Protein extraction, purification, and quantification

C6215 - Lecture 7

Igor Kučera

Protein content in a cell

E. coli

cell density $d = 1.1\ g/cm^3$

water content $w = 0.7$ (70%)

protein fraction of the dry mass $p = 0.55$ (55%)

$\Rightarrow$ protein concentration $c_m = d \times (1 - w) \times p = 1.1 \times (1 - 0.7) \times 0.55 = 0.19\ g/cm^3$

average length of a protein = 300 aa

average molar mass of aa = 110 g/mol

$\Rightarrow$ molar mass of a protein $M = 300 \times 110 = 33\ 000\ g/mol$

$\Rightarrow$ protein concentration $c = (0.19\ g/cm^3) \times (1000\ cm^3/dm^3) / (33\ 000\ g/mol) =$
$= 5.76 \times 10^{-3}\ mol/dm^3$

cell volume = 1 $\mu m^3 = 10^{-15}\ dm^3$

$\Rightarrow$ protein molecules per cell = $(6 \times 10^{23}/mol) \times (5.76 \times 10^{-3}\ mol/dm^3) \times (10^{-15}\ dm^3) = 3.5 \times 10^6$

Compare *H. sapiens* HeLa 2 000 μm^3 => total number is of the order of 10^9

Milo, Bioessays 2013, 35,1050

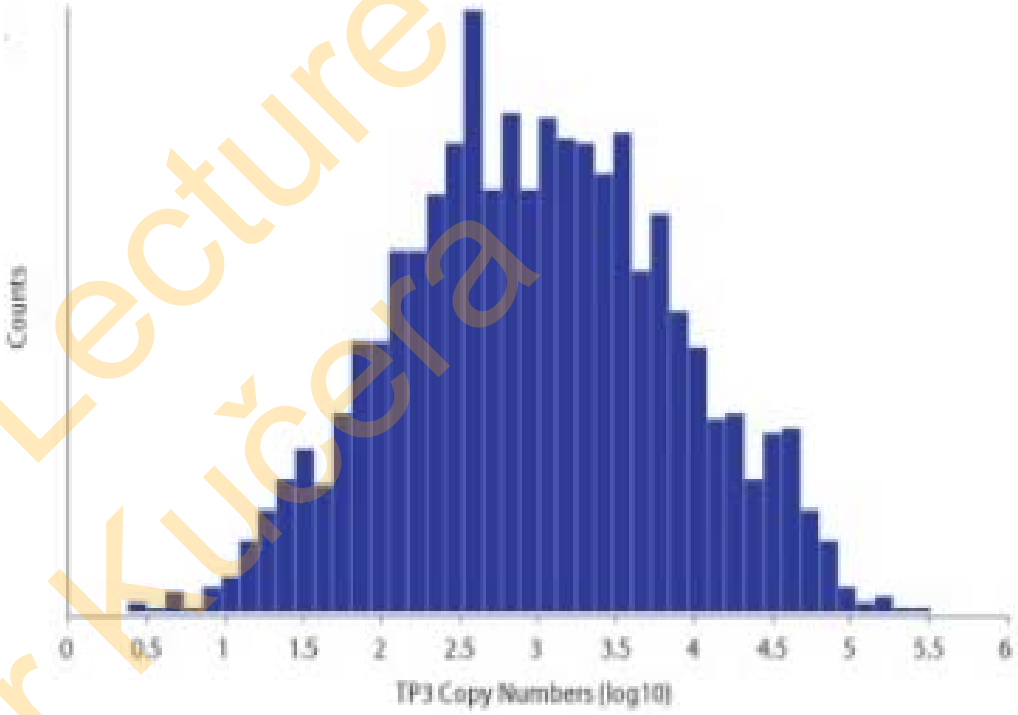
How many different proteins are made in a cell?

E. coli genome contains 4 240 protein coding genes

Soufi et al., Front. Microbiol. 2015, 6, 103

2 303 proteins identified
1 587 proteins absolutely quantified

Protein copy number estimates span across less than six orders of magnitude from approximately 1–300 000 protein copies per cell.



Human genome – about 20 000 protein coding genes. Protein products for about 18 000 of these genes have been detected in at least one human tissue, about 10 000 of these proteins are present in all cells.
Each gene can produce multiple forms of a protein, and these in turn can undergo several post-translational modifications.

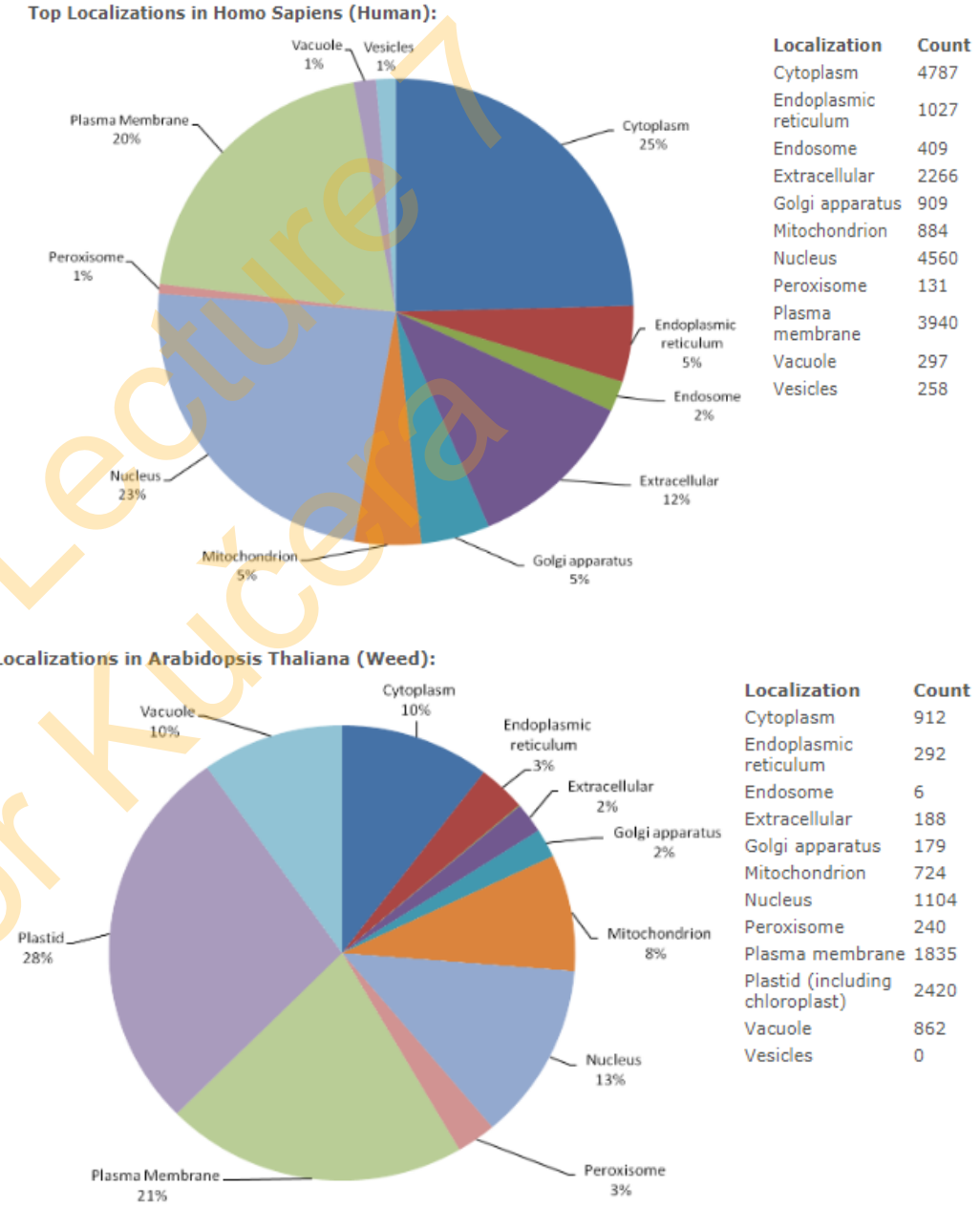
Wilhelm et al., Nature 2014, 509, 582; Salzberg, BMC Biology 2018, 16, 94

Subcellular locations of proteins

Escherichia coli

	Sub-cellular Location	Stepdb nomenlature	# Proteins
Sec Secretory proteins	Peripheral inner membrane protein facing the periplasm	F2	10
	Inner Membrane Lipoprotein	E	21
	Periplasmic	G	295
	Peripheral outer membrane protein facing the periplasm	F3	8
	Outer Membrane Lipoprotein	I	94
	Outer Membrane b-barrel protein	H	64
	Peripheral outer membrane protein facing the extra-cellular space	F4	12
	Extra-cellular	X	1
	Total		**505**
Cytoplasmic	Cytoplasmic	A	1851
	Peripheral proteins	F1	514
	Total		**2365**

http://www.stepdb.eu/info.php



Localization	Count
Cytoplasm	4787
Endoplasmic reticulum	1027
Endosome	409
Extracellular	2266
Golgi apparatus	909
Mitochondrion	884
Nucleus	4560
Peroxisome	131
Plasma membrane	3940
Vacuole	297
Vesicles	258

Localization	Count
Cytoplasm	912
Endoplasmic reticulum	292
Endosome	6
Extracellular	188
Golgi apparatus	179
Mitochondrion	724
Nucleus	1104
Peroxisome	240
Plasma membrane	1835
Plastid (including chloroplast)	2420
Vacuole	862
Vesicles	0

https://www.rostlab.org/services/locDB/statistics.php

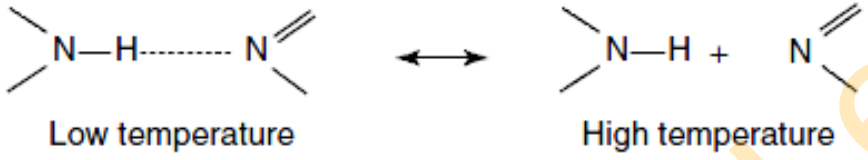
Why purify proteins?

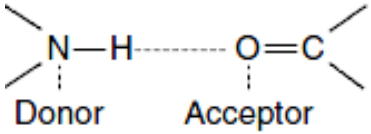
- To establish basic biochemical parameters such as the Michaelis–Menten constant (K_M). Purification would remove conflicting *enzyme* activities which may be present in a crude extract.
- To establish the effects of activators and inhibitors on a protein's function.
- The molecular mass and post-translational modifications can be determined with a purified protein.
- The protein's partial sequence can be used to identify the gene.
- The purified protein can be used to grow crystals for structural studies.
- *Antibodies* can be raised to a purified (or partially purified) protein which can be used to determine cellular location or cross-reactivity with different species.

Molecular interactions and variables that affect them

Important with regard to protein structure and stability; they also take place between an individual protein and other proteins, DNA, or materials used in protein purification.

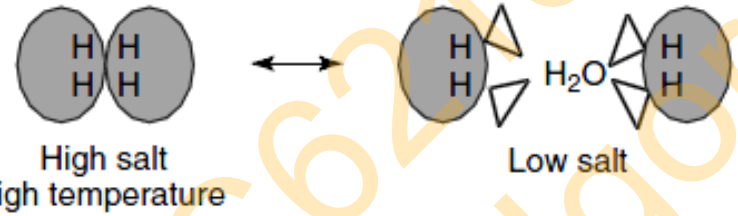
Hydrogen bonds



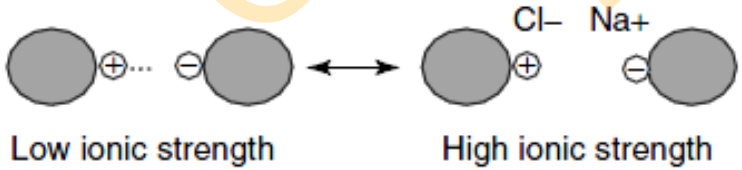


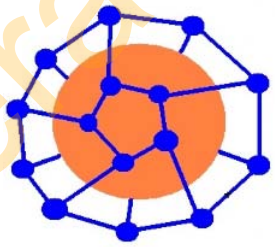
Strength is very dependent on geometry and distance (2.6–3.1 Å)

Hydrophobic interactions (waxy residues: Ileu, Leu, Val, Phe, Trp)

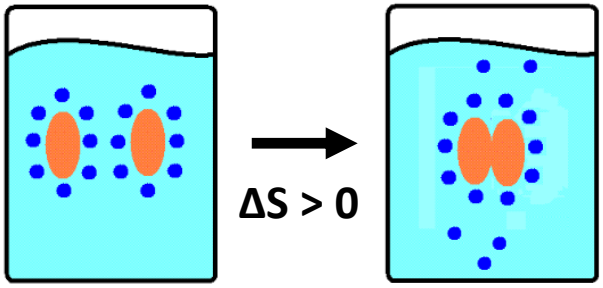


Ionic interactions (charged residues: Asp- Glu- S- Lys+ Arg+ His+)





Water molecules form a ice-like cage structure around the hydrophobe.



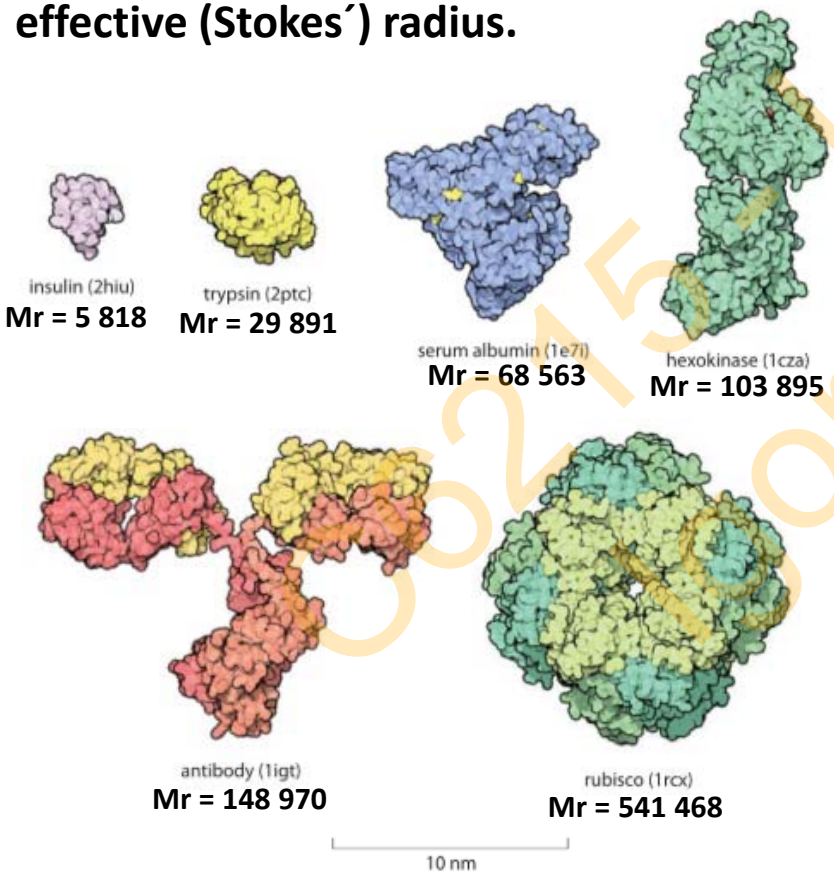
$$W\ (kJ/mol) = 138.94 \times \frac{z_1 \times z_2}{\varepsilon_r \times r\ (\text{in nm})}$$

z_1, z_2 charge numbers, ε_r relative permittivity (dielectric constant): H_2O $\varepsilon_r = 80$, hexane $\varepsilon_r = 2$

Properties of proteins that enable purification

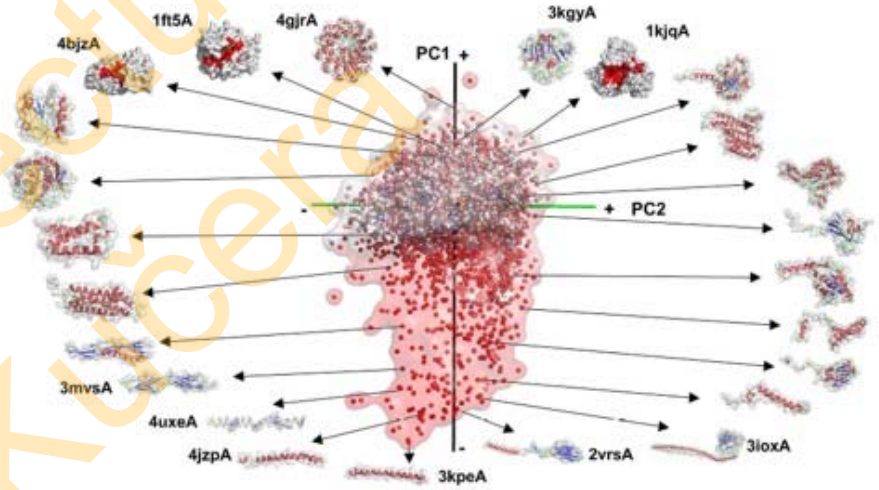
Size

Proteins can vary in size from tens to several tens of hundreds aa.
Most proteins have Mr in the range 10 000 – 150 000.
=> Fractionation on the basis of effective (Stokes´) radius.



Shape

Protein shapes range from approximately spherical (globular) to quite asymmetric.



Han et al., PLoS Comput. Biol. 2019, 15, e1006969

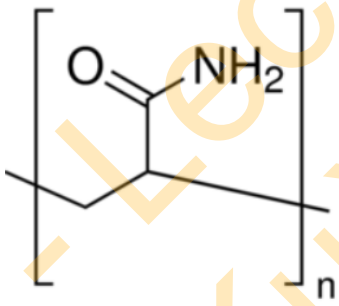
The shape of a protein influences its effective radius.

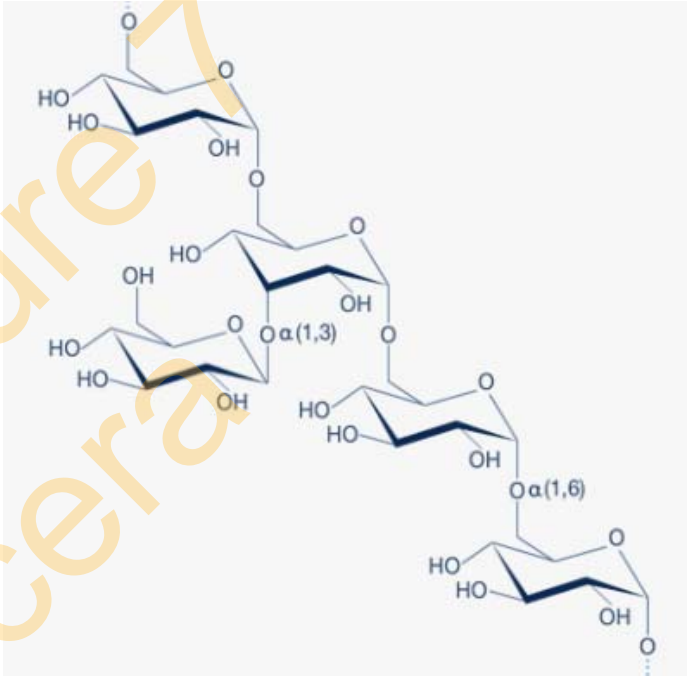
Two protein of the same Mr:

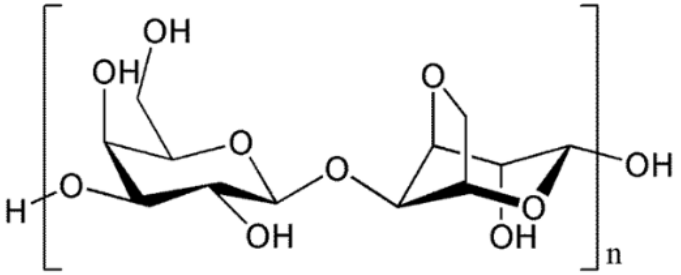
sediments slower => appears smaller
less enters the pores => appears larger

Commonly used gel filtration media

	lower limit	upper limit
dextran gels		
Sephadex G-50	1.5	30
Sephadex G-75	3	80
Sephadex G-100	4	150
Sephadex G-150	5	300
Sephadex G-200	5	600
polyacrylamide gels		
Bio-Gel P-10	1.5	20
Bio-Gel P-30	2.5	40
Bio-Gel P-60	3	60
Bio-Gel P-100	5	100
Bio-Gel P-150	15	150
BioGel P-200	30	200
Bio-Gel P-300	60	400
dextran-polyacrylamide gels		
Sephacryl S-200	5	250
Sephacryl S-300	10	1500
Sephacryl S-400	20	8000
agarose gels		
Sepharose 6B	10	4000
Sepharose 4B	60	20,000
Sepharose 2B	70	40,000
Bio-Gel A-0.5	10	500
Bio-Gel A-1.5	10	1500
Bio-Gel A-5	10	5000
Bio-Gel A-15	40	15,000
Bio-Gel A-50	100	50,000







D-galactose **3,6-anhydro-L-galactose**

https://www.ucl.ac.uk/~ucbcdab/enzpur/gelexcl.htm

Charge

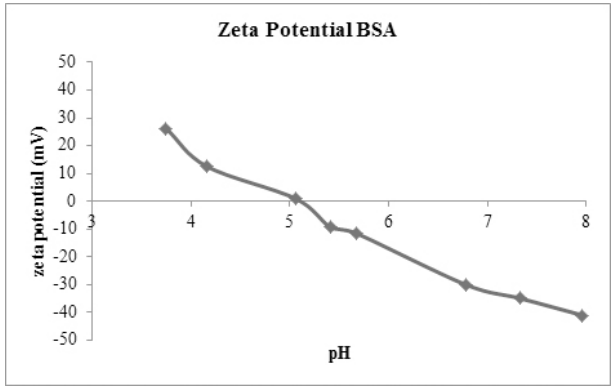
The net charge of a protein is determined by the sum of the positively and negatively charged amino acid residues. If a protein has a net positive (negative) charge at pH 7, it is termed basic (acidic). The charge of an ionizable group is a function of pH.

Ionizable group	pK_a[a]	pH 2										pH 7										pH 12
C-terminal (COOH)	4.0	0	0	0	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Aspartate (COOH)	4.5	0	0	0	0	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glutamate (COOH)	4.6	0	0	0	0	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Histidine (imidazole)	6.2	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0
N-terminal (amino)	7.3	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0
Cysteine (SH)	9.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	–	–	–	–
Tyrosine (phenol)	10.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	–	–	–
Lysine (amino)	10.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
Arginine (guanido)	12.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0

=> Fractionation on anion and cation exchangers (anexes and catexes).
(When the charged residues are not evenly distributed on the surface of the protein, binding to both types of ion exchangers is possible.)

Isoelectric point pI corresponds to the pH in solution at which the net surface charge, and thus the electrophoretic mobility, of a protein equals zero.

pI by electrophoretic light scattering



https://www.entegris.com/content/dam/product-assets/nicompnanodlszlssystems/appnote-isoelectric-point-iep-determination-10528.pdf

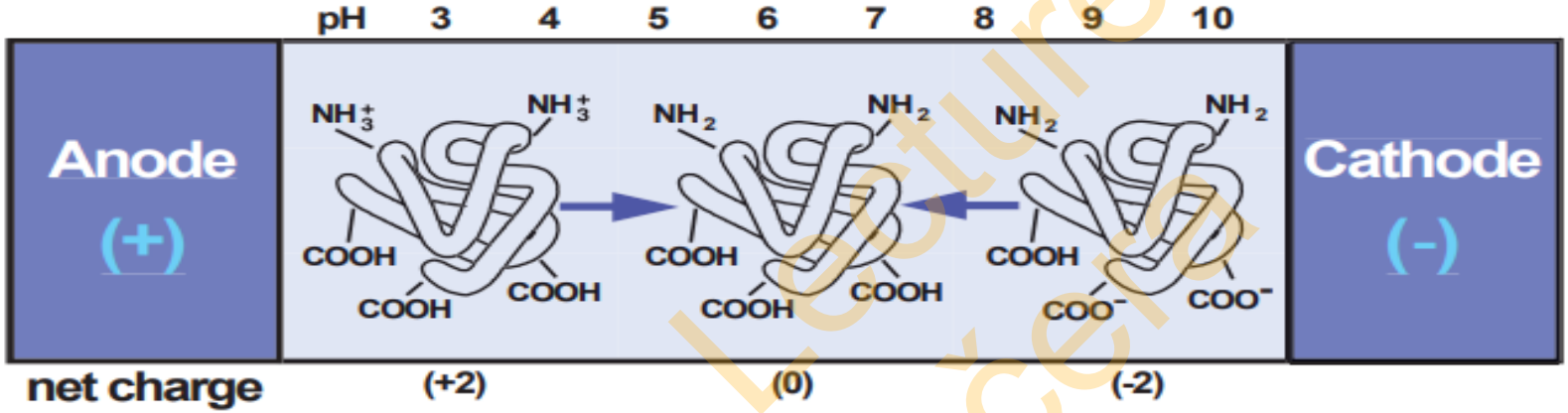
Different cation and anion exchangers with their ionizable groups and features

Name	Type	Ionizable group	Remarks
DEAE cellulose	weakly basic	Diethylaminoethyl $-CH_2CH_2N(C_2H_5)_2$	used to separate acidic and neutral proteins
CM cellulose	weakly acidic	carboxymethyl $-CH_2COOH$	used to separate basic and neutral proteins
P-cellulose	strongly and weakly acidic	Phosphate $-OPO_3H_2$	dibasic binds basic protein strongly
Bio-rex 70	weakly acidic polyststyrene based	Carboxylic acid $-COOH$	used to separate basic proteins and amines
DEAE Sephadex	Weakly basic cross linking dextran gel	Diethylaminoethyl $-CH_2CH_2N(C_2H_5)_2$	Combined chromatography and gel filtration for acidic and neutral protein
SP -Sepharose	Strongly acidic cross linked agarose gel	Methyl sulphonate $-CH_2SO_3H$	Combined chromatography and gel filtration for basic protein
CM Bio Gel A	Weakly acidic cross linked agarose gel	carboxymethyl $-CH_2COOH$	Combined chromatography and gel filtration for basic and neutral protein

Bhatt, Enzymology and Enzyme Technology 2011

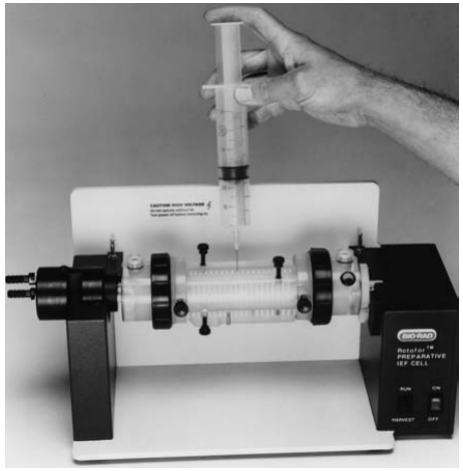
Preparative isoelectric focusing (IEF)

When a protein is electrophoresed through an established pH gradient, it will migrate until it reaches the pH where the net charge on the protein is zero; at that point it will stop migrating and is said to be focused at its isoelectric point or pI.



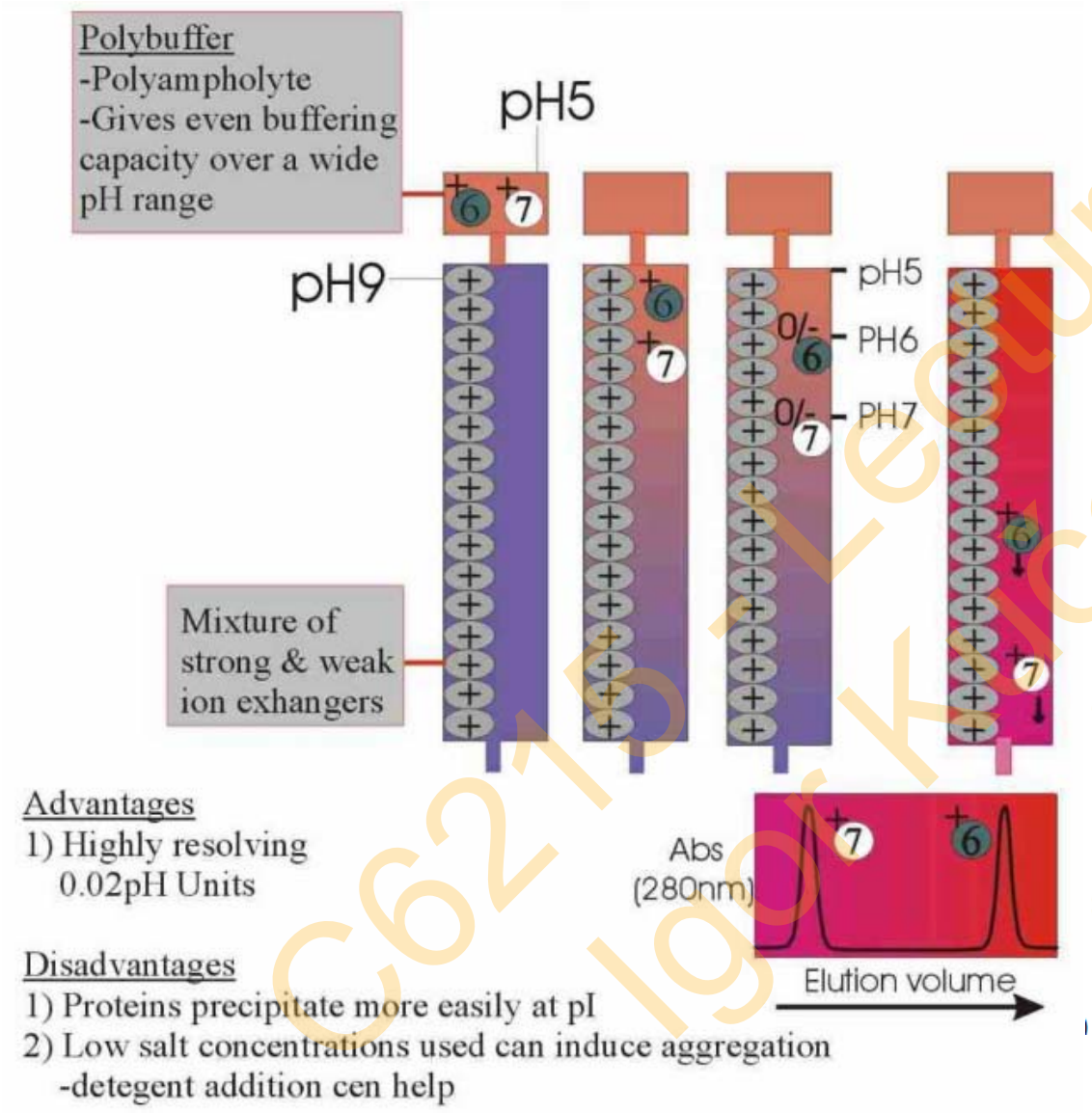
Ampholytes which are small, charged buffer molecules are used to establish the pH gradients increasing in pH from anode to cathode. When voltage is applied to a system of ampholytes and proteins, all the components migrate to their respective pIs. Ampholytes rapidly establish the pH gradient and maintain it for long periods allowing the slower moving proteins to focus.

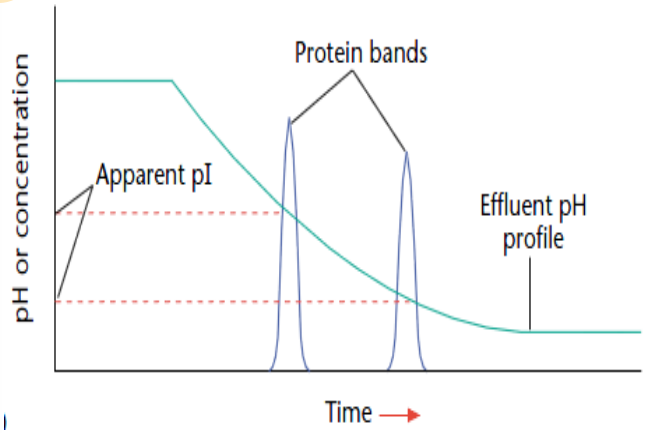
Rotofor system. The separation column is divided into compartments by by means of polyester screens that offer resistance to fluid convection, but do not hinder the flow of current or the transport of proteins. Gravitationally induced convection is inhibited by rotating about the horizontal axis.



Rotofor® System Instruction Manual (BioRad)

Chromatofocusing



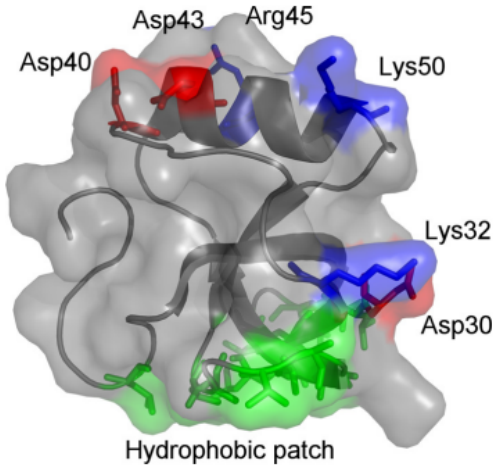


http://macromol.sbcs.qmul.ac.uk/oldsite/expertise/CF3.jpg

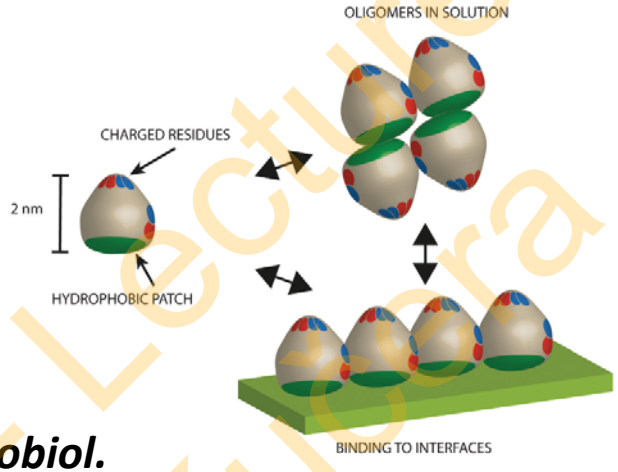
Frey et al., Encyclopedia of Life Sciences 2001

Hydrophobicity

Most hydrophobic amino acid residues are buried on the inside of a protein, but some are found on the surface (hydrophobic patches).



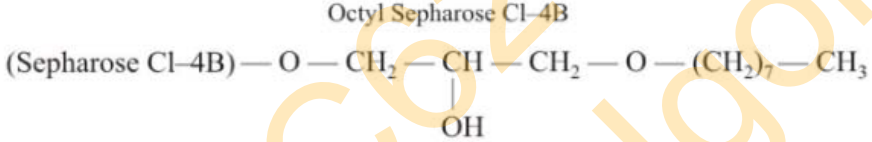
Hydrophobins – amphiphilic fungal proteins



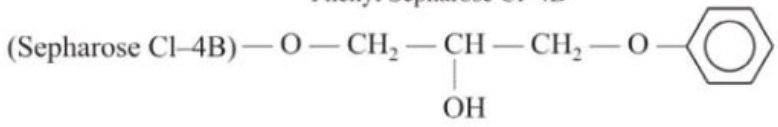
Lienemann et al., Appl. Environ. Microbiol. 2013, 79, 5533

⇒ **Fractionation on hydrophobic column materials**

Octyl Sepharose Cl–4B



Phenyl Sepharose Cl–4B

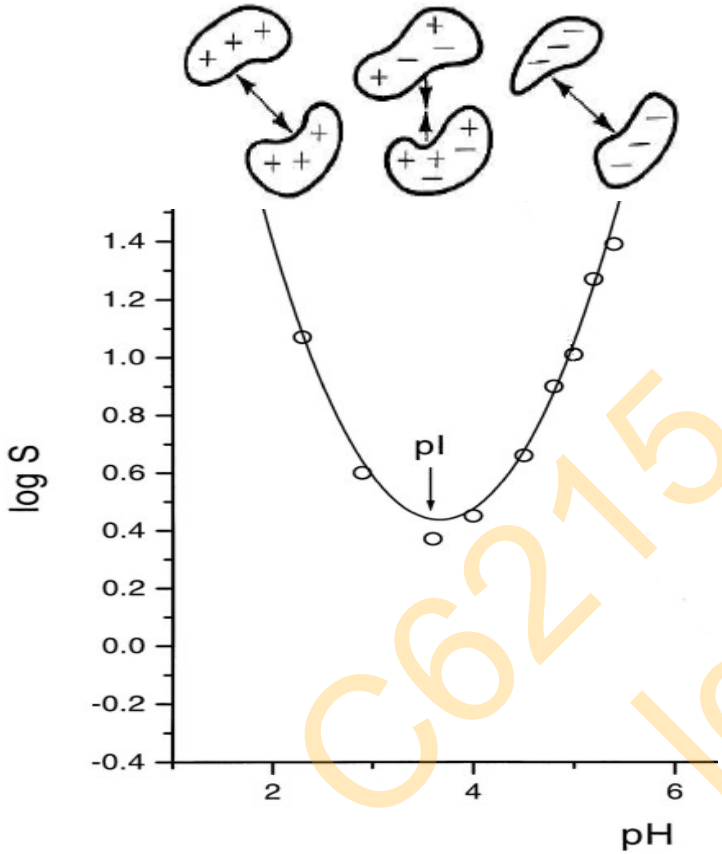


Density

The density of most proteins is between 1.3 and 1.4 g/cm^3. However, some proteins substantially differ, e.g. phosvitin, a phosphoprotein from the egg yolk (density = 1.8 g/cm^3) and β-lipoprotein (density = 1.03 g/cm^3) .

Solubility

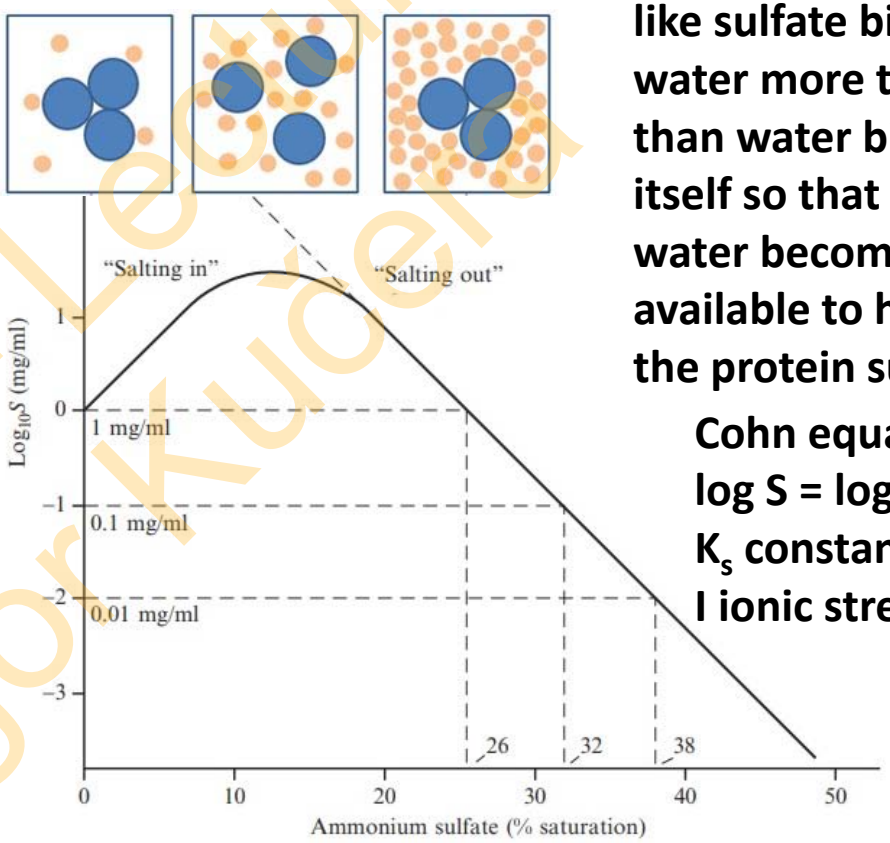
Proteins vary dramatically in their solubility from being essentially insoluble (<10 µg/ml) to being very soluble (>300 mg/ml). Key factors affecting the solubility of a protein include pH, ionic strength, the nature of the ions, temperature, and the polarity of the solvent. Most proteins show minimum solubility at their isoelectric point where there is less charge repulsion.



Solubility S (mg/ml) of RNase Sa as a function of pH.
Shaw et al., Protein Sci. 2001, 10, 1206

Kosmotropic ions like sulfate bind water more tightly than water binds itself so that less water becomes available to hydrate the protein surface.

Cohn equation:
$\log S = \log S_0 - K_s I$
K_s constant
I ionic strength

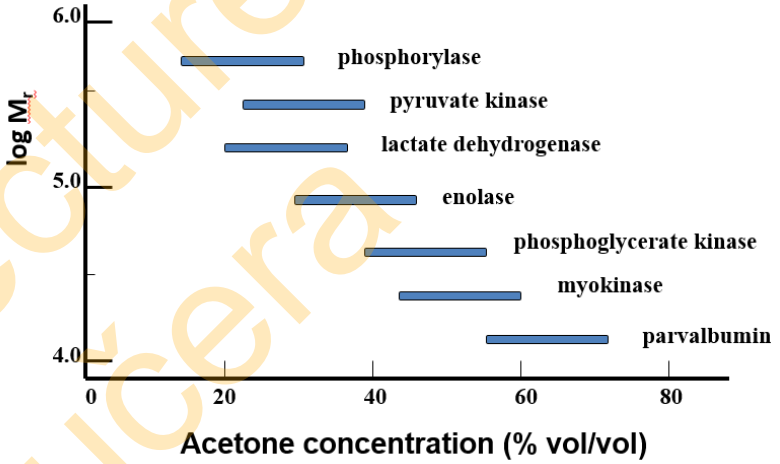


Ammonium sulfate solubility curve for a hypothetical protein (100% saturation = 4.1 M)
Burgess, Meth. Enzymol. 2009, 463, 331

Addition of miscible solvents such as ethanol or acetone causes proteins to precipitate. This is due to decrease of the relative permitivity (and thus the polarity) and dehydration of protein surface. With smaller hydratation layer, the proteins can aggregate by attractive electrostatic and dipol forces. An empirical correlation between the the solubility and relative permitivity is $\log S = \log S_0 - (K_s/\varepsilon_r^2)$.

The size of protein molecule is an important factor for precipitation; the larger the molecule, the lower the percentage of organic solvent required to precipitate it.

Kumar et al., in: Isolation and purification of proteins (Hatti-Kaul, Matthiasson Eds.), Taylor & Francis, 2005.



Thermal stability

Proteins differ in their thermal stability and ability to renature after thermal denaturation. In general, smaller, highly charged proteins are stable to higher temperatures than large, more hydrophobic proteins. Thermal denaturation can be used to precipitate the unwanted proteins while the desired protein remains unaffected.

Table 1
Purification of calmodulin from human red blood cells

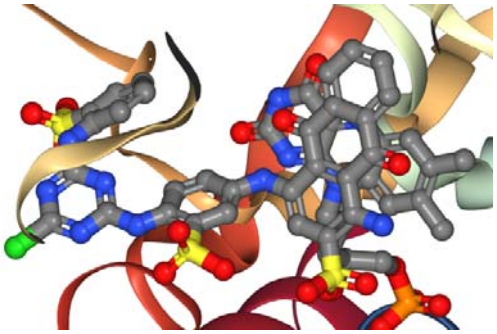
Purification stage	Total protein (mg)	Total units	Yield (%)	Purification (-fold)
Hemolysate	29 800	8.57×10^4	100	–
Conc. DE-11 extract	197.4	8.40×10^4	98	151
Boiling	56	8.25×10^4	96	532
Ca extraction	3.4	7.90×10^4	92	8673
Salt gradient	2.2	7.73×10^4	90	13 545

One activation unit is the protein amount which gives 50% of the maximum activation of the ATP-dependent (Ca+Mg)ATPase activity

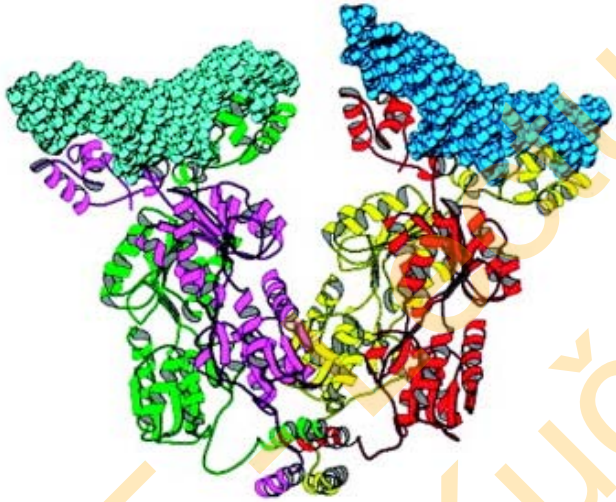
Muallen & Karlish, FEBS Lett. 1979, 107, 209

Ligand binding

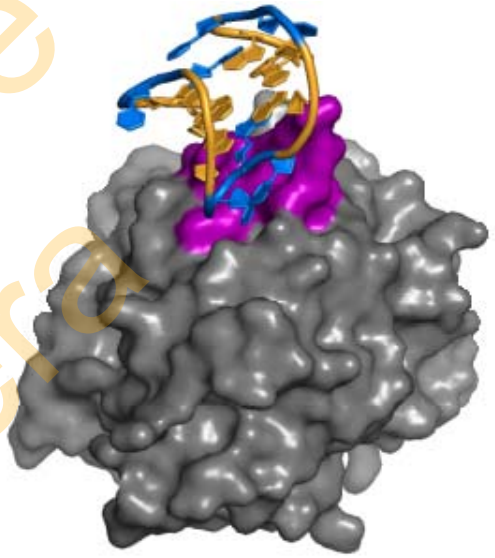
Many proteins tightly and specifically bind substrates, effector molecules, cofactors, or nucleic acid sequences.



Azoreductase AzrC in complex with Cibacron Blue, a biomimetic dye (PDB: 3W78)



Lac repressor complexed to DNA
Lewis, C.R. Biologies 2005, 328, 521



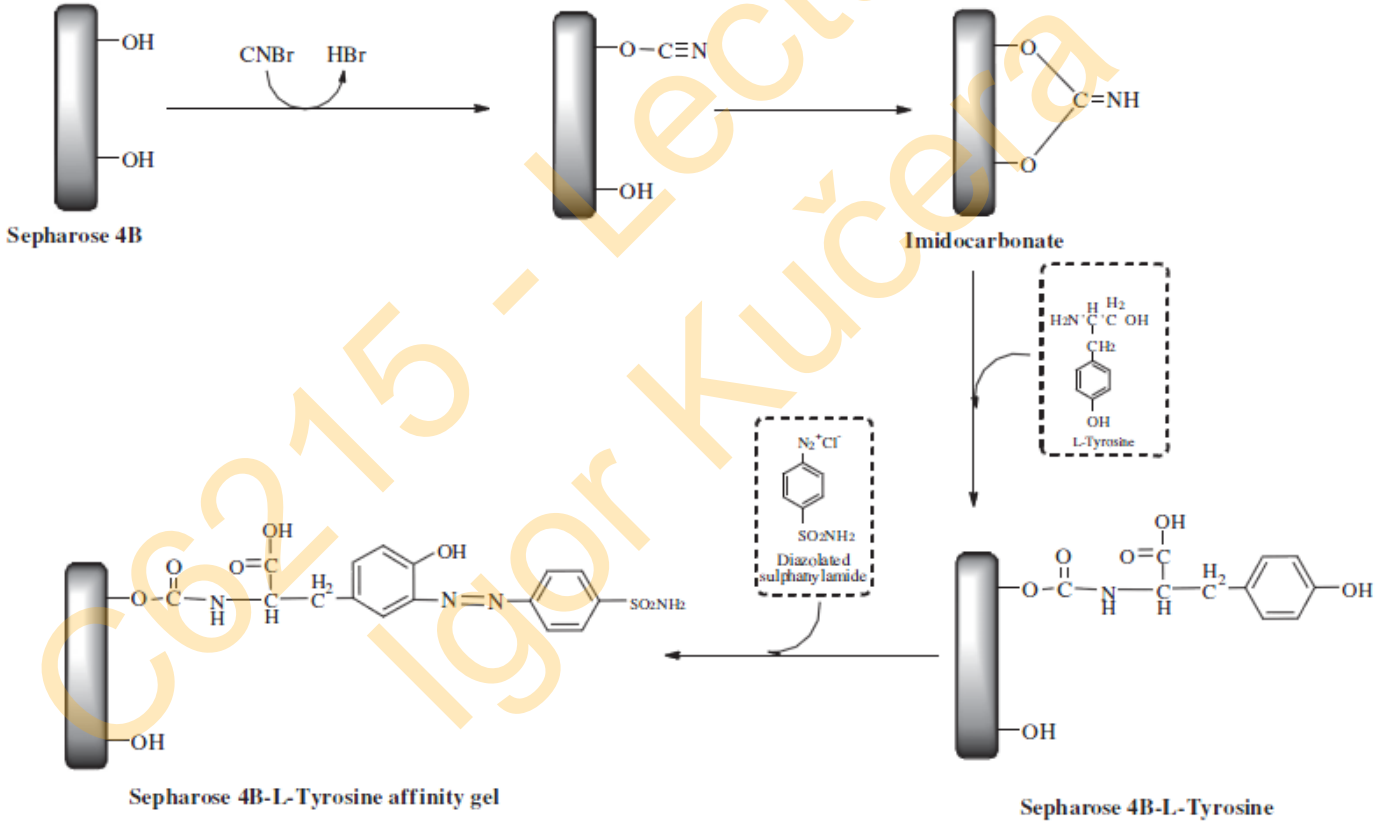
Thrombin aptamer* bound to exosite 1 (magenta) of thrombin
Ruigrok, Int. J. Mol. Sci. 2012, 13, 10537

=> Fractionation on a support to which the appropriate ligand has been immobilized.

***Aptamers (from the Latin *aptus* – fit, and Greek *meros* – part) are oligonucleotide (ssDNA or RNA) or peptide molecules that specifically bind to a predefined target. Usually they are created by selecting them from a large random sequence pool.**
Reviewed in *Mascini, Angew. Chem. Int. Ed. 2012, 51, 1316*

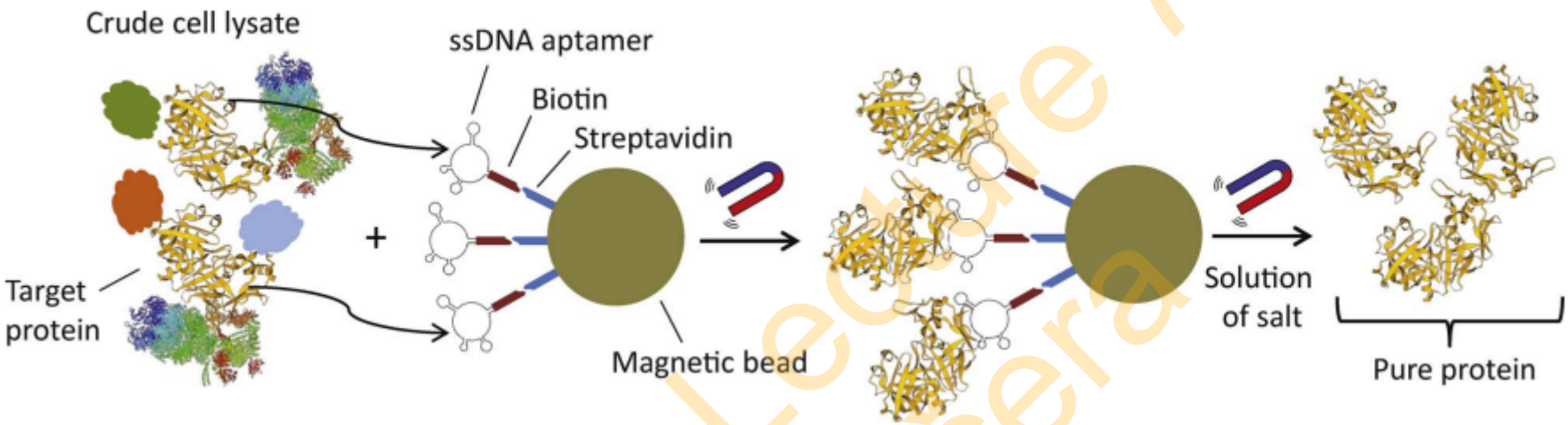
An example of preparation of an affinity matrix

Lactoperoxidase is an oxidoreductase secreted into milk. It catalyses the oxidation of halides and pseudohalides, such as thiocyanate, by H_2O_2 to form potent oxidant and bactericidal agents. Sulphanilamide was found to be an inhibitor of lactoperoxidase, which made it a suitable ligand for constructing a Sepharose 4B-L-tyrosine affinity matrix for LPO purification.



Atasever et al., Food Chemistry 2013, 136, 864

Aptamer-facilitated protein purification



Streptavidin is a homotetrameric protein from the bacterium *Streptomyces avidinii*. It has an extraordinarily high affinity for biotin (K_d on the order 10^{-14} mol/L). Streptavidin-coupled magnetic beads are supplied, e.g., by Thermo Scientific (MagnaBind Streptavidin Beads).

5'-/5BioTEG/CTC CTC TGA CTG TAA CCA CGT GCC TAG CGT TTC ATT GTC CCT TCT TAT TAG GTG ATA ATA GCA TAG GTA GTC CAG AAG CC-3'

Beloborodov et al., J. Chromatogr. B 2018, 1073, 201

Specific sequence or structure

The precise geometric presentation of amino acid residues on the surface of a protein can be used as the basis of a separation procedure. It can serve as an epitope (antigenic determinant), which is recognized by monospecific antibodies.

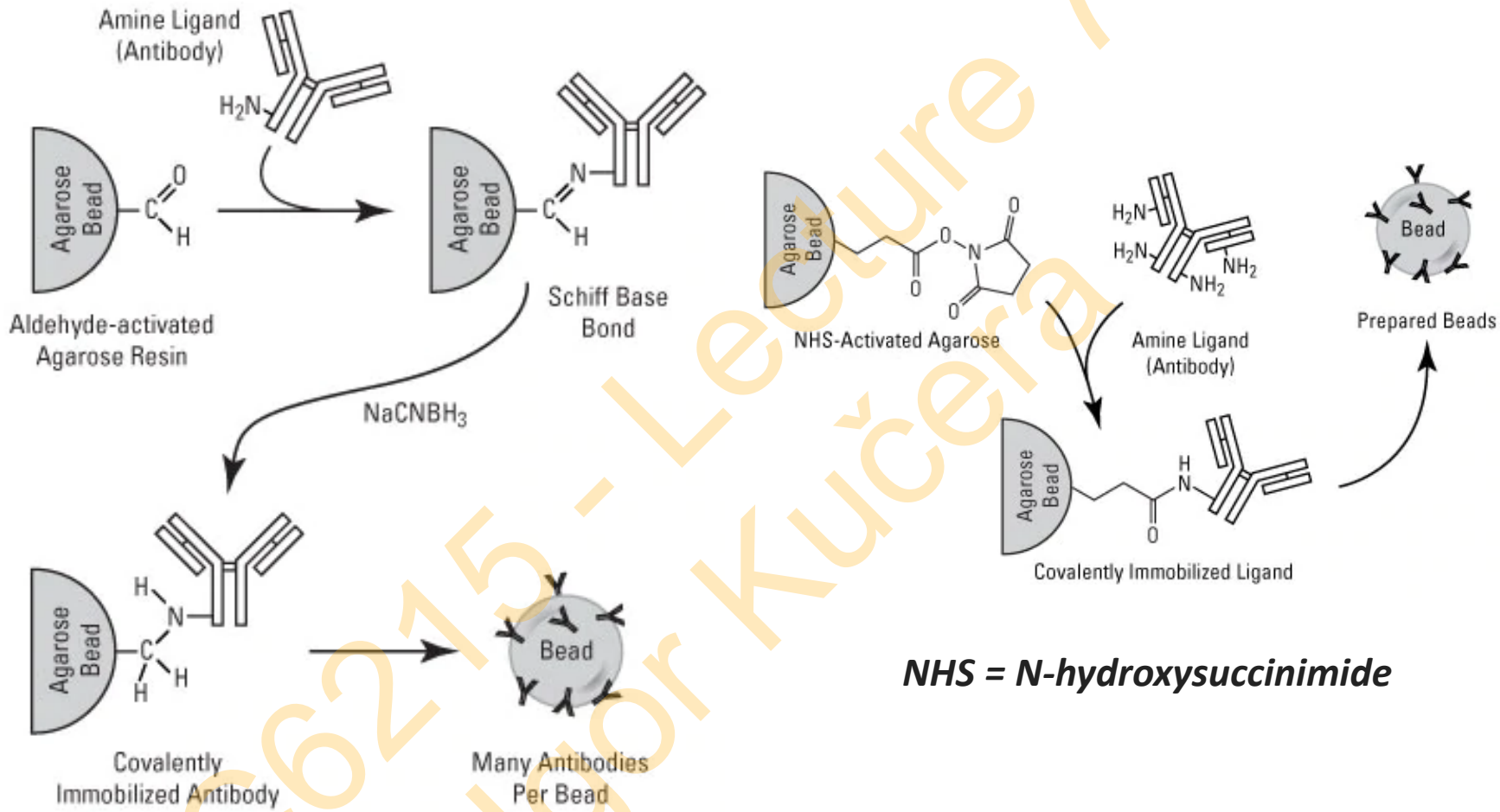
⇒ **Selective separation on a resin with attached monospecific antibody (immunoaffinity chromatography)**

A protein can also be immobilized and used to specifically bind another protein out of a complex protein extract

- **a subunit of an oligomeric protein (subunit exchange chromatography)**
- **a protein interacting with another protein (substrate-channeling enzymes, structural proteins …)**
- **denatured protein (isolation of chaperons)**

Moser, Bioanalysis 2010, 2, 769
Muronetz, J. Biochem. Biophys. Methods 2001, 49, 29

Covalent immobilization of antibodies and other proteins through their amino groups

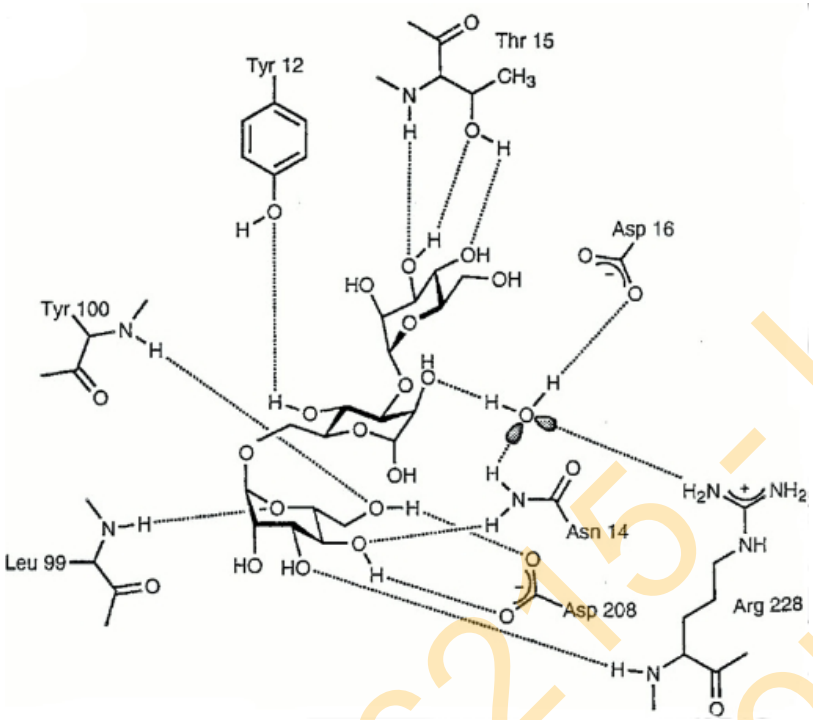


NHS = N-hydroxysuccinimide

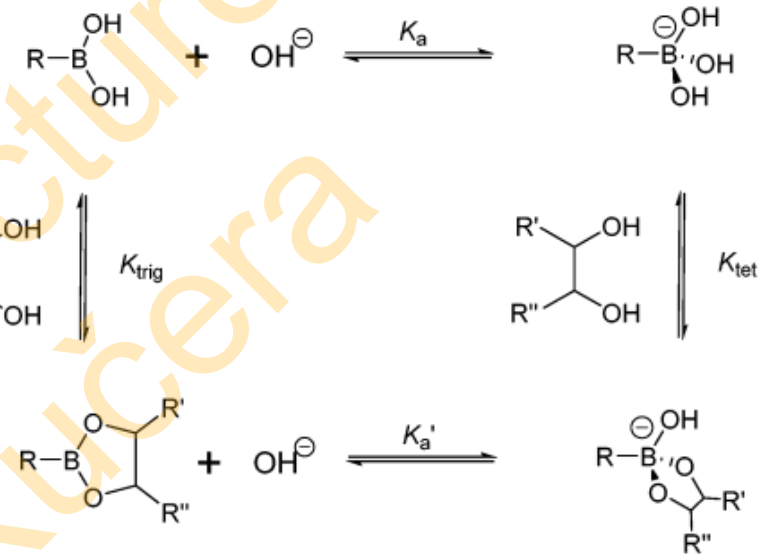
https://www.thermofisher.com/cz/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/covalent-immobilization-affinity-ligands.html

Posttranslational modifications

After protein synthesis, many proteins are modified by the addition of oligosaccharides, acyl groups, phosphate groups, or a variety of other moieties. In many cases, these modifications provide handles that can be used in fractionation.

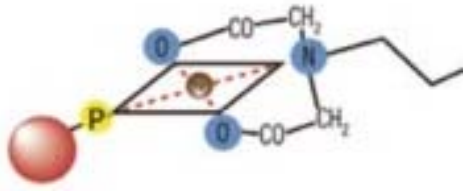


The trimannoside binding site of Concanavalin A, a lectin from jackbean (*Canavalia ensiformis*)
Naismith and Field, J. Biol. Chem. 1996, 271, 972



Boronic acid ionisation and interaction with cis-1,2-diols
Wu et al., Chem. Soc. Rev. 2013, 42, 8032

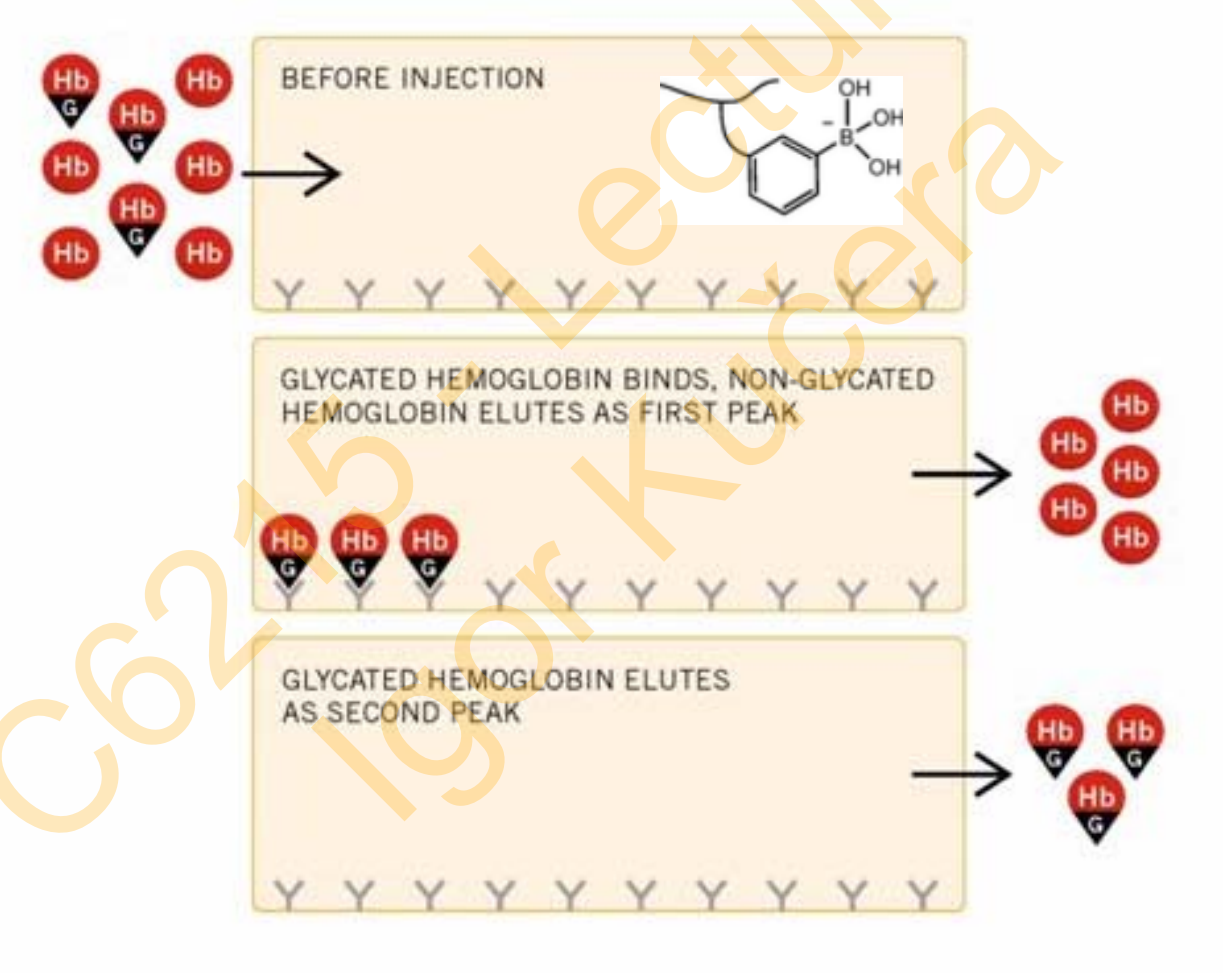
Phosphoproteins



Machida et al., FEBS J. 2007, 274, 1576
https://www.biovision.com/phosphoseektm-phosphoprotein-enrichment-kit.html

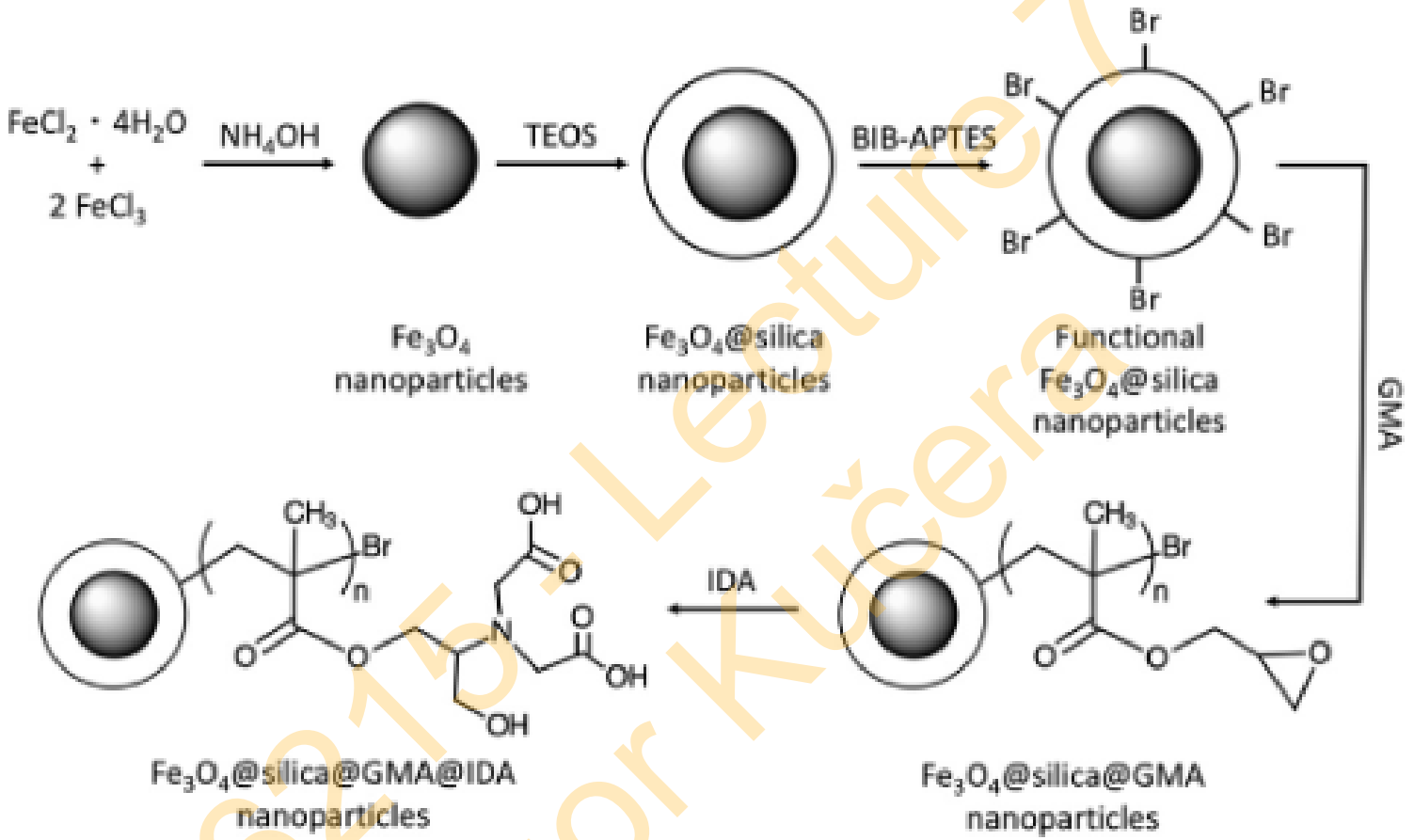
Separation of glycated and non-glycated hemoglobins

When a solution of proteins (hemolysate of red blood cells) is passed through the column, the glycated component is retained by the complexing of its diol groups with the bound phenylboronic acid.

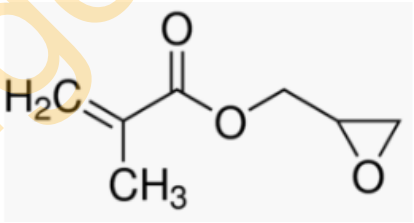


https://www.trinitybiotech.com/haemoglobins/boronate-affinity-chromatography/

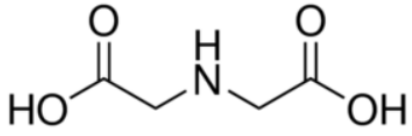
Preparation of Ti-IMAC (Immobilized Metal Affinity Chromatography) magnetic nanoparticles for phosphopeptide enrichment



GMA = glycidyl methacrylate



IDA = iminodiacetic acid (binding site for Ti^{4+})



Capriotti et al., Talanta 2018, 178, 274

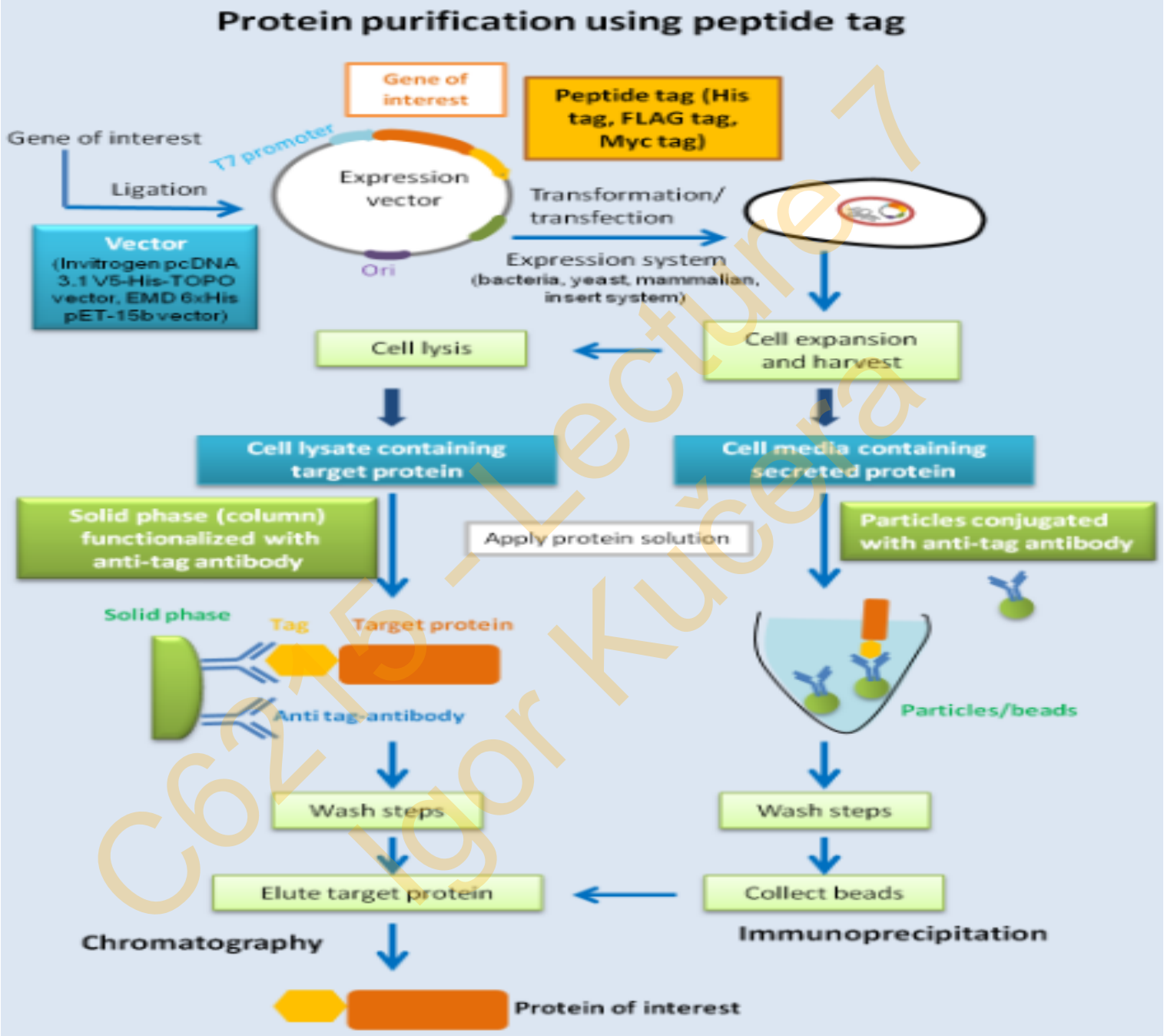
Genetically engineered peptide/protein tags

DNA encoding a given protein is altered to add extra amino acids on the N-terminus or the C-terminus of the protein being expressed. This added "tag" can be used as an effective purification handle. Tags also can increase protein stability and solubility.

Affinity tag	Matrix	Elution condition
Poly-Arg	Cation-exchange resin	NaCl linear gradient from 0 to 400 mM at alkaline pH>8.0
Poly-His	Ni^{2+}-NTA, Co^{2+}-CMA (Talon)	Imidazole 20–250 mM or low pH
FLAG	Anti-FLAG monoclonal antibody	pH 3.0 or 2–5 mM EDTA
Strep-tag II	Strep-Tactin (modified streptavidin)	2.5 mM desthiobiotin
c-myc	Monoclonal antibody	Low pH
S	S-fragment of RNaseA	3 M guanidine thiocyanate, 0.2 M citrate pH 2, 3 M magnesium chloride
HAT (natural histidine affinity tag)	Co^{2+}-CMA (Talon)	150 mM imidazole or low pH
Calmodulin-binding peptide	Calmodulin	EGTA or EGTA with 1 M NaCl
Cellulose-binding domain	Cellulose	Family I: guanidine HCl or urea>4 M Family II/III: ethylene glycol
SBP	Streptavidin	2 mM Biotin
Chitin-binding domain	Chitin	Fused with intein: 30–50 mM dithiothreitol, β-mercaptoethanol or cysteine
Glutathione S-transferase	Glutathione	5–10 mM reduced glutathione
Maltose-binding protein	Cross-linked amylose	10 mM maltose

Tag	Residues	Sequence	Size (kDa)
Poly-Arg	5–6 (usually 5)	RRRRR	0.80
Poly-His	2–10 (usually 6)	HHHHHH	0.84
FLAG	8	DYKDDDDK	1.01
Strep-tag II	8	WSHPQFEK	1.06
c-myc	11	EQKLISEEDL	1.20
S-	15	KETAAAKFERQHMDS	1.75
HAT-	19	KDHLIHNVHKEFHAHAHNK	2.31
3x FLAG	22	DYKDHDGDYKDHDIDYKDDDDK	2.73
Calmodulin-binding peptide	26	KRRWKKNFIAVSAANRFKKISSSGAL	2.96
Cellulose-binding domains	27–189	Domains	3.00–20.00
SBP	38	MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP	4.03
Chitin-binding domain	51	TNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALWQLQ	5.59
Glutathione S-transferase	211	Protein	26.00
Maltose-binding protein	396	Protein	40.00

Terpe, Appl. Microbiol. Biotechnol. 2003, 60, 523



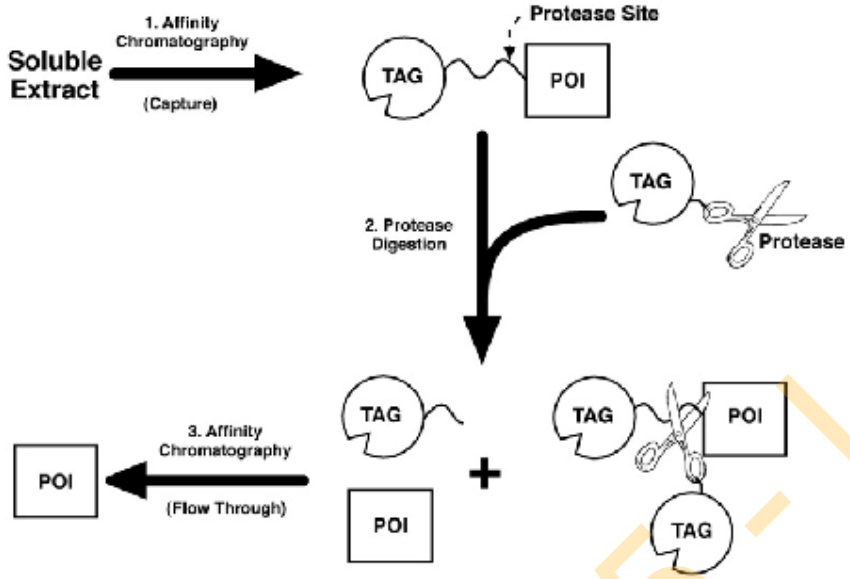
https://www.labome.com/method/Protein-Peptide-Tags.html

Enzymatic reagents for the removal of affinity tags

Enzyme	Source(s)	Molecular weight (kDa)	Tagged forms	Inhibitors	Recognition Site
Endoproteases					
Enteropeptidase	Duodenum *E. coli* *S. cerevisiae*	110 + 35	His_6	Reducing agents	DDDDK↓
Thrombin	Plasma CHO cells	32 + 4.5	None	Reducing agents	LVPR↓GS
Factor Xa	Plasma HEK 293 cells	42 + 17	None	Reducing agents Chelating agents Phosphate ions	LVPR↓GS
TEV Protease	*E. coli*	27	His_6 MBP GST Strep II	Thiol alkylating agents	ENLYFQ↓G
Rhinovirus 3C Protease	*E. coli*	27	His_6 GST His_6-GST	Thiol alkylating agents	LEVLFQ↓GP
Exoproteases					
Carboxypeptidase A	Pancreas *E. coli* *S. cerevisiae* *S. frugiperda* (baculovirus)	33	His_6	Reducing agents Chelating agents	C-terminal amino acids except Pro, Lys and Arg
Carboxypeptidase B	Pancreas *E. coli* *P. pastoris*	35	none	Reducing agents Chelating agents	C-terminal Lys and Arg
DAPase	Kidney *S. frugiperda* (baculovirus)	23 + 16 + 6	His_6	Reducing agents Thiol alkylating agents	N-terminal dipeptides

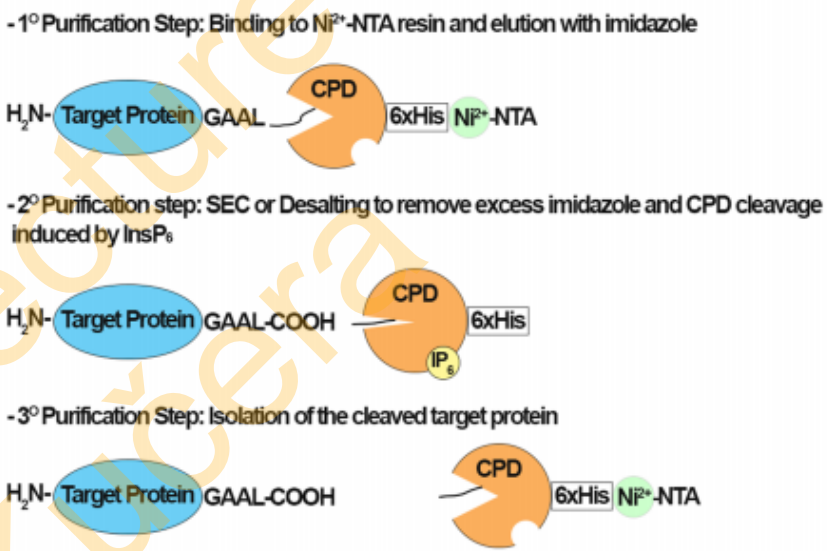
Waugh, Protein Express. Purif. 2011, 80, 283

The use of an affinity tagged endoprotease (POI = protein of interest)



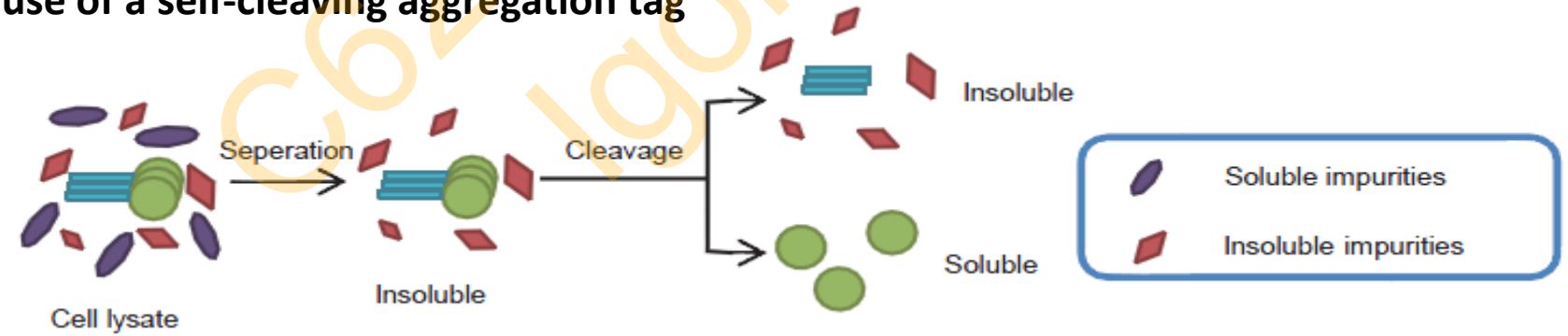
Waugh, Protein Express. Purif. 2011, 80, 283

The use of a self-cleaving tag (CPD = Cysteine Protein Domain of *Vibrio cholerae*, $InsP_6$ inositol hexakisphosphate)



Biancucci et al., BMC Biotechnology 2017, 17,1

The use of a self-cleaving aggregation tag

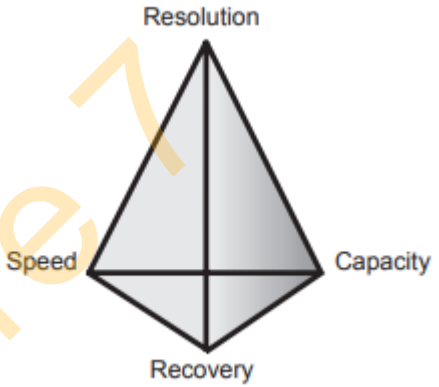


Xing et al., Microbial Cell Factories 2011, 10, 42

	Separation Process	Basis of Separation
Precipitation	Ammonium sulfate	Solubility
	Acetone	Solubility
	Polyethyleneimine	Charge, size
	Isoelectric	Solubility, pI
Phase partitioning	(e.g., with polyethylene glycol)	Solubility
Chromatography	Gel filtration/size exclusion (SEC)	Size, shape
	Ion exchange (IEX)	Charge, charge distribution
	Hydrophobic interaction (HIC)	Hydrophobicity
	Affinity	Ligand-binding site
	DNA affinity	DNA binding site
	Lectin affinity	Carbohydrate content and type
	Immobilized metal affinity (IMAC)	Metal binding
	Immunoaffinity (IAC)	Specific antigenic site
	Chromatofocusing	pI
Electrophoresis	Gel electrophoresis (PAGE)	Charge, size, shape
	Isoelectric focusing (IEF)	pI
Centrifugation		Size, shape, density
Ultrafiltration		Size, shape

Burgess, in: Proteomics of the Nervous System (Nothwang and Pfeiffer Eds.) WILEY-VCH Verlag GmbH & Co., 2008

Every technique offers a balance between resolution, capacity, speed and recovery.



Capacity, in the simple model shown, refers to the amount of target protein loaded during purification. In some cases the amount of sample which can be loaded may be limited by volume (as in gel filtration) or by large amounts of contaminants rather than the amount of the target protein.

Speed is of the highest importance at the beginning of a purification where contaminants such as proteases must be removed as quickly as possible.

Recovery becomes increasingly important as the purification proceeds because of the increased value of the purified product. Recovery is influenced by destructive processes in the sample and unfavourable conditions on the column.

Resolution is achieved by the selectivity of the technique and the efficiency of the chromatographic matrix to produce narrow peaks. In general, resolution is most difficult to achieve in the final stages of purification when impurities and target protein are likely to have very similar properties.

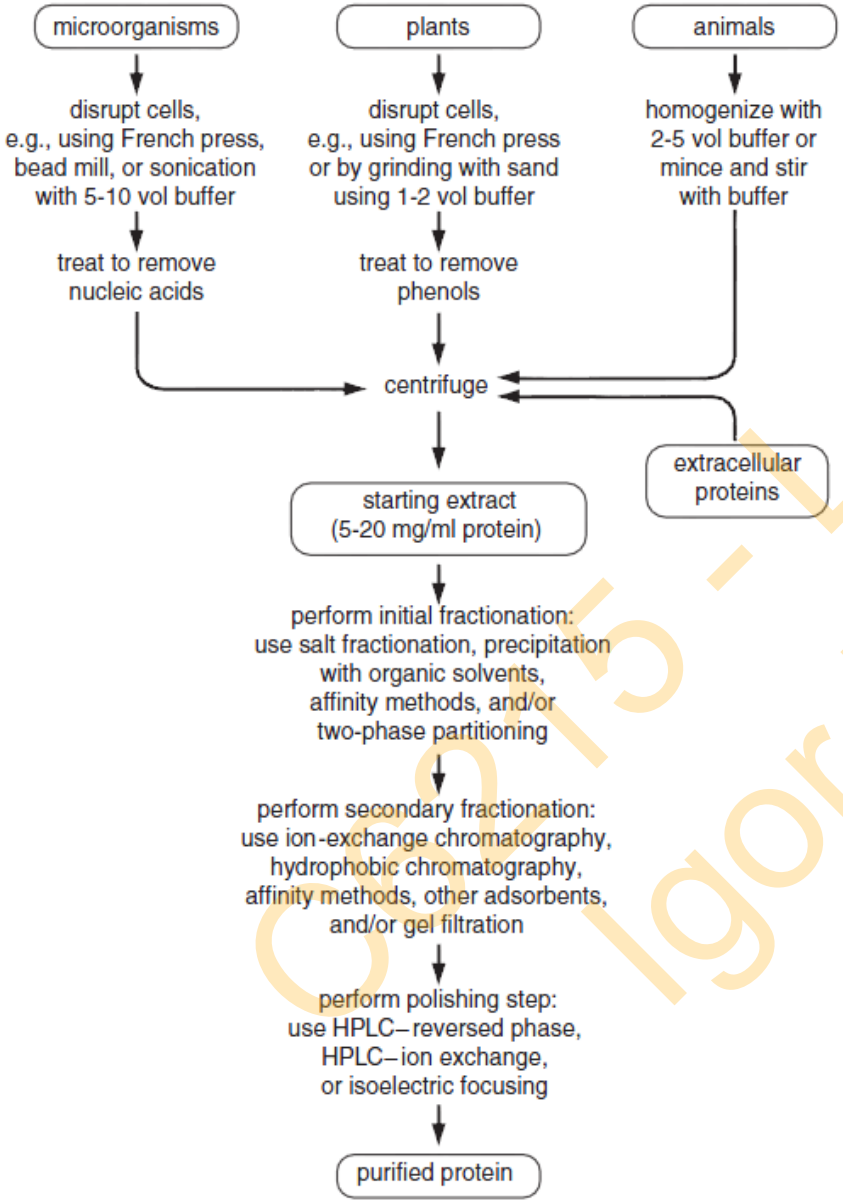
Protein Purification Handbook, Amersham Biosciences

Suitability of chromatographic techniques

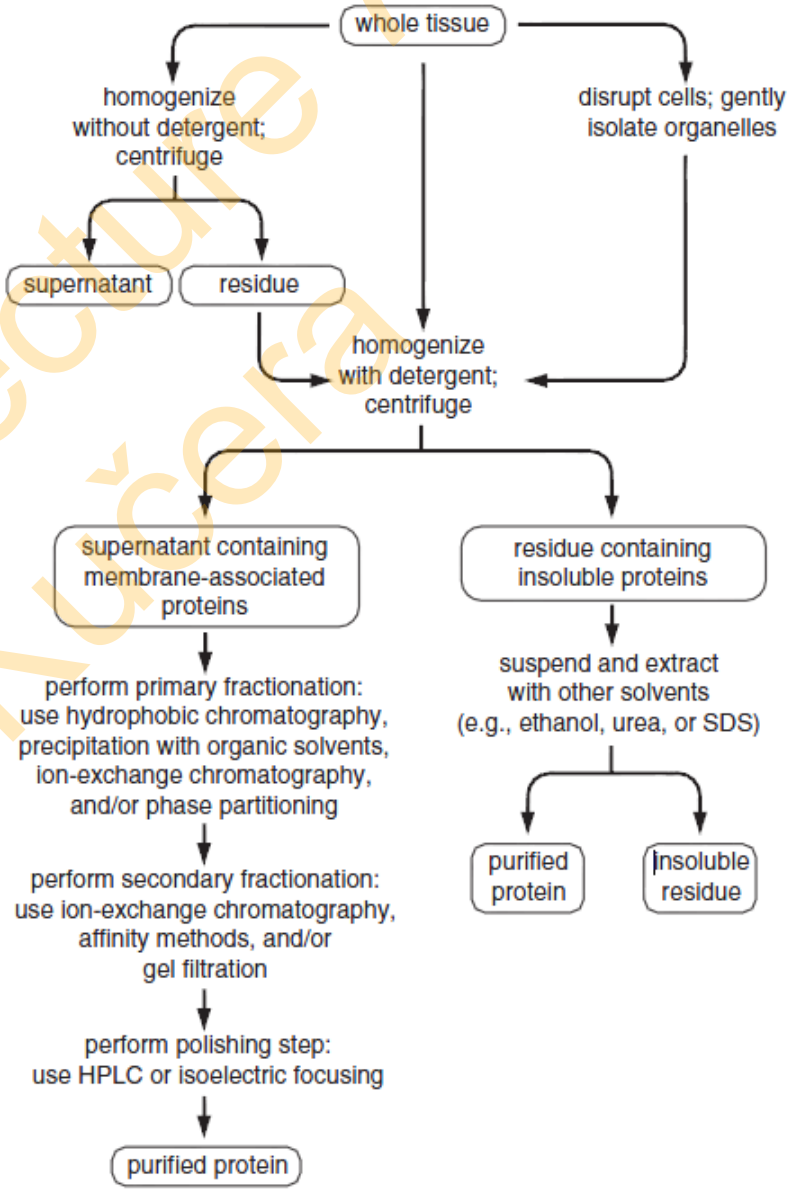
Technique	Main features	Capture	Intermediate	Polish	Sample Start condition	Sample End condition
IEX	high resolution high capacity high speed	★★★	★★★	★★★	low ionic strength sample volume not limiting	high ionic strength or pH change **concentrated**
HIC	good resolution good capacity high speed	★★	★★★	★	high ionic strength sample volume not limiting	low ionic strength **concentrated**
AC	high resolution high capacity high speed	★★★	★★★	★★	specific binding conditions sample volume not limiting	specific elution conditions **concentrated**
GF	high resolution using Superdex™		★	★★★	limited sample volume (<5% total column volume) and flow rate range	buffer exchanged (if required) **diluted**

Protein Purification Handbook, Amersham Biosciences

Soluble proteins present in their natural host cells

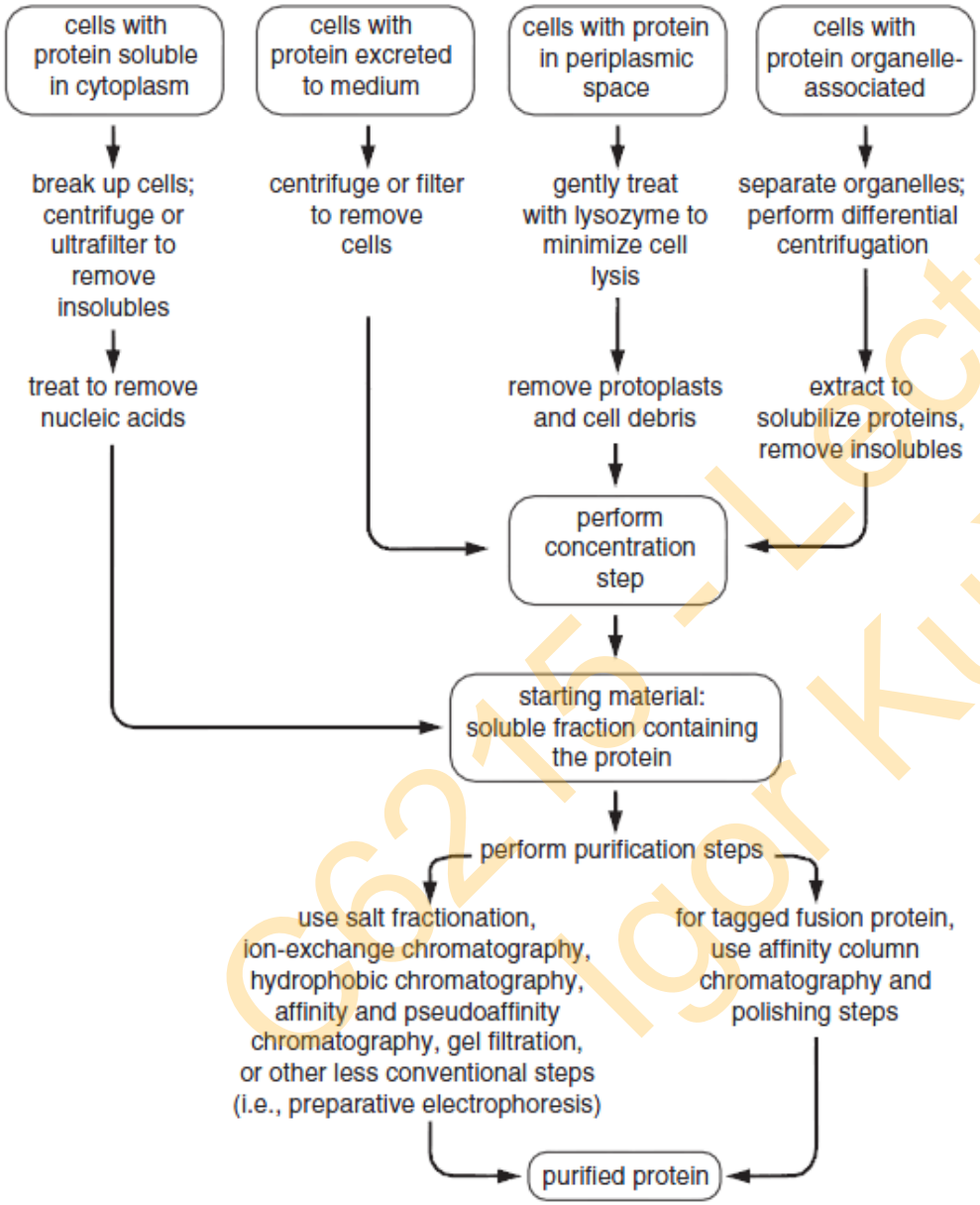


Membrane-associated and poorly soluble proteins (nonrecombinant)

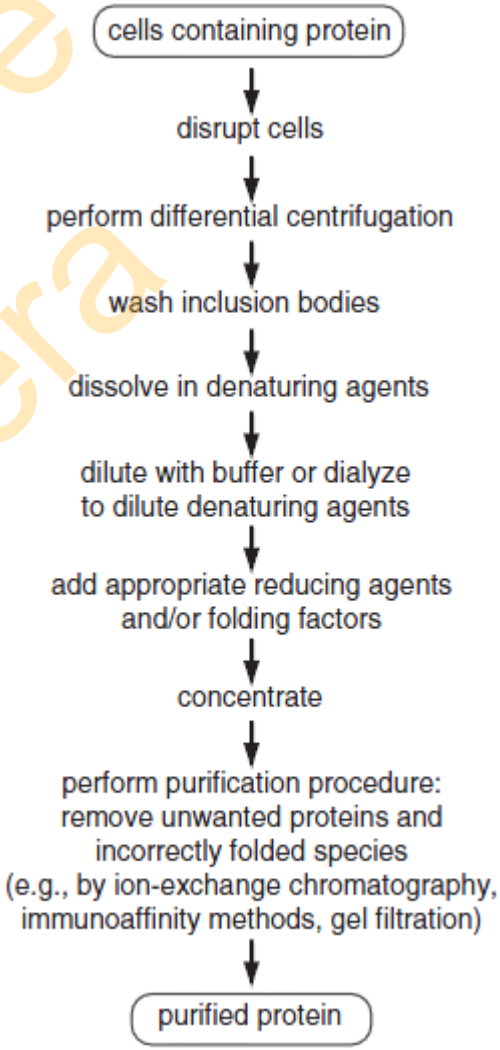


Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons 2003

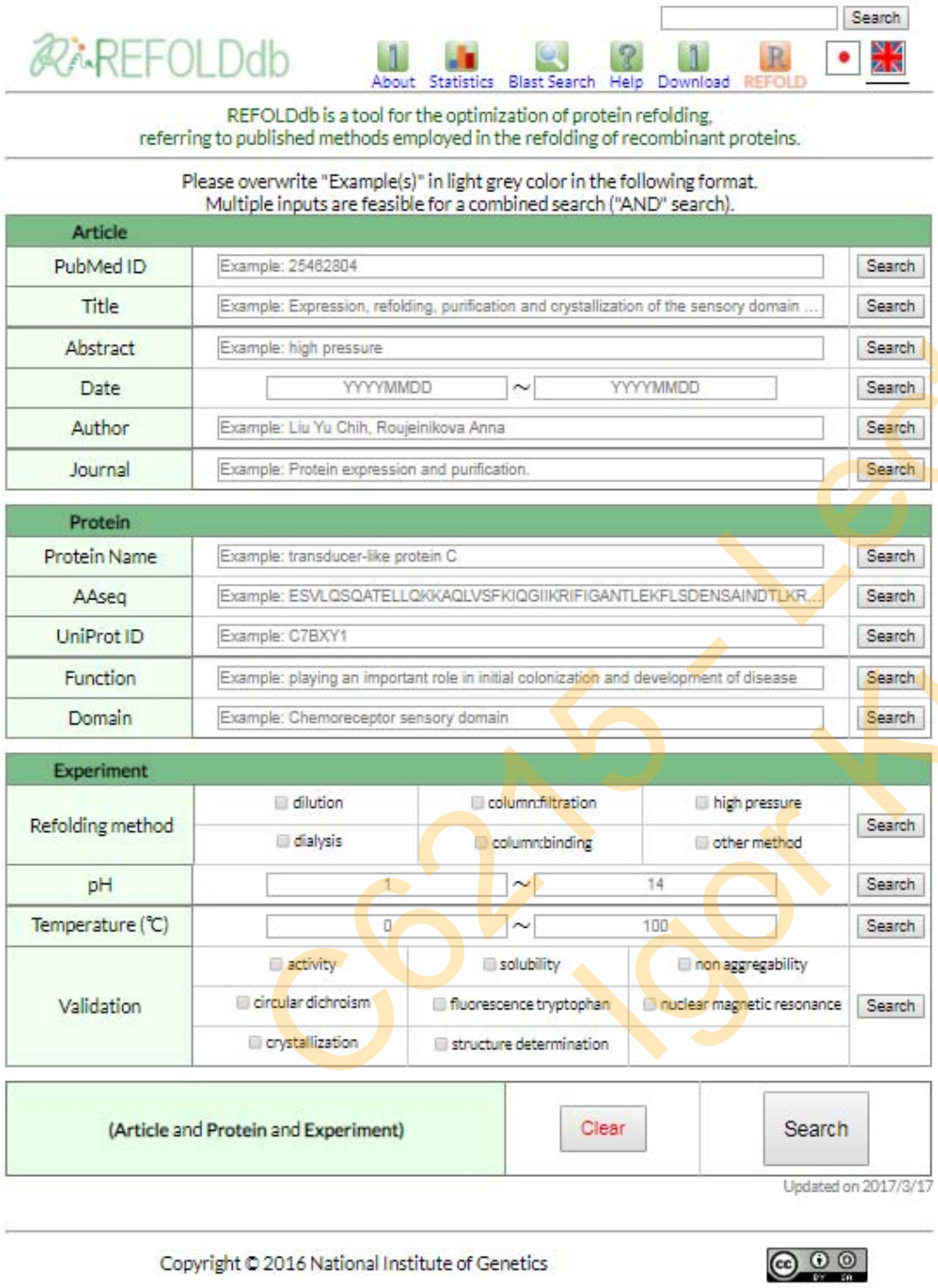
Soluble recombinant proteins



Insoluble recombinant proteins (inclusion bodies)



Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons 2003



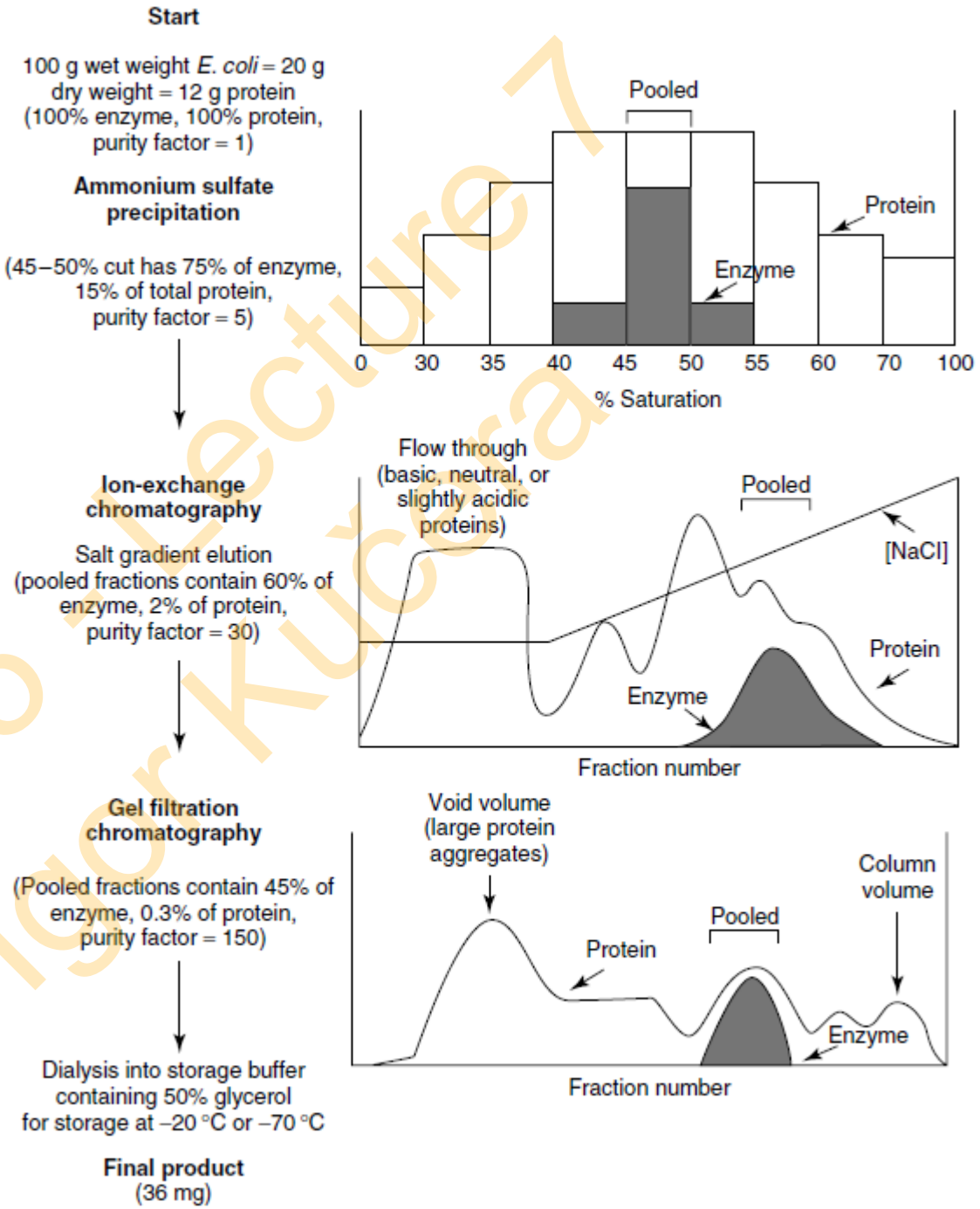
REFOLD database, developed by S. Bottomley and his colleagues at Monash University (Australia), contains proteins that have been successfully refolded and presents protocols and statistics on the frequency of use of various refolding techniques, disruption methods, fusion proteins, and preparation prior to refolding.

Chow et al., Nucleic Acids Res. 2006, 34, D207

http://pford.info/refolddb/

A hypothetical purification scheme for an enzyme

purity factor = 75/15 = 5
5-fold purification
75% recovery



Burgess, in: Proteomics of the Nervous System (Nothwang and Pfeiffer Eds.) WILEY-VCH Verlag GmbH & Co., 2008

Total protein assays

assay	absorption	mechanism	detection limit	advantages	disadvantages
UV absorption	280 nm	tyrosine and tryptophan absorption	0.1-100 ug/ml	small sample volume, rapid, low cost	incompatible with detergents and denaturating agents, high variability
Bicinchoninic acid	562 nm	copper reduction (Cu^{2+} to Cu^{1+}), BCA reaction with Cu^{1+}	20-2000 ug/ml	compatible with detergents and denaturating agents, low variability	low or no compatibility with reducing agents
Bradford or Coomassie brilliant blue	470 nm	complex formation between Coomassie brilliant blue dye and proteins	20-2000 ug/ml	compatible with reducing agents, rapid	incompatible with detergents
Lowry	750 nm	copper reduction by proteins, Folin-Ciocalteu reduction by the copper-protein complex	10-1000 ug/ml	high sensitivity and precision	incompatible with detergents and reducing agents, long procedure

https://www.labome.com/method/Protein-Quantitation.html

NanoOrange® reagent, a merocyanine dye, produces a large increase in fluorescence quantum yield upon interaction with detergent-coated proteins. The NanoOrange assay allows for the detection of 0.01 to 10 µg/mL protein with a standard fluorometer.

Jones et al., BioTechniques 2003, 34, 850

Protein inactivation and ways of preventing it

Reasons for inactivation	How to prevent it
Oxidation, foaming	Add DTT or TCEP, store under argon
Protease degradation	Add protease inhibitors, cooler, purer
Adsorption to container	Use polypropylene tubes, BSA carrier, glycerol, non-ionic detergent, protein more concentrated
Aggregation and precipitation	Store less concentrated, add salt, pH away from pI
Heavy metals	Add EDTA, cleaner tube, reagents
Temperature inactivation	Store cooler, add ligand or glycerol to stabilize
Bacterial growth	Use Tris, EDTA, azide, avoid PO_4, OAc^-
Enzymatic reaction (phosphatase)	Cooler, purer, add specific inhibitor
Dissociation of subunits/cofactors	Store more concentrated
pH changed	Avoid CO_2 in room, Tris changes pH with temperature
Inactive/misfolded conformation	Incubate at 37 °C to anneal the structure

Burgess, in: Proteomics of the Nervous System (Nothwang and Pfeiffer Eds.) WILEY-VCH Verlag GmbH & Co., 2008