# 5.36 Biochemistry Laboratory
Spring 2009

# Affinity Tags for Protein Purification

I. Overview of Protein Expression
   and General Strategies for Protein Purification

II. Affinity tags for Protein Purification

   A. Characteristics of tags

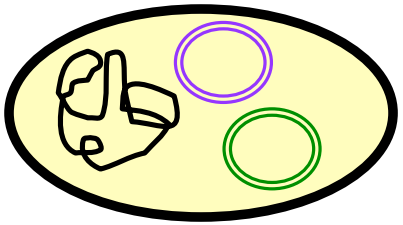
   B. Common affinity tags

   - GST-tag
   - FLAG-tag
   - His-tag
   -

   C. Cleavage of affinity tags

III. SDS gel analysis of purified proteins

Our progress so far for H396P Abl(229-511) expression and isolation:



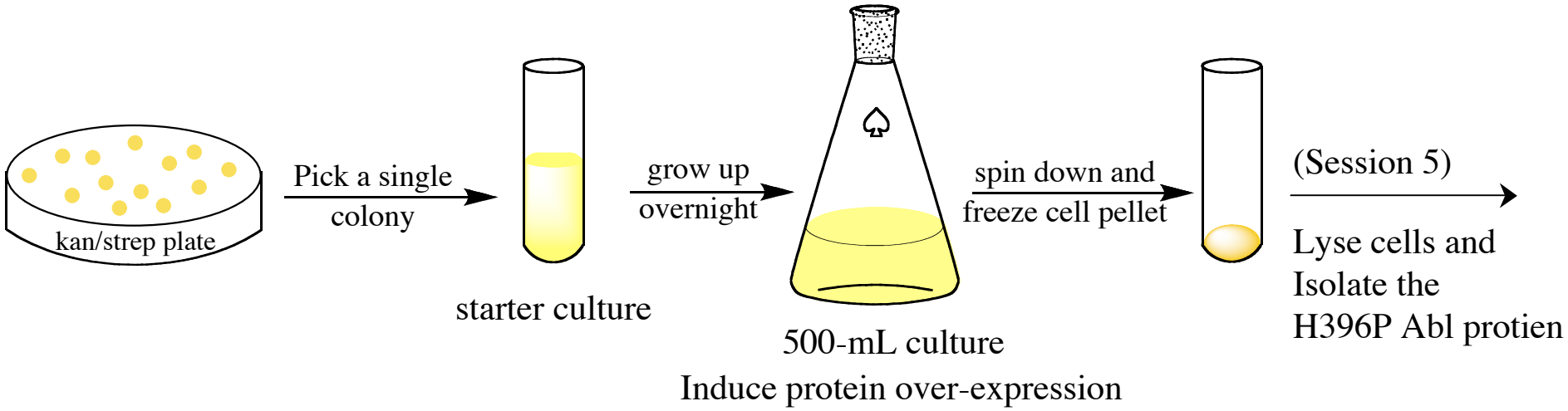
BL21(DE3) cell

Prior to Session 2, BL21(DE3) expression cells co-transformed with

1) an H396P Abl-encoding kan -resistant vector and

2) a YopH Tyr phosphatase-encoding strep -resistant vector

were spread onto a LB-agar plate with antibiotics for colony selection.



# Successful Abl kinase domain expression in bacteria requires co-expression with a phosphatase!

**High yield bacterial expression of active c-Abl and c-Src tyrosine kinases**

Markus A. Seeliger 2 1, Matthew Young 2 1, M. Nidanie Henderson 2 1, Patricia Pellicena 2 1, David S. King 1, Arnold M. Falick 1, John Kuriyan 3 2 1 *

Received: 3 August 2005; Revised: 2 September 2005; Accepted: **4 September 2005**

Seeliger, M.A. et al. *Protein Sci.* **14**, 3135-3139 (2005)

# Successful Abl kinase domain expression in bacteria requires co-expression with a phosphatase!

**High yield bacterial expression of active c-Abl and c-Src tyrosine kinases**

Markus A. Seeliger 2 1, Matthew Young 2 1, M. Nidanie Henderson 2 1, Patricia Pellicena 2 1, David S. King 1, Arnold M. Falick 1, John Kuriyan 3 2 1 *

Received: 3 August 2005; Revised: 2 September 2005; Accepted: **4 September 2005**

**Prior to 2005, expression of active Abl kinase domain was carried out**

- in insect cells. Insect cells yield milligram quantities of protein, but are time consuming and expensive to maintain.
- in bacteria with very low yields (micrograms). While biochemical studies can be carried out with tiny amounts of protein, milligram quantities are required for biophysical and structural studies, such a crystallography and NMR.

# Successful Abl kinase domain expression in bacteria requires co-expression with a phosphatase!

**High yield bacterial expression of active c-Abl and c-Src tyrosine kinases**

Markus A. Seeliger 2 1, Matthew Young 2 1, M. Nidanie Henderson 2 1, Patricia Pellicena 2 1, David S. King 1, Arnold M. Falick 1, John Kuriyan 3 2 1 *

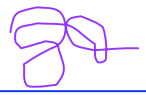
Received: 3 August 2005; Revised: 2 September 2005; Accepted: **4 September 2005**

**Seeliger and co-workers hypothesized that the low yields of soluble Abl in E. coli were due to the toxicity of Tyr kinase activity in bacteria.**

- Phosphatase co-expression prevents high levels of toxic kinase activity.
- YopH is a non-selective Tyr phosphatase, meaning it dephosphorylates most phosphotyrosine (pY) substrates, regardless of the specific sequence.
- Yields of purified Abl K domain protein using the co-expression method in BL21-DE3 cells range from 5 to 15 mg/L.

In Session 5 you will lyse (split open) your BL21-DE3 cells and isolate the H396P Abl protein.

Crude cell lysate has many components. Your mixture will include



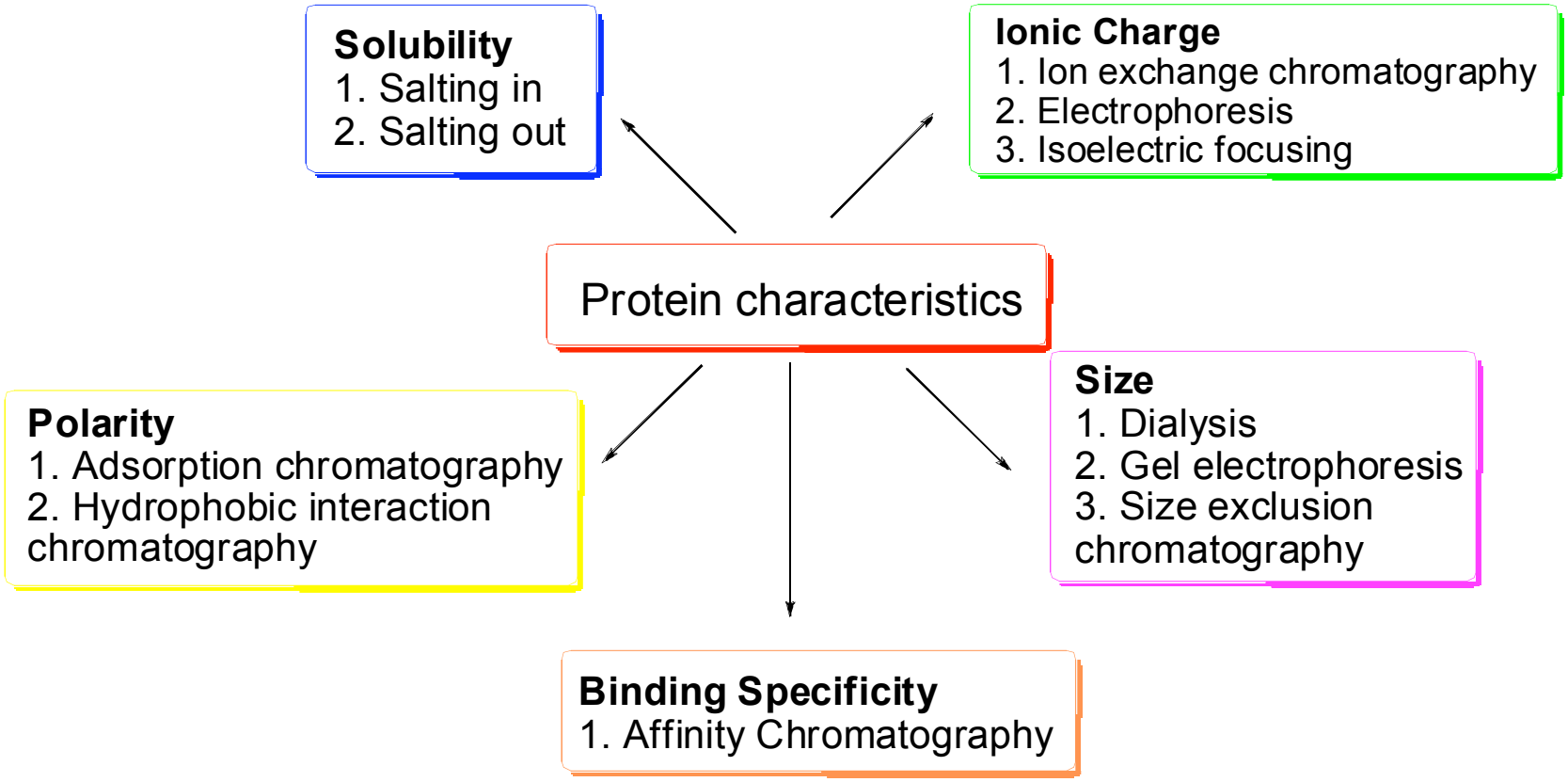
- Overexpressed His-tagged H396Abl-kinase domain ✻

- ~~Overexpressed Yop phosphatase~~ **X**
- ~~E. Coli proteins, DNA, and metabolites~~ **X**

✻ what we want

**X** what we don't want

# Strategies for protein purification



# Affinity Tags for Protein Purification

I. Overview of Protein Expression
   and General Strategies for Protein Purification

**II. Affinity tags for Protein Purification**

   A. Characteristics of tags

   B. Common affinity tags
      - GST-tag
      - FLAG-tag
      - His-tag

   C. Cleavage of affinity tags

III. SDS gel analysis of purified proteins

# Affinity Chromatography

Chromatography involves a mobile phase (ie. cell lysate with overexpressed protein) and a stationary phase (the column with ligand-bound beads)

Three steps in column chromatography:

1) Binding the desired protein to the affinity column
2) Washing away unwanted proteins, DNA etc.
3) Eluting the desired protein

# Affinity Chromatography

Examples of protein/**ligand** pairs

- Avidin/ or streptavidin **--- biotin**
- Glutathione-S-transferase **--- glutathione**
- Dihydrofolate reductase **--- methotrexate**

However,

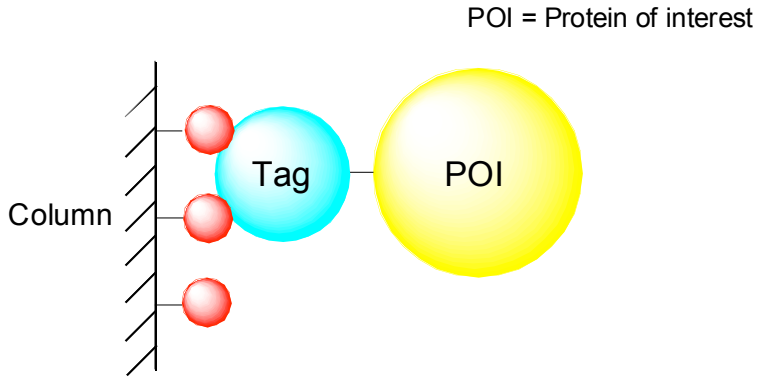
- Not all proteins have known binding partners
- Known binding interactions might not be sufficiently strong to withstand washing steps.
- Raising antibodies for all proteins?—no way.

**Is there a general purification strategy that lends itself to a diverse set of proteins?**

# Affinity Tags

In **affinity tag purification**, the protein of interest (POI) is expressed with a polypeptide affinity "tag" on the N- or C-terminus of the protein.

Purification is based on the highly specific interaction between the polypeptide affinity tag and a binding counterpart that is immobilized on solid support




Tags are introduced at the DNA level, either supplied by the expression vector or using primers.

# Summary of sequence, size, matrices, and elution conditions of commonly-used affinity tags

Figure removed due to copyright restrictions. See tables 1 and 2 from Terpe, K. "Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems." *Appl Microbio Biotech* 60 (2003): 523-533.

Terpe, K. *Appl. Microbio. Biotech.* 2003, 60, 523-533

## An ideal affinity tag:

- requires a simple purification process that is free of harsh reagents.

- does not affect (or only minimally affects) the tertiary structure and biological activity of the POI.

- can be easily and specifically removed from the POI following purification to produce native protein.

- can be used with diverse types of proteins.

Some tags confer additional benefits, such as increasing protein solubility and increasing the yield of recombinant proteins.

# Small tags versus large tags:

## Small peptide tags

- Examples: poly-Arg, FLAG-, poly-His, Strep
- Advantages:
  - Minimal interference with the fused protein
  - Depending on the location and amino acid composition of the tag, removal of the tag may not be required
  - Not as immonogenic as large tags—recombinant proteins can be used directly as an antigen in antibody production

## Large peptide tags

- Examples: GST, MBP
- Can increase solubility increase solubility and expression of the POI
- In most cases, large tags need to be removed after purification.

# Affinity Tags for Protein Purification

I. Overview of Protein Expression

II. General Strategies for Protein Purification

III. Affinity tags for Protein Purification

A. Characteristics, types (maybe not own A)

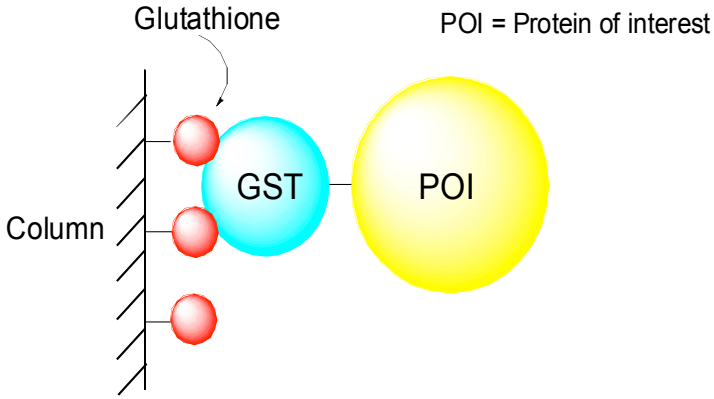
B. Common affinity tags
- GST-tag
- FLAG-tag
- His-tag

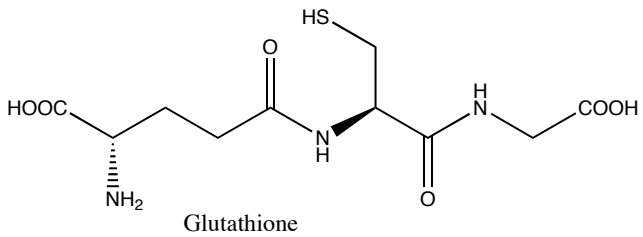
C. Cleavage of affinity tags

IV. SDS gel analysis of purified proteins

# Glutathione S-transferase-tag (GST-tag)

- A 26-kDa (_220_ aa) protein that binds to glutathione (a tripeptide).
- GST-fusion proteins can be purified from crude lysate by affinity chromatography on immobilized glutathione.

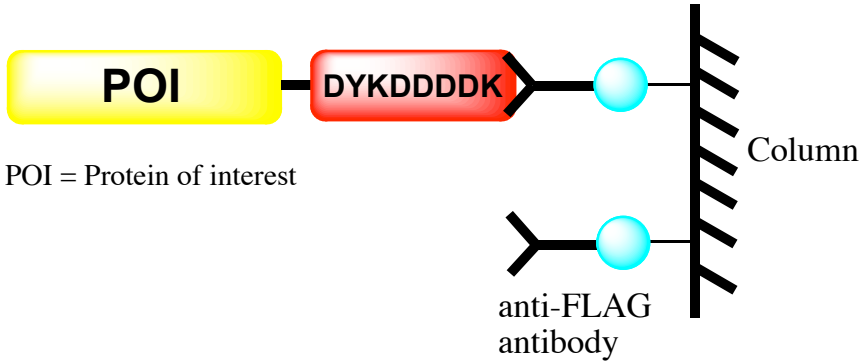



- Bound fusion proteins can be eluted with 10 mM reduced glutathione under non-denaturing conditions.
- Advantages: The tag can help to protect against intracellular protease cleavage and stabilize the recombinant protein.
- Due to its large size, the GST-tag should be cleaved from the fusion protein after purification.

# FLAG-tag

- A short hydrophilic octapeptide, DYKDDDDK
- The FLAG-tag binds to a monoclonal antibody purification matrix (typically anti-FLAG antibody bound to sepharose resin.)



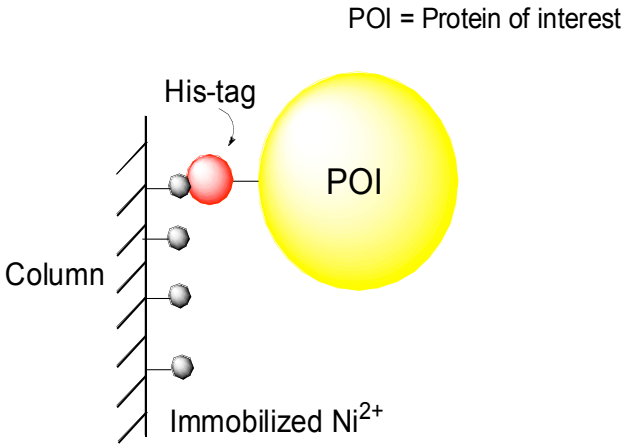

- The tagged protein is commonly dissociated by adding by adding competing FLAG peptide, or by transiently reducing the pH (to ~3.5).

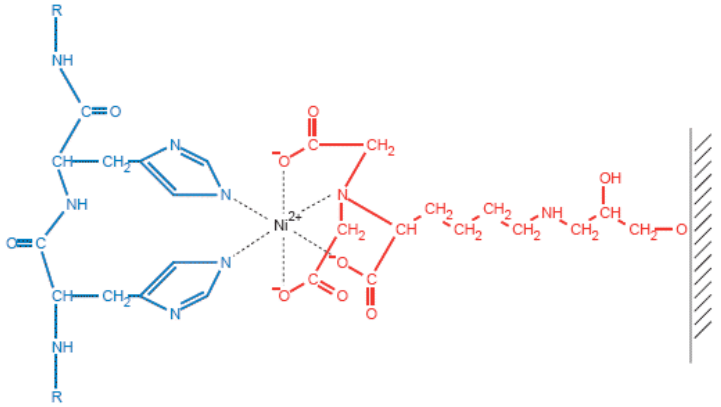
- Advantages: The FLAG tag/antibody interaction is highly specific resulting if highly pure proteins. The tag can be appended to the N or C terminus.

- Diadvantage: cost!

# Polyhistidine tag (His-tag): Binding

- A short polyhistidine peptide (from 4-10 residues; normally 6 )
- His is the amino acid that binds most strongly to metal ions
  - ($Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$)—use column with immobilized metal ions.
  - Common immobilizer is NTA (nitriloacetic acid)



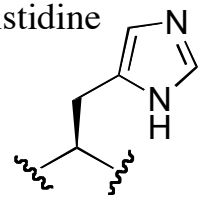
# His-tag: Washing and Eluting

Washing

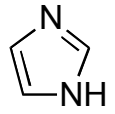
The washing buffer typically contains 10 to 30 mM imidazole.

The imidazole competes with His residues for metal chelation.

Histidine




Imidazole




Elution. There are three options:

- Reduce pH to 4.5-5.3

  Protein can be damaged by reduction in pH
- Use metal chelators (e.g. EDTA)

  Recycling of Ni-NTA resin is much harder when the Ni is chelated

- 100-250 mM imidazole ***

  Mildest conditions. Imidazole can be subsequently removed by dialysis.

## Considerations in using His-tags

- Ni-NTA resin is damaged by high concentrations of strong reducing agents.
- In certain cell systems (e.g. insect), acidic media is required, which can prevent His from binding to Ni-NTA
- Certain proteins have native polyHis patches.

## Advantages to using His-tags.

- Ni-NTA resin is FAR less expensive than FLAG-resin or other anti-body based resins.
- His tags can be added to the N- or C-terminus of a protein and often do not need to be removed after purification.

# The H396P Abl(229-511) protein has an N-terminal hexahistidine ($His_6$) tag


- The tag was introduced by the pET-28a vector (see map).
- The Yop phosphatase is NOT His-tagged.

You will use affinity tag purification to isolate the H396P Abl kinase domain.

Studies have shown that an N-terminal His tag does not *significantly* affect Abl kinase domain activity, so we will not remove the tag.

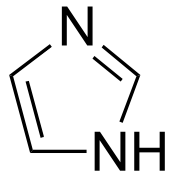
# Dialysis of the purified protein

Long-term storage of certain proteins in high-concentration imidazole buffers can lead to degradation.

Consider the sizes (molecular weights) of the components in you elutions:

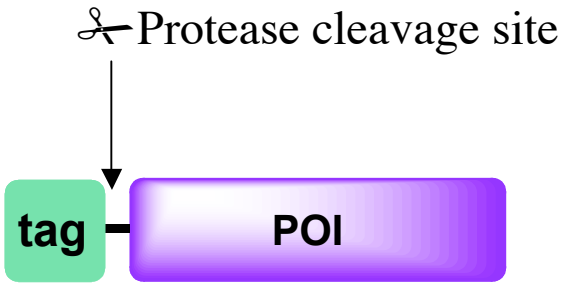
**32 kDa**


**68 Da**




Therefore we'll use a 10 kDa MWCO (molecular weight cut off) dialysis device.

# Cleavage of Affinity Tags




Site specific proteases can be used for tag cleavage.
Commonly used proteases with cleavage sites indicated:

Enterokinase: DDDDK✂X

TEV protease: ENLYFQ✂S(or C).

Thrombin: X4-X3-P-R ✂X1'-X2', where X4 and X3 are hydrophobic aa's and X1', X2' are non-acidic aa's

Factor Xa: I(or E)-DGR✂X

# Affinity Tags for Protein Purification

I. Overview of Protein Expression
   and General Strategies for Protein Purification

II. Affinity tags for Protein Purification

   A. Characteristics of tags

   B. Common affinity tags
      - GST-tag
      - FLAG-tag
      - His-tag

   C. Cleavage of affinity tags

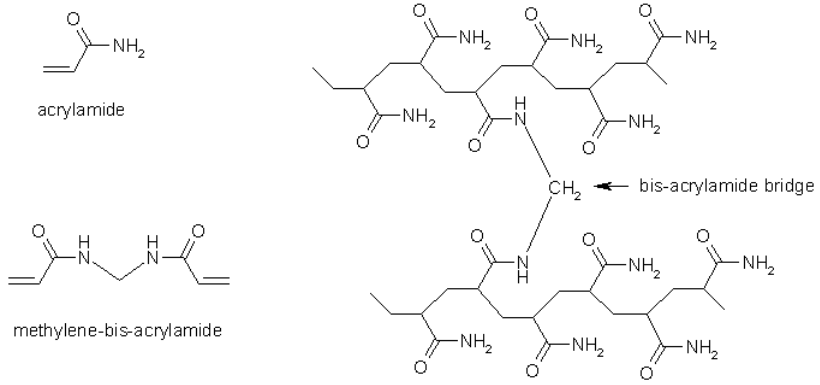
**III. SDS gel analysis of purified proteins**

# SDS-PAGE

- **SDS**: Sodium dodecyl sulfate: a detergent that binds strongly and confers negative charges to proteins

$$H_3C—(CH_2)_{10}CH_2{\cdot}OSO_3^-Na^+$$

- **PA**: Polyacrylamide




- **GE**: Gel Electrophoresis

  Separation (by molecular mass) using a current applied to gel matrix

# Stacking gel vs. Resolving gel

Difference in pore size as a result from different % acrylamide added

**Stacking gel has larger pore size**

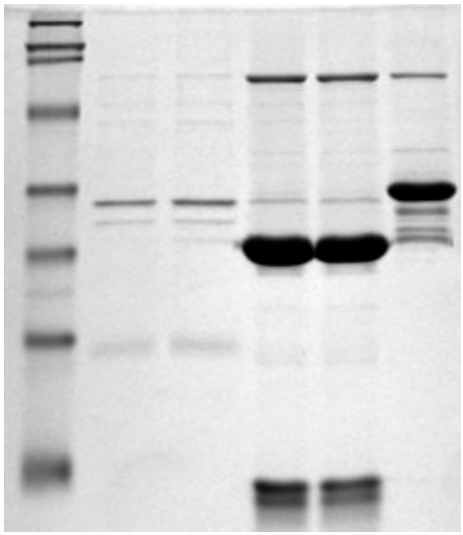
- Proteins are not yet separated by mass. Will be concentrated and thinned.

**Resolving gel allows separation according to size**

- Difference in % acrylamide can be used to separate different ranges of proteins
- 8%-20% gel
- Higher % acrylamide ⇒ _smaller_ pores ⇒suitable for smaller proteins

# Electrophoresis

- Negatively-charged proteins move towards the positive end
- Smaller proteins run faster
- Estimation of size based on the molecular weight markers



- After electrophoresis, the gel can be stained (e.g. by Coomassie stain) or further processed (e.g.Western blot)

Session 4 prepare purification and gel analysis buffers

Session 5 Lyse your cells and isolate the Abl protein. Dialyze.

Session 6, 7, and 8

Run a gel to visualize your protein fractions.

Concentrate your purified and dialyzed protein.

Quantify protein concentration.