Data and Instructions

IDA SepFast
IDA SepFast HighRes

1. Introduction

Immobilised metal affinity chromatography (IMAC) has been widely employed as a powerful separation approach in the purification of a broad range of proteins and peptides. It is based on the specific interactions between certain transitional metal ions, mostly Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} to the exposed amino acid surface chains containing histidine (or cysteine and tryptophane). The presence of several adjacent histidines such as $(His)_6$-tag increases the affinity to immobilised metal ions. Increasingly, chelating resins after charged with suitable metal ions are employed for the purification of histidine-tagged recombinant proteins expressed in bacteria, yeast and mammalian cells. There are other applications of chelating resins to purification of certain native non-tagged proteins as well, such as interferons, lectins, antibodies, serum and plasma proteins, peptides and peptide hormones.

Metal ions can be chelated to the carefully designed porous polysaccharide polymer supports via covalently attached iminoacetic acid (IDA) groups.

2. Product characteristics

IDA SepFast is specifically designed and fabricated for purification of proteins at cost-effective way. The base matrix is made of heavily cross-linked agarose. Its high mechanical strength permits liquid passing through columns at decent flow rates.

Table 1. Product characteristics

Particle size	IDA SepFast: 50 – 150 μm; IDA SepFast HighRes: 20 – 50 μm
Base matrix	Highly cross-linked 6% agarose
Metal ion capacity	Approx. 30 – 50 μmol / ml resin*
Chemical stability	Stable in all the commonly used aqueous buffers; 0.01M HCl, 0.1M NaOH tested for one week.
pH stability	2-14 (<2 h) 3-12 (up to one week)
Storage	20% ethanol at 4°C - 30 °C

*Tested with nickel ion

For higher protein binding capacity, purity or less metal leakage, alternative products are IMAC SepFast and IMAC SepFast HighRes series (i.e. NTA-type chelating ligand)

3. Charging and regenerating of IDA SepFast / IDA SepFast HighRes

Charging of metal ion of best choice can be conducted in the following procedure.

1. Transfer the required amount of resin to a suitable vessel.

2. Prepare a solution containing 0.08 M of the metal ion (e.g. Ni^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} etc) in distilled water. For Zn^{2+} ion, tiny amount of acid is added to reduce the solution pH to 5.5 or slightly less. Load the same volume of the solution to mix with the resin for 5-10 minutes.

3. Wash the resin with at least 5 volumes of distilled water.

4. Afterwards, the resin can be equilibrated with 5 - 10 bed volume of the equilibration buffer before loading protein samples.

After the resin has been used for a few cycles of purification, it may be discarded or regenerated depending on the properties of protein samples and purification conditions etc. It is recommended to use fresh resin if a different type of protein molecule is purified.

For the purpose of regeneration, the resin has to be totally free of any bound metal ion. By incubating the resin in the same volume of 100 mM EDTA + 0.5 M NaCl, pH 7.5, for 10 – 30 minutes with continuous mixing, the bound metal ion can be fully stripped off. This can be done in a gravity column. After removing the EDTA solution, wash the resin with 5 bed volume of 0.5 M NaCl in distilled water then 5 bed volume of distilled water. Afterwards, it is ready to recharge the resin according to the protocol described above.

4. Purification procedures

Please read Section 5 before a purification experiment is designed.

After the choice of metal ion (among the most popular ones are Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}) is charged to the resin, target protein can be directly purified.

4.1 Choice of the operation modes

The ***purification mode***, i.e. batch adsorption, gravity column adsorption, purification with magnetic resin, centrifugal adsorption or packed column adsorption etc, can be selected according to the guidance set in the ***Section 5.2***.

4.2 Preparations before protein purification

Protein expression and cell culturing can be referred to the well established protocols in literature.

Harvest the cells and / or broth after the culture is finished. For intracellularly expressed proteins, the pelleted cells are generally resuspended in PBS, tris-HCl buffer or other suitable buffers for following cell disruption such as freeze-thaw, ultrasonication, homogenisation and bead milling etc. Or the pelleted cells are directly suspended into a self-made or commercial cell lysing solution for releasing of the target protein (refer to the well established protocols in literatures). Pre-conditioning of the cell lysates, such as pH adjustment, addition of salt (e.g. 0.5-1.0 M NaCl) etc, can be done in this step. Clarified or unclarified protein samples can be purified directly. If unclarified sample is loaded, a treatment with DNAse I (e.g. 5 μg/ml of Benzonase with 1 mM Mg^{2+} for 10-15 mins in ice-bath) may be required to reduce the sample viscosity.

Equilibration / binding buffer is recommended as: 20 mM sodium phosphate + 0.5 M (or up to 1.0 M) NaCl, pH 7.4.

Washing buffer can be the same as the binding buffer or may contain additional reagents (e.g. low concentration of detergents) or have low pH value etc, in order to remove as much weakly bound impurities as possible. Refer to the ***Section 5.4*** for more information.

Elution solution should be prepared according to the guidance set in the ***Section 5.5***. The standard one can be 20 mM sodium phosphate (or sodium acetate) + 0.5 M (or up to 1.0 M) NaCl, pH 4.0.

Water and chemicals used for the protein purification process should be of high purity.

5. General considerations and optimisations

5.1 Choice of metal ions

Chelating SepFast BG is supplied free of charged metal ion. The choice of metal ions mainly depends upon the nature of target proteins and the specific application requirements. Cu^{2+} is commonly the first choice for purification of histidine-tagged recombinant proteins. As the strength and selectivity of interaction between a target protein and immobilised metal ion is affected by a few factors including the length and exposed position of the tag, electron distributions of the pair, pH and competitions from other impurities etc, some tagged proteins might be better purified with Zn^{2+}, Co^{2+}, Fe^{3+} or Ni^{2+} etc rather than Cu^{2+}. The similar considerations apply to purification of untagged proteins as well. Cu^{2+} might be used more often than other metal ions. A screening of different charged metal ions in combination with the specific application requirement (e.g. purity or yield or both) and binding conditions is recommended.

5.2 Choice of operational mode

There are mainly the following operational modes for consideration: batch stirred tank mode (including magnetic operation with magnetic particles), gravity flow mode, centrifugal (spin) mode and pressurised (packed) column mode.

For laboratories that have no access to expensive and sophisticated process chromatography systems (e.g. packing columns, pumps and valves, detectors and associated computing machinery etc), or protein purification is only a tool rather than their core research activities, the best choice is among batch stirred tank mode, gravity flow mode and centrifugal (spin) mode.

Batch stirred tank operation is the most versatile and flexible approach. Very little training in chromatography is required. It is particularly attractive in cases when the volume of protein sample is large but the concentration is relatively low or the target protein molecule is large (e.g. over 65K Dalton). Longer contact time can be employed in this mode to allow protein molecules diffusing into the pores, which means better utilisation of the whole resin ligands and subsequently higher binding capacity. Also, there is no limitation from the column side. So, large volume of protein sample can be processed in the same time period as that for small volumes.

Gravity flow operation is a choice if the volume of protein sample is from a few mls to tens of mls and the protein molecule isn't very big. It is readily operated as well. More than one column can be easily operated in parallel. IDA SepFast possesses strong mechanical structure to permit liquid passing through at decent flowrate. The particle size and pore structure are carefully controlled for such operations.

Pressurised (packed) column operation gives the best chromatographic performance. However, expensive and specialised instrument and accessories are required for such kind of operations. Professional training and expertise in process chromatography is essential in order to benefit the most from such operations. Unclarified cell lysates can't be directly processed in packed column, as cell debris will severely block the column.

5.3 Binding conditions

Proteins tagged with one or more 6 x His in either the N-terminus or C-terminus can be strongly bound to the metal charged resin. The interaction doesn't depend on the three-dimensional structure of the protein, as long as two or more than two chelating residuals in the protein can access the immobilised metal ion to form chelating bonds. Host cell proteins (HCP) that contain histidine, cysteine or tryptophan in a close proximity might interact with the resin but the strength is generally much weaker.

Two key factors are commonly optimised for the best binding performance. They are pH and salt. Sodium chloride (or sodium potassium) must be added to the sample and binding buffer to suppress any non-specific interaction caused by electrostatic charges. Commonly 0.5M of NaCl is used but further optimisation might be required to improve the product purity. In some cases, manipulation of pH (particularly reducing pH value in the sample) can improve product purity, as the chelating residues in HCP can't form chelating bond after they are charged.

Phosphate buffer is recommended in most cases (20-50 mM). Tris-HCl buffer at lower concentration (e.g. 10-50 mM) is normally fine but care should be taken if higher concentration is employed, as it might affect the binding in case that the affinity of target protein to the metal ion isn't very strong. Addition of other chelating reagents such as EDTA should be avoided.

The amount of resin used to purify a given amount of target protein can be considered as well. The addition of much excess amount of resin might promote undesired non-specific binding of HCP as more free ligands are available to impurities.

5.4 Washing conditions

Stepwise increase of the washing stringency is recommended for the batch or gravity flow operations. In some cases, longer contact time may help to dissociate the bound impurities from the resin. Increased salt concentration, reduced pH, addition of detergents like Triton and Tween etc are the commonly used approaches to remove the weakly bound impurities. A compromise between the final yield and product purity should be considered to develop the best washing conditions.

5.5 Elution conditions

There are mainly three choices of elution approaches: pH, imidazole and EDTA.

When the pH is reduced from neutral to less than 6 (typically 4.2 to 5.5), the histidine residues in the bound protein are protonated. Under this condition, the chelating bond between the metal ion and the histidine residues is dissociated. As a result, the bound protein is released. Care should be taken to investigate the stability of the target protein under low pH value like 4.5.

Imidazole at concentration of 20-30 mM can effectively compete off the bound protein as well. The best elution concentration has to be determined case by case. This is the mildest approach among the three mentioned here.

EDTA is a very strong chelating reagent. 50 mM EDTA can fully stripe off the immobilised metal ion and therefore release the bound protein as well. It might not be desirable for metal ion being present in the final protein product.

6. Clean-in-place (CIP)

In case that the resin is severely contaminated by strong ionic substances (e.g. proteins and nucleic acids etc), hydrophobic proteins, lipoproteins and lipids etc, the following clean-in-place approaches can be conducted. Before any CIP is pursued, the immobilised metal ions must be removed according to the procedure described in ***Section 3***.

Salt with concentration up to 2 M can be used to clean the impurities bound by ionic interactions.

The contaminants bound by hydrophobic nature can be removed by the following reagents: 1 M NaOH, low percentage detergents (e.g. 0.1 – 2%), 70% ethanol or 30% isopropanol in basic or acidic conditions (e.g. in the presence of acetic acid or phosphoric acid). A combination of the above reagents can be explored as well. In general, the incubation time should be longer (e.g. from 30 minutes to overnight) to ensure full dissociation of the contaminants.

7. Storage

Store the resin in 20% ethanol at 4°C – 30°C. Seal the bottle soon after each use.

8. Further information

Visit www.biotoolomics.com for further information or contact the technical team or sale representatives.

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