

His-tagged protein purification: Insight into a new kit on the block

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Background

There is an explosion in the number of protein biochemists in pharmaceutical and biotechnology research and development, research institutes and universities working on protein structure and function in recent times. These researchers are creating a strong and sustainable need for new tools to rapidly screen, prepare, purify, desalt and concentrate protein samples such as antibodies, soluble native proteins, membrane proteins and recombinant proteins. One of the most widely used methods for protein purification is immobilized metal ion affinity chromatography (IMAC), which was introduced by Porath *et al* in 1975. Metal chelate affinity chromatography is a rapid one-step purification of fusion proteins which removes most contaminants and can achieve purities close to homogeneity. Recombinant protein purification represents one of the major protein purification markets and fusion proteins tethered to histidine tags lend themselves to the standardized, rapid and affinity chromatography kit model.

Proteins are engineered with affinity tags attached to the 5' or 3' end of the target gene. Examples of such tags are hexahistidine (M_r 700) and an 8-residue peptide containing alternating histidines (M_r 900). The matrix is attached to chelating groups that immobilize transition metal ions such as Ni^{2+} , Co^{2+} , Cu^{2+} , Zn^{2+} (Porath and Olin, 1983; Porath, 1988; Sulkowski, 1989). Certain amino acids such as histidine, tryptophan, cysteine and tyrosine can act as electron donors on the surface of the protein and bind reversibly to the transition metal ion. The most common matrix for IMAC purification is iminodiacetic acid (IDA). Ni^{2+} is the most widely used metal ion as most IMAC tags seem to have very high affinity for immobilized Ni^{2+} . The simplicity of IMAC technology is extremely attractive as it lends itself to the bind, wash and elute mode of operation if the appropriate buffer formulations are selected. IMAC can often be used with samples without any pre-treatment. The use of metal chelate affinity is widespread for the selective adsorption of engineered recombinant proteins and has largely superseded non-affinity methods of chromatography for purifying many recombinant proteins. However, many IMAC separations are still based either on lengthy and tedious home brew methods or on expensive and elaborate liquid chromatography systems such as HPLC.

Driven by applications

The Proteus metal chelate Mini and Midi kits are designed for simple, complete and rapid His-tagged recombinant protein purification from bacterial cells, insect vectors, mammalian cells and yeast under native or denaturing conditions. These Proteus metal chelate kits offer distinct innovations over existing technologies for molecular biologists shifting into the world of protein research. Their proteins will mostly be expressed as fusion proteins. These molecular biologists will have a limited knowledge of protein purification techniques. They are already conditioned to using nucleic acid purifications kits and will expect to continue in the same vane. In view of the customer profile of Pro-Chem's IMAC kits, the application drivers are screening expression clones for high levels of His-tagged proteins, scouting mutant clones for recombinant protein expression and multiple preparative purification of His-tagged proteins for activity studies, structural studies or for raising antibodies.

The Proteus kit in perspective

Pro-Chem's rapid protein purification kit products emerged from a clear and comprehensive understanding of the present methods used by researchers to purify recombinant proteins from prokaryotic and eukaryotic expression systems. Currently, the researcher needs a minimum level of experience in protein chromatography to purify their target proteins. Filling plastic or glass columns with loose resin slurry is time-consuming and demands a good understanding of the performance of the resin *in situ*. Gravity or vacuum columns packed with dry and non-compressible silica resins are unfamiliar to the overwhelming number of researchers who work with agarose-based resins. Finally, high pressure chromatography columns marketed as column accessories for expensive and elaborate liquid chromatography (LC) systems fall outside the budget or core competencies of many small research teams. These LC systems are also bottlenecks for researchers because they have to find time to learn the computer software that runs the system and, in many cases, queue in a line to find time to use the shared resource.

Pro-Chem's principal task was to simplify current protein separation methods confronting molecular biology researchers with little or no chromatography knowledge who ideally want to affinity purify their recombinant proteins easily, cost-effectively and quickly. We also prioritized key features demanded by customers. They wanted us to supply a re-usable spin column format which did not require an elaborate LC system but could be used by any laboratory with access to a standard bench top centrifuge. The kit had to be attractive to both inexperienced and experienced protein separation researchers. This, we felt, could be done by including all components such as buffers, desalting columns and centrifuge tubes in a box necessary for the purification process. In terms of performance, the spin columns had to provide high yields and high purities of recombinant proteins from a large amount of contaminants in a relatively short

period of time. To this end, Pro-Chem is heavily committed to method development and extending the knowledge of applications of these spin columns. All protocols are rigorously evaluated against a panel of protein samples and test conditions to ensure that the kits comply with all published specifications and exceed the expectations of researchers.

Pro-Chem's innovation incorporates ready-to-use resin cartridges pre-packed with wet soft agarose resin and stable for a minimum of 2 years. Our research identified that the vast majority of researchers purifying recombinant proteins work repeatedly with agarose resin. We, therefore, employ highly compressible, high quality, robust and reproducible agarose resin in Pro-Chem's spin columns. In this way, these spin columns behave in line with expectations from inexperienced protein researchers and researchers accustomed to working with Ni²⁺ pre-charged agarose resin. The spin column format was chosen because it permits multiple purifications to be performed in parallel and with high reproducibility (e.g. low % CVs). These rapid spin columns can purify up to 24 proteins in a microfuge simultaneously.

The patent extends to the packing of the chromatography cartridge. By precisely controlling how the resin cartridges are packed, we are not only able to standardize the performance of the cartridge but we can ensure that all claims for the spin column made by Pro-Chem are fully endorsed by the end-user (Fig. 3). The column cartridge incorporates a technologically-advanced flow regulator which is designed to control the flow rate of the samples through the active column matrix. Observed yields and purities fluctuate as a direct function of the flow rate of the sample loading step. The flow regulator is pre-set by Pro-Chem to slow down the flow rate to an optimal capture speed. The concomitant increase in the residence time of the target protein with the matrix of the spin column increases substantially the yield and purity of the purified target protein. The flow regulator functions critically in the sample loading step which has been deliberately extended to 30 min for the Midi spin columns and 6 min for the Mini spin columns. However, unlike many other chromatography systems, there are negligible hold-up volumes and His-tagged protein elution to recover the purified target protein is rapid for both Mini (1 min) and Midi (3 min) columns.

The right mix

The Proteus spin columns are pre-charged with nickel to give a marine blue appearance (Fig. 1). The kits contain clear and unambiguous protocols, all buffers and desalting/buffer-exchange columns for purification of histidine-tagged proteins in less than 50 min for the Midi spin columns

and less than 15 min for the Mini spin columns. There is minimal preparation time to make working solutions of the kit buffers and there are no empty columns which require filling with loose resin slurry. These metal chelate kits offer a combination of unique features and benefits such as high binding capacity/high selectivity in a Mini and Midi spin column format for less than £1/mg engineered recombinant protein (Fig. 2). They are very simple to use and are ideal for inexperienced chromatography researchers. The spin columns are highly specific and rapid with often > 90-95 % purity. The kits can isolate pure target protein from a large amount of contaminants and concentrate the target protein simultaneously.

Modus operandi

Cells harvested from a cell culture are disrupted by sonication on ice or homogenization either with or without lysozyme treatment. The lysate is centrifuged and the culture pellet is resuspended in lysis buffer at a pH close to pH 7.4-8.0 using a similar concentration of buffer, imidazole and NaCl to that of a pre-equilibration buffer used for metal chelate chromatography. The spin columns are pre-equilibrated with binding buffer by centrifuging the columns at 500 g for 3 min. The lysate is filtered through a 0.2 µm filter and the cleared cell lysate is loaded on to the spin column at an optimal flow rate to ensure efficient capture of the target protein to the Ni metal chelate matrix. The spin column is washed up to three times at 500 g for 3 min to remove unbound proteins and the purified His-tagged protein is eluted by centrifuging the column with an imidazole elution buffer at 500 g for 3 min. The most common elution conditions for IMAC separations involve the use of a competitive counter-ligand such as imidazole. This is the preferred elution method for purifications under native conditions. When a recombinant protein is expressed at high levels in *E. coli*, the protein elutes as insoluble aggregates called inclusion bodies. Denaturants such as 6-8 M urea or 6 M guanidinium HCl completely unfold the target protein making the His-tag much more accessible for interaction with the immobilized Ni²⁺ matrix. For purifications under denaturing conditions, elution is performed either using imidazole in the presence of denaturant such as 8 M urea or by a reduction in elution pH from pH 7.4 to pH 4.5. The Proteus IMAC spin kits contain protocols for His-tagged protein purifications under both native and non-denaturing conditions. Fig. 4. shows the purity of a His-tagged wild-type protein and six site-specific mutants purified from a bacterial expression system under non-denaturing conditions using the Proteus IMAC spin columns.

The Proteus metal chelate kits provide high purity of recombinant proteins in a single step. The unique pre-packed metal chelate plugs are simply inserted into the spin columns and placed in a centrifuge. There is no mess, no filling columns, no attachment of accessories, no pumps and no

lengthy equilibrations. The protocols provided in the operating handbook permit fast recovery and high yields of pure recombinant proteins.

References:

Porath J., Carlson J., Olsson I. and Belfrage G. (1975) *Nature*, 258, 598.

Porath J. and Olin B. (1983) *Biochemistry*, 22, 1621.

Porath J. (1988) *Trends Anal. Chem.*, 7, 254.

Sulkowski E. (1989) *BioEssays*, 10, 170.

Figure legends:

Fig. 1. showing the modus operandi of the Mini and Midi spin columns.

Fig. 2. highlighting features and benefits of the Proteus IMAC spin columns and kits.

Fig. 3. showing the specifications of the Proteus IMAC Mini and Midi spin columns.

Fig. 4. showing high purity and multiple purification of His-tagged wild type and site specific mutant recombinant proteins from *E.coli*. In Fig. 4A, lane 1 corresponds to molecular weight markers, lane 2 corresponds to the sample wash and lanes 3 and 4 correspond to the 1st and 2nd eluates of the wild type protein. In Fig. 4B, lane 1 corresponds to molecular weight markers, lane 2 corresponds to the purified wild type protein and lanes 3 to 8 correspond to six pure mutant his-tagged proteins.

Fig. 1.



Fig. 2

Features	Benefits
Centrifugal spin column format	Simple to use. No HPLC or FPLC equipment has to be used. Every laboratory has a centrifuge.
Uses centrifugal g force as driving force	Extremely fast. Separation times are short. No hold up volume or spluttering. High recovery during fast elution step.
Many samples can be centrifuged in parallel	Multiple samples can be purified at the same time.
Economical	Low cost purification. Less than £1 per mg recombinant protein.
Binding capacity range from µgs to 10s of mgs	Variable volume and amounts can be loaded and recovered with ease.
No need for extensive equilibration	Fast pre-equilibration step!
Negligible dead volume	No loss of critical and precious samples.
No need to de-gas or de-fine the resin slurry	Saves time and mess.
Can purify and concentrate the sample in a single step	Saves time and less steps.
Complete elution of proteins	No carry-over of proteins or contaminants from previous step.
Stable for up to 2 years	No need to store in bactericide.

Fig. 3

Supporting Proteus matrix	Covalently coupled to agarose resin
Charged metal ion	Ni ²⁺
Max. sample volume per load	0.65 ml (Mini, Fixed angle rotor) 20 ml (Midi, Swing bucket rotor)
Resin bed volume	0.23 ml (Mini) 1.6 ml (Midi)
Bead size range	45-165µm
Recommended working pH	pH 2-12
Typical number of uses per plug	2 (assuming no further Ni ²⁺ charging)
Typical binding capacity per bind-wash-elute cycle	1 mg His-tagged protein (Mini) 10 mg His-tagged protein (Midi)
Chemical stability	High
Plastic construction	Polypropylene
Color coded end-caps	Black

Fig. 4

