

Lecture 7: Affinity Chromatography-II

Key words: Chromatography; Affinity chromatography; Protein Purification

We have studied basics of affinity purification during last lecture. The current lecture is continuation of last lecture and we will cover following:

1. Few specific examples of affinity purification in detail
2. Application of affinity chromatography in Proteomics

Because of advancement in Molecular Biology now it is possible to identify gene of a given protein. It is also possible to transfer the gene to other organism (for example *E. coli*) in a vector (simply a vehicle to transfer foreign gene into organism or another cell) and express in the same organism. Such proteins are called recombinant protein. Genetic manipulations are not scope of this course but students might have studied this in Molecular Biology course. Since purification of a protein can be a complex and time-consuming process so that the expression vectors are designed for higher level of expression of recombinant proteins with tags to facilitate the further purification. The DNA sequence codes for the protein are cloned in expression vectors at multiple cloning sites in continuation with tags either at N-terminal or C-terminal (Fig.1). These tags are also DNA sequences which code a small peptide or even a small protein to facilitate the purification of the recombinant protein. e.g. 6x His Tag or GST Tag are commonly used for purifying recombinant proteins.

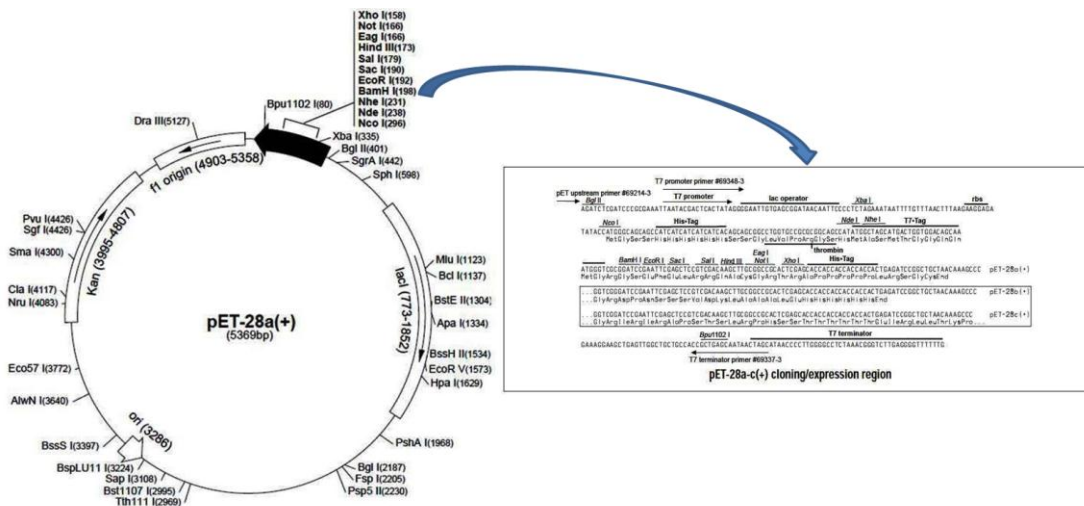


Figure 1: pET 28(a) expression vector and detailed view of multiple cloning sites

His-Tag for Purification of Recombinant Proteins: A hexa-His sequence is called a His-Tag. It has been shown that an amino acid sequence consisting of 6 or more His residues in a row will also act as a metal binding site for a recombinant protein. The 6xHis affinity tag facilitates binding to immobilized Ni. 6xHis coding sequence can be placed at the C- or N-terminus of the protein of interest and recombinant protein with 6xHis residues can be expressed. The tag is poorly immunogenic, and at pH 8.0 the tag is small, uncharged, and therefore does not generally affect secretion, compartmentalization, or folding of the fusion protein within the cell. In most cases, the 6xHis tag does not interfere with the structure or function of the purified protein as demonstrated for a wide variety of proteins, including enzymes, transcription factors, and vaccines. Sometime, a protease cleavage site is inserted between protein sequence and His-tag. After His-tag affinity purification, the purified protein is treated with the specific protease to remove His-tag. A very common example is His-tagged Proteins with Thrombin, a protease, cleavage site. Thrombin recognizes the consensus sequence Leu-Val-Pro-Arg-Gly-Ser, cleaving the peptide bond between Arg and Gly. This is utilised in many vector systems which encode such a protease cleavage site allowing removal of an upstream domain (Fig. 2)

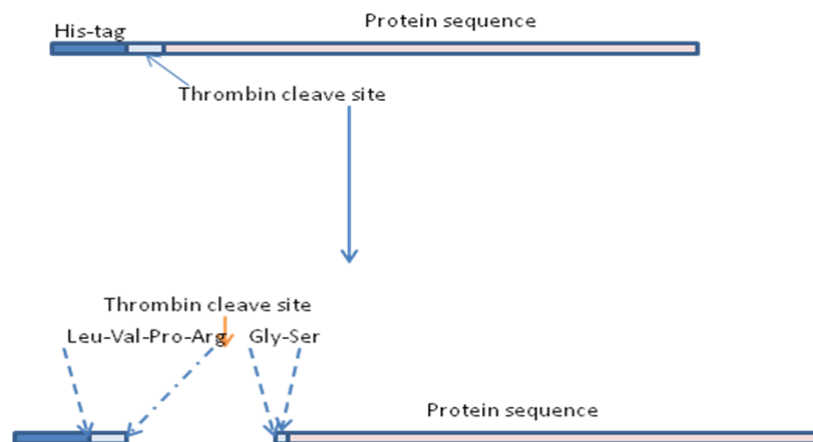


Figure 2: His-tagged Proteins with Thrombin cleavage site. First, his-tagged protein is purified by Immobilized-metal affinity chromatography. After purification, tag is removed by thrombin cleavage. After thrombin cleavage, the sample is again passed through Immobilized-metal affinity chromatography column. The cleaved protein will come in elution while the tag will remain bound to column.

Immobilized-metal affinity chromatography (IMAC) was first used to purify proteins in 1975 by Porath and group using the chelating ligand iminodiacetic acid (IDA, Figure). IDA was charged with metal ions such as Ni^{2+} , and then used to purify a variety of different proteins and peptides. IDA has only 3 metal-chelating sites and cannot tightly bind metal ions. Weak binding leads to ion leaching upon loading with strongly chelating proteins and peptides or during wash steps. This results in low yields, and metal-ion contamination of isolated proteins (Fig.3 and Fig. 4).

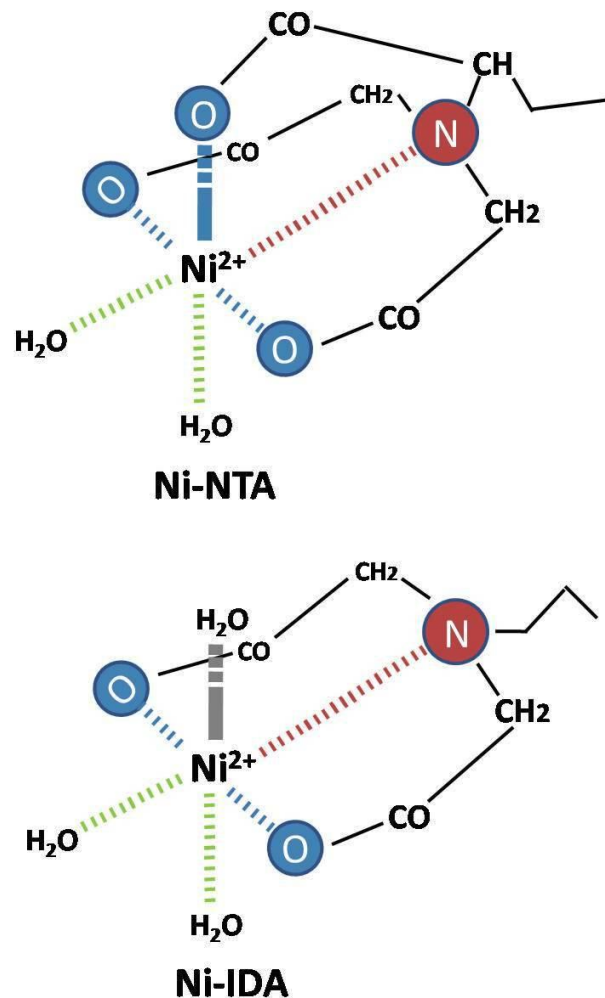


Figure 3: Comparison of interaction of different metal chelate matrices with nickel ions.

Nitrilotriacetic acid (NTA), is a tetradentate chelating adsorbent developed by Hoffmann-La Roche that overcomes these problems. NTA (Fig. 3) occupies four of the six ligand

binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag. NTA binds metal ions far more strongly than other available chelating resins and retains the ions under a wide variety of conditions, especially under stringent wash conditions. The unique, patented NTA matrices can therefore bind 6xHis-tagged proteins more tightly than IDA matrices, allowing the purification of proteins from less than 1% of the total protein preparation to more than 95% homogeneity in just one step.

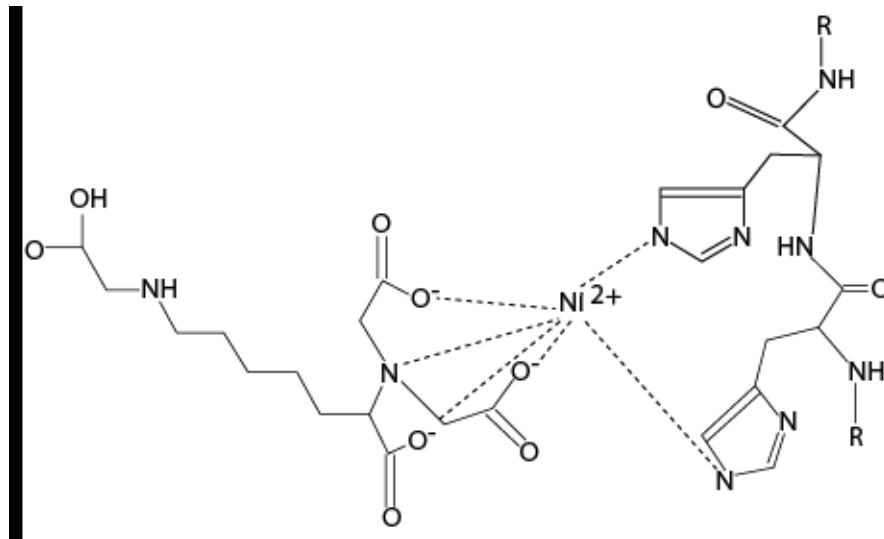


Figure 4: Binding of His-tag with Ni-NTA groups.

Protein binding: Proteins containing one or more 6xHis affinity tags, located at either the amino and/or carboxyl terminus of the protein, can bind to the Ni-NTA groups on the matrix with an affinity far greater than that of antibody–antigen or enzyme–substrate interactions. Binding of the 6xHis tag does not depend on the three-dimensional structure of the protein. Even when the tag is not completely accessible it will bind as long as more than two histidine residues are available to interact with the nickel ion; in general, the smaller the number of accessible histidine residues, the weaker the binding will be. Untagged proteins that have histidine residues in close proximity on their surface may also bind to Ni-NTA, but in most cases this interaction will be much weaker than the binding of the 6xHis tag. Any host proteins that bind nonspecifically to the NTA resin itself can be easily washed away under relatively stringent conditions that do not affect the binding of 6xHis-tagged proteins. Binding can be carried out in a batch or column

mode (**Supplementary section**). If the concentration of 6xHis-tagged proteins is low, or if they are expressed at low levels, or secreted into the media, the proteins should be bound to Ni-NTA in a batch procedure, and under conditions in which background proteins do not compete for the binding sites, i.e. at a slightly reduced pH or in the presence of low imidazole concentrations (10–20 mM). At low expression levels under native conditions, binding can be optimized for every protein by adjusting the imidazole concentration and/or pH of the lysis buffer. If high levels of background proteins are still present, equilibrating the Ni-NTA matrix with lysis buffer containing 10–20 mM imidazole prior to binding is recommended. The matrix is thus “shielded”, and non-specific binding of proteins that weakly interact is significantly reduced.

Supplementary section: Proteins may be purified on Ni-NTA resins in either a batch or a column procedure. The batch procedure entails binding the protein to the Ni-NTA resin in solution and then packing the protein–resin complex into a column for the washing and elution steps. This strategy promotes efficient binding of the 6xHis-tagged protein especially when the 6xHis tag is not fully accessible or when the protein in the lysate is present at a very low concentration. In the column procedure, the Ni-NTA resin is first packed into the column and equilibrated with the lysis buffer. The cell lysate is then slowly applied to the column. Washing and elution steps are identical in the batch and column procedure.

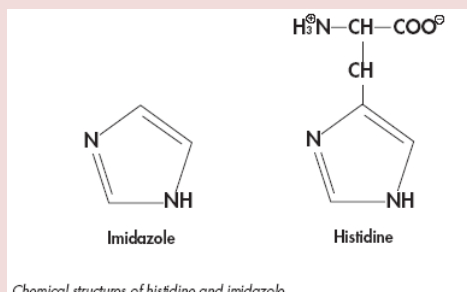
Wash: Endogenous proteins with histidine residues that interact with the Ni-NTA groups can be washed out of the matrix with stringent conditions achieved by adding imidazole at a 10–50 mM concentration.

Protein elution : The histidine residues in the 6xHis tag have a pKa of approximately 6.0 and will become protonated if the pH is reduced (pH 4.5–5.3). Under these conditions the 6xHis-tagged protein can no longer bind to the nickel ions and will dissociate from the Ni-NTA resin. Similarly, if the imidazole concentration is increased to 100–250 mM, the 6xHis-tagged proteins will also dissociate because they can no longer compete for binding sites on the Ni-NTA resin. Elution conditions are highly reproducible, but must be determined for each 6xHis-tagged protein being purified. Reagents such as EDTA or EGTA chelate the nickel ions and remove them from the NTA groups. This causes the

6xHis-tagged protein to elute as a protein–metal complex. NTA resins that have lost their nickel ions become white in color and must be recharged if they are to be reused. Whereas all elution methods (imidazole, pH, and EDTA) are equally effective, imidazole is mildest and is recommended under native conditions, when the protein would be damaged by a reduction in pH, or when the presence of metal ions in the eluate may have an adverse effect on the purified protein (**Supplementary section**).

Supplementary section: In bacterial expression systems, the recombinant proteins are usually expressed at high levels, and the level of copurifying contaminant proteins is relatively low. Therefore it is generally not necessary to wash the bound 6xHis-tagged protein under very stringent conditions. In lysates derived from eukaryotic expression systems the relative abundance of proteins that may contain neighbouring histidines is higher; the resulting background problem becomes more critical especially when nondenaturing procedures are employed. In these instances it becomes necessary to use imidazole gradient.

Additional points: As low affinity binding with other contaminant protein is also possible, low concentrations of imidazole in the lysis and wash buffers (10–20 mM) are recommended. The imidazole ring is part of the structure of histidine (Figure shown below). The imidazole rings in the histidine residues of the 6xHis tag bind to the nickel ions immobilized by the NTA groups on the matrix. Imidazole itself can also bind to the nickel ions and disrupt the binding of dispersed histidine residues in nontagged background proteins. At low imidazole concentrations, nonspecific, low affinity binding of background proteins is prevented, while 6xHis-tagged proteins still bind strongly to the Ni-NTA matrix. Therefore, adding imidazole to the lysis buffer leads to greater purity in fewer steps. For most proteins, up to 20 mM imidazole can be used without affecting the yield. If the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1–5 mM.



Binding of tagged proteins to Ni-NTA resin is not conformation-dependent and is not affected by most detergents and denaturants. The stability of the 6xHis–Ni-NTA interaction in the presence of low levels of β -ME (up to 20 mM) in the lysis buffer can be used to prevent the copurification of host proteins that may have formed disulfide bonds with the protein of interest during cell lysis. Detergents such as Triton X-100 and Tween 20 (up to 2%), or high salt concentrations (up to 2 M NaCl), also have no effect on binding, and may reduce nonspecific binding to the matrix due to nonspecific hydrophobic or ionic interactions. Nucleic acids that might associate with certain DNA and RNA-binding proteins are also removed without effecting the recovery of the 6xHis-tagged protein.