Methods for the study of protein-protein interactions

Biochemical methods

Protein-protein interactions

- They are fundamental in the structural and functional organization of the cell
- Stable interactions: protein complexes
- Transient interactions: control most cellular processes

Binding equilibria

- BP (binding protein) and L (ligand) form a complex BPL
- Association costant K_A and dissociation constant K_D define the strength (affinity) of the interaction

$$[BP]+[L] \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} [BPL]$$

$$k_{+1}[BP][L] = k_{-1}[BPL]$$

$$\frac{k_{-1}}{k_{+1}} = K_D = \frac{[BP][L]}{[BPL]} \qquad \frac{k_{+1}}{k_{-1}} = K_A = \frac{[BPL]}{[BP][L]}$$

How to identify and characterize a protein-protein or protein-ligand interaction?

Biochemical methods:	affinity chromatography gel-filtration chromatography affinity blot (overlay) co-immunoprecipitation cross-linking
Genetic methods:	two-hybrid system and its variants (protein complementation assay) phage display
Biophysical methods:	FRET: Fluorescence Resonance Energy Transfer SPR: Surface Plasmon Resonance ITC: Isothermal Titration Calorimetry

Affinity chromatography

- The method is based on the reversible formation of the complex **BPL** between ligand **L** ('bait' **protein**) and 'binding protein' **BP** ('prey' **protein**)
- The ligand **L** is immobilized on a chromatographic support

Complexes with $K_D > 10^{-4}$ M are difficult to identify

Complexes with $K_D < 10^{-10}$ M are difficult to elute in native conditions

Affinity chromatography

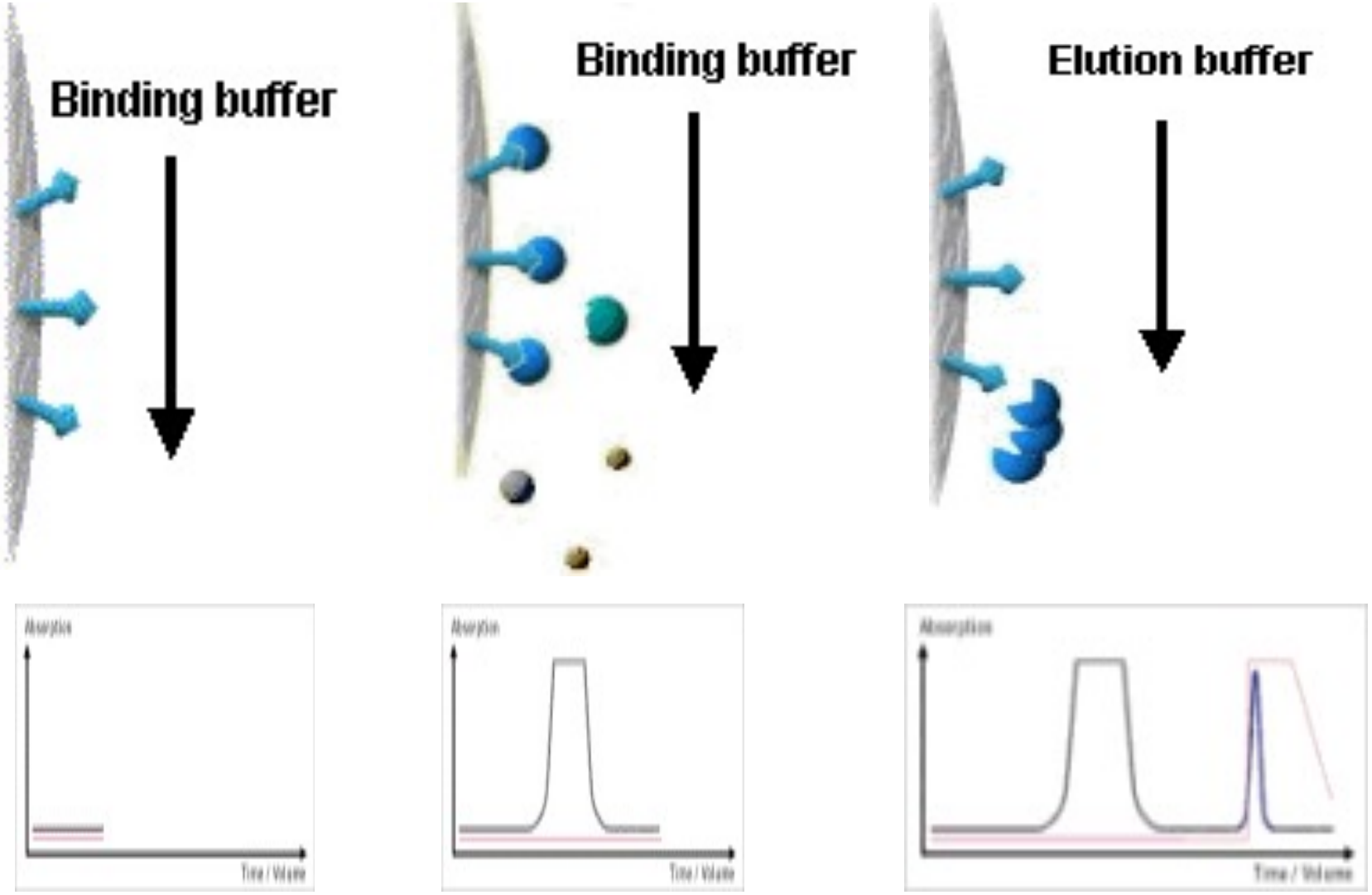
Factors that influence efficacy of affinity chromatography

- Purity of the immobilized 'bait' protein
- Influence of post-translational modifications and cofactors
- Concentration of the immobilized protein (>K_D)

- Very sensitive technique
- It is possible to use complex protein mixtures

- It can be used to identify new partners of the 'bait' protein and also to define interaction regions ➔ it is possible to use mutants

Affinity chromatography

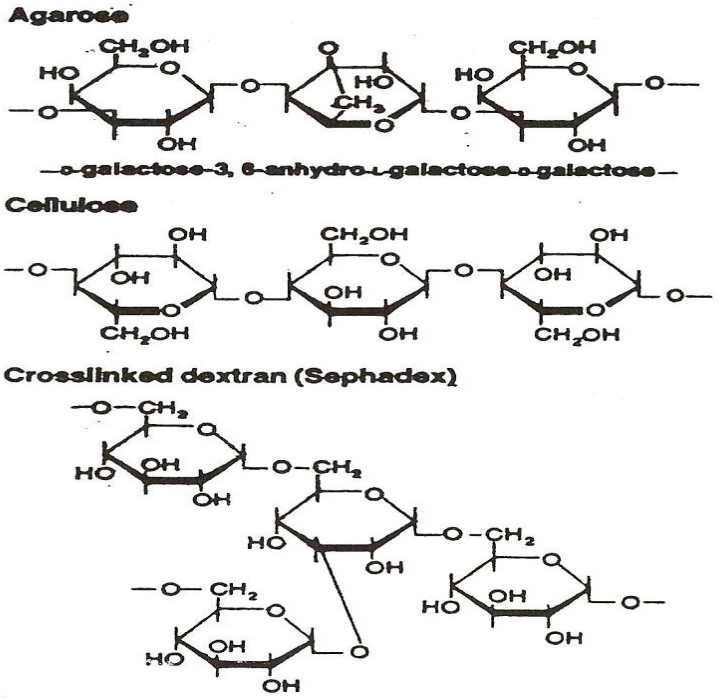


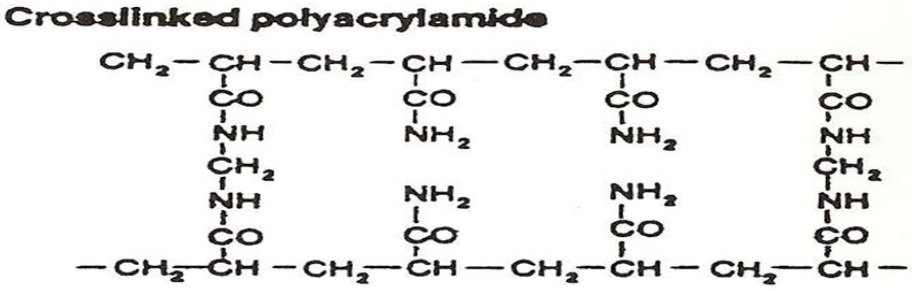
Matrix: an ideal matrix should have the following features:

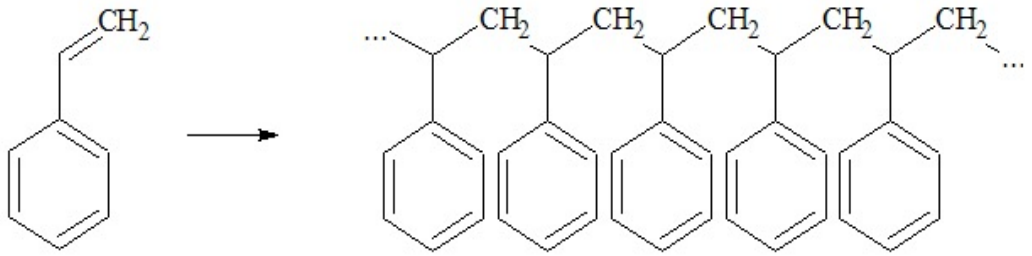
- It should ontain chemical groups adequate for covalent binding
- It should be stable in binding conditions
- It should be inert

Matrices can be divided in two main categories:

NATURAL POLYMERS: polysaccharides (agarose, dextran, cellulose)
SYNTHETIC POLYMERS: polystirene, polyacrylates





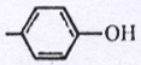
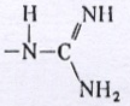
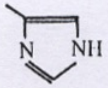
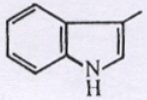


Ligands

- Must have adequate groups for covalent binding, not required for interaction with the binding protein.
- The most common groups are –NH_2, COOH, SH, OH

Reactive residues in proteins

Table 5.8. Reactive residues of proteins[a]

Residue	Originating amino acid
$-NH_2$	ε-Amino of L-lysine and *N*-terminus amino group
$-SH$	Thiol of L-cysteine
$-COOH$	Carboxyl of L-aspartate and L-glutamate and *C*-terminus carboxyl group
	Phenolic of L-tyrosine
	Guanidino of L-arginine
	Imidazole of L-histidine
$-S-S-$	Disulphide of L-cystine
	Indole of L-tryptophan
CH_3-S-	Thioether of L-methionine
$-CH_2OH$	Hydroxyl of L-serine and L-threonine

Usually a spacer arm of variable length is added to increase accessibility of the ligand to the binding protein

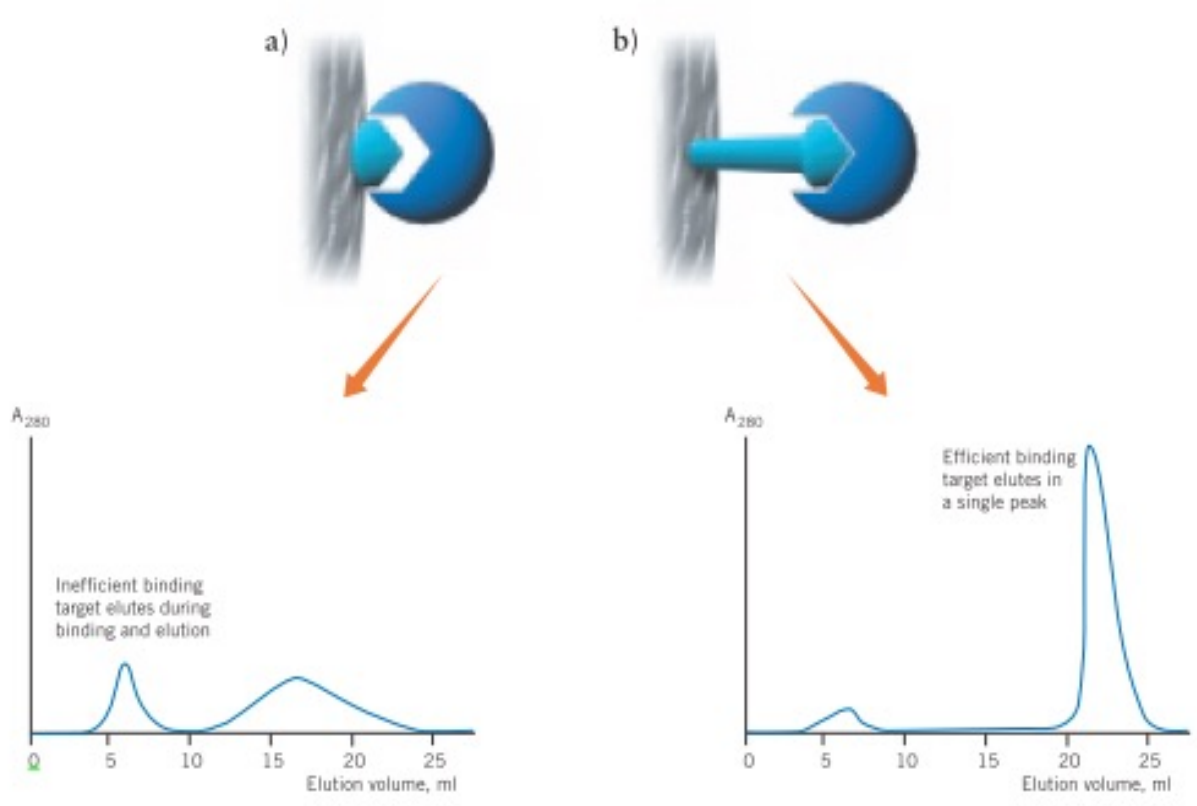
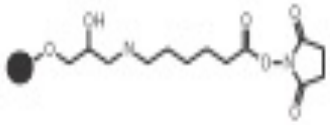
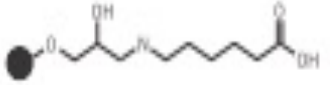
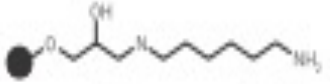
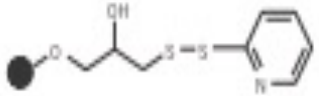
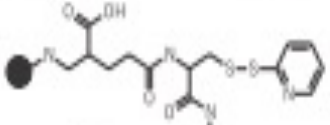
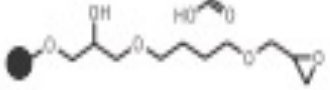
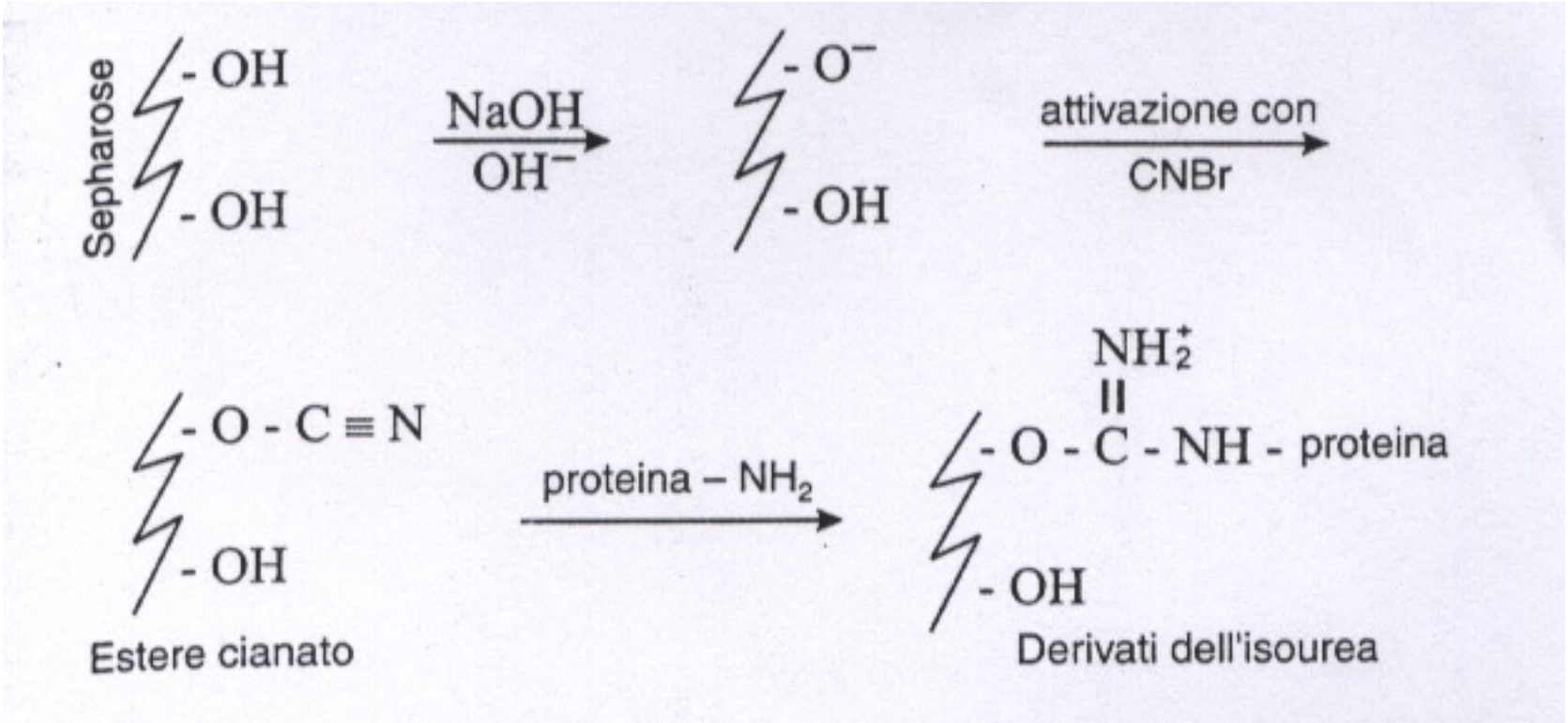


Table 8.

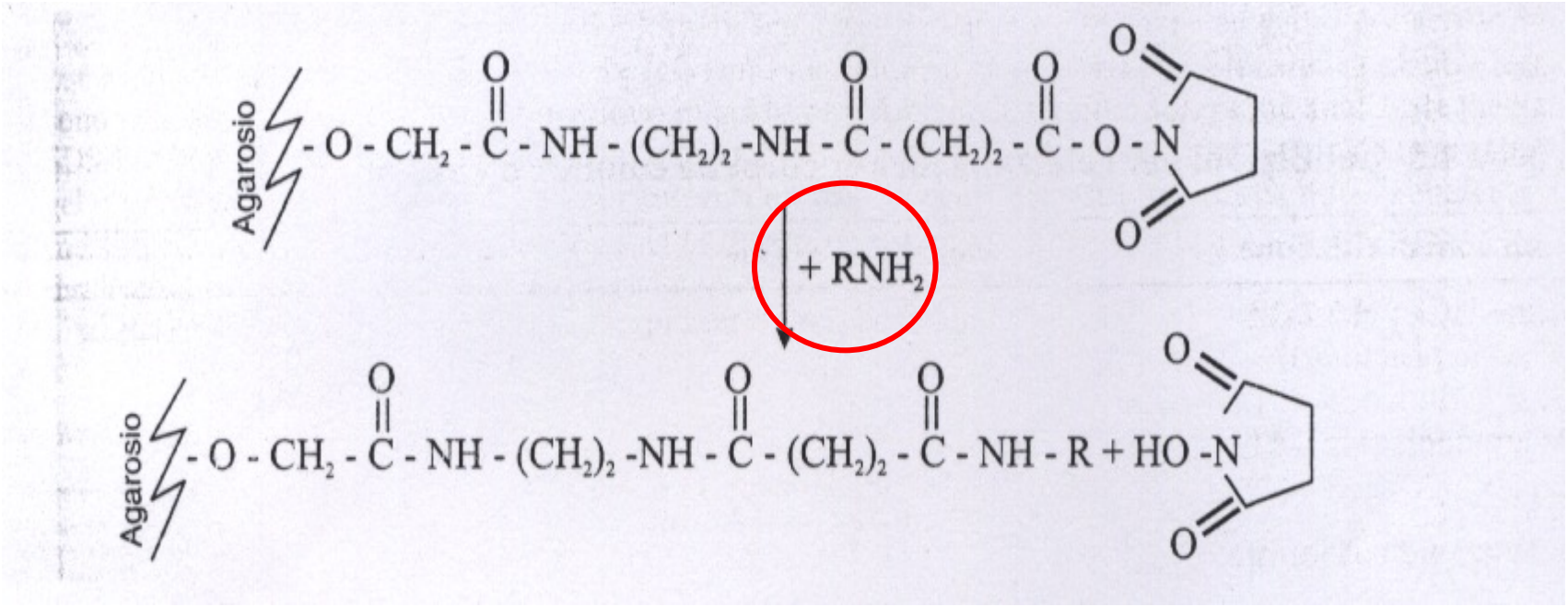
Chemical group on ligand	Length of spacer arm	Structure of spacer arm	Product
Proteins, peptides, amino acids			
amino	10-atom		HiTrap NHS-activated HP NHS-activated Sepharose 4 Fast Flow
	None	–	CNBr-activated Sepharose 4B CNBr-activated Sepharose 4 Fast Flow
	10-atom		ECH Sepharose 4B
carboxyl	11-atom		EAH Sepharose 4B
thiol	4-atom		Thiopropyl Sepharose 6B
	10-atom		Activated Thiol Sepharose 4B
	12-atom		Epoxy-activated Sepharose 6B

Cyanogen bromide coupling procedure for amino groups

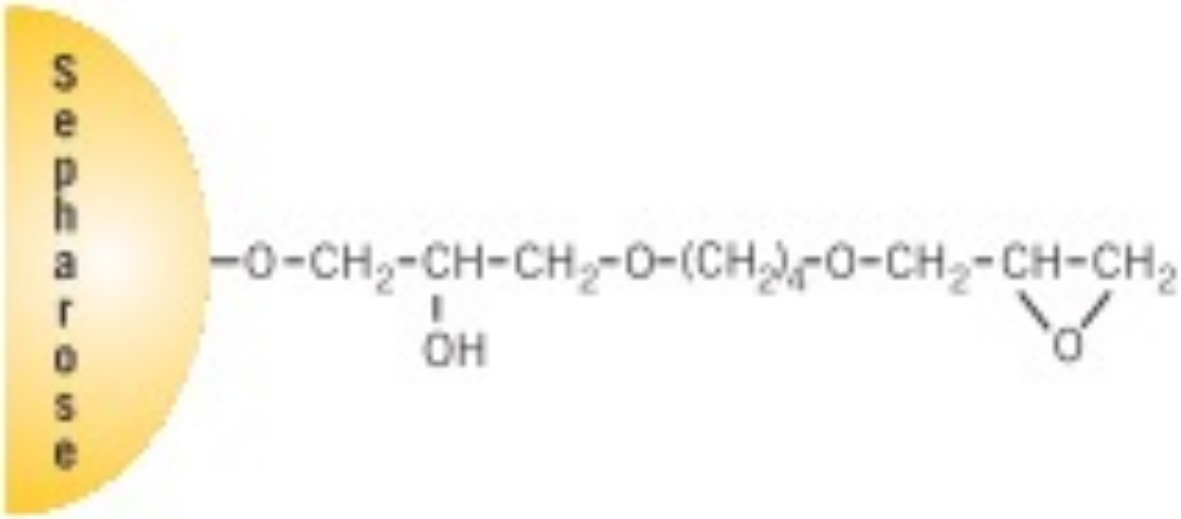
- The resin is activated at alkaline pH
- The 'bait' protein (ligand) is added
- Residual active groups on the resin are blocked
- The resin is washed and equilibrated for use



N-hydroxysuccinimide coupling procedure for amino groups

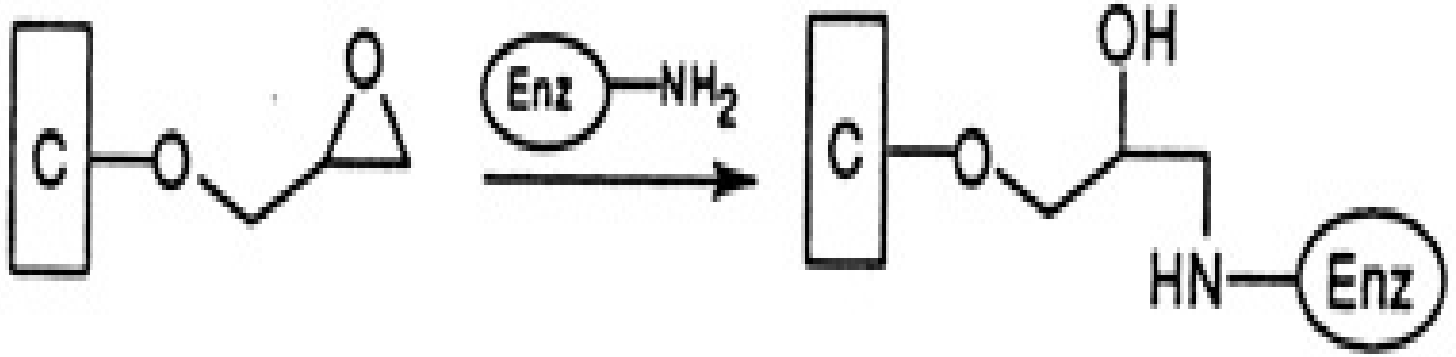


Epoxide coupling procedure for NH_2, SH, OH groups at basic pH

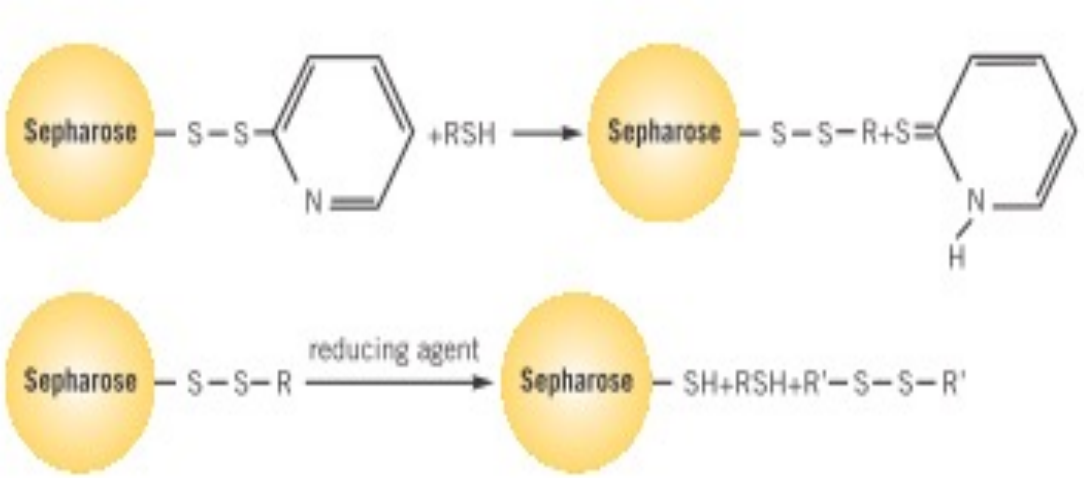


L-NH_2
L-SH
L-OH

Nucleophilic substitution reaction



Coupling procedure for proteins containing SH groups

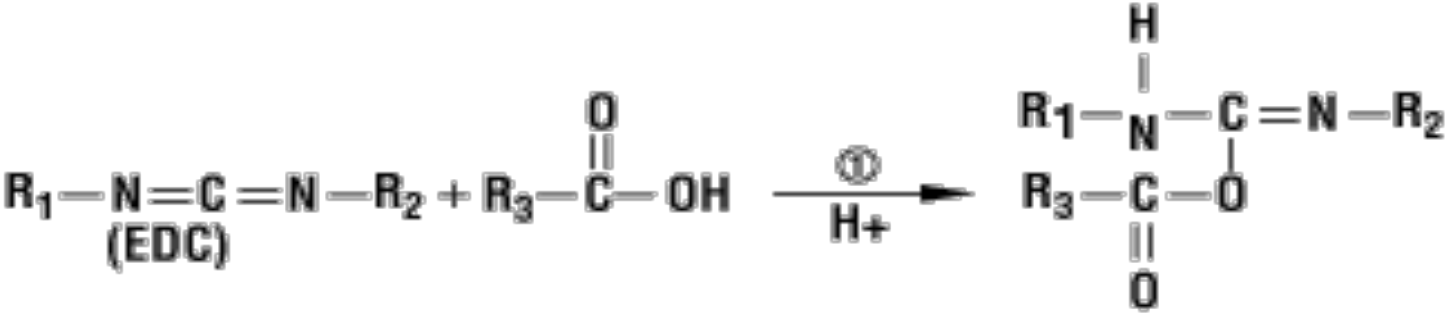


2 pyridyl-thione absorbs at 343 nm

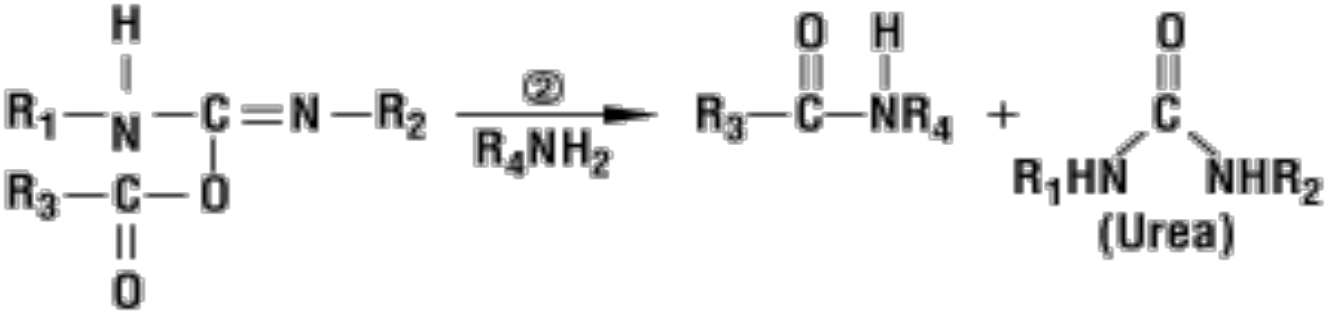
Coupling requires thiol exchange

- Formation of disulphide bonds between the protein and the matrix
- Release of 2 pyridyl-thione

Coupling between carboxyl and amino groups by carbodiimides (Formation of amide bonds)



Carbodiimide reacts with the carboxyl group at pH 4.5 to form oxyacylurea



The carbonyl group of oxyacylurea undergoes nucleophilic attack by the amino group to form an amide bond with release of urea

Recombinant proteins with 'affinity tags': 'Pull-down' affinity chromatography

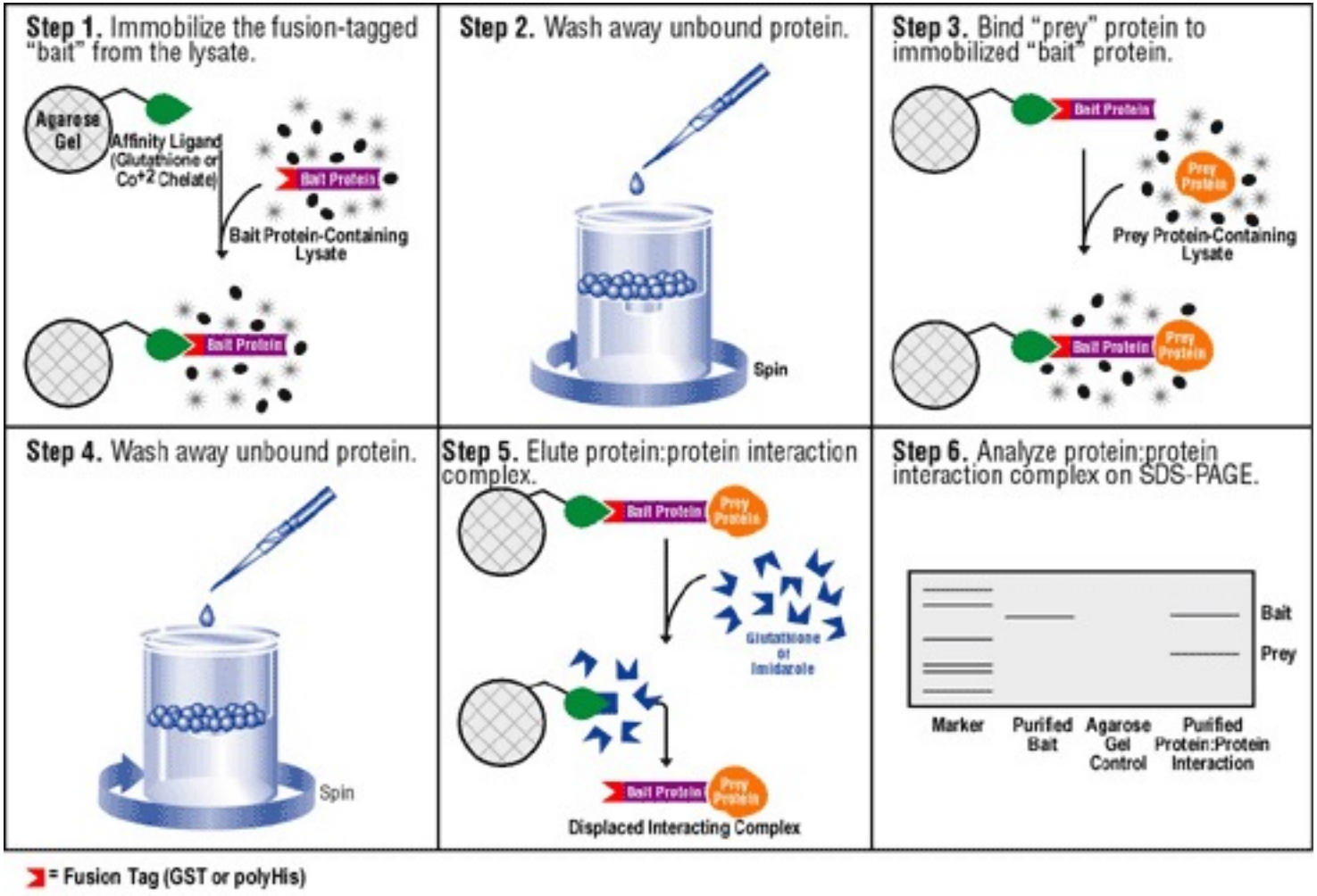
- Peptides or protein tags that can be purified by interaction with small molecules immobilized on a matrix. E.g., His-Tag, glutathione *S*-transferase, maltose binding protein are captured by resins with a bound metal, glutathione or maltose

- Peptide tags that can be purified on resins with immobilized partner proteins (calmodulin binding peptide CBP binds a resin with immobilized calmodulin)

- Peptide tags that can be purified on resins with immobilized antibodies (FLAG peptide, c-myc peptide etc)

➔ **Immobilization of the 'bait' protein is mediated by the tag**

Recombinant proteins with affinity tags
'Pull-down'



Tandem Affinity Purification (TAP)

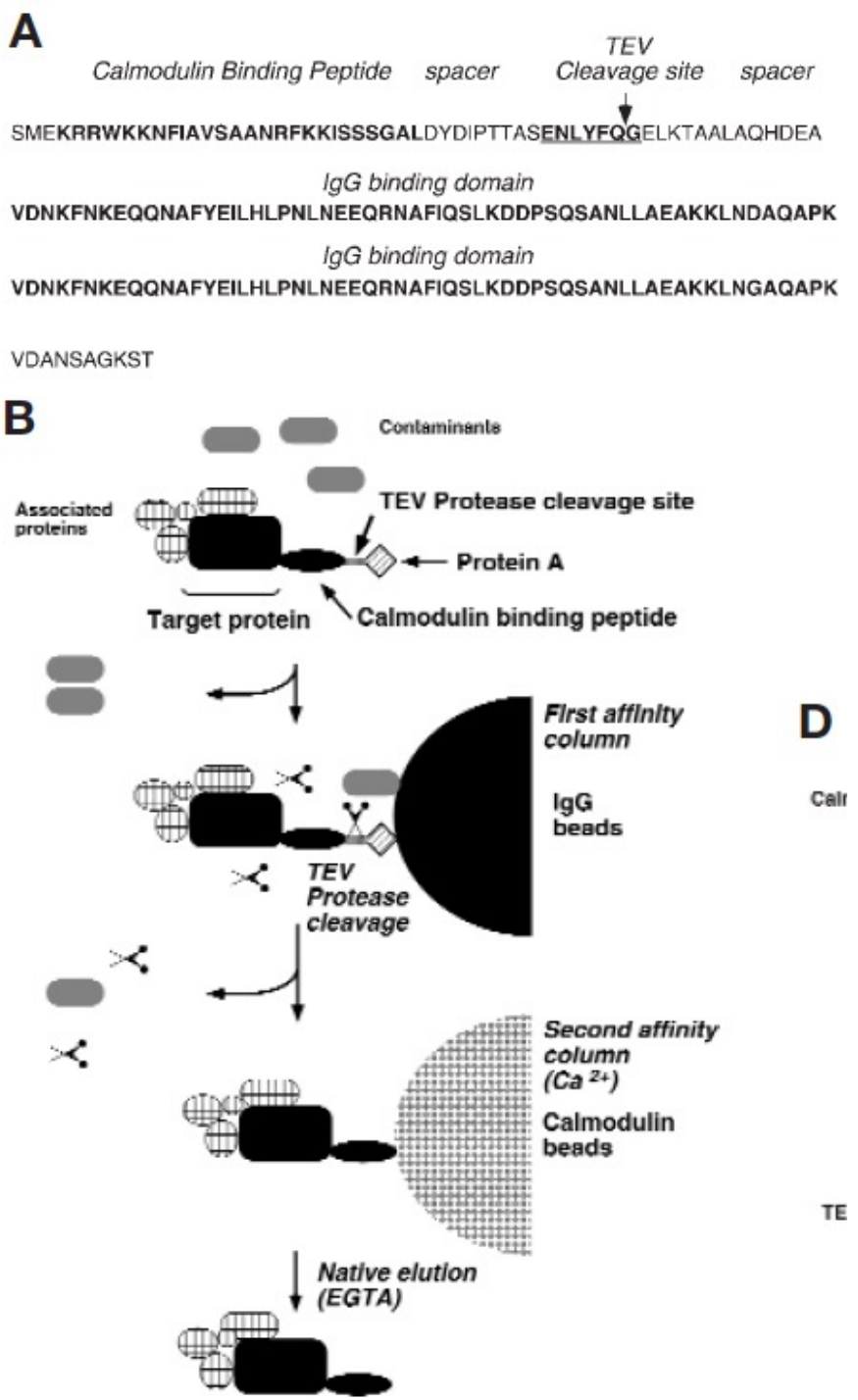
Double tag based on a region of protein A (binds IgG) and a calmodulin-binding peptide separated by linker with a TEV protease cleavage site

Chromatografy on

1. IgG
2. calmodulin (+ Ca^{2+})

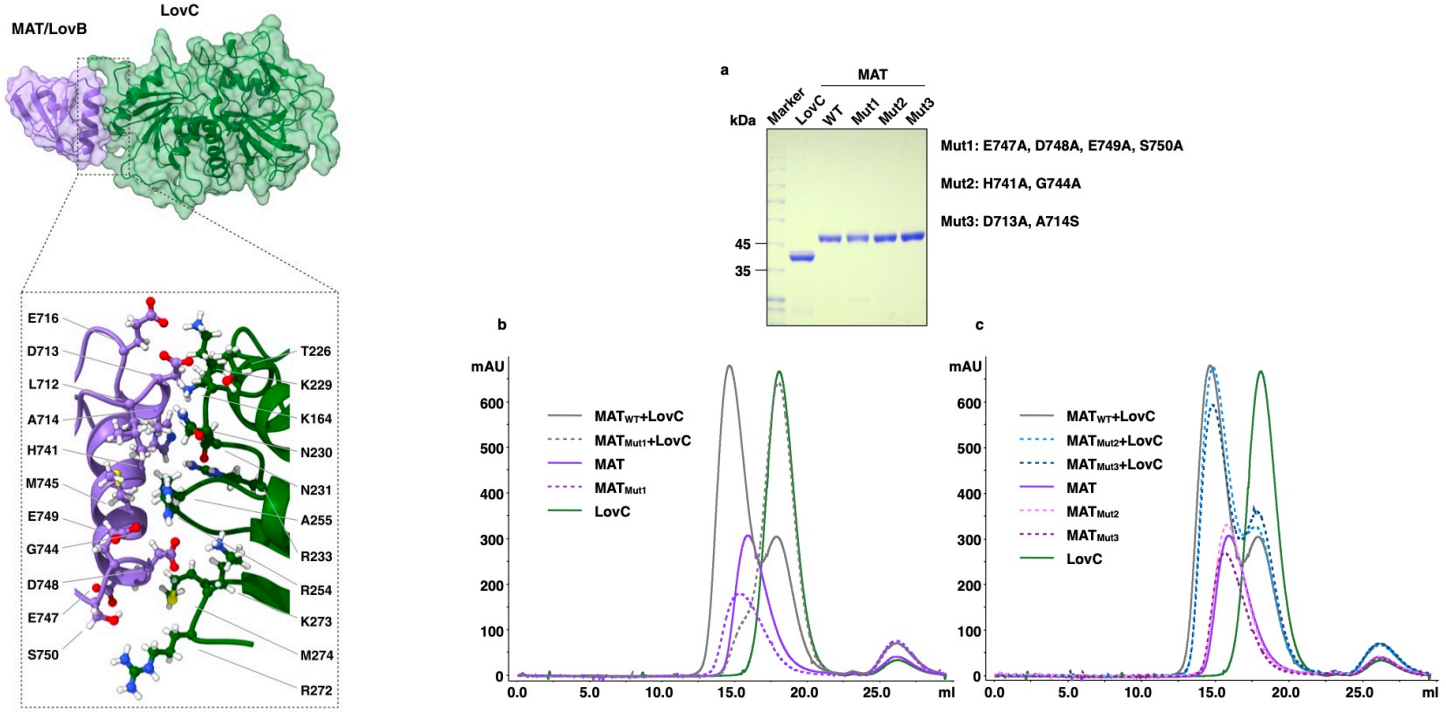
Elution

1. TEV cleavage
2. EGTA (Ca^{2+} chelator)



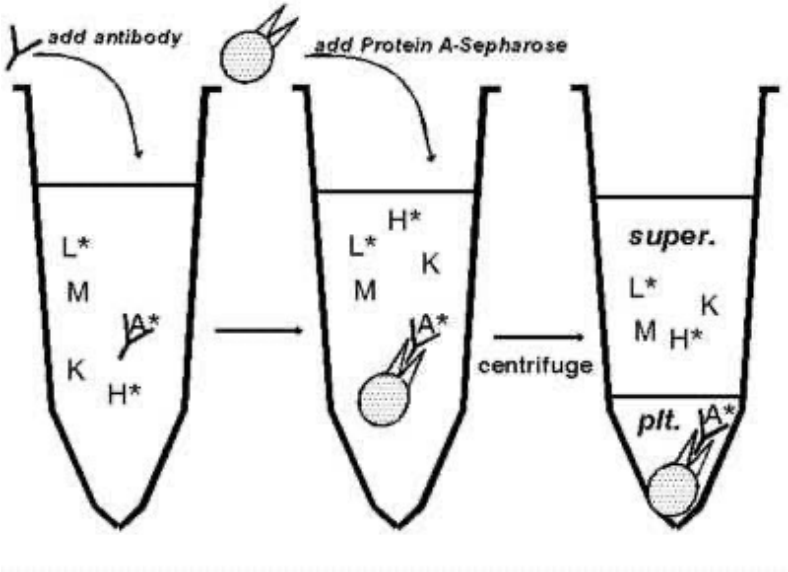
Size-exclusion chromatography (SEC)

- Requires purified proteins and allows analysis of mutants
- The molecular weight difference between isolated partners and the complex must be sufficiently large
- The complex must be sufficiently stable and it must not dissociate during chromatography



IMMUNOPRECIPITATION

It may be used to purify antigens



- An antibody (monoclonal or polyclonal) specific for the bait protein is added to the sample
- Immobilized protein A (a bacterial protein that binds the constant region of IgG) is added
- The immunocomplex is 'precipitated' by centrifugation
- Proteins not captured by the protein A matrix are washed away
- Bound proteins are eluted at low pH

CO-IMMUNOPRECIPITATION

The immunoprecipitation may 'co-precipitate' macromolecules that interact with the antigen

Evaluation of pull-down and co-immunoprecipitation

Components are eluted and analized by SDS-PAGE followed by mass spectrometry or Western blot to verify the identity of the captured proteins

It is necessary

- To verify that the 'prey' protein does not interact with the resin or the antibody in the absence of the 'bait' protein
- To determine if the interaction is direct or indirect
- To determine that the interaction is physiological and it is not a consequence of cell lysis

Affinity blot Overlay or Far Western blotting

The method requires:

- Separation of proteins by SDS-PAGE
- Blotting on nitrocellulose or PVDF
- Incubation with the probe ('bait' protein).

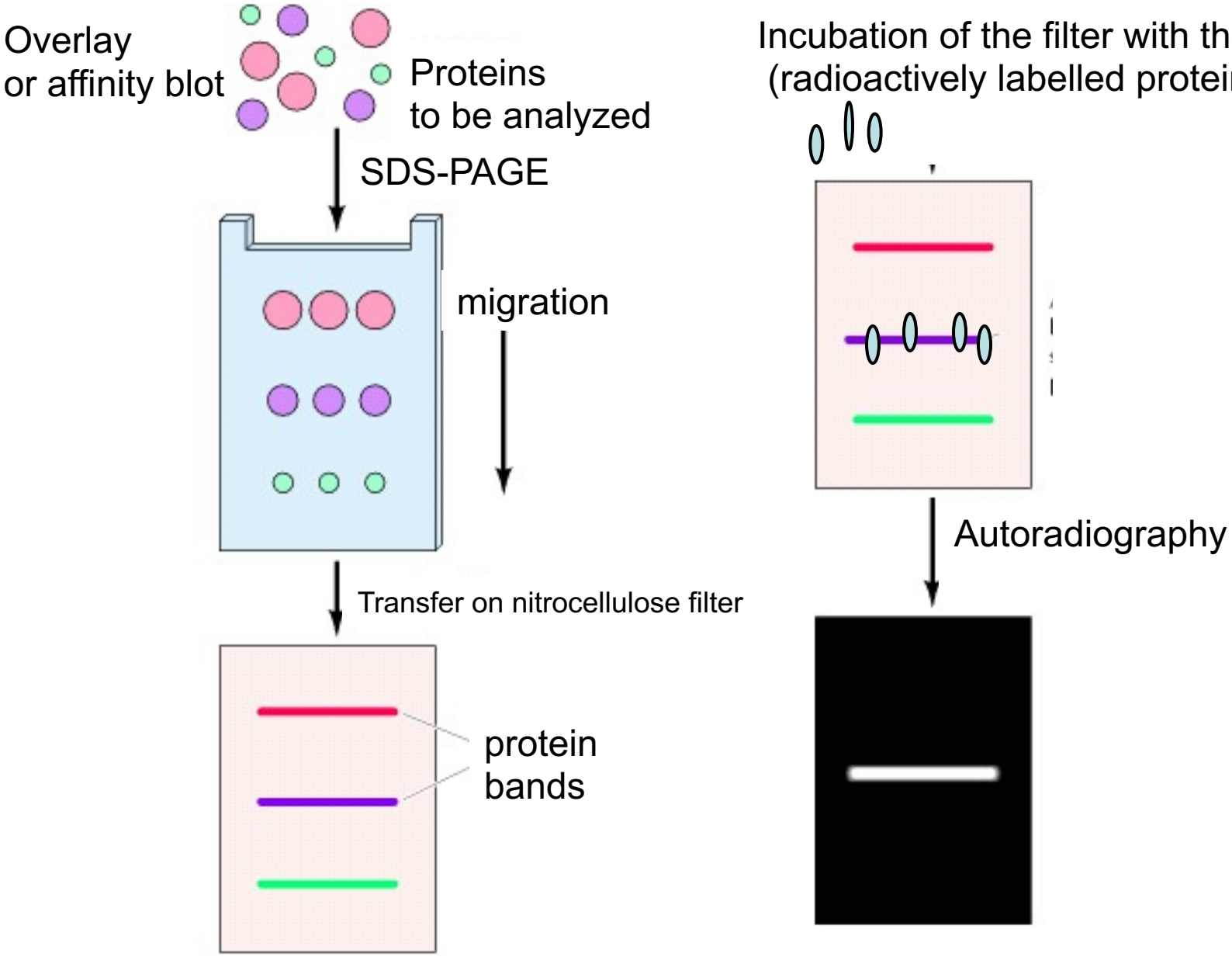
To visualize the 'bait' protein:

- Radioactive labelling
- Biotin labelling
- Antibody-mediated detection

Fusion tagged proteins are often used because antibodies against the TAG are available

Limits:

SDS-PAGE is performed in reducing and denaturing conditions and this may cause loss of protein-protein interactions → renaturation protocols


Overlay
or affinity blot
Proteins
to be analyzed
SDS-PAGE
migration
Transfer on nitrocellulose filter
protein
bands
Incubation of the filter with the probe
(radioactively labelled protein)
Autoradiography

Cross-linkers

- Cross-linkers allow to covalenty bind two proteins that interact
- Cross-linkers are molecules that contain two reactive groups separated by a spacer arm

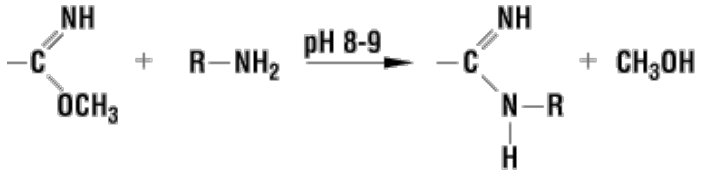
Protein groups that are able to react with cross-linkers: amino- carboxyl- sulphydryl- groups, carbohydrates (carbonyl)

Cross-linker reactive groups: mostly the same used for immobilization

Cross-linker reactivity. Amino groups

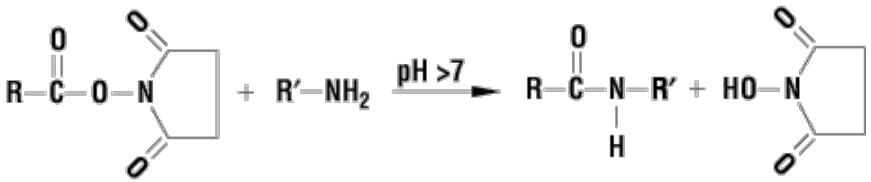
Imidoesters

Unstable at neutral pH



N-hydroxy-succinimide esters

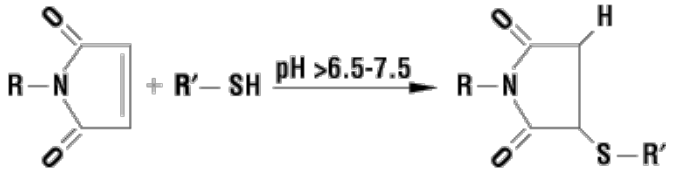
Efficient at neutral pH



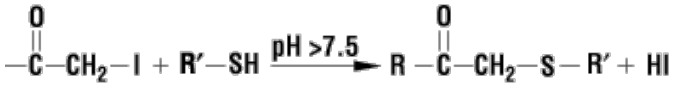
Cross-linker reactivity. Sulfhydryl groups

Maleimides

Form stable thioether bonds at neutral pH

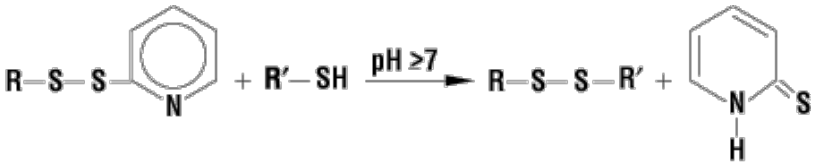


Alkyl halides



Pyridyl-disulfides

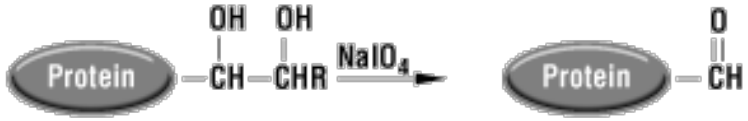
Form disulfide bridges. 2-pyridyl-thione absorbs at 343 nm.



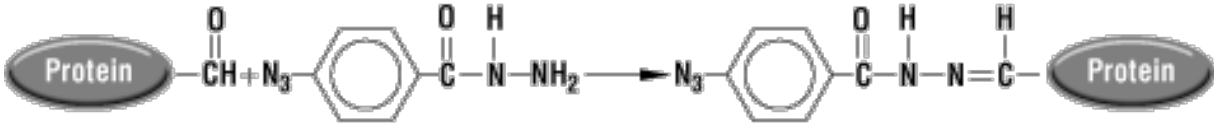
Cross-linker reactivity. Carbonyl groups

Hydrazides

Reactive with carbonyl groups derived from oxidation of carbohydrates



The oxidation of a Protein Carbohydrate (*cis*-diol) to an aldehyde.

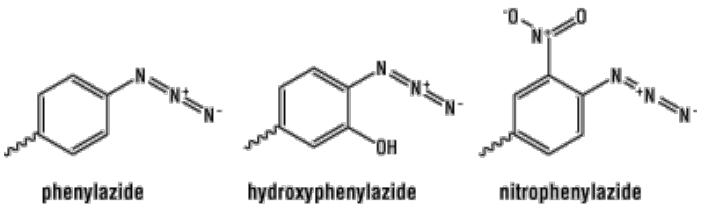


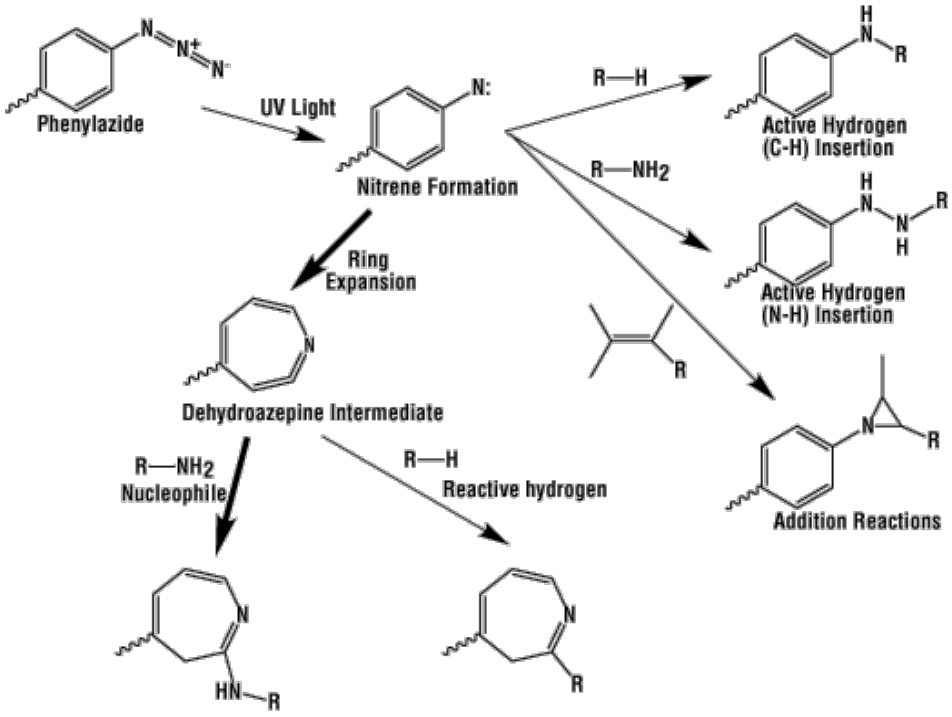
ABH, or Azidobenzoyl Hydrazide, reacts with the aldehyde on the protein to form an arylazide activated protein.

Cross-linker reactivity. Photoreactive cross-linkers

Aryl-azides

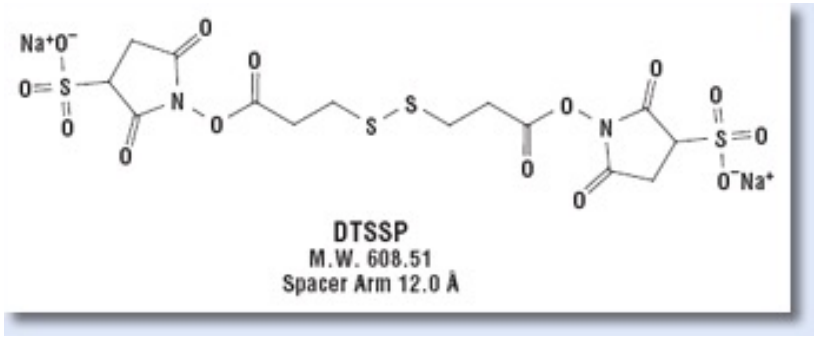
are chemically inert and activated by UV light (250 nm), the reactive nitrene group reacts with double bonds, C-H and N-H bonds





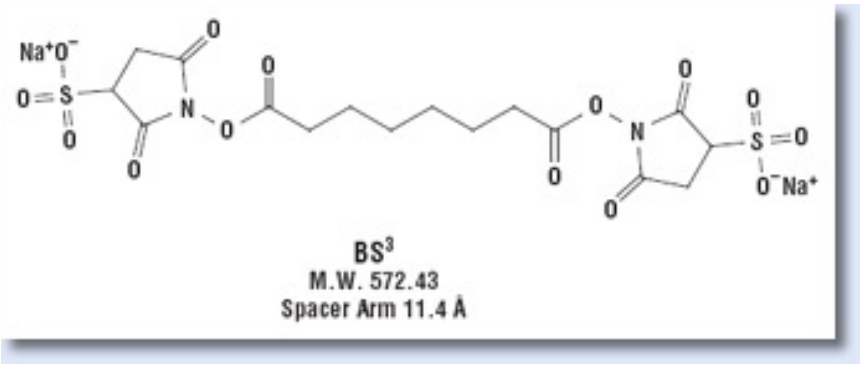
Homobifunctional cross-linkers

DTSSP 3,3´-Dithiobis(sulfosuccinimidylpropionate)



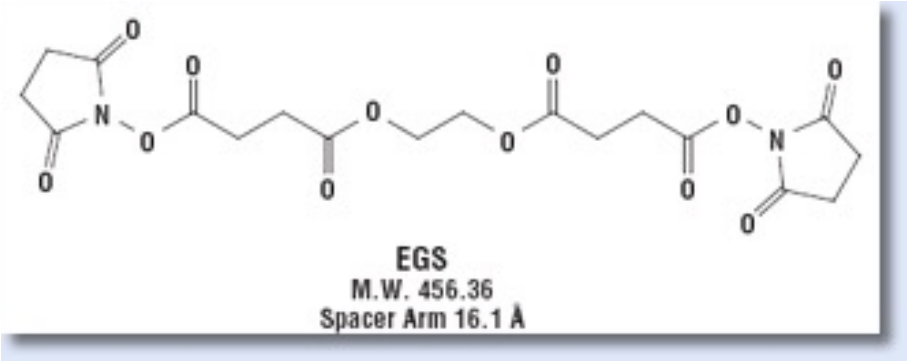
Cross-link is reversible with DTT

BS^3 Bis(Sulfosuccinimidyl)suberate



Cross-link is irreversible

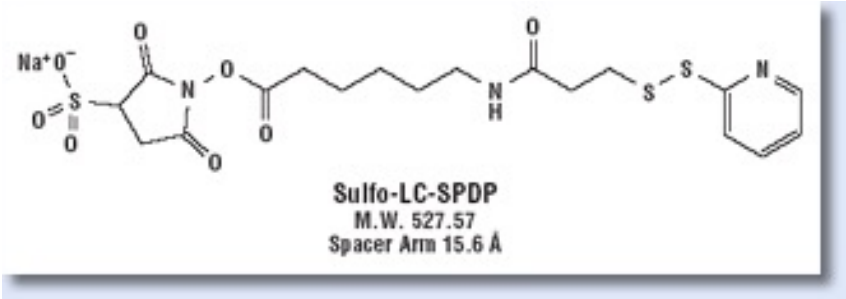
EGS (Ethylene glycol bis[succinimidylsuccinate])



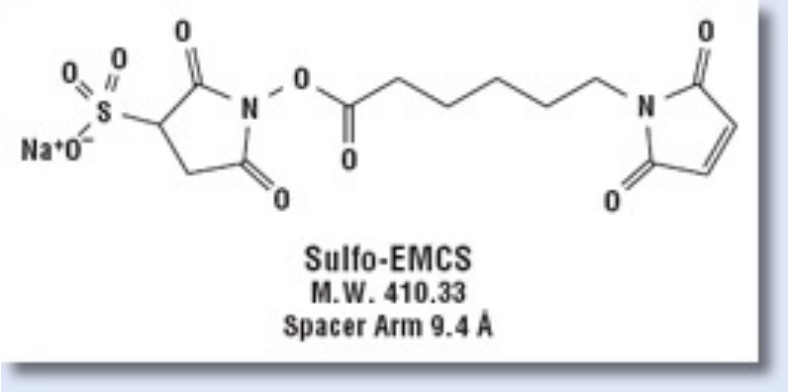
Cross-link is reversible at pH 8.5 with hydroxylamine

Heterobifunctional cross-linkers

Sulfo-LC-SPDP (Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate)



Sulfo-EMCS ([N-e-Maleimidocaproyloxy] sulfosuccinimide ester)

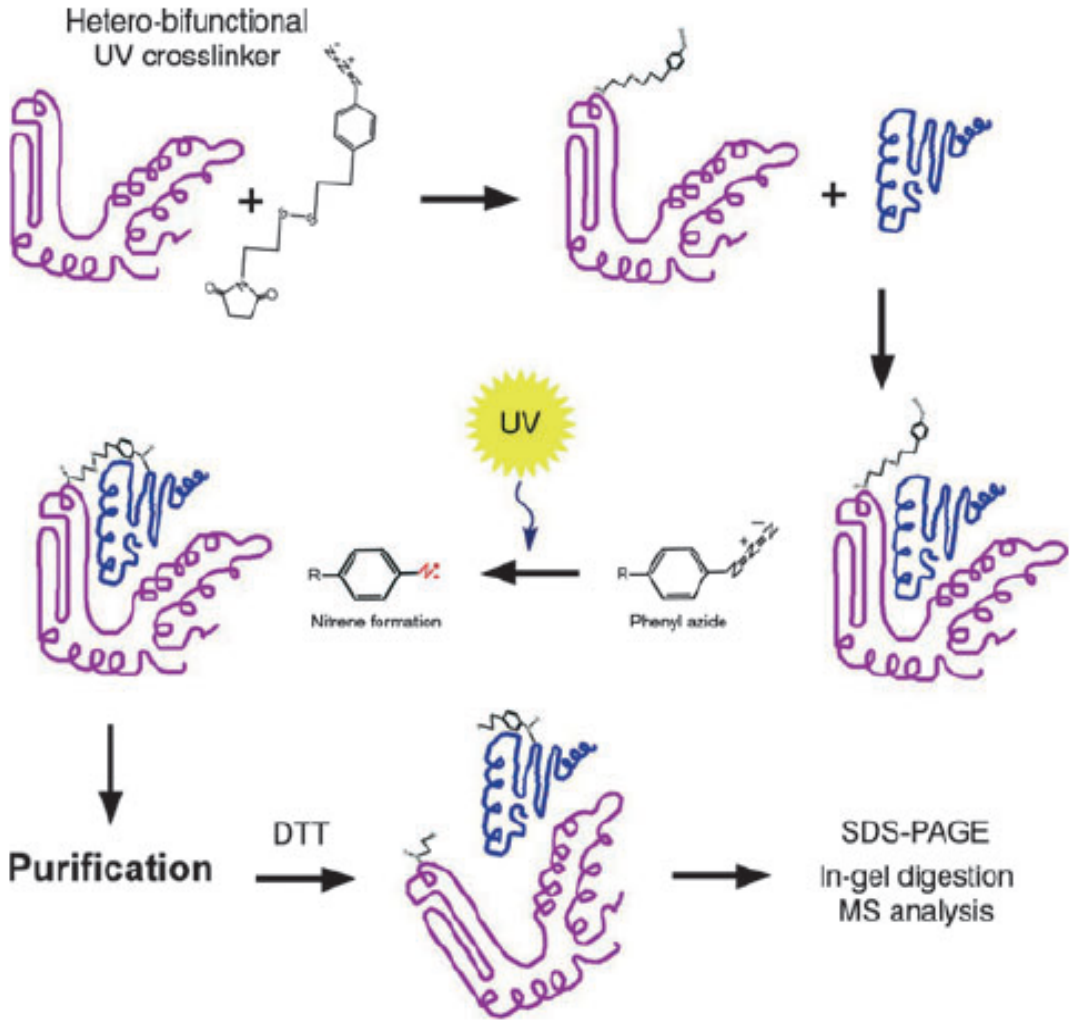


How to choose a cross-linker?

- Chemical specificity
- Spacer arm length
- Solubility in water and membrane permeability
- Reactive groups (homobifunctional or heterobifunctional)
- Reversible or irreversible
- Possibility of two-step cross-link

Cross-linkers for characterization of the molecular architecture of a complex

- Purified protein complex (not strictly necessary)
- Two-step cross-linking
- Proteolysis
- Separation and identification of modified peptides, by mass spectrometry (LC-MS)



The transcriptional repressor Fep1 forms homodimers: pull-down

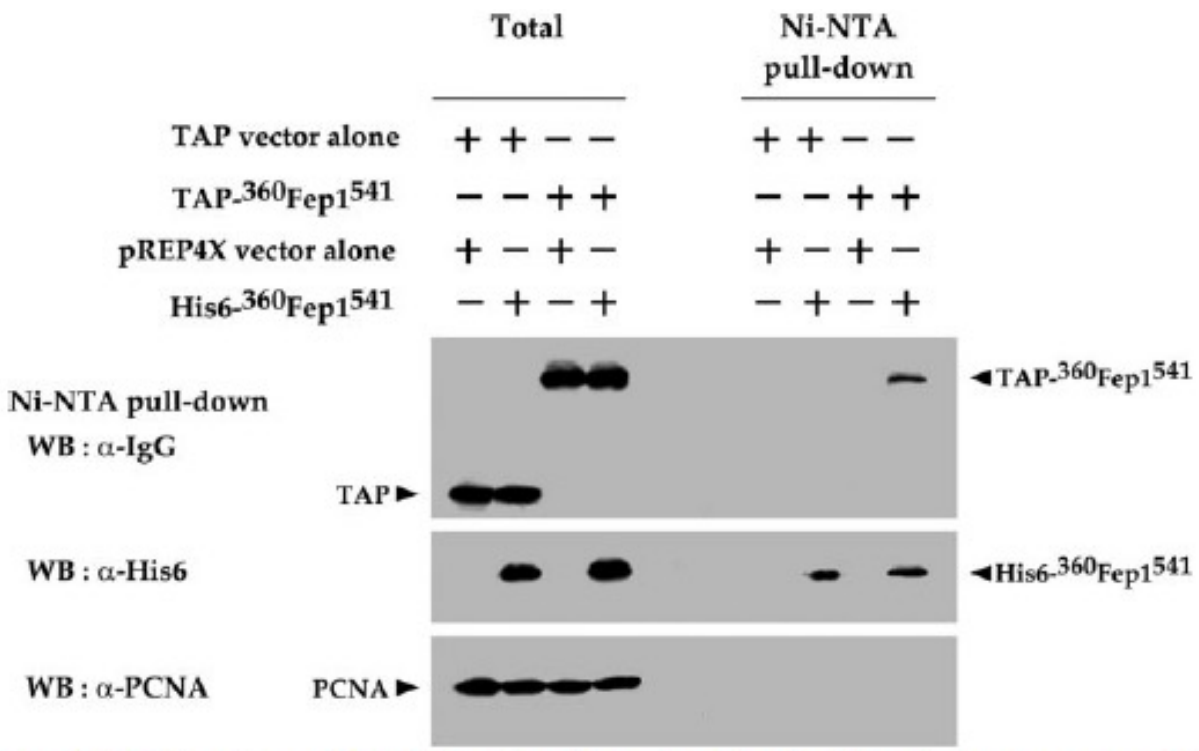
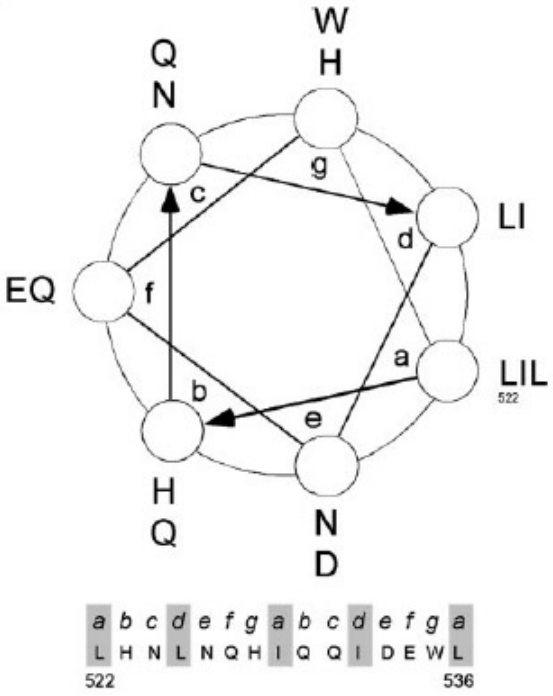


FIG. 8. **Self-association of the C terminus of Fep1 in native *S. pombe* extracts.** Cell extracts were prepared from *fep1*Δ mutant cells coexpressing the *TAP-*^{360}*fep1*$^{+541}$ and *His6-*^{360}*fep1*$^{+541}$ alleles. The cell extracts were incubated with a Ni^{2+} affinity resin and washed, and the bound fraction was eluted with 150 mM imidazole (nickel-nitrilotriacetic acid pull-down). A portion (~2%) of the total cell extract was also included to monitor the presence of the proteins prior to chromatography (*Total*). All samples were subjected to immunoblotting with the indicated antibodies. *WB*, Western blot.

The transcriptional repressor Fep1 forms homodimers: cross-link



An amphipathic helix in position 522-536 is responsible for dimerization of Fep1

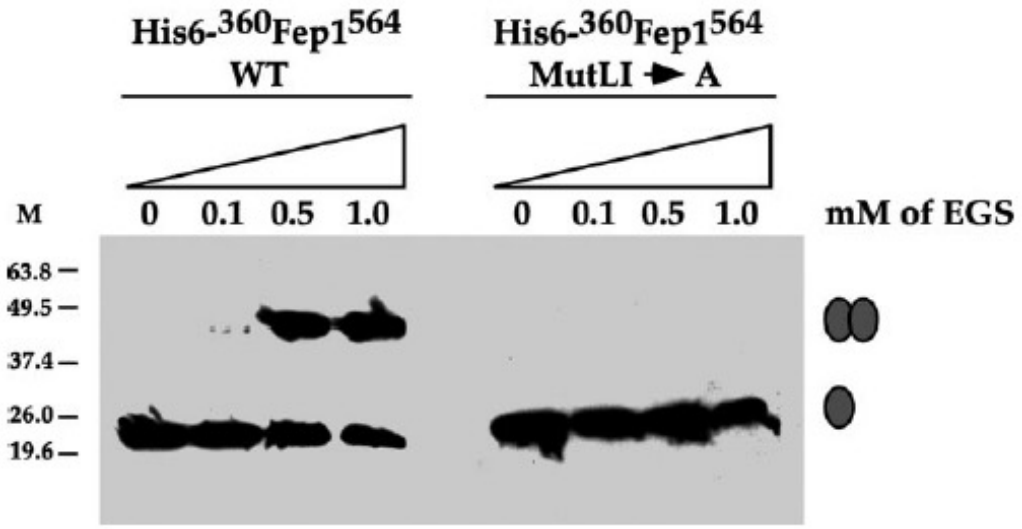


FIG. 10. **C terminus of Fep1 assembles as a dimer.** Purified His6-^{360}Fep1^{564} or His6-^{360}Fep1^{564} Mut LI → A was incubated with 0, 0.1, 0.5, and 1.0 mM EGS for 30 min at room temperature. The EGS-cross-linked complexes were analyzed by SDS-polyacrylamide gel electrophoresis and subjected to Western blotting using anti-His monoclonal antibody. Monomeric (~22-kDa, *1 oval*) and dimeric (~44-kDa, *2 ovals*) forms were detected. *M*, reference marker.