5.36 Biochemistry Laboratory
Spring 2009

5.36 Lecture Summary #3 Thursday, February 12, 2009

CI-M workshop on Feb. 19th: Bring two hard copies of your mini-review outline and an annotated bibliography w/ at least 3 primary research reports (from 2005 or later).

HHMI Summer Research Fellowship in Chemical Biology: http://web.mit.edu/cld/hhmi/

Next Laboratory Session: #4

Topics: **Affinity Tags for Protein Purification**

I. Overview of protein expression and general protein purification strategies

II. Affinity tags for protein isolation
- **A.** Characteristics of tags
- **B.** Common affinity tags (GST, FLAG, and His)
- **C.** Cleavage of affinity tags

III. SDS gel analysis of purified proteins

Please note that we will complete our discussion of Quickchange DNA mutagenesis (continued from Lecture #2 notes) in our March 5th lecture, prior to lab sessions #9-11.

I. PROTEIN EXPRESSION AND GENERAL PURIFICATION STRATEGIES

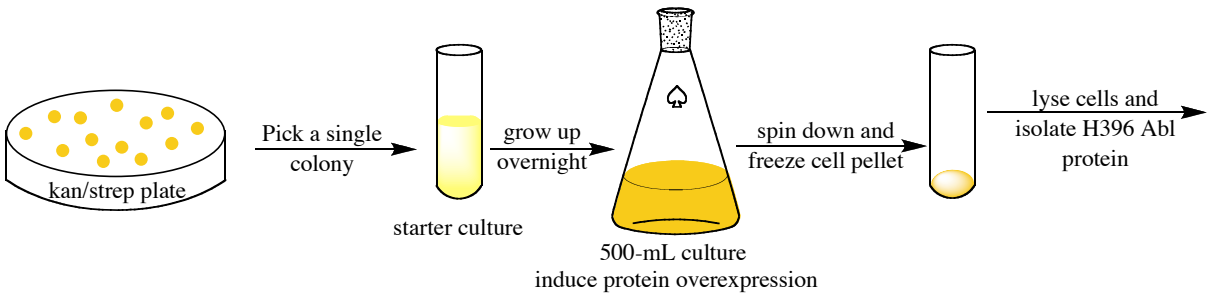
Consider our progress so far for H396P Abl(229-511) expression and isolation:

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

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

2) a YopH Tyr _____-encoding _____-resistant vector

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



Successful Abl kinase domain expression in _____ requires co-expression with a phosphatase! (Seeliger, M.A. et al. *Protein Sci.* 14, 3135-3139 (2005))

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

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

The low yields of soluble Abl in E. coli may be due to the _____ 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 (____) substrates, regardless of the specific sequence.
- Yields of purified Abl K domain protein using the co-expression method in BL21-DE3 cells range from ___ to ____ mg/L.

PROTEIN PURIFICATION

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 lysate mixture will include

- Overexpressed _____-tagged H396Abl-kinase domain
- Overexpressed Yop phosphatase
- E. Coli proteins, DNA, and metabolites

(∗ = what we want, × = what we don't want)

Strategies for protein purification rely on exploiting unique protein characteristics

- **Solubility** (1) Salting in. (2) Salting out
  By changing salt concentrations, you solubilize or precipitate out protein.
- **Ionic charge** (1) Ion exchange chromatography. (2)Electrophoresis (3) Isoelectric focusing
  The isoelectric point (pI) of a protein is the ____ at which the overall charge of a protein is _____. When pH > pI, the protein is _____ -charged. A negatively-charged protein will bind to a positively-charged column (anion exchange chromatography).
- **Polarity** (1) Adsorption chromatography. (2) Hydrophobic-interaction chromatorgraphy
- **Size** (1) Dialysis. (2) Gel electrophoresis. (3) Size exclusion chromatography.
- **Binding specificity**. Affinity chromatography

II. AFFINITY TAGS FOR PROTEIN ISOLATION

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

Three steps in column chromatography:

1) _____ the desired protein to the affinity column.

2) _____ away unwanted proteins, DNA etc.

3) _____ the desired protein.

Examples of protein / ligand pairs include:

Avidin/ or streptavidin ---- biotin

Glutathione-S-transferase ---- _____

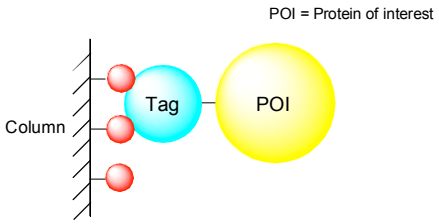
Dihydrofolate reductase ---- methotrexate

However, not all proteins have _____ binding partners, and some known binding interactions are not sufficiently strong to withstand washing steps.

Is there a general purification strategy that lends itself to a diverse set of proteins? Yes! Affinity tag purification.

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

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



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

For a summary of sequence, size, matrices, and elution conditions of commonly-used affinity tags, see Terpe, K. *Appl. Microbio. Biotech.* 2003, 60, 523-533.

A) CHARACTERISTICS OF AFFINITY TAGS

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 _____ of the POI.
- can be easily and specifically _____ 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 PEPTIDE TAGS

Examples: poly-Arg, FLAG, poly-His, Strep

Advantages:

- _____ interference with the fused protein
- Depending on the location and amino acid composition of the tag, removal of a small tag may _____ be required.
- Small tags are far less immonogenic than larger tags, so tagged recombinant proteins can be used directly as antigens in antibody production.

LARGE PEPTIDE TAGS

Examples: GST, MBP

- Advantage- These tags can increase solubility and expression of the POI.
- Disadvantage- In most cases, large tags need to be removed after purification.

Considerations for selecting the best fusion system for a protein of interest include

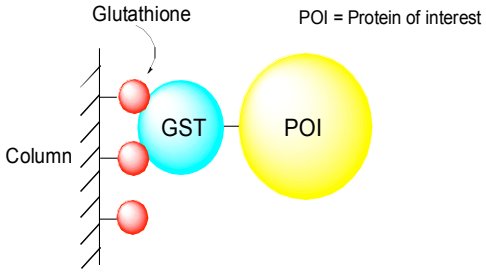
- Properties of the target protein, such as stability, hydrophobicity, etc.
- The expression system. There can be differences in expression efficiency depending on the system.
- The application of the purified protein

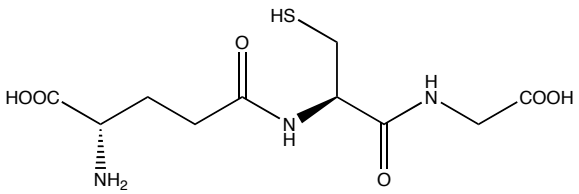
B) COMMON AFFINITY TAGS

Glutathione S-transferase-tag (_____-tag)

A 26-kDa (_____ aa) protein that binds to glutathione (a tripeptide).

GST-fusion proteins can be purified from crude cell lysate by affinity chromatography on immobilized glutathione.





Glutathione (reduced form)

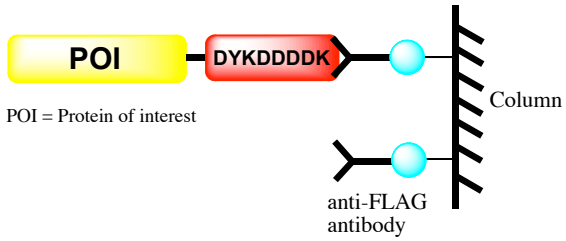
Bound fusion proteins are eluted with 10 mM reduced glutathione under _____-denaturing conditions.

The GST tag can help to protect against intracellular protease cleavage and stabilize the recombinant protein. However, due to its large size, the GST-tag should be cleaved from the fusion protein after purification.

FLAG-tag

A short hydrophilic octapeptide, _____

The FLAG-tag binds to a monoclonal _____ purification matrix (typically anti-FLAG antibody bound to sepharose resin.)



The tagged protein can be dissociated by adding chelating agents, by adding competing FLAG peptide, or by transiently reducing the pH (to ~_____).

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

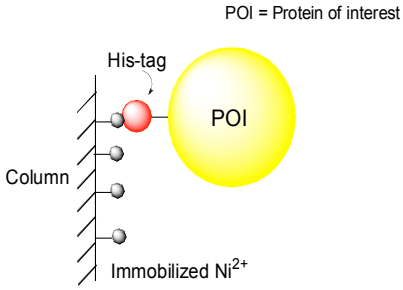
Diadvantage: _____

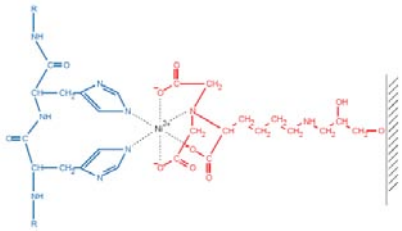
Poly-Histidine Tag (His-tag)

A short polyhistidine peptide (from 4-10 residues; normally ____)

His is the amino acid that binds most strongly to metal ions (Co^{2+}, _____ , Cu^{2+}, Zn^{2+}), and His-tag affinity purification uses a column with immobilized metal ions.

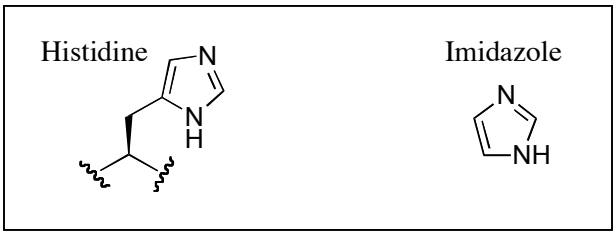
A common His-affinity column is Ni^{2+} immobilized by _____ (nitriloacetic acid).





The washing buffer typically contains 10 to 30 mM _____.

The imidazole competes with His residues for metal chelation, washing away untagged proteins. Note that 10 mM is not a sufficient imidazole concentration to compete off a His_4 tag.



Elution. There are three options for elution of a His-tagged target protein.

- Lower buffer pH (to 4.5-5.3). pKa of His is _____, so lowering pH results in nitrogen protonation and prevents chelation.
  Possible drawback: Some proteins can be damaged by reduction in pH.

- Use metal chelators (e.g. EDTA)
  Drawback: Recycling the resin is more difficult with the Ni chelated by EDTA

- Elute with _____ to _____ 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 _____ to Ni-NTA.
- Certain proteins have _____ 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).

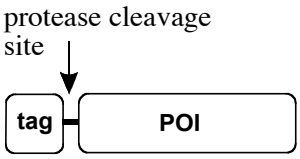
The Yop phosphatase is _____ His-tagged. You can thus use affinity tag purification to isolate only the H396P Abl kinase domain. Studies indicate that an N-terminal His tag does not *significantly* effect Abl kinase activity, so we will not remove the tag.

Dialysis of the purified protein

Consider the sizes (molecular weights) of the components in your elutions: the hexahistidine tagged Abl kinase domain is _____, and the imidazole is _____. Therefore using a 10 kDa _____ (molecular weight cut off) dialysis device, you can easily remove the imidazole from the protein solution.

C) CLEAVAGE OF AFFINITY TAGS

Site specific proteases can be used for tag cleavage.



Commonly used proteases with cleavage sites indicated:

Enterokinase: DDDDK✂X

_____ protease: ENLYFQ✂S(or C).

Thrombin: X4-X3-P-R(or K)✂X1'-X2', where X4 and X3 are hydrophobic amino acids and X1', X2' are non-acidic amino acids.

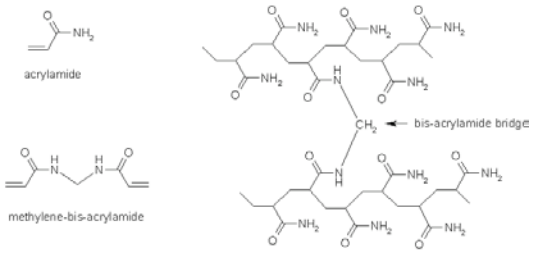
_____: I(or E)-DGR✂X

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 (both components make up a single gel)

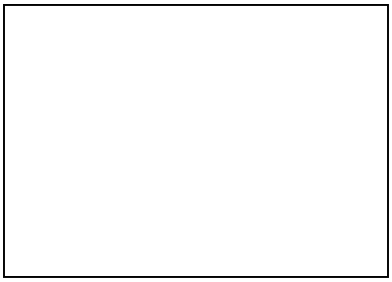
Difference in pore size as a result from different _____ acrylamide added

Stacking gel has _____ 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% acrylamide
- Higher % acrylamide ⇒_____ pores ⇒suitable for smaller proteins



Electrophoresis

- Negatively-charged proteins move toward the positive end.
- Smaller proteins run _____.
- Estimation of size based on the molecular weight markers.
- After electrophoresis, the gel can be stained (e.g. by _____ stain) or further processed (e.g.Western blot).

Upcoming laboratory sessions:

Session 4: prepare buffers for protein purification and gel analysis

Session 5: Lyse your cells and isolate the H396P Abl(229-511) protein. Dialyze.

Sessions 6, 7, and 8 (you can combine these or have several short days):

Run a gel to visualize your protein fractions.

Concentrate your purified and dialyzed protein.

Quantify protein concentration.

The shorter lab days are scheduled to give ample time to work on your mini-review. Draft 1 is due on March 5th (with an outline, introduction and annotated bibliography due earlier.)