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A new IMAC medium for scaling up purification of Histidine-tagged proteins

Jon Lundqvist, Lars C Andersson, Ann Bergh, Ellinor Ancker, Marianne Carlsson, Anna Heijbel, Helena Lindgren, Karin Torstenson and Katarina Öberg

GE Healthcare, SE-751 84 Uppsala, Sweden

Introduction

The (Histidine)₆-tag has become the most used affinity tag due to its small size, strong metal ion binding and ability to bind under denaturing conditions. Immobilized Metal Ion Affinity Chromatography (IMAC) with Ni²⁺ is the standard method for purifying histidine-tagged proteins. Here, a new medium optimized for scaling up purification, Ni Sepharose™ 6 Fast Flow, is presented.

Ni Sepharose 6 Fast Flow

- Pre-charged with Ni²⁺ and available in different formats, including pre-packed 1-ml and 5-ml HisTrap™ FF and 20-ml HisPrep™ FF 16/10 columns.
- The bead-size (~90-μm) makes it possible to scale-up purification maintaining high target protein concentration in the eluted peak.
- Compatible with a wide range of buffers and additives, including denaturants, detergents and reducing agents.

Conclusions

The following results were obtained with Ni Sepharose 6 Fast Flow:

- Easy to scale-up purification from 1-ml via 5-ml to 20-ml columns.
- Low leakage of Ni²⁺ and compatibility with the reducing agent DTT.
- A number of Histidine-tagged proteins from different:
 - expression systems
 - expression levels
 - with different M_r
 - with different lengths of the Histidine-tagwere easily purified.



Material and Methods

Chromatography conditions were, unless otherwise stated:

Medium: Ni Sepharose 6 Fast Flow pre-packed in 1-ml and 5-ml HisTrap FF, and 20-ml HisPrep FF 16/10 columns

Samples: Cell extracts from *E. coli* or *P. pastoris* were clarified by centrifugation and filtration; containing 0.5 M NaCl and imidazole at a concentration appropriate for each target protein.

Flow rate: 1 ml/min

Binding buffer: 20 mM sodium phosphate, 0.5 M NaCl, x mM imidazole, pH 7.4 (x = optimized for each target protein)

Optimization: The optimal imidazole concentration to obtain the best purity and yield differs from protein to protein. A small number of screening runs, including a run with a linear imidazole gradient from 5 mM, will facilitate finding a suitable imidazole concentration for optimal results.

Elution buffer: 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4

Purifications were performed on ÄKTAexplorer™ 10 or 100 systems. Elution was done either with a step or a linear imidazole gradient. SDS-PAGE was performed with ExcelGel™ SDS Gradient 8–18 Gels.

Purifications of Histidine-tagged proteins in the presence of DTT

Column: HisTrap FF 1-ml
Sample: (Histidine)₆-tagged Maltose Binding Protein in *E. coli* extract (conc. ~1 mg/ml, M_r ~43 000)

Sample volume: 7 ml

Binding buffer: 2 or 5 mM DTT, 20 mM sodium phosphate, 25 mM imidazole, 500 mM NaCl, pH 7.4

Elution buffer: 2 or 5 mM DTT, 20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4

Flow rate: 1 ml/min

Note: A blank run without DTT was performed before the sample application.

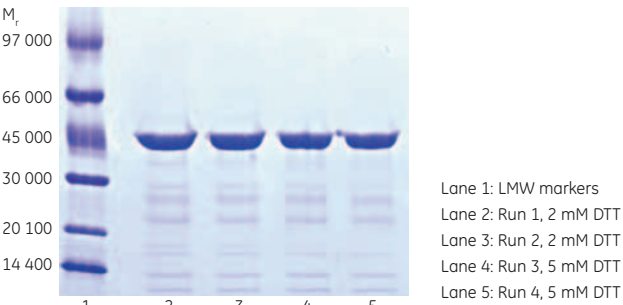


Fig 1. Purity and recovery of repeated purification runs on Ni Sepharose 6 Fast Flow without Ni²⁺ recharging.

Results:

- Purity and recovery were not affected by DTT.

Six repeated purifications

without stripping, cleaning or Ni²⁺ recharging the column

Column: HisTrap FF 1-ml

Sample: (Histidine)₆-tagged Maltose Binding Protein in *E. coli* extract

Sample volume: 7 ml

Binding buffer: 20 mM sodium phosphate, 5 mM imidazole, 500 mM NaCl, pH 7.4

Elution buffer: 20 mM sodium phosphate, 5–200 mM imidazole, 500 mM NaCl, pH 7.4 (linear gradient)

Flow rate: 1 ml/min

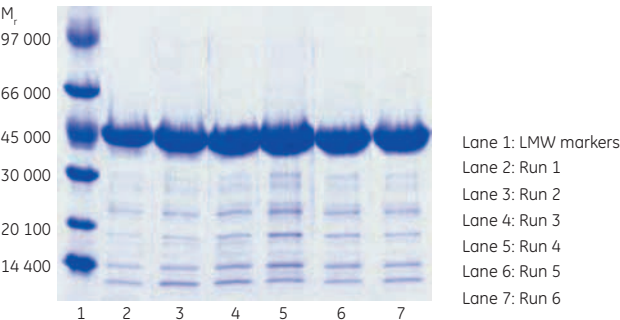


Fig 2. SDS-PAGE analysis under nonreducing conditions of pooled fractions from different runs.

Table 1. Summary of purification results from the six repeated purification runs on Ni Sepharose 6 Fast Flow shown in Figure 2.

Run	Amount eluted protein (mg)	Yield (%)	Molar ratio Ni ²⁺ /protein
1	7.5	88	0.14
2	7.4	87	0.11
3	7.4	87	0.08
4	7.2	85	0.07
5	7.5	88	0.07
6	7.5	88	0.06

Results:

- Excellent reproducibility in purity and recovery without the need for stripping, cleaning, or Ni²⁺ recharging the medium.
- Low Ni²⁺-leakage during purifications (low leakage minimizes potential problems such as Ni²⁺ induced oligomerization and precipitation of target protein, and loss of binding capacity).

Purification of a low expressed (Histidine)₆-tagged hydrolase from *Pichia pastoris*

Column: HisTrap FF 1-ml
Sample: *Pichia pastoris* extract with low expression of a (putative) *Saccharomyces cerevisiae* hydrolase ($M_r \sim 34\ 000$)
Sample volume: 50 ml (corresponding to 20 g cells)
Binding buffer: 20 mM sodium phosphate, 50 mM imidazole, 500 mM NaCl, pH 7.4
Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, pH 7.4, 25 ml linear gradient, 50–300 mM imidazole
Flow rate: 1 ml/min

Results:

- High-purity target protein was obtained despite low expression.
- ~ 90% of the target protein applied on the column was recovered.

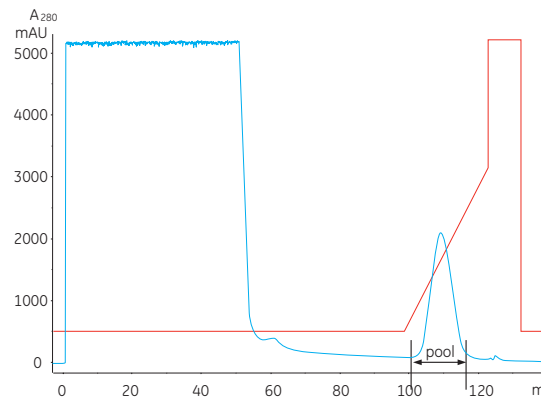


Fig 3. Black lines in the chromatogram indicate the final pool.

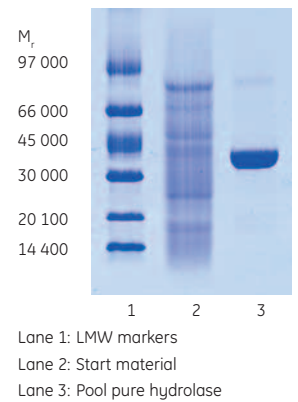


Fig 4. Analysis by SDS-PAGE.

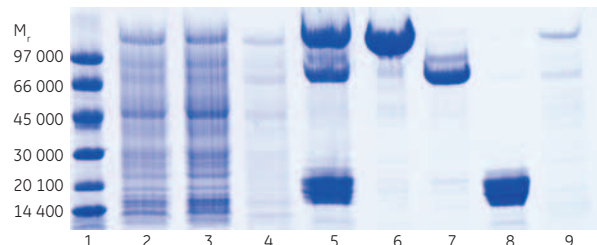
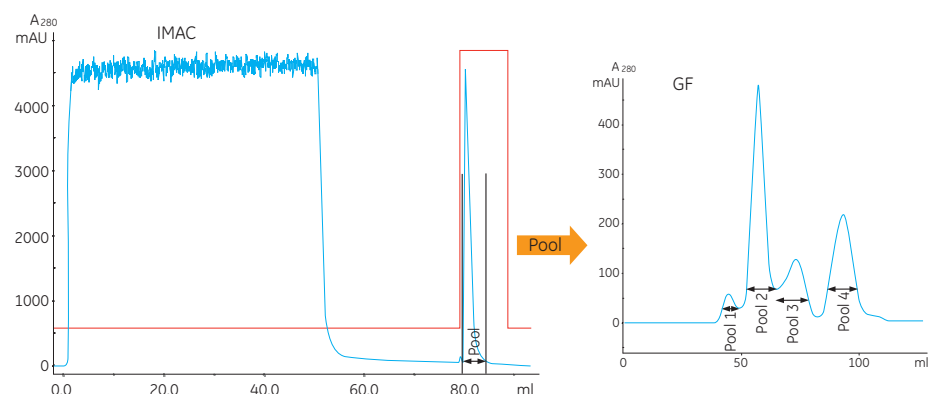
Two-step purification of a high molecular weight (Histidine)₁₀-tagged protein expressed in *E. coli*

Columns: IMAC: HisTrap FF 1-ml
 Gel filtration (GF): HiLoad™ 16/60 Superdex™ 200 pg
Sample: *E. coli* extract with Histidine-trx-p450, a large (Histidine)₁₀-tagged protein ($M_r \sim 130\ 000$)
Sample volume: 50 ml
Binding buffer: IMAC: 20 mM sodium phosphate, 60 mM imidazole, 500 mM NaCl, pH 7.4
Elution buffer: IMAC: 20 mM sodium phosphate, 500 mM NaCl, pH 7.4 and 500 mM imidazole
 GF: 20 mM phosphate, 0.28 M NaCl, 6 mM KCl pH 7.4
Flow rate: IMAC: 1 ml/min
 GF: 0.5 ml/min

Results:

- Three major bands were detected after IMAC purification.
- A very good separation between the contaminants and (His)₁₀-trx p450 was obtained by gel filtration.

Note: Contaminants after IMAC were truncated forms of (His)₁₀-trx-p450 (as determined by N-terminal sequencing, data not shown).



Lane 1: LMW markers
 Lane 2: Start material, 1:30
 Lane 3: Flowthrough IMAC, 1:20
 Lane 4: Wash IMAC, 1:10
 Lane 5: Pool IMAC, 1:10
 Lane 6: Pool 2, GF, 1:2
 Lane 7: Pool 3, GF, 1:2
 Lane 8: Pool 4, GF, 1:2
 Lane 9: Pool 1, GF

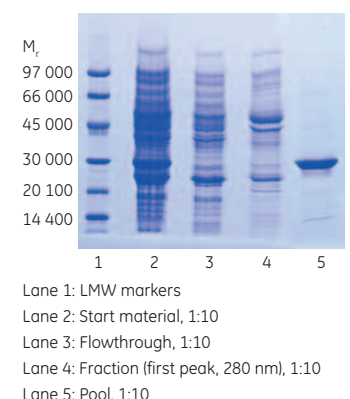
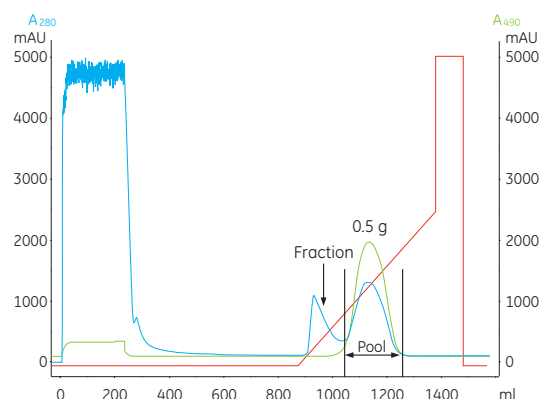
Fig 5. Two-step purification by IMAC and gel filtration. SDS-PAGE analysis under reduced conditions.

Purification of (Histidine)₆-tagged protein on a 20-ml HisPrep FF 16/10 column

Column: HisPrep FF 16/10, 20-ml
Sample: (Histidine)₆-tagged Green Fluorescent Protein (GFP-(His)₆, $M_r \sim 28\ 000$) in *E. coli* extract
Sample volume: 230 ml
Binding buffer: 20 mM sodium phosphate, 5 mM imidazole, 500 mM NaCl, pH 7.4
Elution buffer: 20 mM sodium phosphate, 500 mM NaCl, pH 7.4, 500 ml linear gradient, 5–250 mM imidazole
Flow rate: 5 ml/min

Results:

- 0.5 g GFP-(His)₆ was purified on HisPrep FF 16/10.
- GFP-(His)₆ was eluted in 180 ml (conc. 2.7 mg/ml).



Lane 1: LMW markers
 Lane 2: Start material, 1:10
 Lane 3: Flowthrough, 1:10
 Lane 4: Fraction (first peak, 280 nm), 1:10
 Lane 5: Pool, 1:10

Fig 6. Total protein monitored at 280 nm (blue line) and GFP-(His)₆ monitored at 490 nm (green line). Black lines in the chromatogram indicate the fraction and pool collected for SDS-PAGE analysis.

Scaling up purification of (Histidine)₆-tagged protein

Column: HisTrap FF 1-ml, HisTrap FF 5-ml, HisPrep FF 16/10 20-ml

Sample: (Histidine)₆-tagged Maltose Binding Protein (M_r ~43 000) in *E. coli* extract

Binding buffer: 20 mM sodium phosphate, 25 mM imidazole, 500 mM NaCl, pH 7.4

Elution buffer: 20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4

Flow rates: HisTrap FF 1-ml 1 ml/min
HisTrap FF 5-ml 5 ml/min
HisPrep FF 16/10 5 ml/min

Results:

- Scaling up provided highly consistent results.
- Scaling up from a 1-ml to a 20-ml column did not significantly affect the purity and recovery.

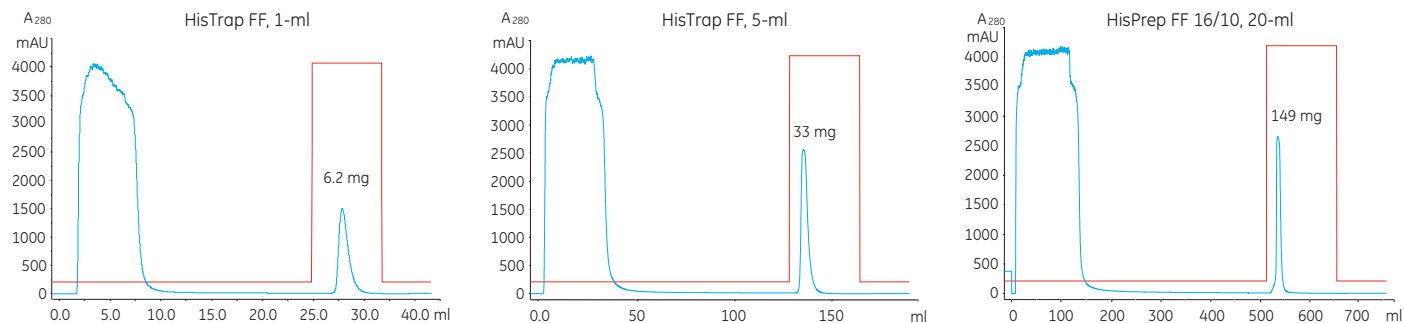


Fig 7. Scaling up from HisTrap FF 1-ml via HisTrap FF 5-ml to a HisPrep FF 16/10, 20-ml prepacked column. The samples loaded contained approximately 8, 40, and 160 mg MBP-(His)₆ respectively. Yield in milligrams is shown in each chromatogram.

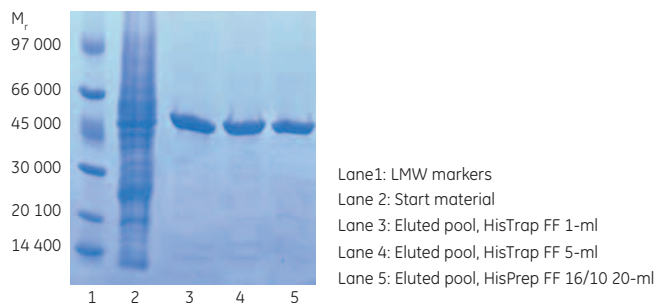


Fig 8. SDS-PAGE analysis under nonreducing conditions.

Acknowledgement

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GE Healthcare
Amersham Biosciences AB
Björkgatan 30
751 84 Uppsala
Sweden

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Purification and preparation of fusion proteins and affinity peptides comprising at least two adjacent histidine residues may require a license under US pat 5,284,933 and US pat 5,310,663, including corresponding foreign patents (assignee: Hoffman La Roche, Inc).

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