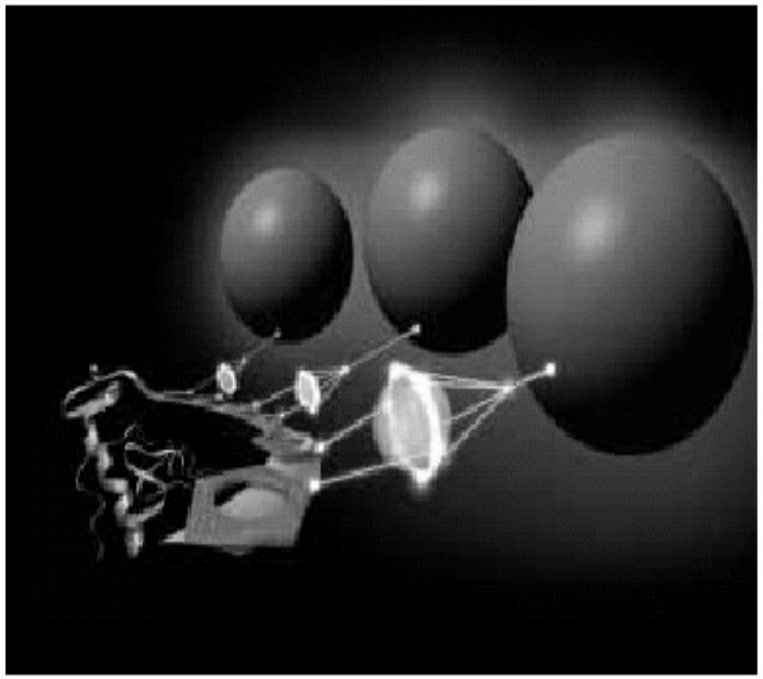
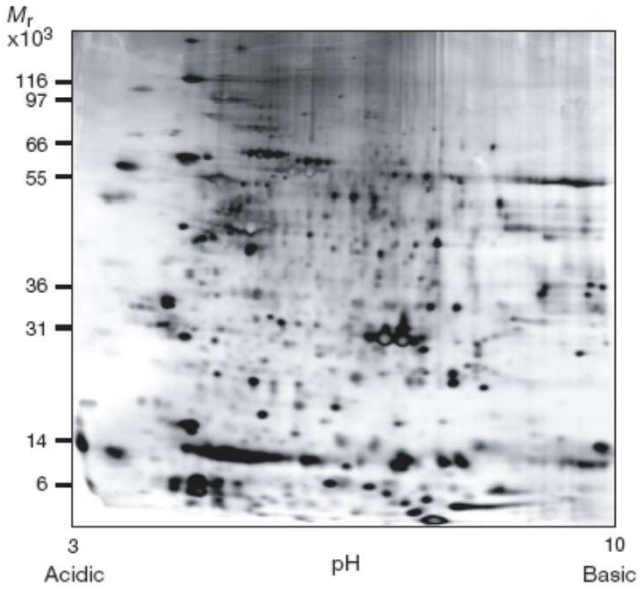
Purification of recombinant proteins



Protein purification from a complex mixture of macromolecules present in a biological sample



2D electrophoresis of the cell extract

• Several thousand proteins with different properties (~ 5000-8000) and in different amounts (actin ~ 10%, unique transcription factor <0.001% of total proteins)

• DNA, RNA, polysacharides, lipids

Biomas desintegration

Physical methods: sonication by ultrasound, pressure in a French press, osmotic shock, shear forces in various types of grinders and homogenizers
- must be cooled during sonication or mechanical methods!

Chemical: detergents, chelators in lysis buffers, organic solvents
- substances may interfere with the subsequent purification method

Enzymatic: must be chosen according to the expression system
Lysozyme for bacteria, lyticase or zymolase (glucanase) for yeasts.

In all lysis procedures, the cells are destroyed and their contents are released, including proteolytic enzymes. Therefore, it is advisable to add protease inhibitors to the lysis buffer, which will prevent degradation of the product during disintegration and other steps.

Before starting……

1. Why??? **For what purpose?**

2. How??? **How to analyze target protein?**

3. What??? **What features has target protein?**

1. Why??? For what purpose?

Aplication	Amount	Purity
Identification	0,002-0,2 μg	High >95%
Antibody production	μg-mg	middle-high
Enzymology	1-5 mg	High > 95 %
Biofysical studies	mg-g	High (>95%)
3D structure (crystalization, NMR)	10-20 mg	High (>95%)
Farmaceutical purposes	mg-kg	high (99,9%)

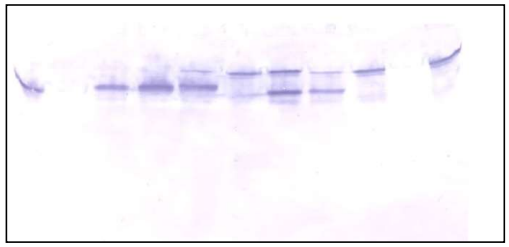
2. How??? How to analyze the protein?

1. Polyacrylamide gel electrophoresis with specific detection:

Detection of target protein during purification

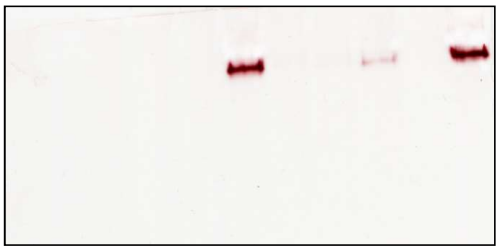
• Using antibodies

SDS PAGE followed by western bloting with antibody detection



Biological activity monitoring during purification

• For enzymes e.g. gel staining using chromogenic substrates (or specific constant determination in complex samples)



Native PAGE followed by substrate staining, zymogram gel

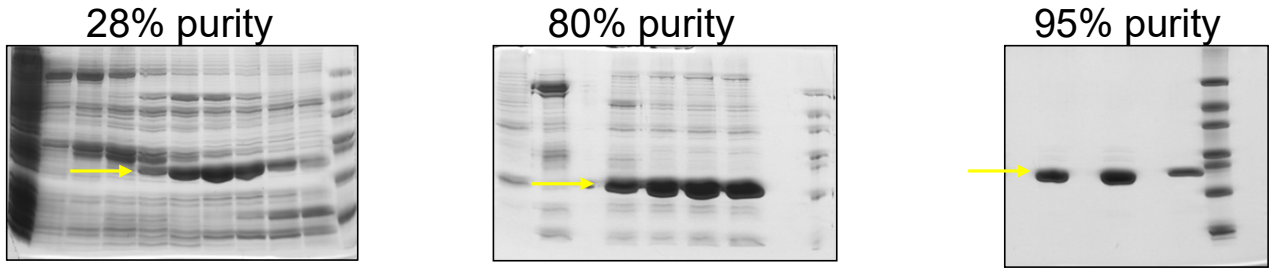
2. How??? How to analyze the protein?

2. Polyacrylamide gel electrophoresis with nonspecific detection

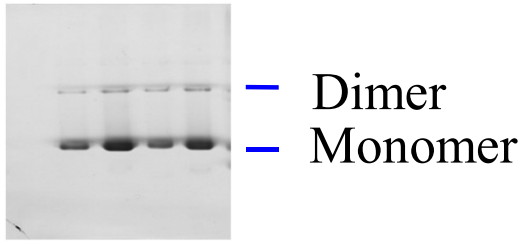
Protein purity and homogeneity monitoring

- e.g. coommasie blue, silver staining

SDS PAGE



Native PAGE



3. Determination of protein concentration

- e.g. Bradford, Lowry methods

What??? What features has target protein?

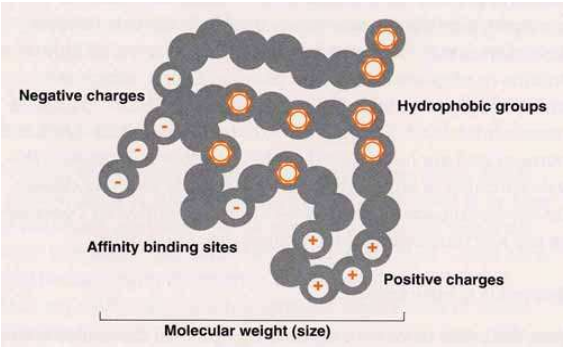
Information about target protein and related proteins from databases, literature or from pilot experiments

- Protein size (SDS PAGE, gel filtration nebo analytical centrifugation)
- Izoelectric point (izoelectric focusing)
- Stability (pH, temperature, presence of salts, proteases, additives ensuring protein solubility)
- Purification strategy (methods, buffers, protein stability, …..)

2D and native PAGE

- Sample complexity, features of target protein and other contaminating proteins

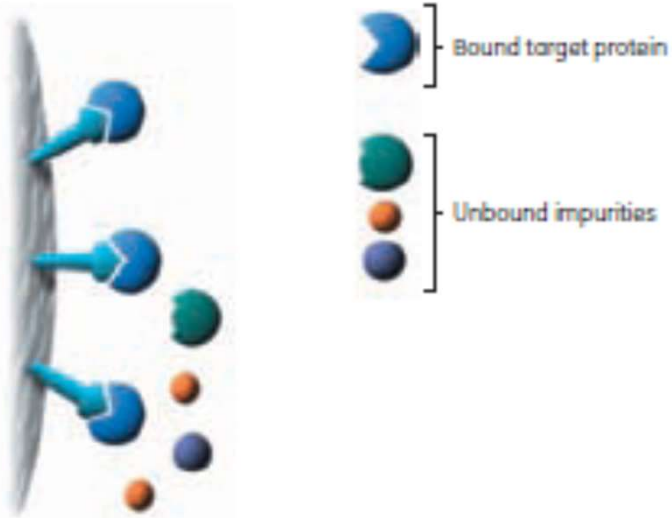
Properties/purification methods



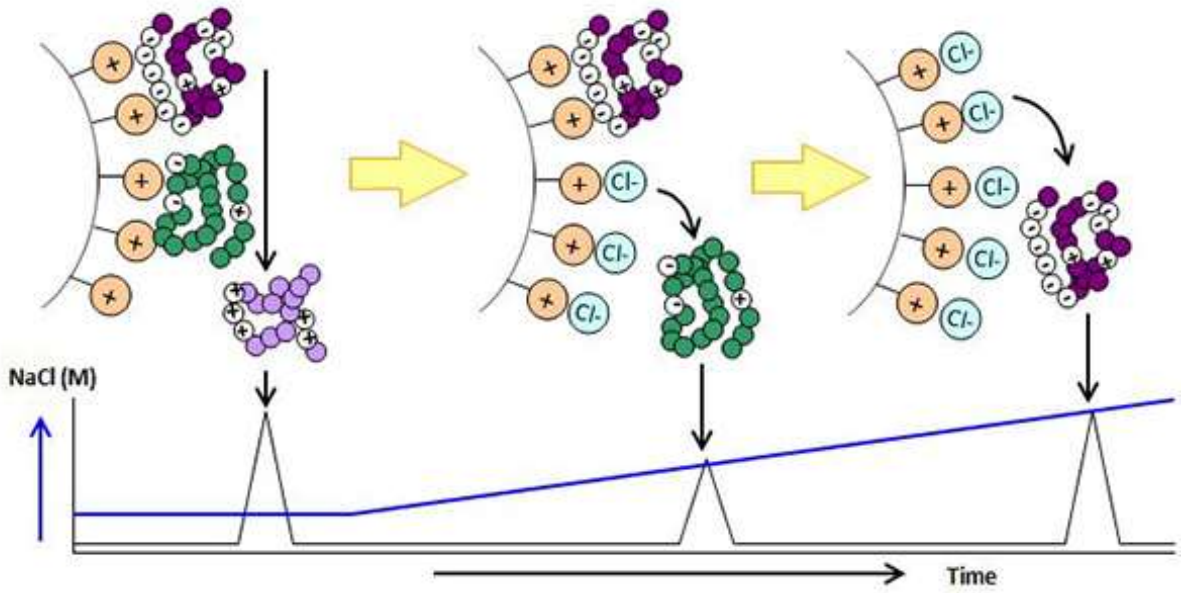
Solubility	precipitation e.g. ammonium sulphate, low/high pH
Stability	thermal precipitation
Size	gel filtration (gel permeation chromatography)
pI (surface charge)	ion exchange chromatography
Hydrophobicity	hydrophobic or reverse phase chromatography
Specific binding site	afinity chromatography

Affinity Chromatography

- A type of adsorption chromatography, in which the molecule to be purified is specifically and reversibly adsorbed to a complementary binding substance immobilized on an insoluble support.
- Mostly it is a specific interaction of affinity fusion tags (eg. polyhistidine, glutathione-S-transferase, etc.) with ligands (eg. metal, glutathione, etc.) in chromatographic matrix.



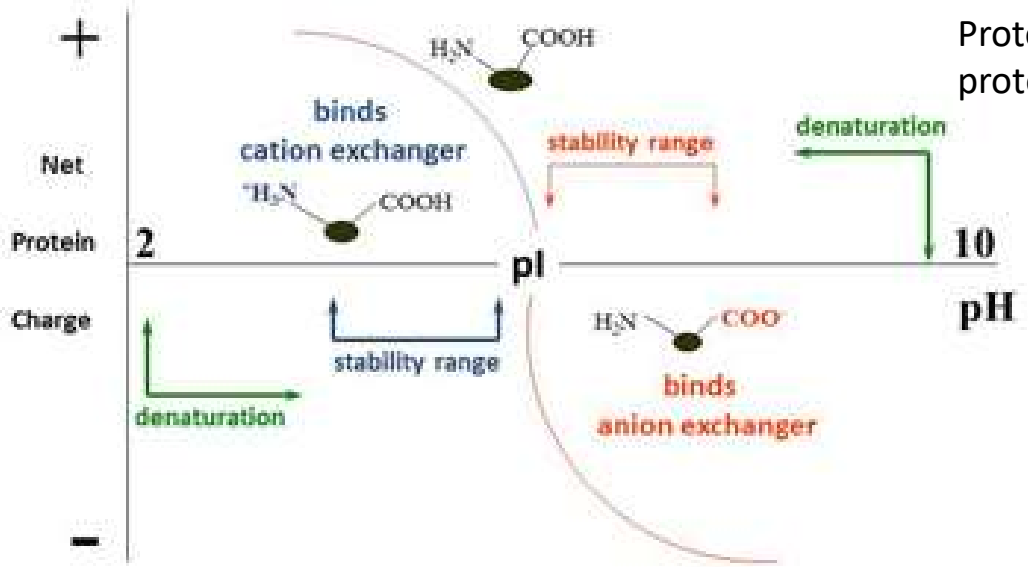
Ion Exchange Chromatography



Ion exchange chromatography involves the separation of ionizable molecules based on their total charge. Generally, media, which have cationic or anionic groups, are used as stationary phases and the counter-ion added buffers are used as mobile phases

In the sample application step, molecules with opposite charge to the media bind to them by ionic interaction. Next, in the elution step, by increasing the concentration of the counter-ions in the mobile phase, molecules with the lowest net charge are eluted first and those with higher charge are eluted later.

Ion Exchange Chromatography



Protein stability and ion exchange media binding vary with total protein charge, which depends on pH.

Resin Type	Cation Exchanger	Anion Exchanger
Net charge of molecule of interest	+	−
Charge of resin	−	+
Running conditions	0.5–1.5 pH units below the pI of the molecule of interest	0.5–1.5 pH units above the pI of the molecule of interest

Functional groups used in ion Exchange chromatography media:

Anion exchanger

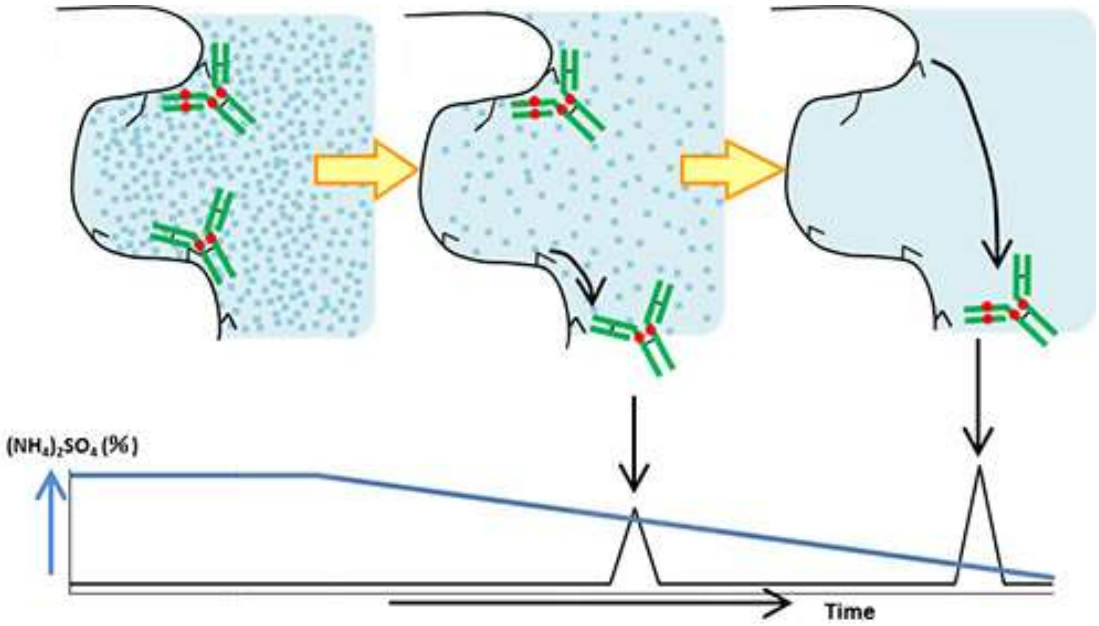
Quaternary ammonium (Q)	strong	-CH2-N+-(CH3)3
Diethylaminoethyl (DEAE)*	weak	-CH2-CH2-N+-(CH2-CH3)2
Diethylaminopropyl (ANX)*	weak	-CH2-CHOH-CH2-N+-(CH2-CH3)2

Cation exchanger

Sulfopropyl (SP)	strong	-CH2-CH2-CH2-SO3-
Methyl sulfonate (S)	strong	-CH2-SO3-
Carboxymethyl (CM)	weak	- CH2-COO-

A “**weak**” **exchanger** is ionized over only a limited pH range, while a “**strong**” **exchanger** shows no variation in **ion exchange** capacity with changes in pH. ... **Strong** exchangers do not vary and remain fully charged over a broad pH range, which can make optimization of separation simpler than with **weak** exchangers..

Hydrophobic Chromatography



Ligand used in purification matrix

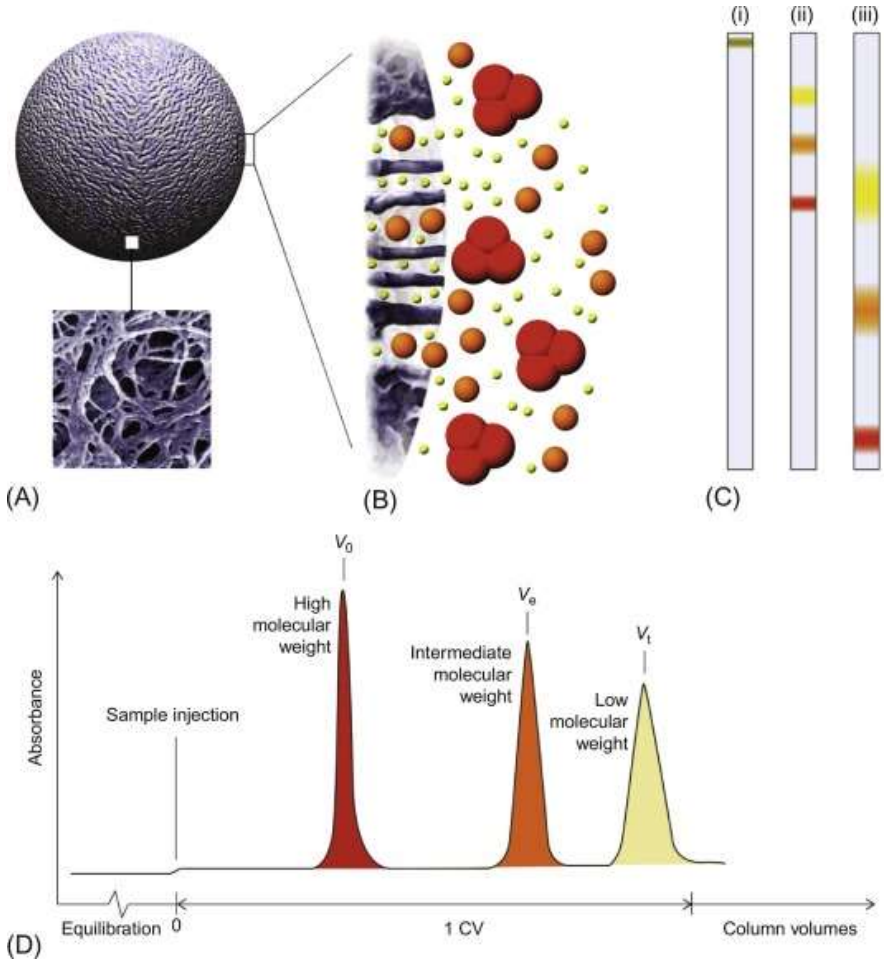
Phenyl	–O– 
Butyl-S	$-S-(CH_2)_3-CH_3$
Butyl	$-O-(CH_2)_3-CH_3$
Octyl	$-O-(CH_2)_7-CH_3$
Ether	$-O-CH_2-CHOH-CH_2-OH$
Isopropyl	$-O-CH-(CH_3)_2$

Gel permeation Chromatography (gel filtration)

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

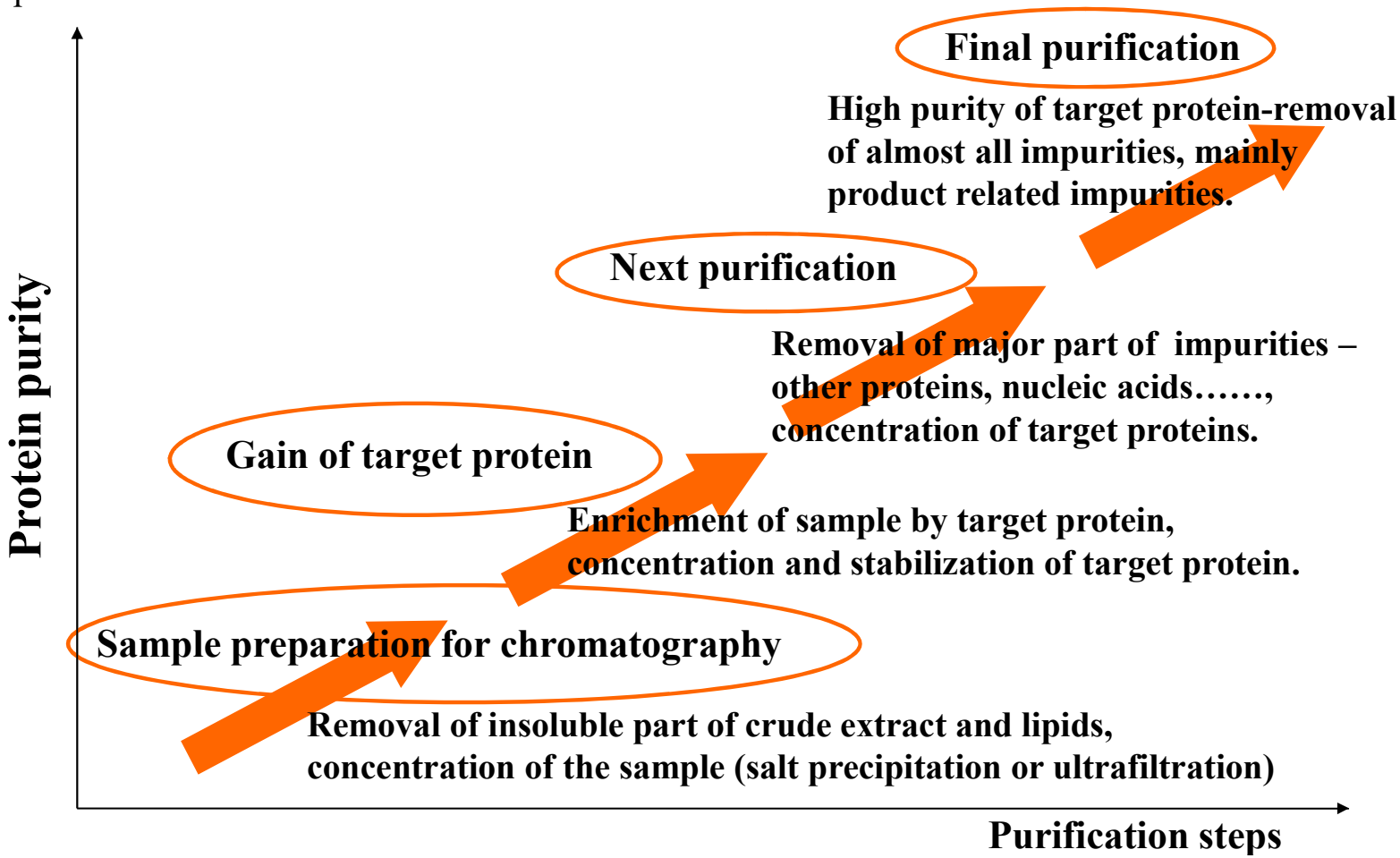
• Molecules move through a bed of porous beads. Smaller molecules diffuse further into the pores of the beads and therefore move through the beads more slowly, while larger molecules enter less or not at all and thus move through the beads more quickly.

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



How many steps are needed?

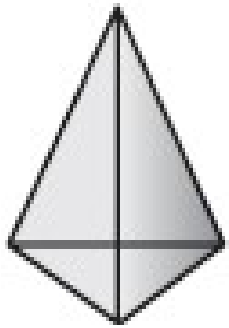
The number of steps used will always depend on the purity requirements and purposed use for the target protein. For most laboratory-scale work a two- or three-step purification protocol will be sufficient. Difficult purifications may require several additional steps.



Logic combination of purification steps

Every separation technique should be evaluated with regard to different parameters such as:

Resolution
how efficiently proteins are *separated* from each other

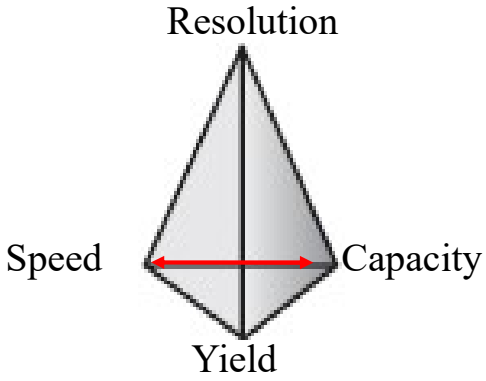


Speed is important with respect to the protein degradation by proteases in the complex sample

Capacity (max.)
the sample amount that can be handled.

Yield-minimalization of protein loss

Gain of target protein



Goal: fast isolation, stabilization and concentration.

Purification techniques: affinity chromatography
ion exchange chromatography
hydrophobic chromatography

Column: rProtein A Sepharose Fast Flow, XK16/20, bed height 4.8 cm (9.6 ml)
Sample: 600 ml clarified cell culture containing 87.6 mg of IgG_{2a}
Starting buffer: 20 mM sodium phosphate, pH 7.0
Elution buffer: 20 mM sodium citrate, pH 4.0
Flow rate: 5 ml/min (150 cm/h)

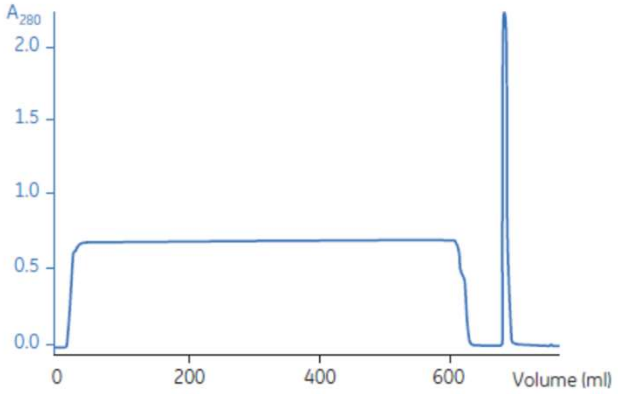
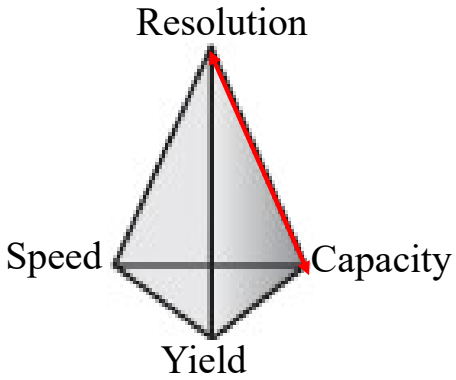


Fig 4.5. Example of capture step: Purification of IgG_{2a} from clarified cell culture.

Next purification of protein



Goal: Purification and concentrating.

Purification techniques: ion exchange chromatography
hydrophobic chromatography

Column:	XK 16/20 Butyl Sepharose 4 Fast Flow
Sample:	5 ml of partially purified Annexin V expressed in *E. coli*
Buffer A:	20 mM Sodium phosphate, pH 7.0, 1 M $(NH_4)_2SO_4$
Buffer B:	20 mM Sodium phosphate, pH 7.0
Flow rate:	100 cm/h
Gradient:	0 to 50% B, 20 column volumes

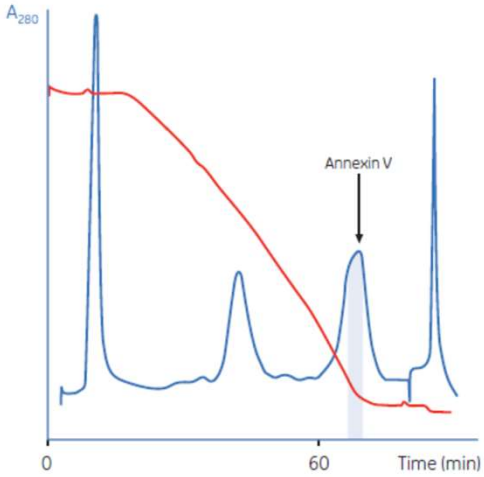
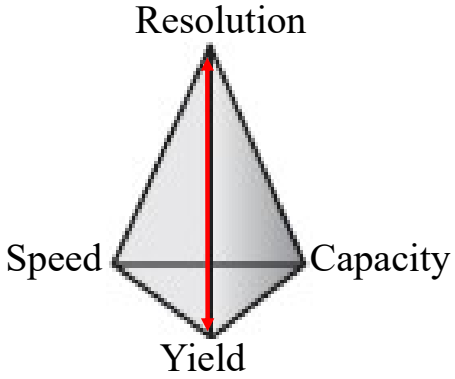


Fig 4.7. Example of an intermediate purification step: Purification of recombinant Annexin V by HIC.

Final purification and adjustment of conditions for target protein storage (pH, salts, additives)



Goal: Product in high purity.

Purification techniques: gel permeation chromatography

ion exchange chromatography

hydrophobic chromatography

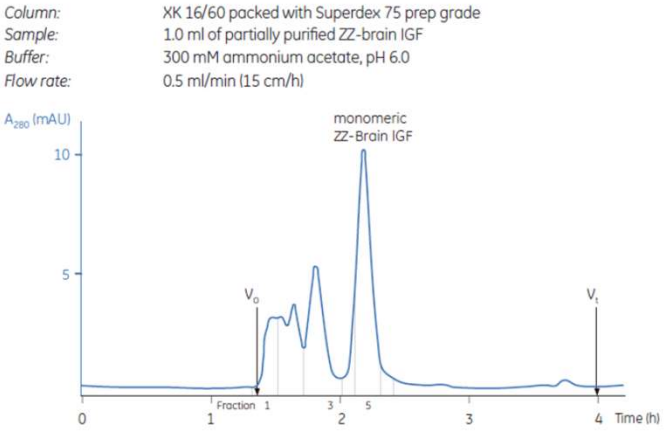


Fig 4.9. Example of polishing step: removal of dimers and multimers by GF.

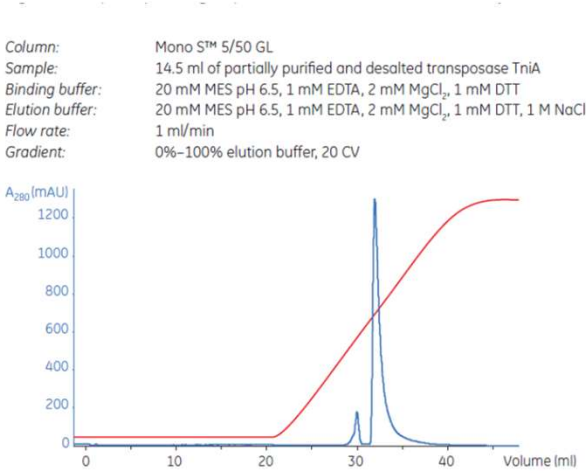
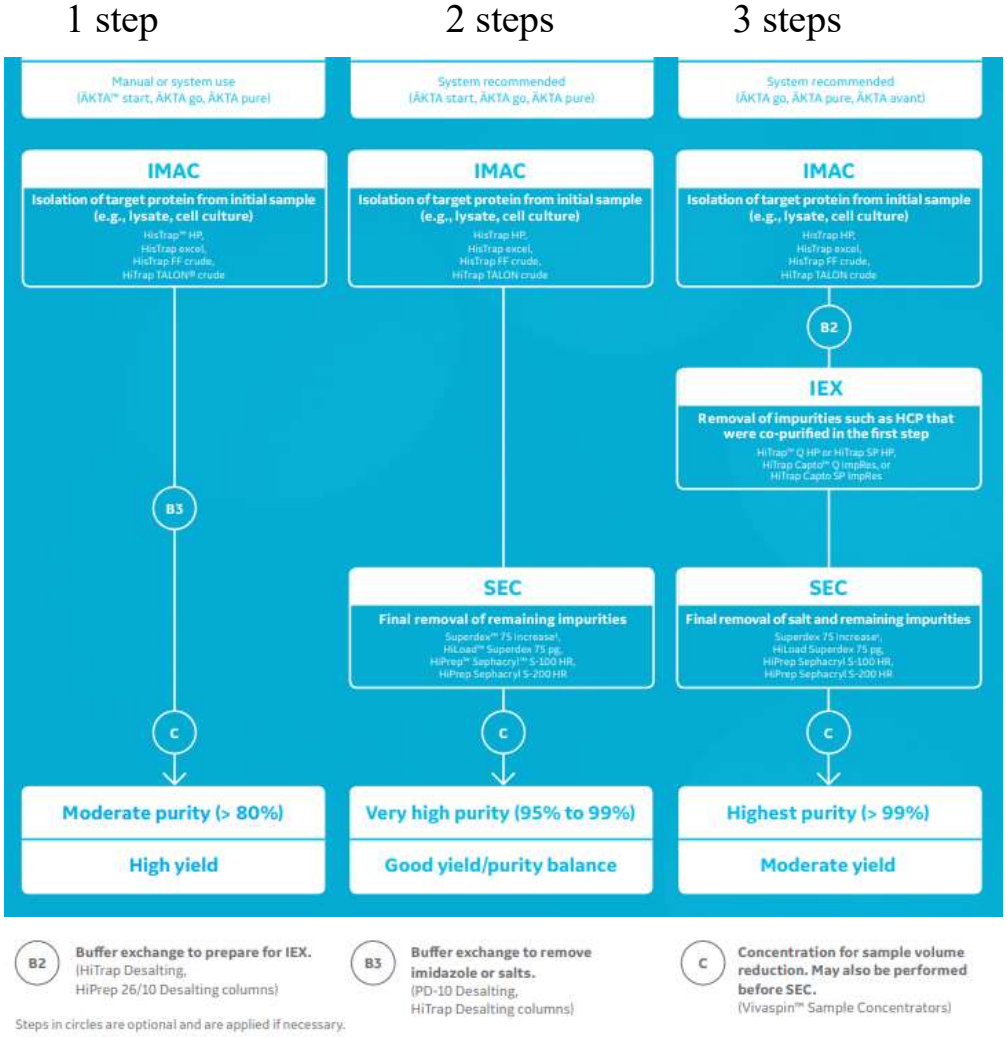


Fig 4.10. Example of polishing: removal of trace contaminants by high-resolution CIEX. Purification of the transposase TniA.

His-tagged protein purification



Basic rules for order of steps in recombinant protein purification

Method	Typical characteristics		Purification phase			Sample start conditions	Sample end conditions
	Resolution	Capacity	Capture	Intermediate	Polishing		
AC	+++ or ++	+++ or ++	+++	++	+	Various binding conditions	Specific elution conditions
IMAC	+++	++	+++	++	+	For purifying histidine-tagged proteins using Ni Sepharose columns:: 20-40 mM imidazole; pH > 7; 500 mM NaCl; no chelators Other proteins: low concentration of imidazole	High concentration of imidazole, pH > 7, 500 mM NaCl
GF	++	+	+		+++	Most conditions acceptable, limited sample volume	Buffer exchange possible diluted sample
IEX	+++	+++	+++	+++	+++	Low ionic strength. pH depends on protein and IEX type	High ionic strength or pH changed
HIC	+++	++	++	+++	+++	High ionic strength, addition of salt required	Low ionic strength

Basic rules for order of steps in recombinant protein purification

- In the early stages, the methods characterized by high capacity and low yield and resolution are needed → high amount of input material.

- Later, methods characterized by high resolution and yield are important, capacity is less relevant → amount of protein is smaller.

- The method should be rank rationally, without intermediate steps like changes of buffers between two separation techniques

e.g. after precipitation by ammonium sulfate or after ion exchange chromatography (protein is eluted in high salt concentration) to order hydrophobic chromatography (sample is injected on the column in high salt concentration).

- Individual separation methods not to repeat.

- The fewer steps, the higher yield of protein.

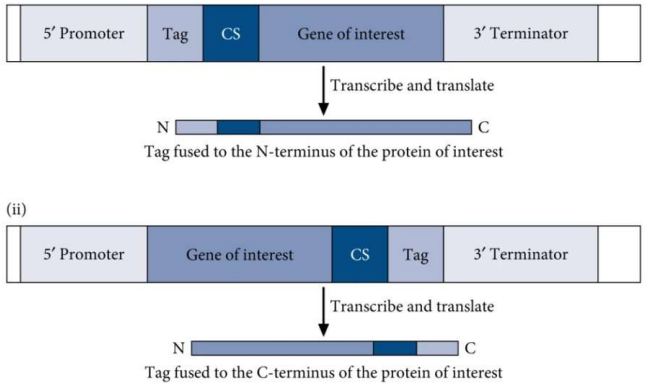
Fusion proteins

Translation fusion of sequences coding a recombinant protein and tag.

Tags:

a) **short peptides** [ex. $(His)_n$, $(Asp)_n$, $(Arg)_n$ ...].

b) **protein domains, entire proteins** [ex. MBP, GST, thioredoxin …].



- Facilitating the purification of recombinant proteins (purification uniformity)
- Increasing the yield of recombinant proteins
- Enhancing the solubility of recombinant proteins
- Improving protein detection
- Enabling secretion
- Tag can be selectively removed.

Fusion partner (tag)	Size	Tag placement	Uses
His-tag	6, 8, or 10 aa	N- or C-terminus	Purification, detection
Thioredoxin	109 aa (11.7 kDa)	N- or C-terminus	Purification, solubility enhancement
Calmodulin-binding domain (CBD)	26 aa	N- or C-terminus	Purification
Avidin/streptavidin *Strep*-tag	8 aa	N- or C-terminus	Purification, secretion
Glutathione *S*-transferase (GST)	26 kDa	N-terminus	Purification, solubility enhancement
Maltose binding protein (MBP)	396 aa (40 kDa)	N- or C-terminus	Purification, solubility enhancement
Green fluorescent protein (GFP)	220 aa (27 kDa)	N- or C-terminus	Localization, detection, purification
Poly-Arg	5-16 aa	N- or C-terminus	Purification, solubility enhancement
N-utilization substance A (NusA)	495 aa (54.8 kDa)	N-terminus	Solubility enhancement

Increasing the yield of recombinant proteins using fusion technology

Yield enhancing tags are proteins and peptides which can be involved in:

- **Increasing the efficiency of translation initiation (e.g. GST, MBP, NusA…)**
  - Advantage of N-terminal tags
  - Providing a reliable context for efficient translation initiation
  - Ribosome efficiently initiates translation at the N-terminal methionin of the tag
  - Deleterious secondary structures are more likely to occur in conjunction with short N-terminal tags because short RNA-RNA interactions tend to be more stable than long-range interactions.
- **Protection against proteolytic degradation**

- Several studies have shown that the nature of terminal residues in a protein can play a role in recognition and subsequent action by proteases and in some cases affinity tags might improve the yield of recombinant proteins by rendering them more resistant to intracellular proteolysis.

- **Helping to properly fold their partners leading to increased solubility of the target protein (*in vivo* and *in vitro*).**

Enhancing the solubility of recombinant proteins

Solubility-enhancing tags

- Advantage of N-terminal tags

- Rather proteins (highly soluble proteins) than peptides

-Fusion with a soluble fusion partner often helps to properly fold their fusion partners leading to improved solubility (*in vivo and in vitro*) of the target protein.

-The choice of a fusion partner is still a trial-and-error experience.

- Fusion partners do not perform equally with all target proteins, and each target protein can be differentially affected by several fusion tags (Esposito and Chatterjee, 2006)

➢PROTEINS

Some commonly used solubility-enhancing fusion partners

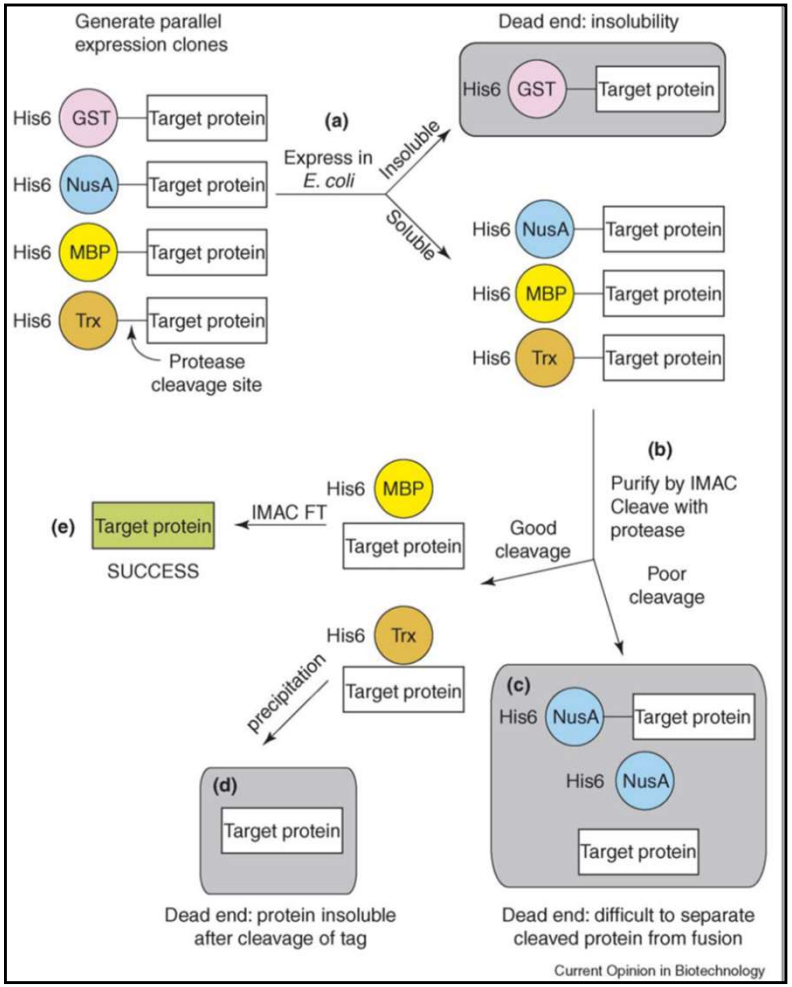
Tag	Protein	Source organism
MBP	Maltose-binding protein	*Escherichia coli*
GST	Glutathione-*S*-transferase	*Schistosoma japonicum*
Trx	Thioredoxin	*Escherichia coli*
NusA	N-Utilization substance	*Escherichia coli*
SUMO	Small ubiquitin-modifier	*Homo sapiens*
SET	Solubility-enhancing tag	Synthetic
DsbC	Disulfide bond C	*Escherichia coli*
Skp	Seventeen kilodalton protein	*Escherichia coli*
T7PK	Phage T7 protein kinase	Bacteriophage T7
GB1	Protein G B1 domain	Streptococcus sp.
ZZ	Protein A IgG ZZ repeat domain	*Staphylococcus aureus*

Adopted from Esposito and Chatterjee, 2006

➢ PEPTIDES

Poly-Arg

Poly-Lys



Schematic representation of the pathway from protein expression to purification using solubility tags (Esposito and Chatterjee, 2006).

Solubility-enhancing tags - the mechanism of action

-The mechanism by which partners exert their solubilising function is not fully understood and possibly differs between fusion proteins.

Examples of possible mechanisms

Maltose binding protein (MBP) has an intrinsic chaperone-like activity. MBP might bind reversibly to exposed hydrophobic regions of nascent target polypeptide, steering the polypeptides towards their native conformation by a chaperone like –mechanism.

N-utilization substance (NusA) decreased translation rates by mediating transtriptional pausing, that might enable critical folding events to occur.

MBP and N-utilization substance (NusA) attract chaperones. The fusion tag drives its partner protein into a chaperone-mediated folding pathway. MBP and N-utilization substance (NusA) interact with GroEL in E. coli (Huang and Chuang, 1999).

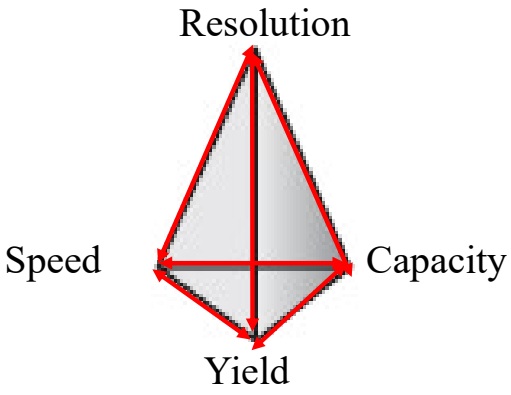
Small ubiquitin related modifier (SUMO) promotes the proper folding and solubility of its target proteins possibly by exerting chaperoning effects in a similar mechanism to the described for its structural homolog Ubiquitin (Ub; Khorasanizadeh et al., 1996).

Negative charged tags (highly acidic peptide) inhibit aggregation by increasing electrostatic repulsion between nascent polypepdides (Zhang et. 2004) .

Purification tags

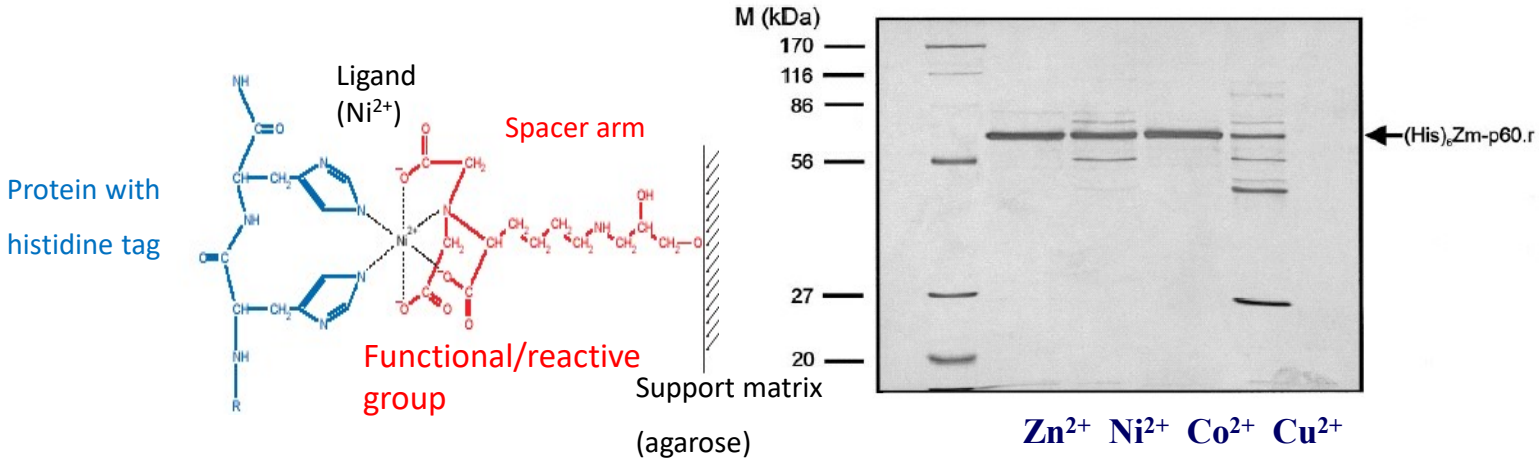
Tag	Chromatographic technique	Principle of separation technique
poly [His]	afinity	Bind to metal
IgG binding domain	afinity	Bind to metal
Poly [Asp]	ion exchange	Bind to anion binding matrix
Poly [Phe]	hydrophobic	Bind to hydrophobic matrix
Strep-tag	afinity	Bind to streptavidin
Poly [Arg]	ion exchange	Bind to cation binding matrix

These separation techniques are characteristic by equilibrium of all parameters.



Immobilized metal affinity chromatography (IMAC)

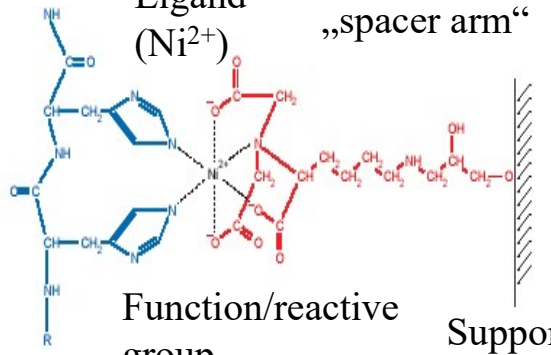
- The most common purification tag is typically composed of six consecutive histidine residues.
- Histidine, cysteine, and tryptophan residues are known to interact specifically with divalent transient metal ions such as Ni^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+}.
- Histidine is the amino acid that exhibits the strongest interaction with immobilized metal ion matrices as the electron donor groups on the histidine imidazole ring readily form coordination bonds with an immobilized transition metal.
- IMAC can be used under native and/or denatured conditions.
- A highly purified protein can often be obtained in one or, at most, two purification steps.



Bond strength: $Cu^{2+} > Ni^{2+} > Zn^{2+} \sim Co^{2+}$

Metal Chelate Affinity Chromatography Matrix

Protein with polyhistidin tag



Ligand (Ni^{2+})

„spacer arm“

Function/reactive group

Support matrix (porous polysacharide beads)

„spacer arm“

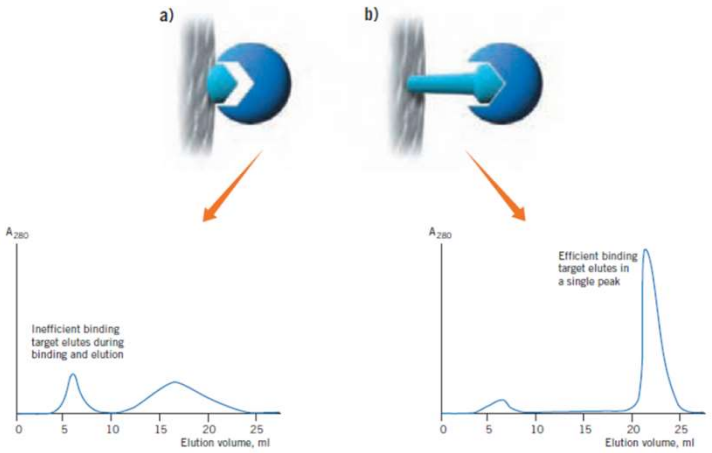


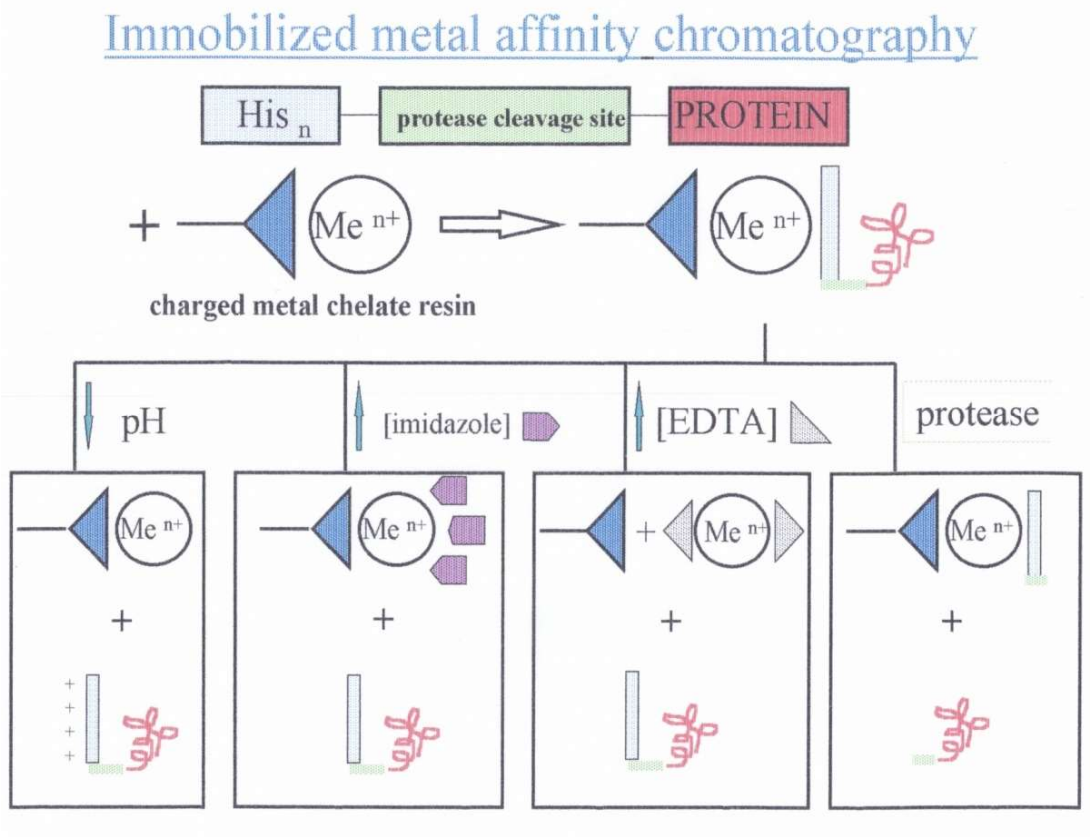
Fig. 56. Using spacer arms. a) Ligand attached directly to the matrix. b) Ligand attached to the matrix via a spacer arm.

➢The introduction of a spacer arm between the ligand and the matrix minimizes this steric effect and promotes optimal adsorption of the target protein to the immobilized ligand.

Immobilized metal affinity chromatography (IMAC)

Purification under native conditions

- Optimal binding of recombinant protein with metal ion is achieved at pH 7–8.
- Buffers with a high salt concentration (0.5–1 M NaCl) reduce nonspecific electrostatic interaction.
- Nonionic detergents or glycerol reduce nonspecific hydrophobic interactions.
- Elution of contaminating proteins can be achieved by lowering the pH or using low concentrations of imidazole.
- Elution of tagged protein is achieved at high imidazole concentrations (0–0.5 M), by strongly decreasing the pH, or by using EDTA.



His-tagged protein and IMAC under denatured conditions

– Purification of proteins expressed in inclusion bodies.
– Purification in a high concentration of urea or guanidine chloride.
– Result is a pure protein, but in a denatured form (sufficient for immunization).

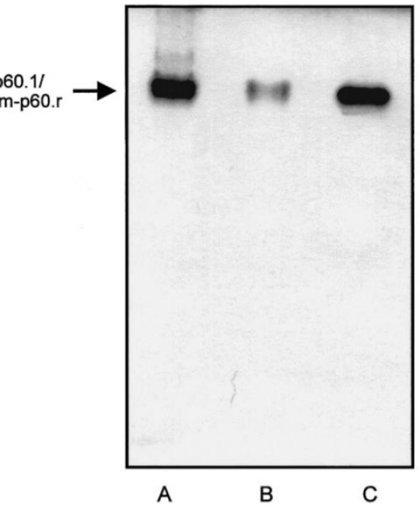
Recovery of native conformers (necessary for functional and structural analysis):

➢ Binding to the column under strong denaturing conditions (8 M urea)

➢ **Two possibilities of renaturation**:

1. The protein is eluted from the column and renatured by dialysis or rapid dilution in renaturing buffers.

2. Renaturation of the protein bounded to the column (matrix assisted refolding procedure): gradient from denatured to renatured buffers or pulsion renaturation (8-0M urea).



(Zouhar et al., 1999)

Identification of properly refolded $(His)_6$Zm-p60.1 (maize β-glucosidase) using 10% native PAGE, followed by activity in gel staining:

A = crude protein extract prepared from maize seedlings containing the native enzyme

B = $(His)_6$Zm-p60.1, renatured product (matrix assisted refolding procedure – 23 renaturing cycles)

C = $(His)_6$Zm-p60.1 purified by native IMAC

K_M $(His)_6$Zm-p60.1 purified by native IMAC: **0.64 ± 0.06 mM**

K_M $(His)_6$Zm-p60.1 renatured product: **0.6 ± 0.08 mM**

Determination of v_{max} and k_{cat} was hampered by the fact that the refolding process yielded a number of improperly folded polypeptides.

Removal of fusion tags- the Achilles' heel of the fusion approach

All tags, whether small or large, have the potential to interfere with the biological activity of a protein, impede its crystallization (presumably due to the conformational heterogeneity allowed by the flexible linker region), be too large for NMR analysis, cause a therapeutic protein to become immunogenic or otherwise influence the target protein`s behavior.

The fusion tags can be removed by:

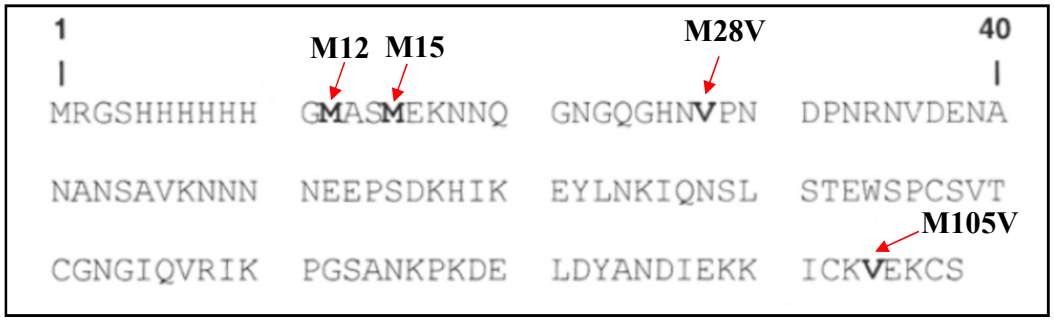
- **Chemical cleavage**
- **Self - cleavage**
- **Enzymatic cleavage**

Removal of fusion tags – chemical cleavage

➢ Rarely used.

Cyanogen bromide Met/X

Hydroxylamine Asn-Gly



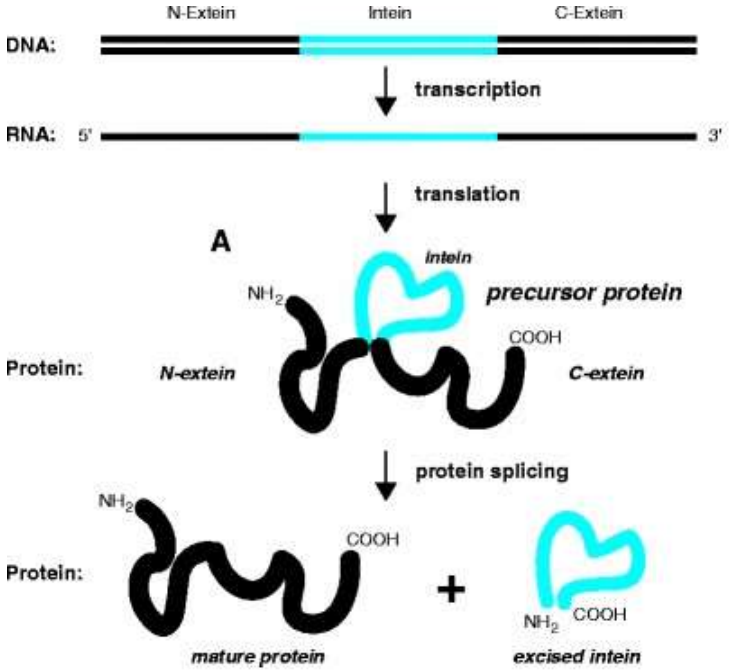
Amino – acid sequence of the *P. falciparum* C-terminal segment of CSP (PfCSP C-ter) fused to a purification tag (*Rais-Beghdadi et al., 1998*).

Chemical cleavage is a harsh method, efficient, but rather non-specific and may lead to unnecesary denaturation or modification of the target protein.

Removal of fusion tags - self - cleaving

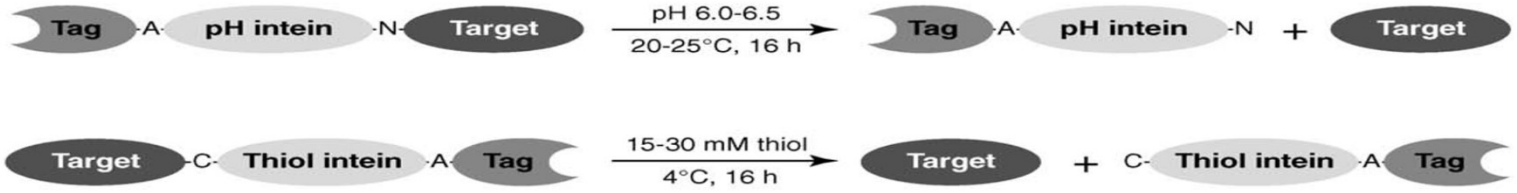
➢ **Use of self-cleaving fusion tags**

1. Inteins



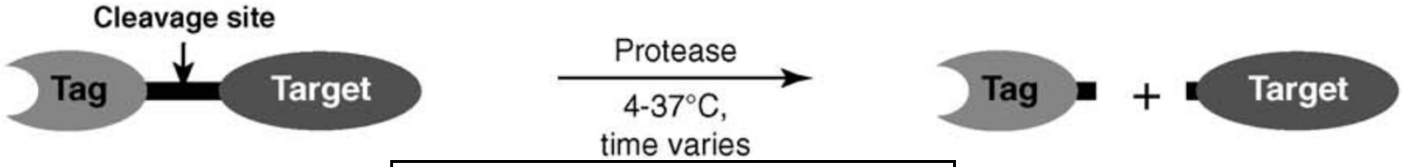
Inteins (***int****ervening prot****eins****)* are protein segments that can excise themselves from protein precursors in which the are inserted and rejoin the flanking regions.

➢ Self - splicing inteins can be mutated at the N- or C-terminal splice junction to yield self cleaving inteins, which can be used to mediate self cleaving of various tags.



Perler, (2005)

Removal of fusion tags – enzymatic cleavage



Site-specific proteolytic cleavage:

- **Exopeptidases**
- **Endopeptidases**

Exopeptidases (aminopeptidases and carboxypeptidases):

DAPase (TAGZyme)	Exo(di)peptidase	Cleaves N-terminal. His-tag (C-terminal) for purification and removal
Aeromonas aminopeptidase	Exopeptidase	Cleaves N-terminal, effective on M, L. Requires Zn
Aminopeptidase M	Exopeptidase	Cleaves N-terminal, does not cleave X-P
Carboxypeptidase A	Exopeptidase	Cleaves C-terminal. No cleavage at X-R, P
Carboxypeptidase B	Exopeptidase	Cleaves C-terminal R, K

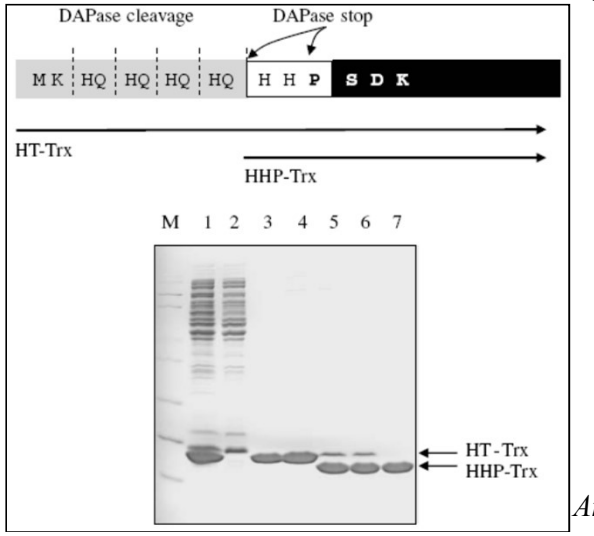
➢APM, CPA and CPB release sequentially a single amino-acid from the N- or C- terminus of a protein until the stop site is reached.

TAGZyme system (Qiagen):

➢ DAPase (dipeptidyl aminopeptidase I)

TAGZyme stop points

Amino acid	DAPase stop point (↓) sequence*
Lysine (Lys, K)	Xaa-Xaa...Xaa-Xaa ↓ Lys-Xaa ...
Arginine (Arg, R)	Xaa-Xaa...Xaa-Xaa ↓ Arg-Xaa ...
Proline (Pro, P)	Xaa-Xaa...Xaa-Xaa ↓ Xaa-Xaa Pro-Xaa...
Proline (Pro, P)	Xaa-Xaa...Xaa-Xaa ↓ Xaa-Pro Xaa-Xaa...
Glutamine (Gln, Q)'	Xaa-Xaa...Xaa-Xaa ↓ Gln-Xaa...



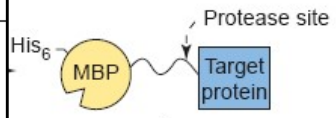
Arnau et al., 2006

Removal of fusion tags - enzymatic cleavage

Endopeptidases

➢ The enzymatic cleavage site has to be placed between the fusion tag and the target protein.

Enzyme	Cleavage site	Comments
Enterokinase	DDDDK*	Secondary sites at other basic aa
Factor Xa	IDGR*	Secondary sites at GR
Thrombin	LVPR*GS	Secondary sites. Biotin labeled for removal of the protease
PreScission	LEVLFQ*GP	GST tag for removal of the protease
TEV protease	EQLYFQ*G	His-tag for removal of the protease
3C protease	ETLFQ*GP	GST tag for removal of the protease
Sortase A	LPET*G	Ca^{2+}-induction of cleavage, requires an additional affinity tag (e.g., his-tag) for on column tag removal
Granzyme B	D*X, N*X, M*N, S*X	Serine protease. Risk for unspecific cleavage



Enterokinase **Asp-Asp-Asp-Asp-Lys/X**

Table 4 Cleavage (%) of enterokinase through densitometry (Hosfield and Lu 1999) based on the amino acid residue X_1. The sequence....-GSDYKDDDDK-X_1-ADQLTEEQIA-... of a GST-calmodulin fusion protein was tested using 5 mg protein digested with 0.2 Uof enterokinase for 16 h at 37 °C

Amino acid in position X_1	Cleavage of enterokinase (%)
Alanine	88
Methionine	86
Lysine	85
Leucine	85
Asparagine	85
Phenylalanine	85
Isoleucine	84
Aspartic acid	84
Glutamic acid	80
Glutamine	79
Valine	79
Arginine	78
Threonine	78
Tyrosine	78
Histidine	76
Serine	76
Cysteine	74
Glycine	74
Tryptophan	67
Proline	61

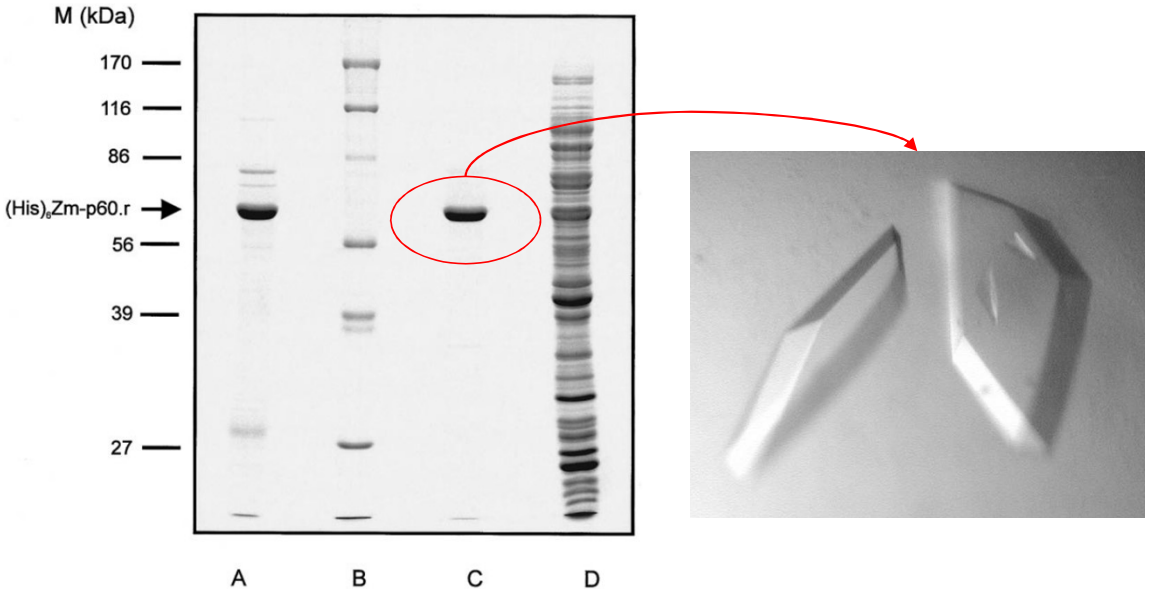
Removal of fusion tags - enzymatic cleavage

- **Optimization of protein cleavage conditions** (mainly enzyme-to-substrate ratio, temperature, pH, salt concentration, length of exposure).
- **Cleavage efficiency** (Optimization is needed. The efficiency varies with each fusion protein in an unpredictable manner, probably due to aggregation or steric issues; the problem can be solved by introducing short linkers between the protease site and the fusion tag).
- **Unspecific cleavage** (SOLUTION: optimization of protein cleavage conditions or using re-engineered proteases with increased specificity such as ProTEV and AcTEV proteases). Product of cleavage is reccomended to verify using mass spectrometry.
- **Precipitation of the target protein when the fusion partner is removed** (so-called soluble aggregates; SOLUTION: another approach for protein solubilization has to be found).
- **Target protein modification** (some proteases like thrombin, TEV, Precision leave one or two amino-acids on the target protein near the cleavage site).
- **Re-purification step** is needed to separate the protease from target protein.

His-tagged protein and IMAC under native conditions

One-step purification of maize β-glucosidase

- Perfusion matrix: POROS MC/M
- Functional group: iminodiacetate, metal ion Zn^{2+}
- Removing contaminated proteins: linear gradient of imidazole (0–50 mM) and pH (pH 7–6.1)
- Protein elution: 0.1 M EDTA
- 80% recovery, 95 fold purification
- Common production and isolation of wild type protein and soluble mutant form for enzymatic measurements and crystallization.



(Zouhar et al., 1999)

Purification of AHP2 protein (Arabidopsis histidin phosphotransfer protein 2)

4L cell culture

Immobilized metal affinity chromatography

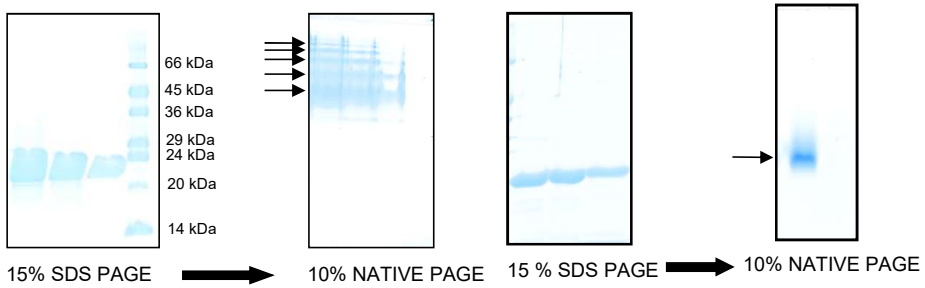
Buffer: 50 mM Tris pH 7.9, 300 mM NaCl, 10 % glycerol, 20 mM imidazole, 3.9 mM mercaptoethanol
Gradient elution: 20 -500 mM imidazole

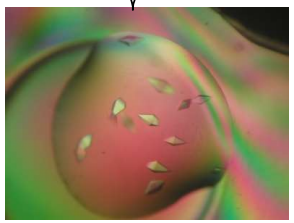
Size exclusion chromatography

Buffer: 20 mM Tris pH 7.9, 250 mM NaCl
Isocratic elution

Anion exchange chromatography

Buffer: 20 mM Tris pH 7.9
Gradient elution: 0 - 1M NaCl





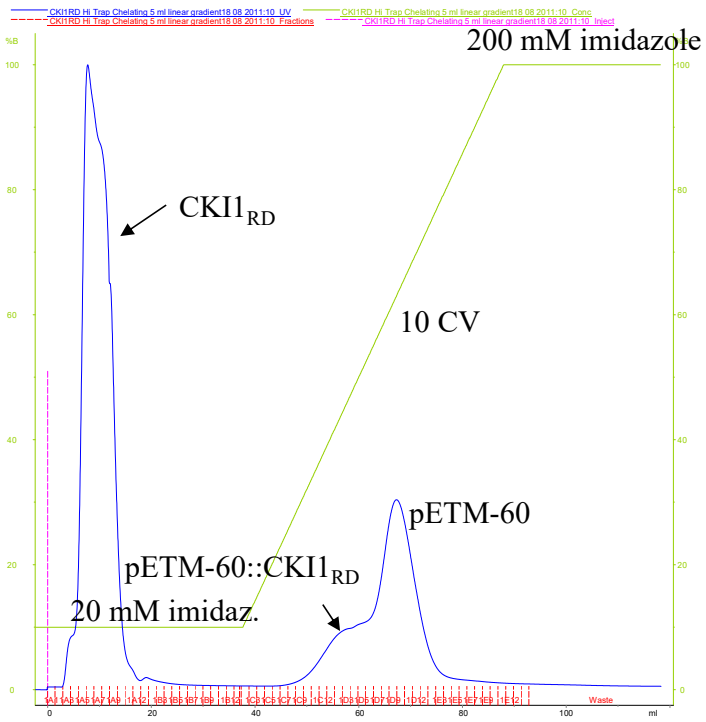
His-tagged protein and IMAC under native conditions

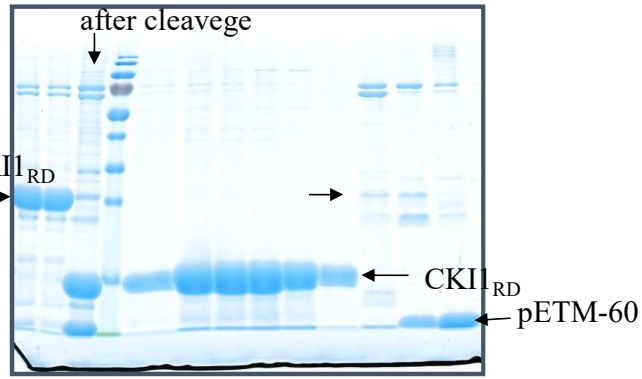
Four-step purification of *Arabidopsis* $CKI1_{RD}$

1. Affinity purification (MCAC)
2. Tag removal (TEV protease)
3. Affinity purification (MCAC)
4. Size exclusion chromatography

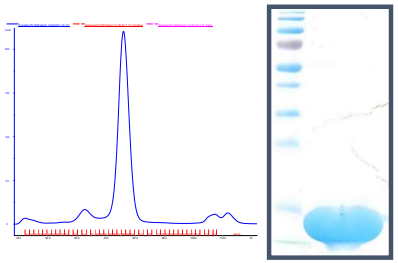
Ub-SGSG-HisTag-SA-TEV-AME-CKI1

3. Affinity purification after TEV cleavege





4. Size-exclusion chromatography



1 L→ ~10-20 mg for TB and M9

Pekárová B.