			
ITC			
ELISA			
Enzymatic assay			
Protein refolding			
Immunoprecipitation			
Immunofluorescence			
Immunohistochemistry			
Proximity ligation			

The corresponding pictures (SDS-gels, chromatographic profiles, fluorimeter spectra,.....) would be embedded in the metadata form.

Abbreviations: Aggregation Index (AI) (16), Size Exclusion Chromatography (SEC), Dynamic Light Scattering (DLS), Circular Dichroism (CD), Fourier-Transform InfraRed (FT-IR), Nuclear Magnetic Resonance (NMR).

Standards in Genomic Sciences (2011) 5:195-197 DOI:10.4056/sigs.1834511

Recombinant protein quality evaluation: proposal for a minimal information standard

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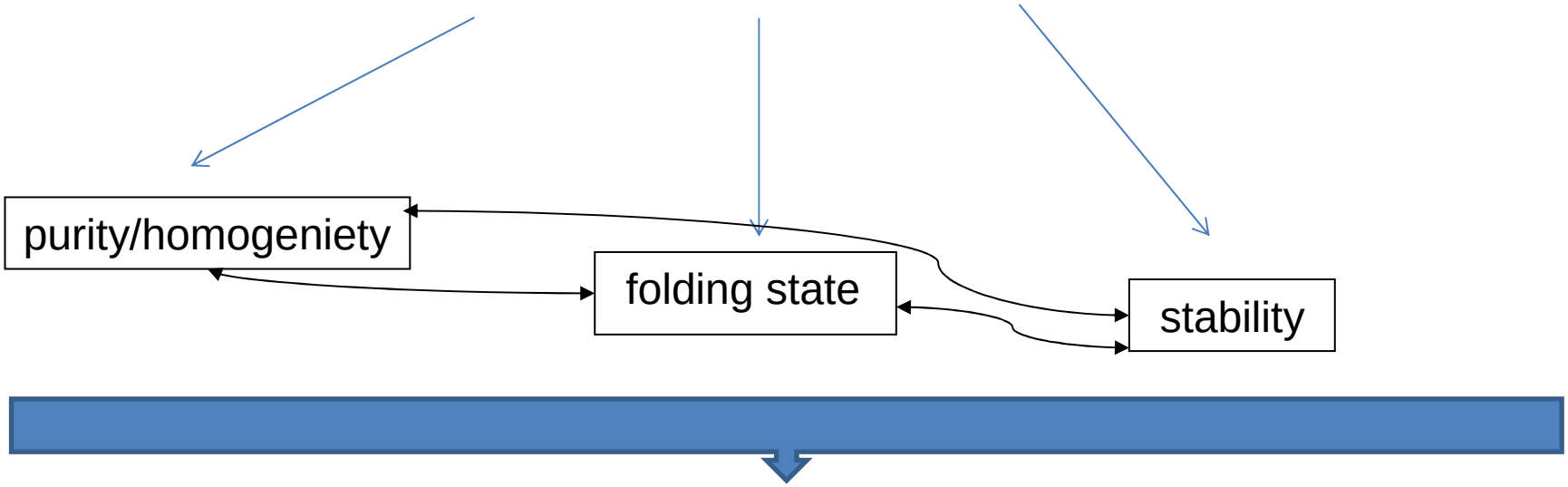
We consider that information concerning aggregation status and secondary structure should be reported as a minimal requirement for publication under Supplementary Material. These controls should be available when authors describe protein production as well as protein interaction experiments (pull-down,surface plasmon resonance, antibody/protein microarrays, and isothermal titration calorimetry).

Source of protein → Extraction → Separation → Quality evaluation

Quality parameters of produced protein



High quality of produced protein

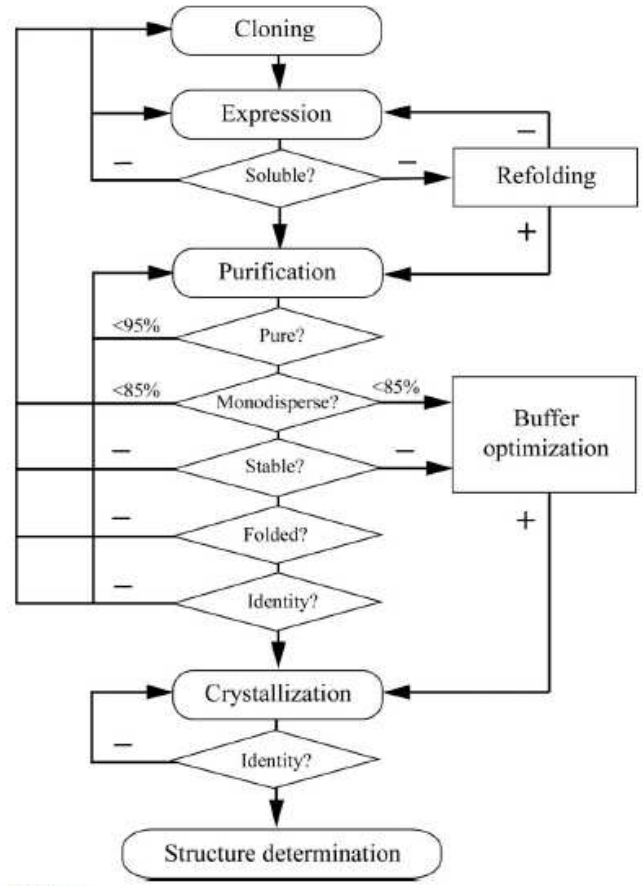
3D structure determination
Detail function analysis

Quality assessment of protein preparation

Structural Proteomics In Europe (SPINE) consortium

- 3 year project, over 20 laboratories
- investigation of the effect of the biophysical properties of purified proteins on their crystallizability

Scheme for quality assessment of purified protein



The strategy is based on the experiences within SPINE and the results of quality assessment survey.

Geerlof et al., 2006

Important biophysical properties for quality assessment of a protein preparation and the most frequently used methods for their determination.

Biophysical property	Methods used for its determination
Purity	SDS-PAGE, size-exclusion chromatography, mass spectrometry
Monodispersity, aggregation state	Dynamic light scattering, size–exclusion chromatography, analytical ultracentrifugation
Conformational state	Static light scattering, analytical ultracentrifugation, size–exclusion chromatography
Folding state	Nuclear magnetic resonance spectroscopy, circular dichroism, Fluorescence emission spectroscopy, Fourier-transformed infrared spectroscopy
Stability	Differential scanning calorimetry, circular dichroism, thermal shift assay
Identity, modification	Mass spectrometry

PROTEIN PURITY/HOMOGENIETY

Purity = protein is without contaminations by other proteins

Homogeniety/heterogeniety

– heterogeniety in pure macromolecules = **MICROHETEROGENIETY** of the sample

POSSIBLE SOURCE OF MICROHETEROGENIETY in protein sample:

- variation in primary structure (genetic variation, degradation)
- variation in folding (errors in folding or partial unfolding)
- variation in quarternary structure (oligomerization)
- variation in posttranslation modifications
- various aggregate states
- oxidation (e.g. sulfuhydryl groups in proteins)
- aging (e.g. deamination in proteins)

PROTEIN PURITY/HOMOGENIETY

How to determine protein purity/(micro)homogeniety?

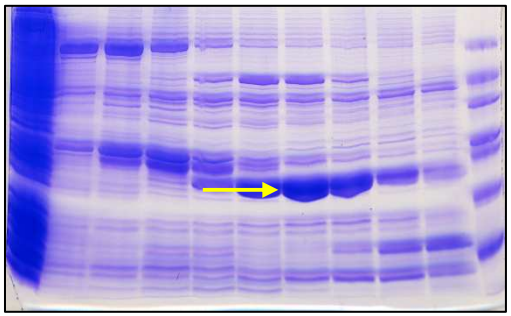
- **electrophoresis (SDS, native)**
- **size- exlusion chromatography**
- **mass spectrometry (MS)**
- **dynamic light scattering**
- **analytical centrifugation**

PROTEIN PURITY

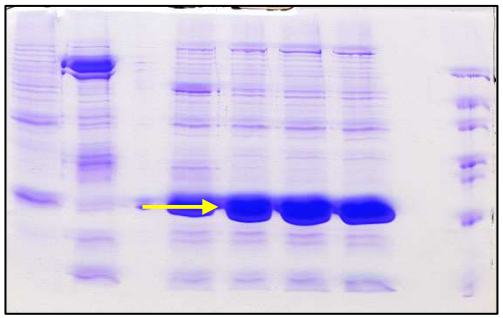
SDS-PAGE

- For a pure protein, only one band is expected (20 μg of protein/lane of the gel).
- Mw determination (information about protein subunits)
- Protein purity is evaluated in SDS-PAGE using various software (e.g. Quantity One from Bio-RAD).
- Definition of protein purity: a quantity of particular band as measured by its intensity, expressed as a percentage of the total intensity of all bands in the lane of the gel.

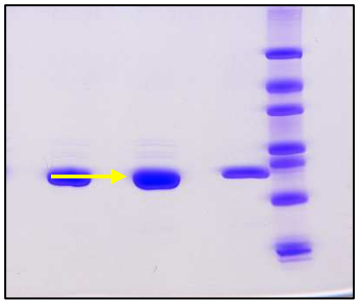
28% purity



80% purity

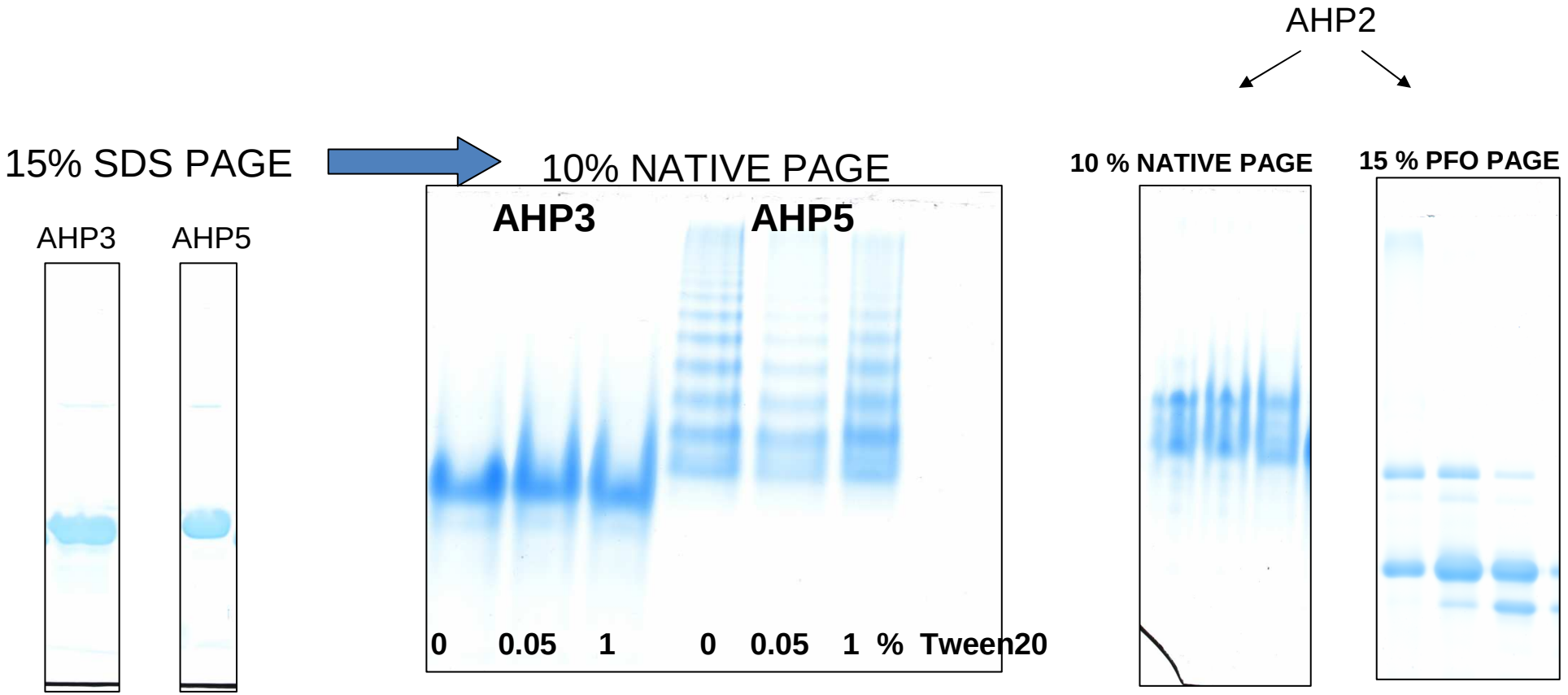


95% purity



PROTEIN PURITY/HOMOGENIETY

- **NATIVE PAGE**
- **PFO (pentadecafluorooctanic acid) NATIVE PAGE**

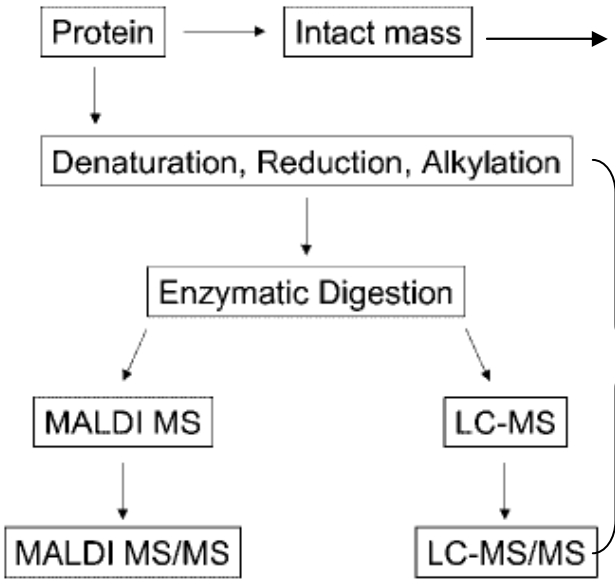


Mass spectrometry (MS)

- Technique separates molecules according to their mass and charge.

- Mass spectrometer are composed of three parts: ionization source, an analyser, and a detector. The mass analyzer separates protein ions according to their mass (m) to charge (z) ratios (m/z). Protein ions are generated within the ionization source; the mostly common ionization technique are electrospray ionization and matrix-assisted laser desorption ionization (MALDI).

- Application: Evaluation of recombinant protein heterogeniety resulting from posttranslation modifications sequence variation generated from proteolysis, or tranctriptional/translation errors, and degradation products, which are formed during processing and final product storage.



Analysis of intact proteins: complete sequence coverage, assessment of overall protein heterogeniety, identification of labile PTMs.

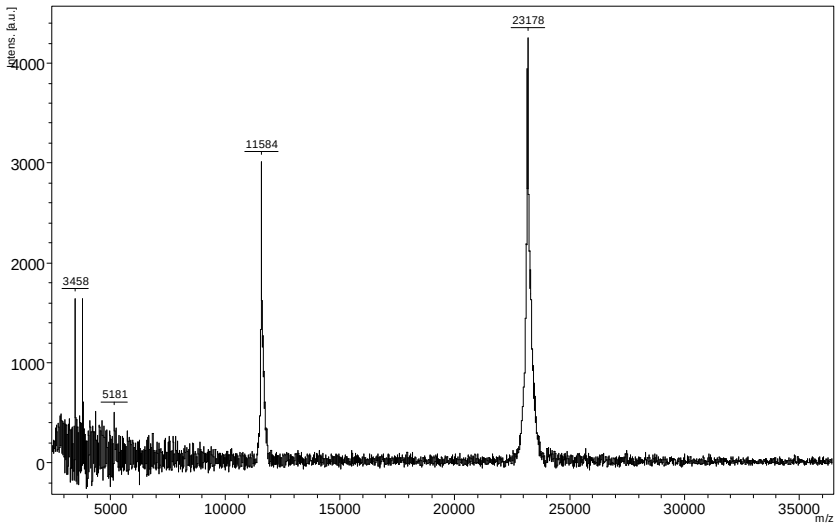
Analysis of protein digest mixture: location of PTMs, amino acid degradation products, glycosylation sites, and disulfide linkages with specific regions of protein sequence.

Mass measurement accuracy ranging from 0.02% (ESI) to 0.1% (MALDI) was routinely achieved.

Mass spectrometry (MS) – intact analysis

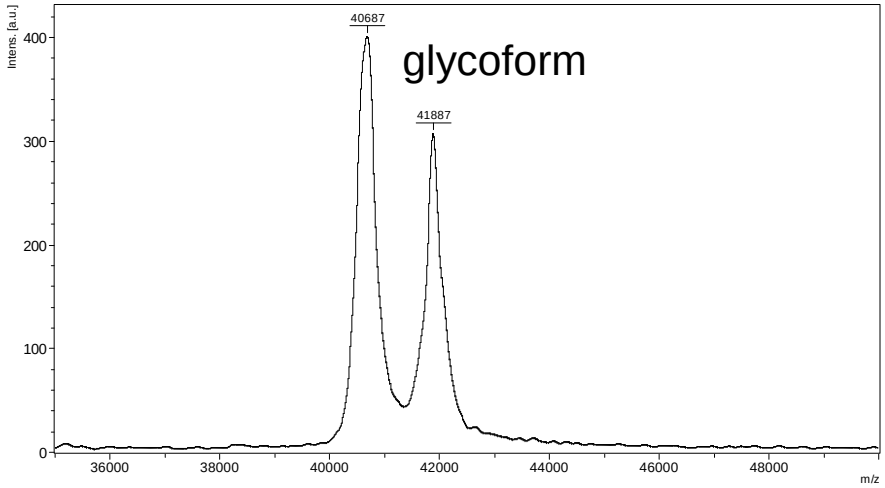
- Measurement of total mass of purified protein
  - verification of construct
  - information about degradation products, contaminants, posttranslation modifications

Purity



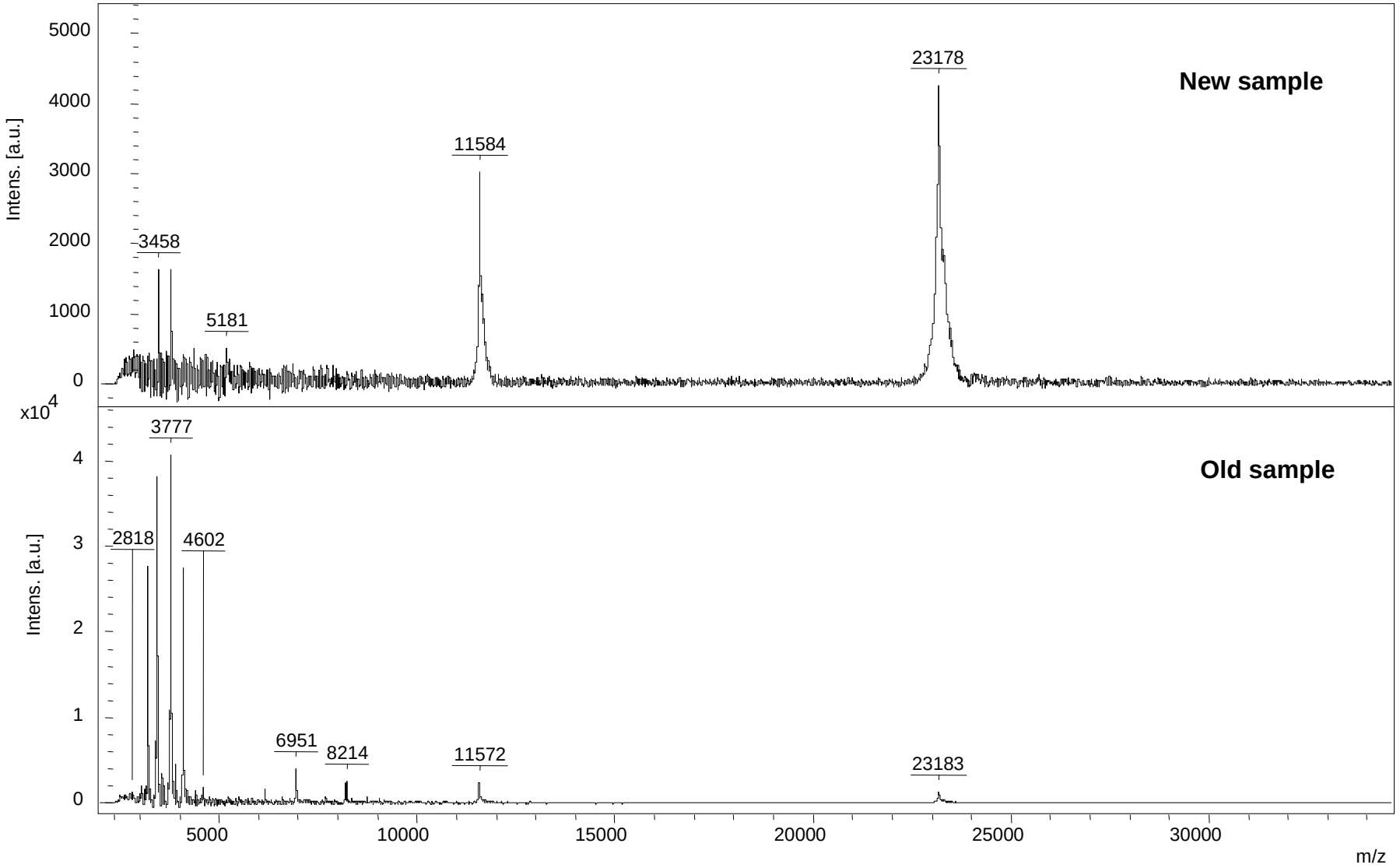
Posttranslation modification

Non-modified protein



Mass spectrometry (MS)-intact analysis

- information about protein degradation

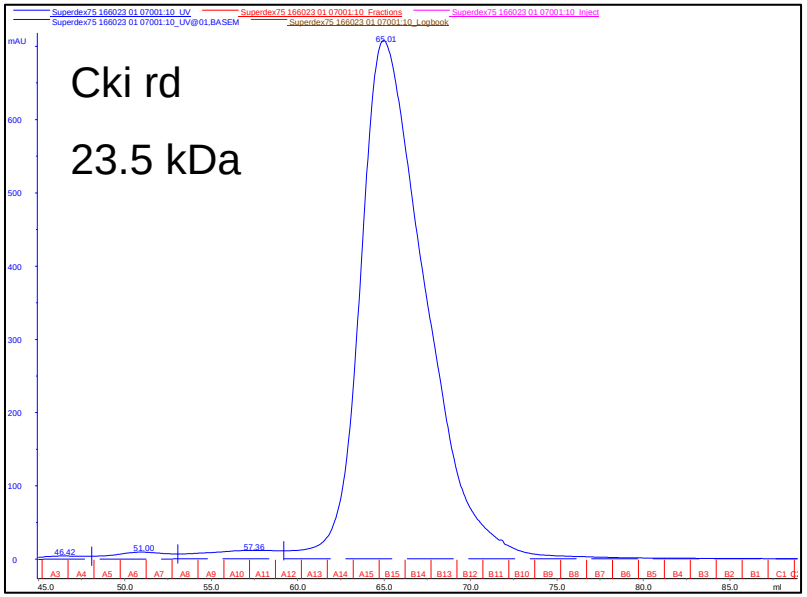


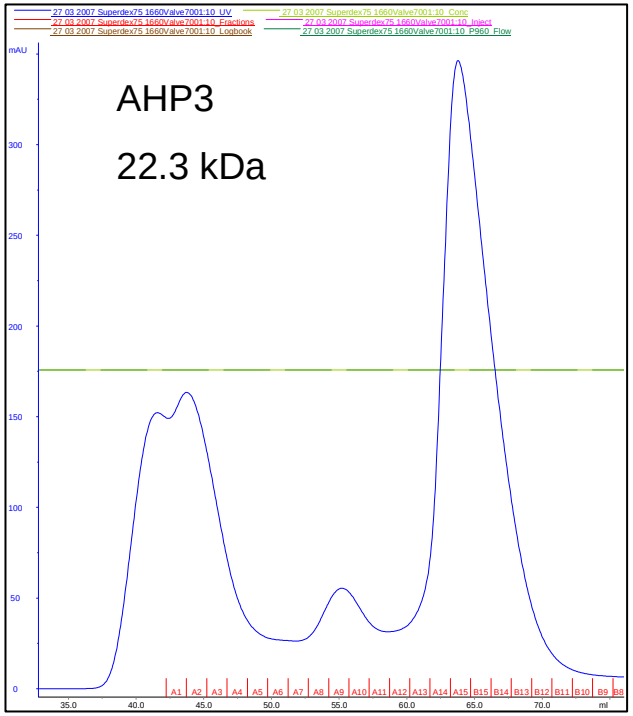
Size-exclusion chromatography (Gel filtration)

- Size-exclusion chromatography separates proteins on the basis of size.

- Molecules move through a bed of porous beads. Smaller molecules diffuse further into the pores of the beads and therefore move through the bed more slowly, while larger molecules enter less or not at all and thus move through the beads more quickly.

- Both molecular weight and three-dimensional shape contribute to the degree of retention.





Dynamic light scattering (DLS)

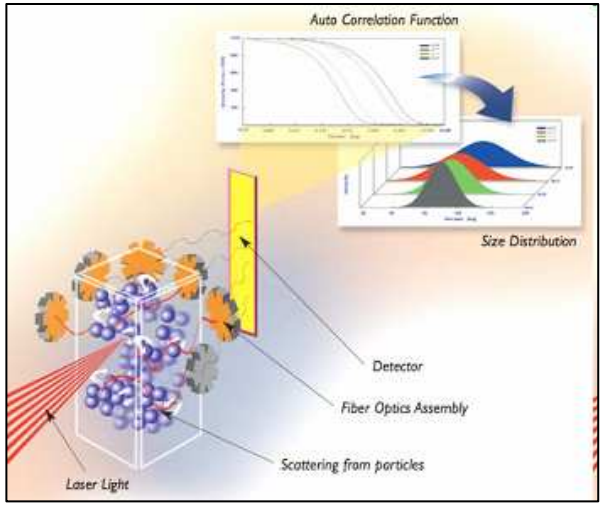
• DLS is a method to determine hydrodynamic size, polydispersities and aggregation effects of protein samples.

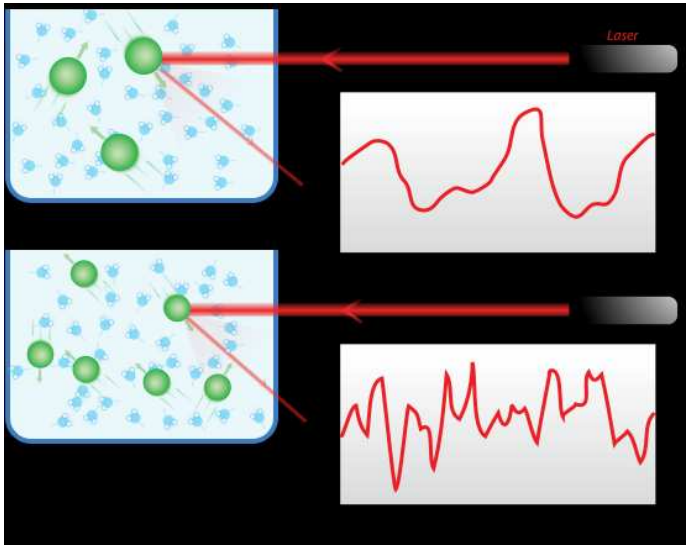
• Measurement of the velocity of the Brownian motion of molecules and/or particles.

• The laser is focused in the sample cell and the particles scatter the light in all directions.

• DLS measures time dependent fluctuation in the intensity of the scattered light which occurs because the particles are undergoing random Brownian motion. Analyses of these intensity fluctuation anables the determination of the distribution of diffusion coefficients of the particles, which are converted into a size distribution using Stokes-Einstein equation.

• Larger particles produce a very strong scattering intensity signal (detection of very small amounts of higher mass species).

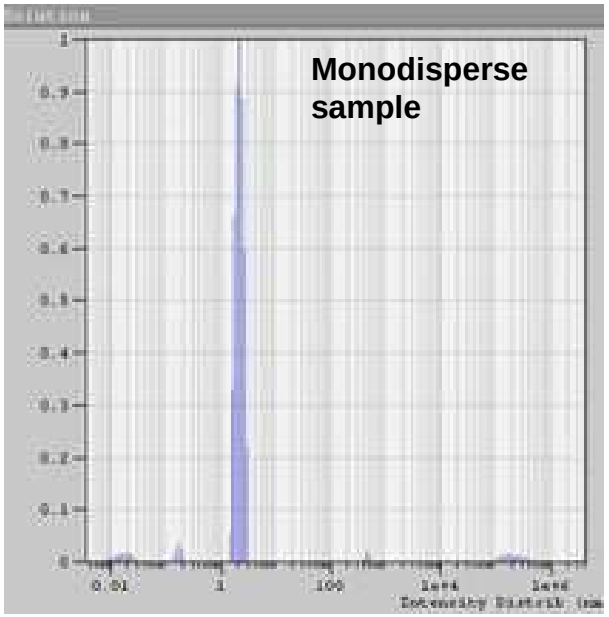




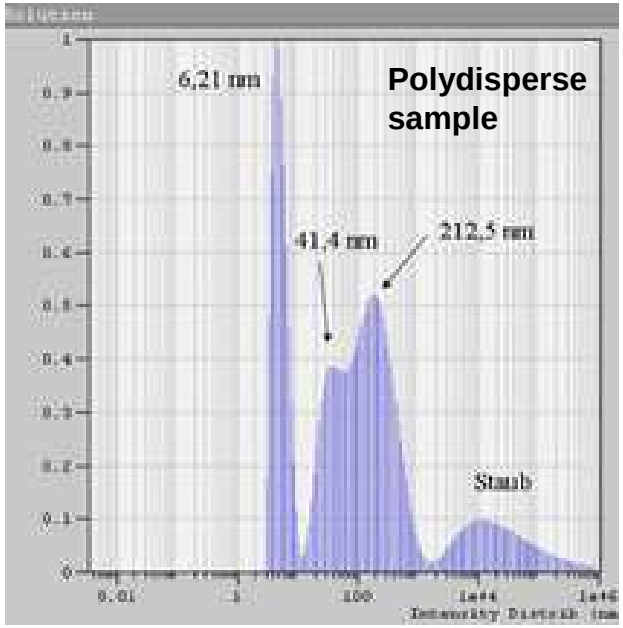
Applications:

• Analysis of samples containing very broad distribudions of species of widely different sizes (e.g. native protein and various sizes of large aggregates).

Dynamic light scattering (DLS)

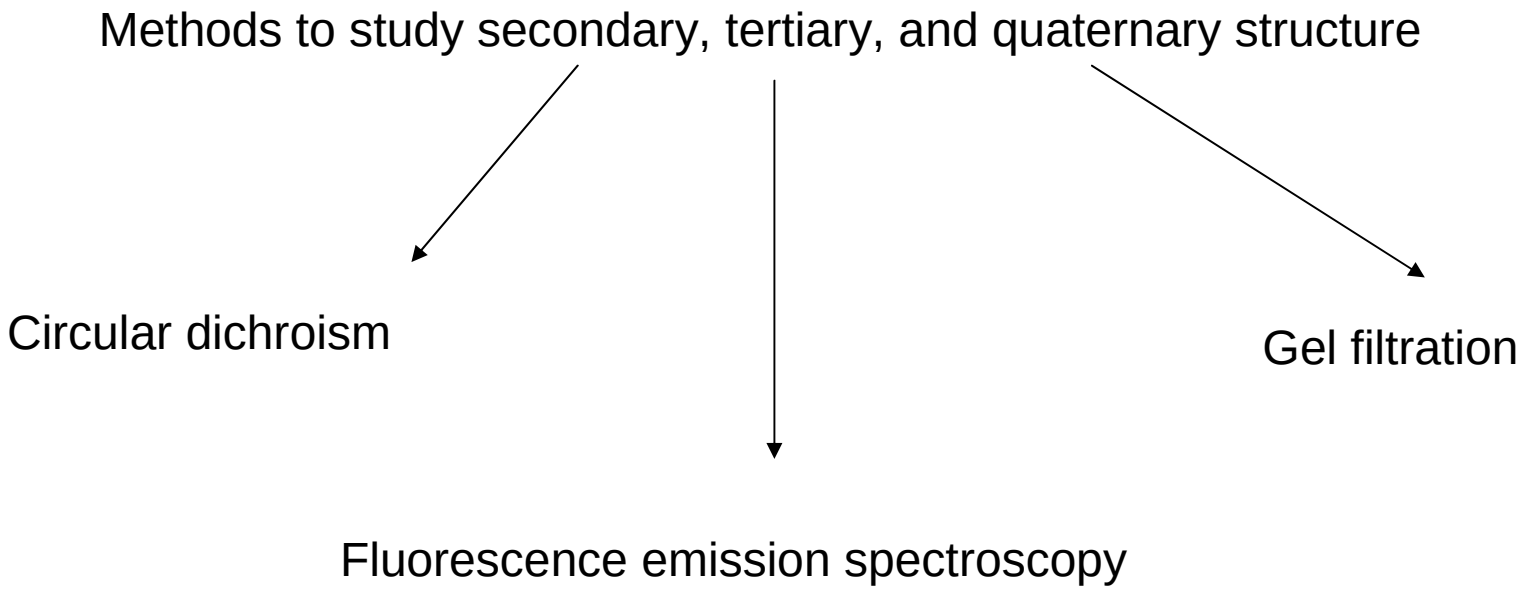


Correlation function (average from 10 measurements) and calculated hydrodynamic radius for a lysozyme sample. Results: Rh = 1,95 nm, Polydispersity = 19,1%.
No protein aggregates are present. Such a DLS result indicates a good chance for crystal growth, but it is not a guarantuee.



Results: A small peak is observed with a hydrodynamic radius of 6,21 nm (polydispersity 28,9%). Further broad peaks are visible at 41,4 nm and 212,5 nm and some dust particle in the micrometer range.
The chances to get crystals from this protein sample are small. For this sample a filtration step would be necessary to remove the large particles that interfere with the smaller ones.

Folding


Methods to study secondary, tertiary, and quaternary structure
Circular dichroism
Fluorescence emission spectroscopy
Gel filtration

Fluorescence emission spectroscopy

• Amino acids with intrinsic fluorescence properties: phenylalanine, tyrosine, tryptophan; but only tyrosine and tryptophan are used experimentally because their quantum yields (emitted photons/excited photons) are high enough to give a good fluorescence signal. So this technique is limited to proteins having either Trp or Tyr, or both.

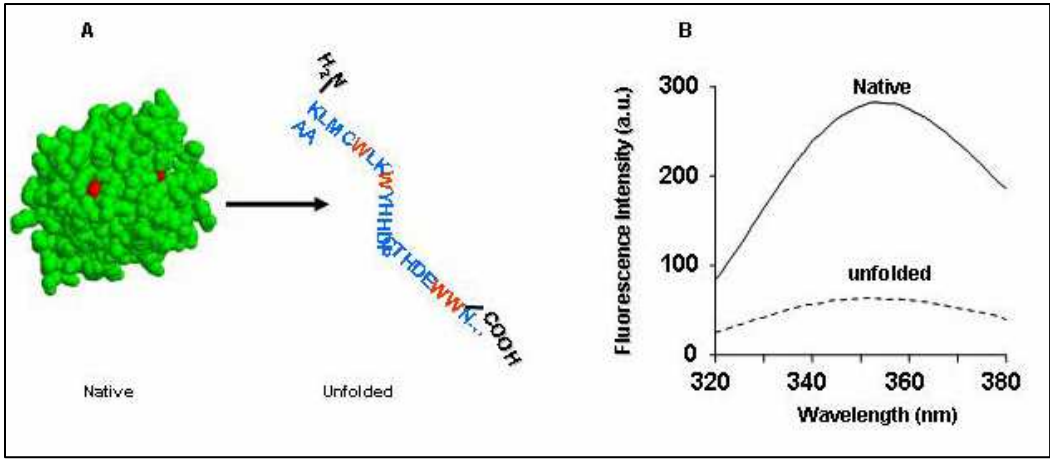
• At an excitation wavelength of 280 nm, both Trp and Tyr will become excited. To selectively excite Trp only, a 295 nm wavelength must be used.

• Trp and Tyr residues can be used to follow protein folding because their fluorescence properties (quantum yields) are sensitive to environment, which changes when a protein folds/unfolds. In the native folded state, Trp and Tyr are generally located within the core of the protein, whereas in a partially folded or unfolded state they become exposed to solvent.

Two modes of measurement

1. In a hydrophobic environment (buried within the core of the protein), Tyr and Trp have a high quantum yield and therefore a high fluorescence intensity. In a hydrophilic environment (exposed to solvent), on the other hand, their quantum yields decrease, leading to low fluorescence intensity.

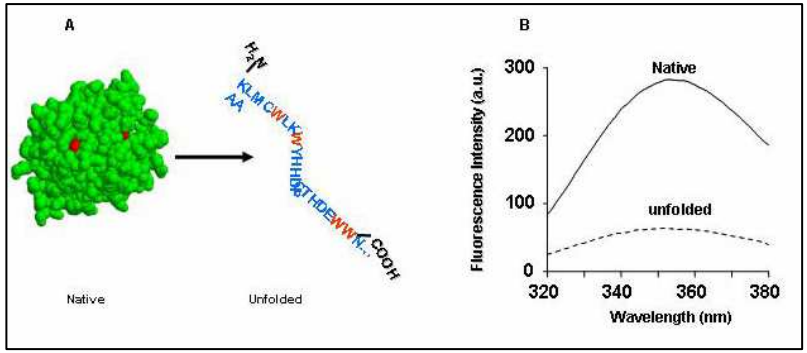
2. The wavelength of the emission maximum also reflects the hydrophobic (λ em 308 nm for azurin indicating that Trp is deeply buried within the core of the protein) and hydrophilic (λ em 352 nm for glucagon indicating that Trp is exposed to the solvent) environment.



Measurement of fluorescence intensity

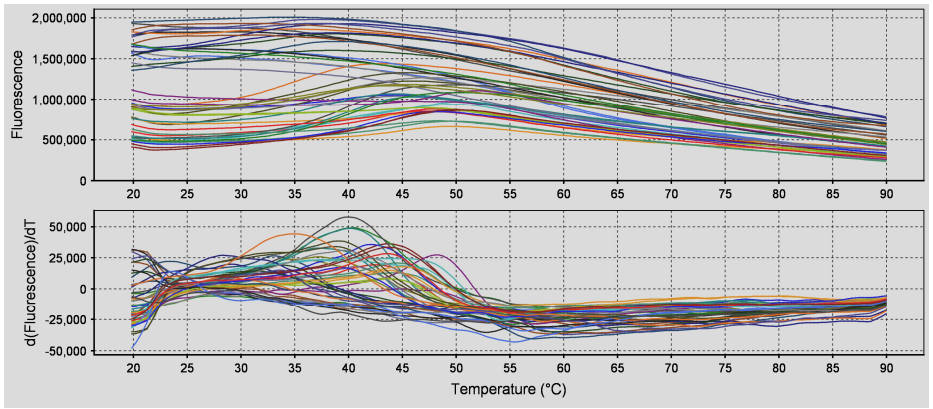
➢ As mentioned, the previous technique is limited to proteins containing Trp or Tyr residues. It is also possible to use probes that bind specifically to hydrophobic protein residues (Sypro Orange, Nie red, dapoxyl sulfonic acid, bis-ANS, 1,8-ANS). Those surfaces are hidden in a native protein but exposed in partially folded or fully unfolded proteins.

NATIVE FLUORESCENCE



X

Fluorescence of probe – Sypro Orange

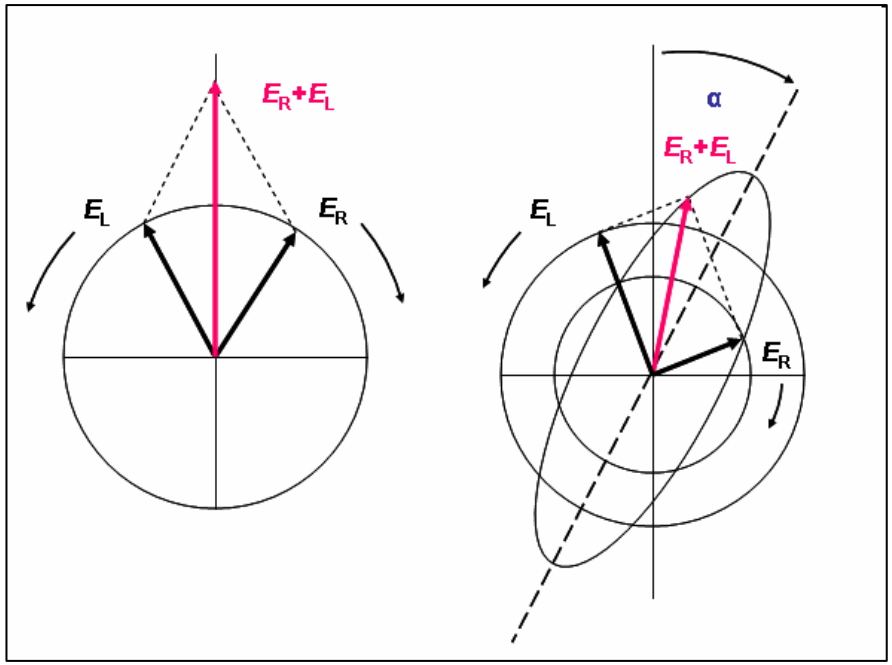


The thermal shift assay result for the initial screen of 23 different buffers with pH range 4.5 - 9.

More in protein stability part….

Circular dichroism spectroscopy

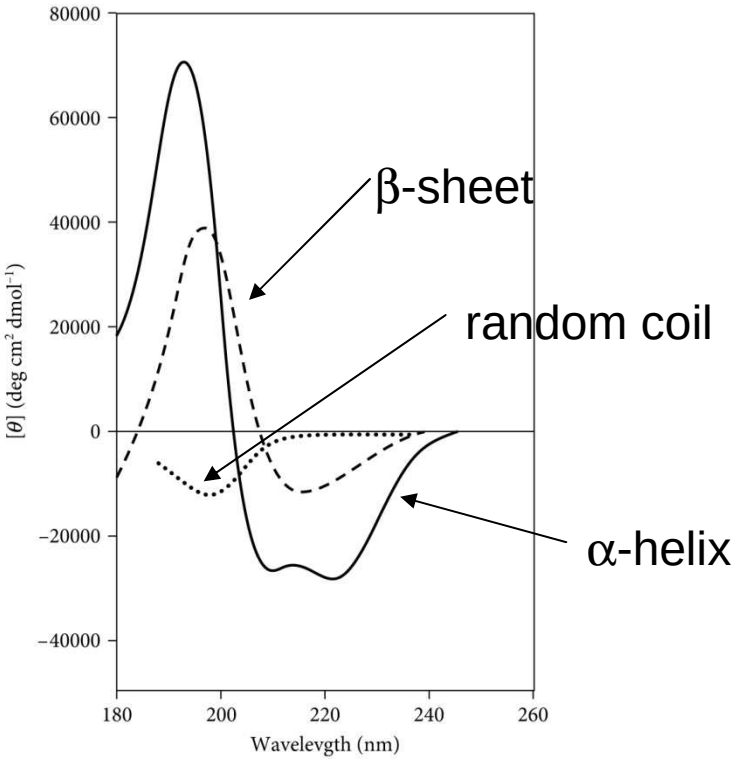
- CD spectra are used to assess the proper folding and the secondary structure of proteins



The technique depends on the difference in absorbance between left and right circularly polarized light beams, which arise due to structural asymetry.

The relevant spectral region is in far UV, i.e. from 240 nm down to 180 nm, where peptide bonds absorb light. In this region, the different types of regular secondary structure, such as alpha-helix and beta-sheet, exhibit a characteristic spectral pattern.

Circular dichroism spectroscopy – exploring the secondary structure of proteins



α-helix spectrum shows the characteristic two negative minima at 208 and 222 nm and a large-magnitude positive maximum at 193 nm.

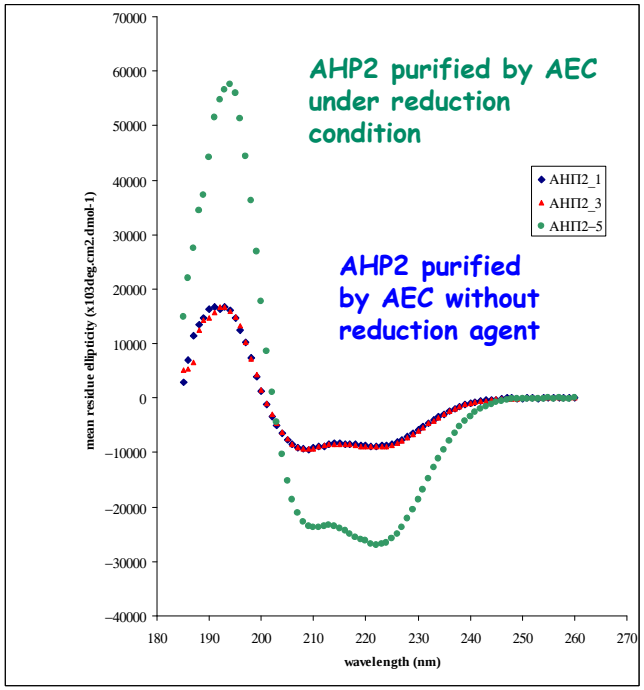
β–sheet spectrum shows a single negative minimum at about 215 nm and a positive maximum at 196 nm (both of these are much smaller than the signal for α-helix).

The random coil (or unordered) shows only a very small signal above 210 nm and a small negative minimum at about 198 nm.

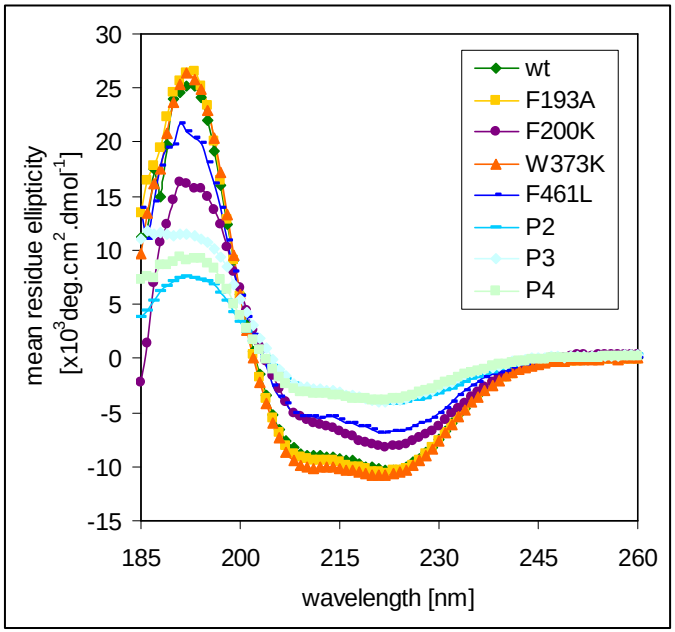
The absence of regular structure results in zero CD intensity.

Circular dichroism spectroscopy – applications

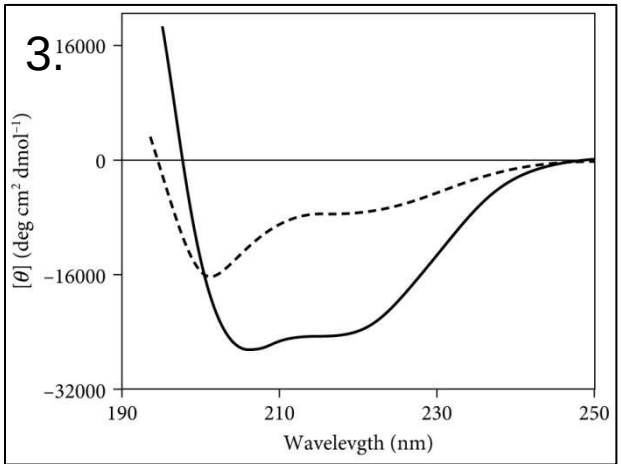
1. Determining whether a protein is correctly folded. (*Arabidopsis thaliana* histidine phosphotransfer protein 2, structure bundle of 6 α-helices). Comparison of AHP2 purified either under reducing or non reducing conditions.



2. Comparing the structures of different mutants of the same protein (maize β-glucosidase its mutants, structure $(\beta/\alpha)_8$ barrel).



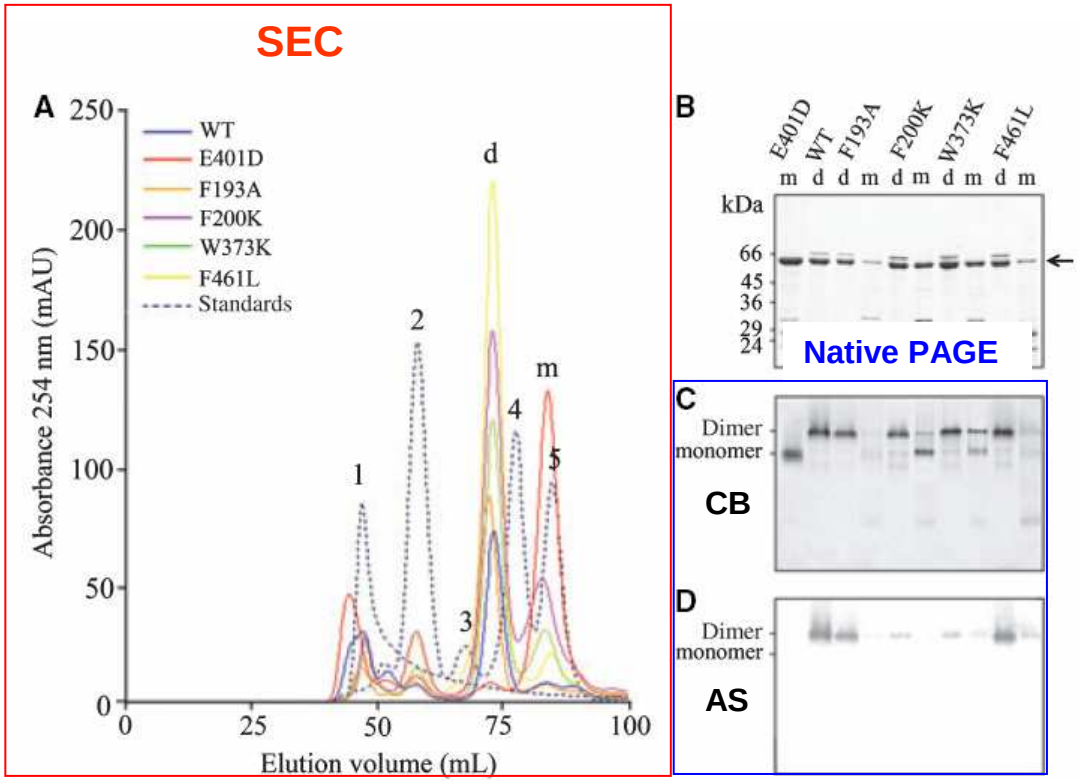
3. Studying the secondary structure under different conditions. Far-UV spectra of CGRP (calcitonin gene related peptide) in phosphate buffer of pH 7 (dashed line) and in the same buffer + 50% trifluoroethanol (solid line).



Determination of quaternary structure – size exclusion chromatography (SEC)

- Size exclusion chromatography may be used to analyze the molecular size of macromolecules after column calibration.
- Both molecular weight and three-dimensional shape of proteins contribute to the degree of their retention.

maize β-glucosidase and its mutant forms



Enzyme	*Apparent molecular weight (kDa)*	
	Dimer	*Monomer*
WT	**107**	**41**
E401D	**114**	**42**
F193A	**117**	**43**
F200K	**109**	**46**
W373K	**109**	**44**
F461L	**109**	**40**

Theoretical Mw:

Dimer/monomer 120 kDa/ 60 kDa

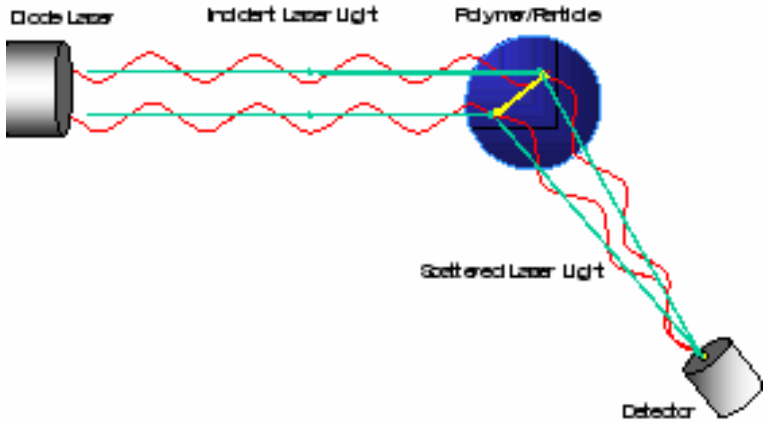
Fig. 5. Quaternary structure of wild-type and mutant Zm-p60.1 β-glucosidases. (A) Elution profiles of wild-type and mutant Zm-p60.1 β-glucosidases from the HighLoad 16/60 Superdex 200 column. A sample (1.5 mL) of each enzyme purified by metal chelate affinity chromatography was applied to the column and eluted with elution buffer (50 mM Tris/HCl, 500 mM NaCl; pH 7.00). Fractions corresponding to peaks d and m were collected and analyzed by (B) Coomassie Brilliant Blue-stained SDS/PAGE, (C) Coomassie Brilliant Blue-stained native-PAGE and (D) in-gel activity staining of native-PAGE gels. Peaks 1, 2, 3, 4 and 5 correspond to Blue Dextran 2000, ferritin (M_r 440 kDa), aldolase (M_r 158 kDa), BSA (M_r 67 kDa) and ovalbumin (M_r 43 kDa), respectively, used as standards. Arrow marks positions of the wild-type and mutant Zm-p60.1 polypeptides in SDS/PAGE.

„Classical“ light scattering

• Also known as "**static**" or "Rayleigh" scattering.

• Provides a direct measure of average molecular mass of the sample.

• Static Light Scattering a beam of polarized light is focused onto a polymer/particle and the scattered light is detected with a photodiode detector.



• The intensity of this scattered light is measured, which is proportional to the molar mass and the concentration of protein(s) in solution (I_{LS} ~ Mw * c).

Applications:

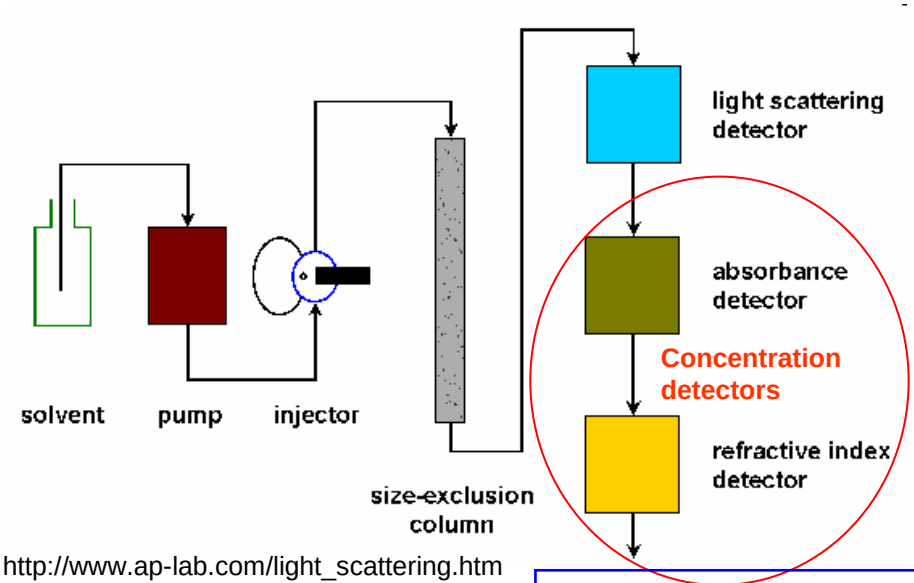
• Determining whether the native state of a protein is a monomer or a higher oligomer.

• Measuring the masses of aggregates or other non-native species.

• The accurate determination of molecular weight from SLS data is absolutely dependent on the purity of the protein sample.

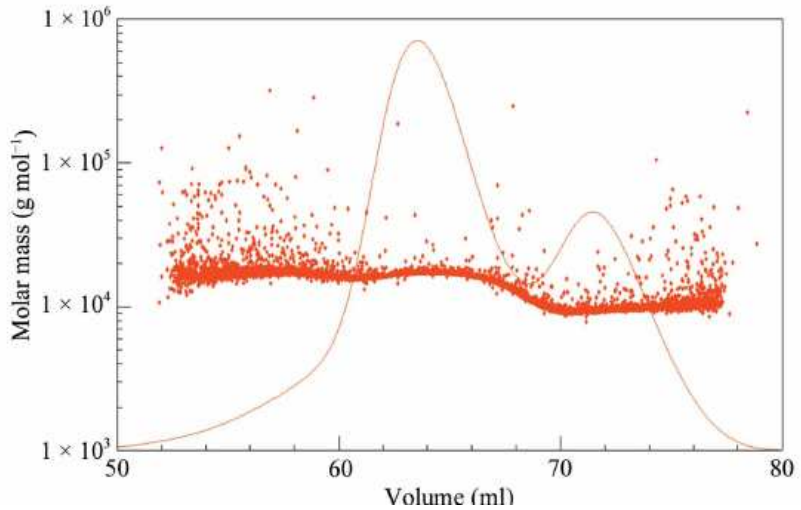
Static light scattering (SLS) in combination with size-exclusion chromatography (SEC)

- SLS in non-invasive method
- mass determination accuracy: 2-5%



http://www.ap-lab.com/light_scattering.htm

Molecular mass of each protein fraction coming off the column



- SEC elution profile shows two distinct species of the protein.
- Comparison of elution peaks with standard curve results in estimated Mws of proteins that do not correspond well to defined defined oligomeric states.
- SLS clearly demonstrate that the two peaks correspond well to monomeric (7.3 kDa) and dimeric (13.7 kDa) forms of the protein.

- SLS measures the average Mw of the sample, the use of SLS data allows selection of elution fractions that contains only single species, rather than mixture of monomer and dimer.

(Geerlof et al., 2006)

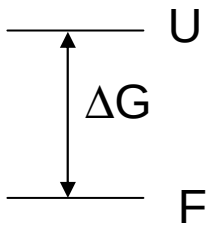
Protein Stability

- Protein stability is the net balance of forces, which determine whether a protein will be in its native folded conformation or a denatured state.

- Protein stability normally refers to the physical (thermodynamic) stability, not the chemical stability (loss of integrity due to bond cleavage, oxidation of several residues etc.).

Principle of thermodynamic stability

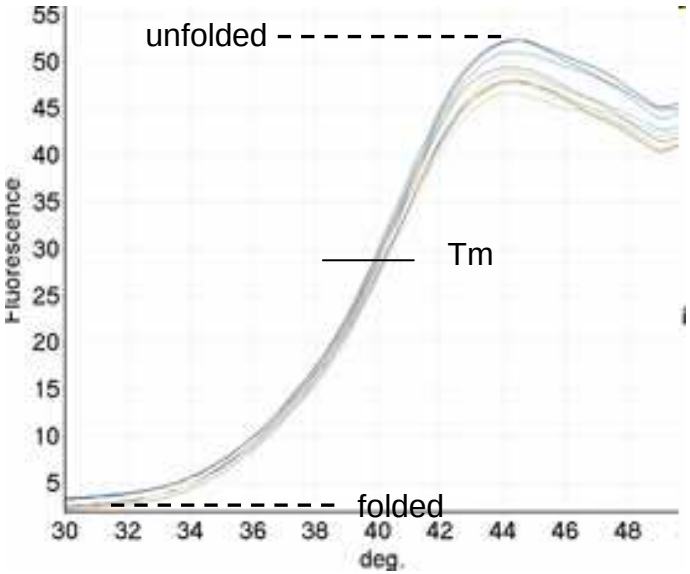
$P_{folded} \rightleftharpoons P_{unfolded} \rightarrow A$



• The net stability of a protein is defined as the difference in free energy between the native and denatured state.

• The energy difference between these two states in physiological conditions is quite small, about 5-15 kcal/mol.

ΔG is temperature – dependent



Protein stability profiling assays monitor the amount of unfolded protein in solution as function of temperature.

The midpoint temperature Tm of the unfolding transition is defined as the temperature at which concentration of native and non-native protein is equivalent: $[P_{folded}] = [P_{unfolded}]$.

Then the equilibrium is

$$K = [P_{folded}] / [P_{unfolded}] = 1$$

Thus the free energy: $\Delta G = 0$ ($\Delta G = - RT\ln K$)

Methods used to test protein stability:

• **Differential scanning calorimetry**: measures the enthalpy (ΔH) of protein unfolding due to heat denaturation.

• **Circular dichroism**: monitors specific changes of protein structure with increasing temperature at single wavelength (220 nm).

• **Differential static light scattering**: monitors the denaturation and subsequent aggregation of unfolded proteins.

• **Differential scanning fluorimetry** (Tm shift assay**,** protein stability shift assay, thermal shift or thermofluor): uses an environmentally sensitive dye to monitor protein unfolding.

Methods used to test protein stability

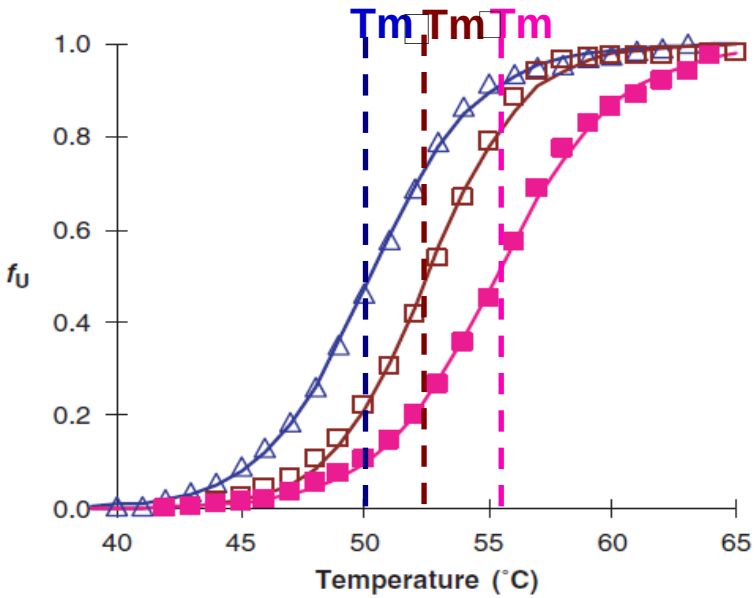
Protein stability in solution is influenced by:

- **temperature**
- **pH**
- **buffer type**
- **salt type and its concentration**
- **metal ions**
- **surfactants**
- **ligands….**

25 mM PIPES pH 6.5, 100 mM NaCl

25 mM PIPES pH 7, 100 mM NaCl

25 mM PIPES pH 7, 500 mM NaCl



- Protein will be most stable at the conditions where T_m is the highest.
- ΔT_m larger than 2℃ is considered as significant.
- A positive ΔTm can be coupled to an increase in structural order and a reduced conformational flexibility.
- A negative ΔTm indicates that buffer induces protein structural changes towards a more disordered conformation or it can be a sign of misfolding.

High-throughput method is necessary to analyze the conditions which stabilize/destabilize the protein.

Monitoring solution-dependent changes in protein stability

Thermal shift assay

Instrumentation:

• Fluorescent Plate reader with heated stage

• Real-time PCR machine

Reagents:

• Enviromentally sensitive fluorescence dye, such as Sypro Orange

Principle:

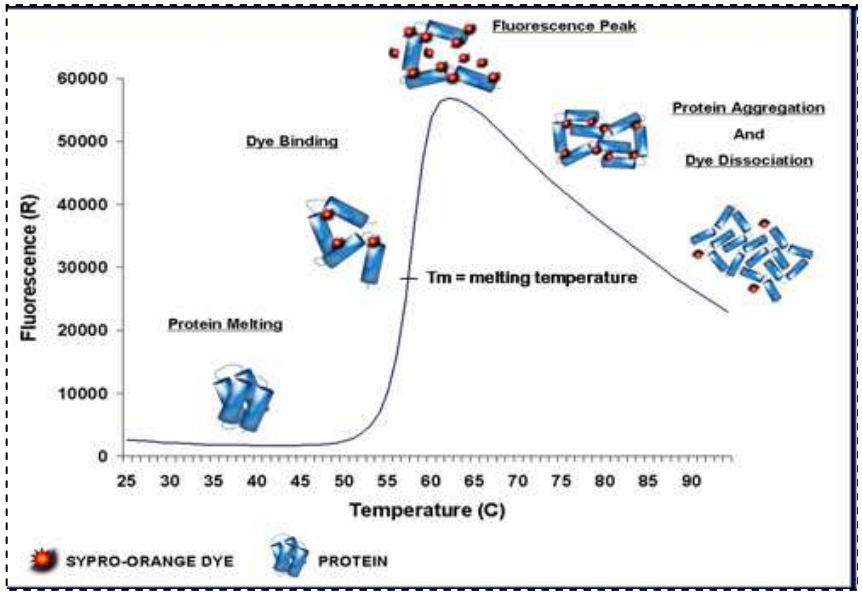
• Sypro Orange fluorescence is quenched in an aqueous environment.

• As the temperature rises, the protein undergoes thermal unfolding and exposes its hydrophobic core regions.

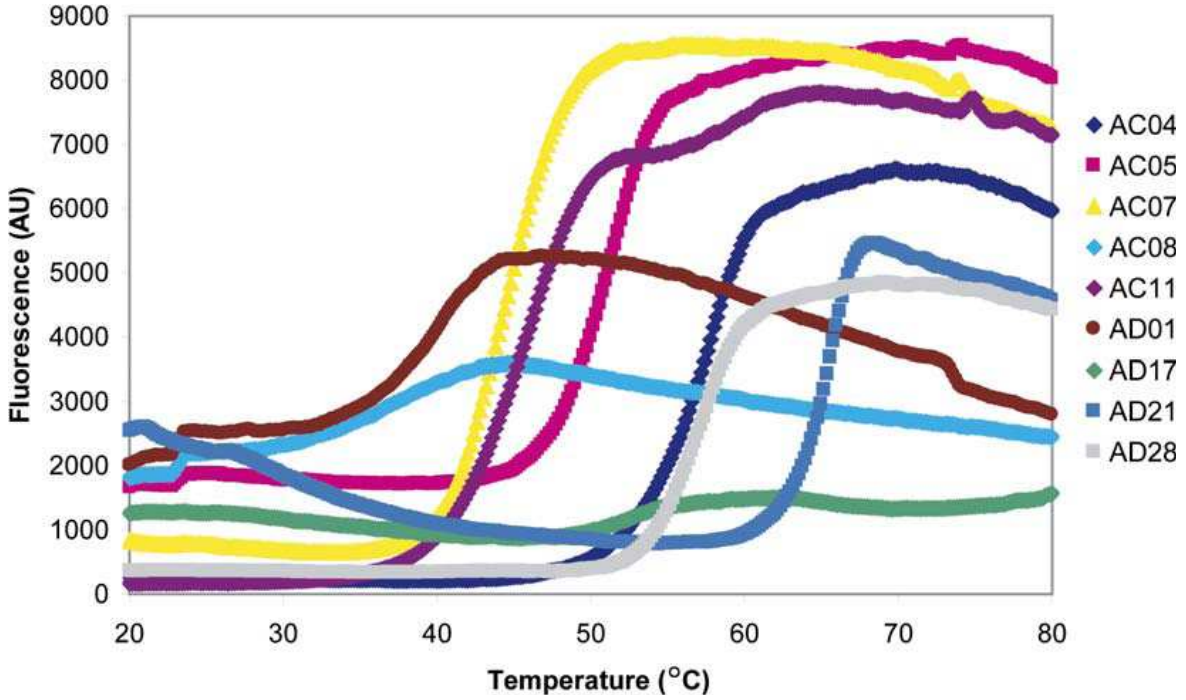
• Sypro Orange then binds to the hydrophobic regions and becomes unquenched.

• Fluorescence is monitored and plotted versus temperature.

• The midpoint of the protein unfolding transition is defined as the Tm.



Thermal shift assay result for the initial screen of different proteins
- information about the condition of proteins



A significant difference in the shape of the curves could be observed between the proteins that have crystallized (e.g., AC04, AC07, AD28) and the proteins that have not crystallized (e.g., AC08, AD01, AD17).

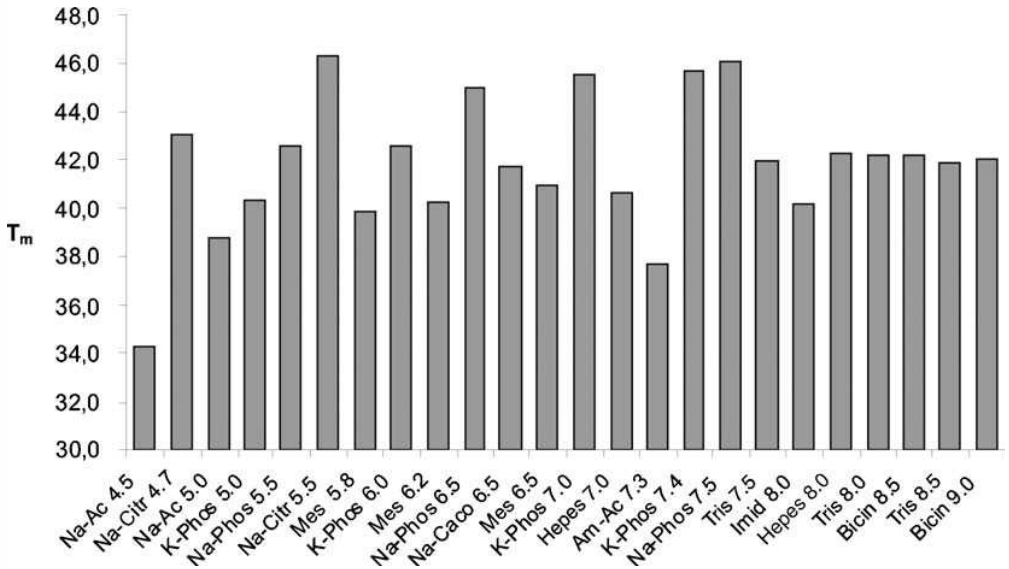
Ericsson et al. 2006

Thermal shift assay results

Melting temperatures of the AC07 protein in the presence of different buffers

List of the buffers and their pH values used in the screen

Number	Buffer (100 mM)	pH
1	Sodium acetate	4.5
2	Sodium citrate	4.7
3	Sodium acetate	5.0
4	Potassium phosphate	5.0
5	Sodium phosphate	5.5
6	Sodium citrate	5.5
7	Mes	5.8
8	Potassium phosphate	6.0
9	Mes	6.2
10	Sodium phosphate	6.5
11	Sodium cacodylate	6.5
12	Mes	6.5
13	Potassium phosphate	7.0
14	Hepes	7.0
15	Ammonium acetate	7.3
16	Sodium phosphate	7.5
17	Tris	7.5
18	Imidazole	8.0
19	Hepes	8.0
20	Tris	8.0
21	Bicine	8.0
22	Tris	8.5
23	Bicine	9.0

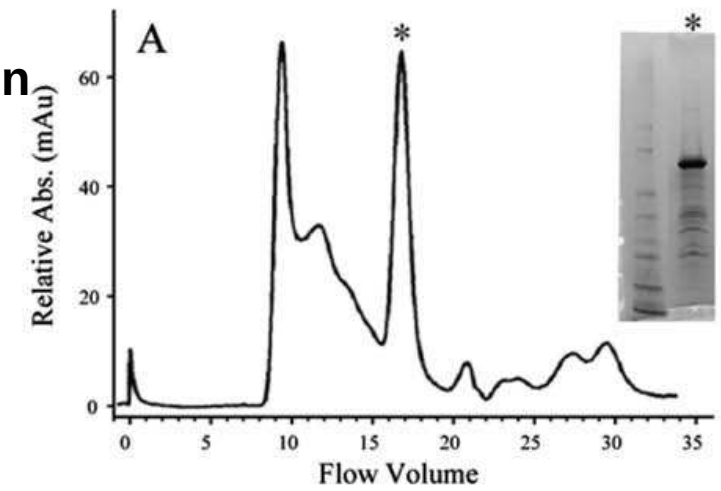


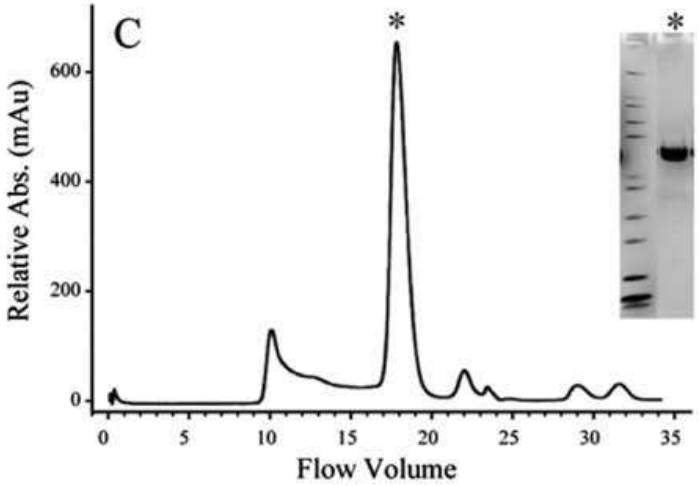
Ericsson et al. 2006

Protein purification and aggregation before and after stability profiling –optimized conditions

Size-exclusion chromatography and SDS PAGE were used to gauge protein quality, purity and tendency to aggregate.

cFMS protein





Original purification conditions:

Ni NTA Superflow resin

Lysis/column wash buffer: **25 mM HEPES pH 7.5**, 400 mM NaCl, **10% glycerol**, 1 mM glutathione, **20 mM imidazole**, 0.1 mM PMSF, 1x EDTA free protease inhibitors

Aggregation was the biggest challenge.

Altered purification conditions:

TALON metal affinity resin

Column wash buffer: **25 mM KH_2PO_4 pH 7.5, 5% glycerol**, **5 mM imidazole**.

Aggregation was minimized.

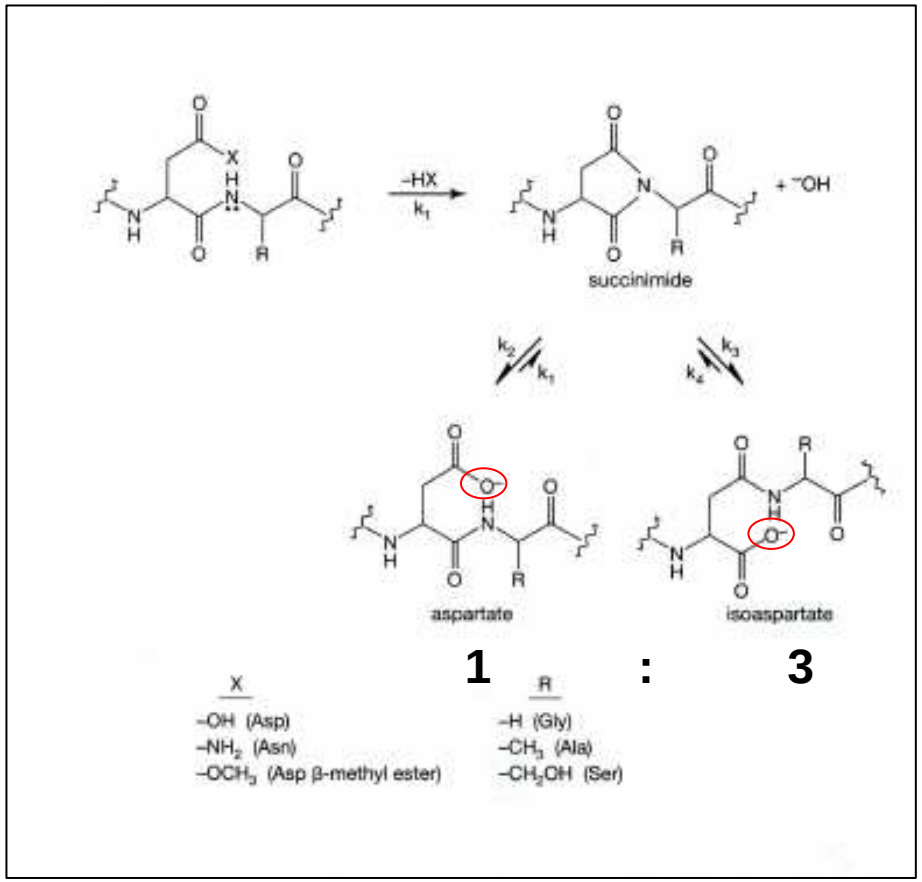
Mezzasalma et al., 2007

Chemical Stability

- Chemical stability involves loss of integrity due to bond cleavage.
- Examples of some degradation reactions in proteins:
  - deamination of asparagine and/or glutamine residues
  - succiinimidation
  - hydrolysis of the peptide bond of Asp residues at low pH
  - oxidation
  - proteolysis
  - disulfide bond breakage and formation
  - disulfide interchange at neutral pH

Succinimidation following deamination

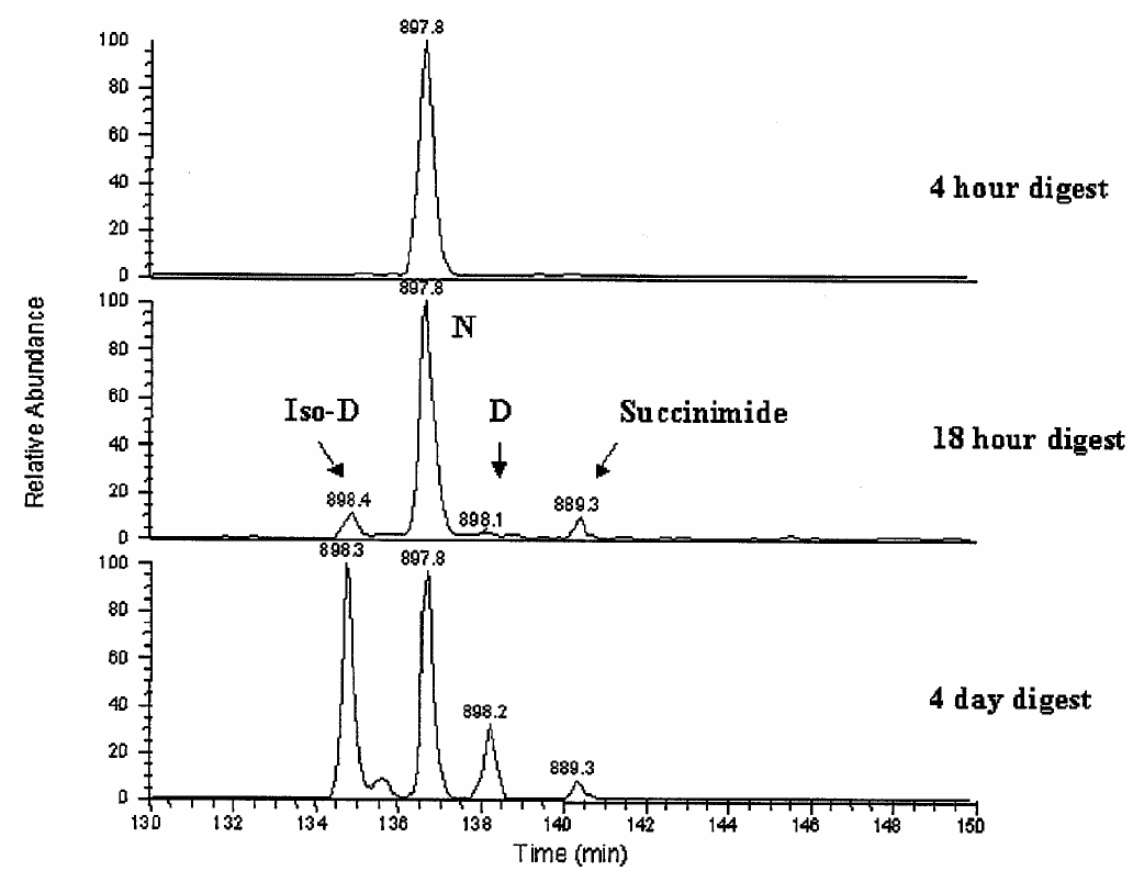
• Formation of β-isoaspartate as a result of deamination of asparagyl residues or isomerization of aspartyl residues is a major source of instability in proteins and peptides, especially **at neutral and alkaline pH after prolonged storage**.



For instance, the disposition of an asparagine residue followed by residue such as glycine, serine or threonine in a polypeptide chain can lead to deamination of an asparagine and formation of α-aspartic acid and/or β-isoaspartate via an intramolecular rearrangement that produce a succinimide (cyclic imide, unstable under physiological conditions) intermediate. This phenomenon occurs at a rapid rate and conveys extra negative charge to the protein.

Analysis of succinimidation following deamination

LC/MS analysis



The relative elution order of the iso-Asp, Asn, Asp, and succinimide forms of the $_{300}$VVSVLTVVHQDWLNGK$_{315}$ tryptic peptide from a recombinant human monoclonal antibody (Chelius, Rehder, &Bondarenko, 2005). The data demonstrate **the effect of incubation time on succinimide and iso-Asp/Asp formation forthe 300–315 tryptic peptide following incubation in 100 mM Tris, pH 7.5 at 37℃ for 4 hr, 18 h r, and 4 days**. **Note the approximately 3:1 ratio of iso-Asp to Asp in the 18 hr and 4 day data.**

(Srebalus Barner and Lim, 2006)

OTHER CHEMICAL REACTIONS AFFECT PROTEIN STABILITY

Hydrolysis

• Asp-Y bond may be at least 100 times more labile than other peptide bonds in dilute acid. Cleavage is particularly rapid at Asp-Gly and Asp-Pro.

• Specific cleavage at Asp-Pro peptide bonds in a polypeptide chain can occur by exposure to acid conditions (e.g. 10% acetic acid with pH 2.5, 70–75% formic acid) at moderate temperatures (37℃, 40℃) for periods u p to 120 hours.

Pyroglutamate formation

• Cyclization of amino terminal glutamine residues to pyroglutamyl residues under mild acidic conditions is a frequent cause of protein modification.

Protein oxidation

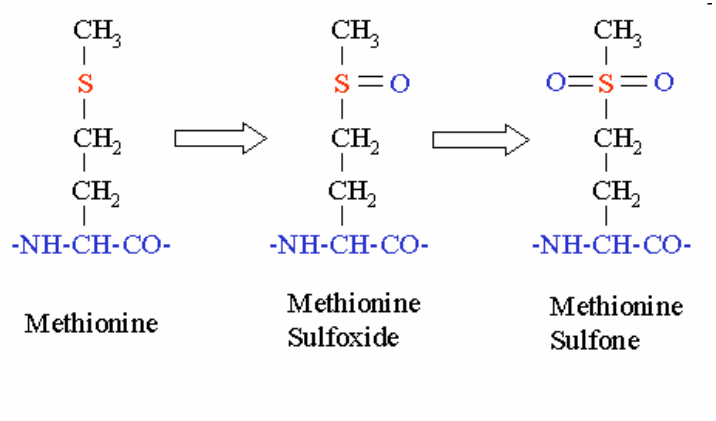
• The side chain of cysteine, methionine, histidine, tyrosin and tryptophan residues are potential sites of oxidation.

• Oxidation at these sites can be catalyzed by trace amount of transition metal ions (site-specific process) or enhanced by oxidants or upon exposure to light (non-specific process).

• Oxidation can lead to protein aggregation, inactivation, increasing immunogenicity and encourage proteolysis.

• The most easily oxidizable sites are thio groups on Met and Cys.

• Met residues in protein can be oxidized by atmospheric oxygen in vial containin only 0.4 % oxygen. The rate of Met oxidation depend on the position in protein and pH.



http://www.ionsource.com/Card/MetOx/metox.htm

Detection of oxidized Met species by MS

Amino Acid	Residue Composition	Residue Monoisotopic Mass	Delta Mass
Methionine	C_5H_9NOS	131.0405	0
Methionine Sulfoxide	$C_5H_9NO_2S$	147.0354	15.9949
Methionine Sulfone	$C_5H_9NO_3S$	163.0303	31.9898
Sulfur	S	31.9721	-

Mass spectrometry (MS)- Analysis of disulfide linkage

Example: In a properly folded IgG antibody, all cysteins are involved in disulfide bonding and thus are responsible for maintaining the characterisitic 3D structure of IgG antibody. Incomplete disulfide linkage or free SH groups can lead to (a) the formation of antibody fragments that do not bind antigen and to (b) antibody aggregation.

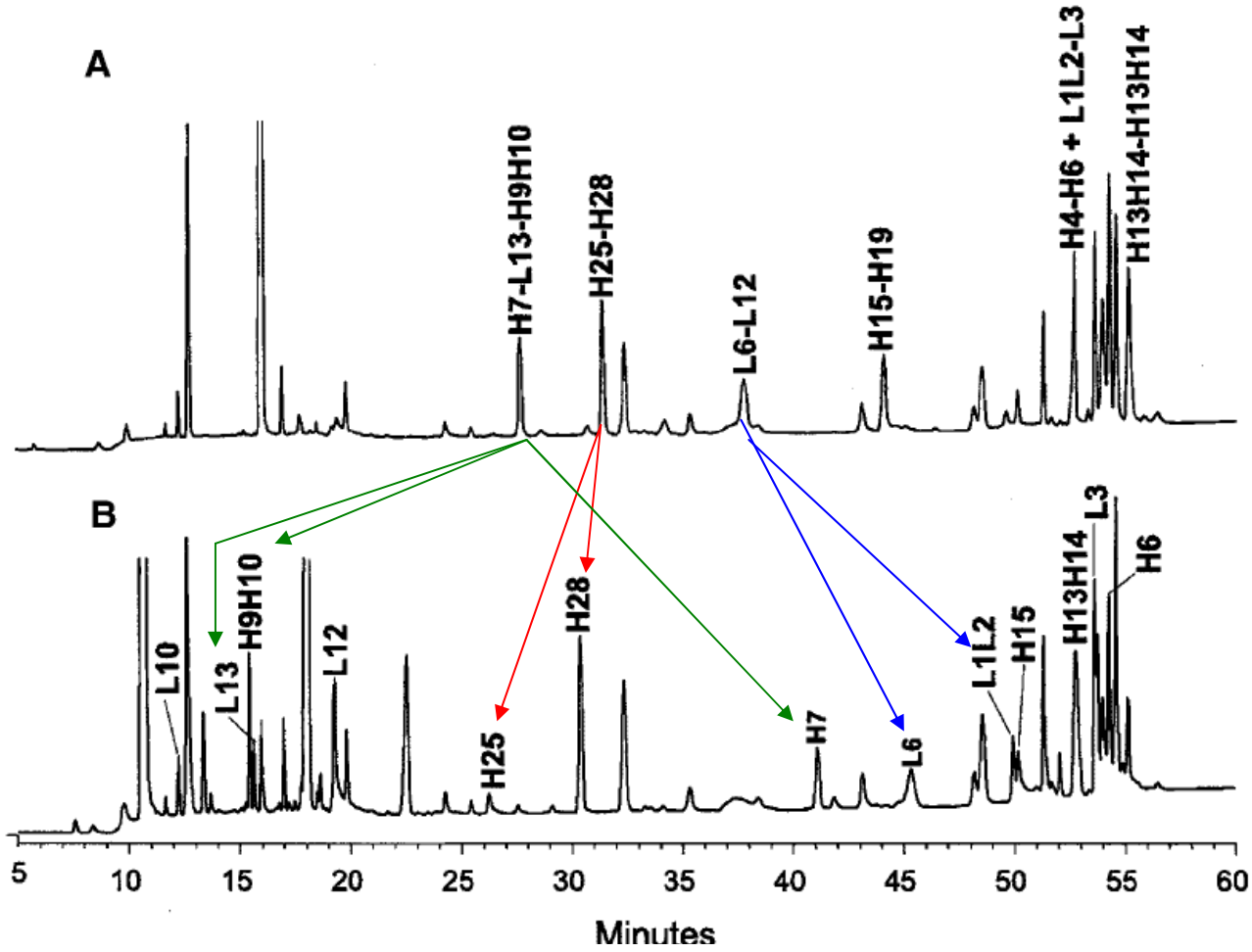
LS/MS analysis

(A)Non- reduced peptide mixture:

1. Alkylation of free sulfhydryl groups.
2. Digestion with protease

(B) Reduced peptide mixture:

1. Alkylation of free disulfide groups.
2. Digestion with protease
3. Reduction of disulfide bonds with DTT



''H'' and ''L'' denote heavy chain and light chain of IgG, respectively.

(Barnes and Lim, 2006)

Protein oxidation-reducing agents

• Reducing agents that are primarily used to protect free SH-groups from oxidation (particularly of cysteine and methionine residues): dithiothreitol (DTT) or 2-mercaptoethanol (2-ME), *tris*(2-carboxyethyl)phosphine (TCEP), EDTA (or EGTA).

• The higher the pH and temperature, the shorter the half-lives of the thiol reagents.

	Half-life of thiol compounds in solution (hours)	
Conditions	**2-mercaptoethanol**	**DTT**
pH 6.5, 20℃	**> 100**	**40**
pH 7.5, 20℃	**10**	**10**
pH 8.5, 20℃	**4**	**1.4**
pH 8.5, 0℃	**21**	**11**
pH 8.5, 40℃	**1**	**0.2**
pH 8.5, 20℃ + 0.1 mM Cu^{2+}	**0.6**	**0.6**
pH 8.5, 20℃ + 1 mM EDTA	**>100**	**4**

All thiols compounds were dissolved in 0.1 M potassium phosphate buffer.

• TCEP is a more stable, faster and stronger reductant than DTT at pH values below 8.0. It resists air oxidation and is stable over a wide pH range (1.5–8.5).

• TCEP is recommended for long-term storage of proteins in the absence of metal chelators (EDTA, EGTA), because Fe3+ and Ni2+ catalyzes DTT oxidation.

• Metal chelators such as EDTA at final concentation of 1-5 mM avoid metal (Cu, Fe, and Zn) -induced oxidation of SH groups and helps to maintain the protein in reduced state.

PROTEOLYSIS

• Proteolysis decreases a protein size, make it less stable and modify its charge, hydrophobicity, activity as a catalyst or immunogenic properties.

A simple test for proteolytic degradation

Incubatation of sample (extract or purified protein) at 30℃-37℃ at various time points

↓

Take aliquats at various time points and assay them for biological activity (Caution: as the target protein may be partially degradated, yet retain full biological activity) or protein size and microheterogeneity have to be monitored by SDS-PAGE and following western blotting and/or by mass spectrometry.

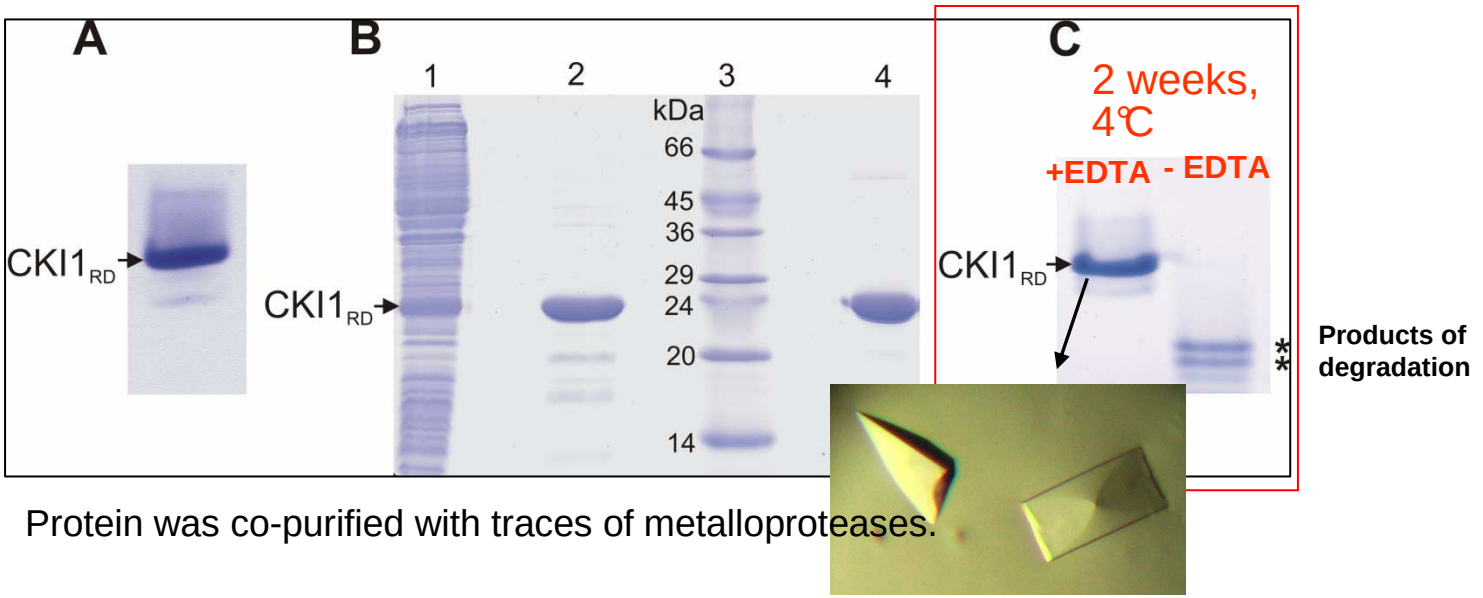
↓

If the proteolysis is a problem, then protease inhibitors must be added to the sample and during all future attempts at purification of that target protein.

PROTEOLYSIS

Table 2. Common Protease Inhibitors

Protease Inhibitor	Target Protease	Working Concentration
PMSF (Phenylmethylsulfonyl fluoride)	Serine proteases	0.1-1 mM
Benzamidine	Serine proteases	1 mM
Pepstatin A	Acid proteases	1 µg/ml
Leupeptin	Thiol proteases	1 µg/ml
Aprotinin	Serine proteases	5 µg/ml
Antipain	Thiol proteases	1 µg/ml
EDTA and EGTA	Metalloproteases	0.1-1 mM



Protein was co-purified with traces of metalloproteases.

PROTEIN CONCENTRATION AFFECTS STABILITY

- In general, proteins are **less stable** at low concentrations (**<50 μg/ml**). Under these conditions, multiple subunit proteins and cofactors tend to dissociate.

- At the protein concentration falls below ~**50 μg/ml**, protein loss can occur due to strong and irreversible adsorption of the protein to a variety of surfaces, including glass, plastic, and various types of filtration media used for concentrating, clarifying, or sterilizing proteins.

- It is **advisable** to keep protein concentration as high as possible (**e.g. >1 mg/ml**) during purification and storage. This is relatively easy to achieve during the early stages of a purification procedure; however, it becomes more difficult during the later stages of purification or in the case of purification of low abundant proteins isolated from natural sources.

- Protein solutions lower than 1 mg/ml should be concentrated as rapidly as possible using methods such ultrafiltration or lyophilization or precipitation using TCA or $(NH_4)_2\ SO_4$ and after dilution in small volume.

- When the rapid concentration of a dilute protein solution is not possible, the addition of ~1 mg/ml bovine serum albumin (BSA) or solvent additives, such as polyethylene glycol (0.05% v/v) and nonionic detergents (0.02% w/v, Triton-X-100, or Tween-20) to all buffers and eluents used in the purification procedure has been shown to be very effective.

- On the other hand, **protein aggregation is generally concentration dependent**.

Protein aggregation – a major event of physical instability of proteins

• Protein aggregation is the assembly of individual protein molecules into amorphous, multimeric states, which results in many cases from intermolecular association of partially denatured protein chains.

• Protein aggregation may be induced by a variety of physical factors, such as temperature, ionic strength, foaming, protein concentration, pH shift, vortexing, etc.

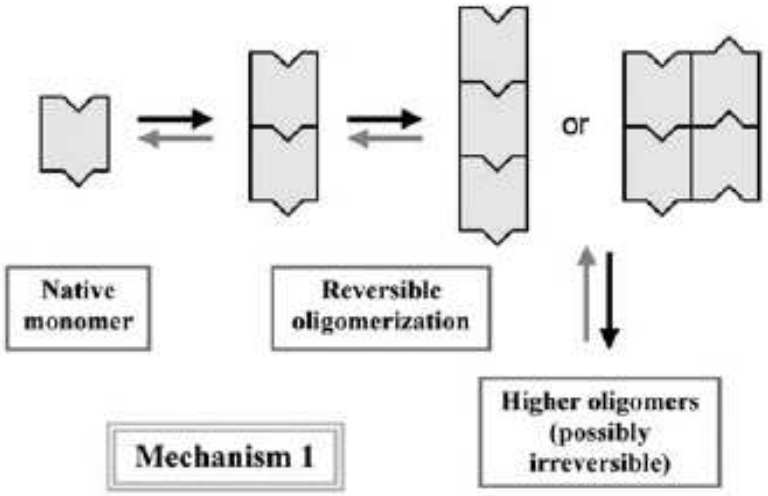
• Protein aggregates may have reduced or no activity, solubility, and altered immunogenicity.

• Proteins aggregate to minimize thermodynamically unfavorable interactions between a solvent and exposed hydrophobic protein residues.

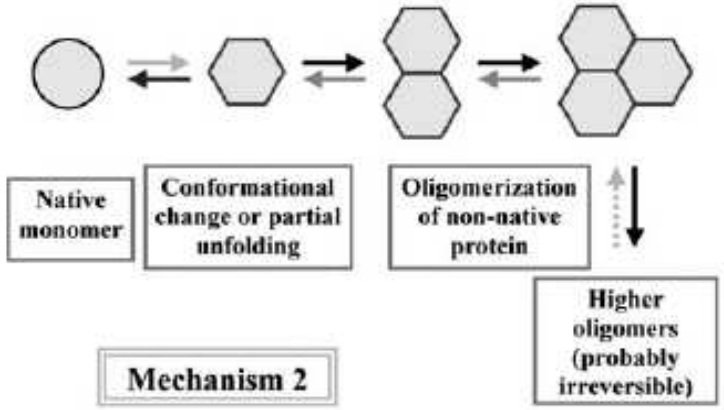
• Hydrophobic interaction, i.e. the reluctance of non-polar groups to be exposed to water, is considered to be a major driving force for both protein folding and aggregation. Both protein aggregation and folding represent a balance of exposed and buried hydrophobic surface areas. The balance is so delicate that a change of one amino acid in a protein may substantially change its aggregation behavior.

• Other possible mechanisms: formation of non-native disulfide bonds, electrostatic interactions.

Mechanisms of protein aggregation



Mechanism 1: The tendency to reversible associate is intrinsic to the native form of the protein. There may be complementary patches on the monomer surface to form reversible small oligomers. **With increasing concentration** larger and larger oligomers form, over time these larger aggregates often become irreversible.



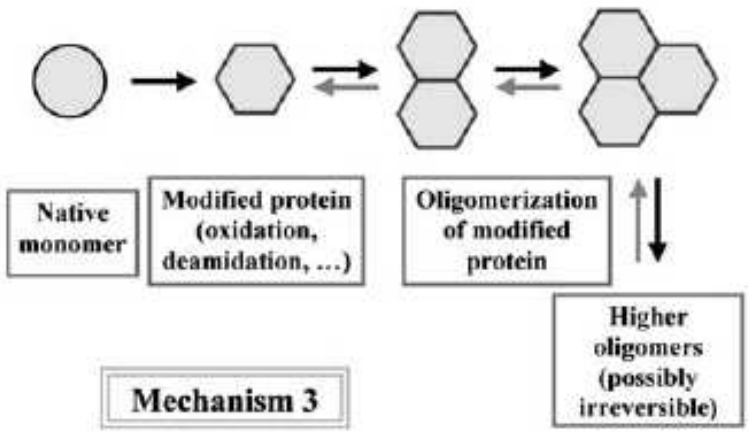
Mechanism 2: The native monomer transiently undergoes a conformational change or partial unfolding. Altered conformation of monomer associates strongly. This aggregation mechanism appears to be the dominant one for many protein.

Aggregation wil be promoted by stress such as heat or shear.

This type of aggregation will be inhibited by excipients or conditions that stabilize the native conformation.

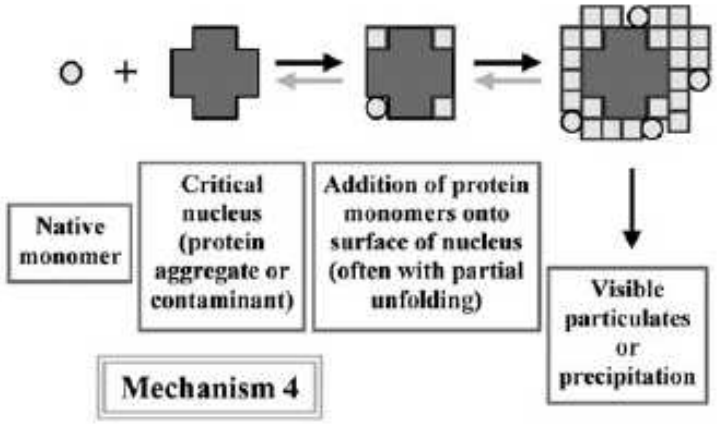
Philo and Arakawa, 2009

Mechanisms of protein aggregation



Mechanism 3: The change in protein conformation that precedes aggregation is caused by differences in covalent structure. This difference is usually caused by chemical degradation such as oxidation of methionin, deamination, or proteolysis. Chemical changes may for example create a new sticky patch on the surface, or change the electric charge in a way that reduces electrostatic repulsion between monomers.

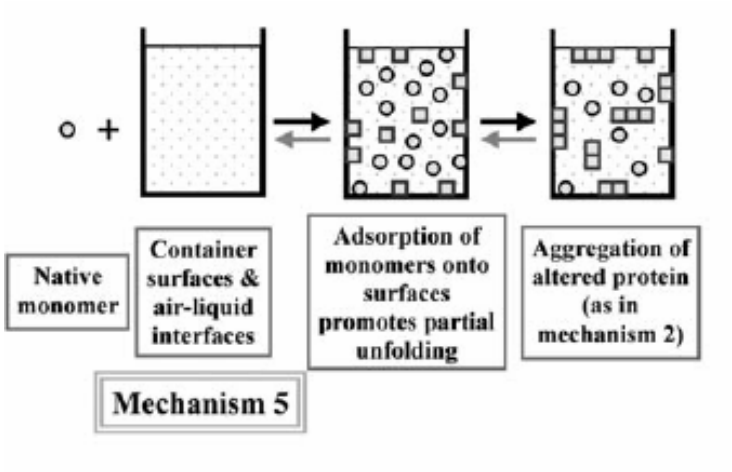
Improving chemical stability will reduce aggregation



Mechanism 4: Nucleation- controlled aggregation is a common mechanism for formation of visible particulates or precipitates. In this mechanism the native monomer has a low tendency to form small or moderately- sized oligomers. However if an aggregate of sufficient size manages to form, then the growth of "critical nucleus" through addition of monomers is strongly favored and the formation of much larger species is rapid.

Philo and Arakawa, 2009

Mechanisms of protein aggregation



Mechanism 5: Surface- induced aggregation starts with binding of the native monomer to a surface. In the case of air/liquid interface that binding would be driven by hydrophobic interactions, but for a container favorable electrostatic interactions might also be involved. After this initial binding event the monomer undergoes a change in conformation and the it is the same as mechanism 2.

To avoid protein aggregation the experimental conditions have to be test very carefully with regards to all type of aggregation mechanisms.

Philo and Arakawa, 2009