

# Purification of (His)<sub>6</sub>-tagged proteins using HiTrap Chelating HP columns charged with different metal ions

## Summary

Three important parameters—the nature of the protein, the metal ion used for charging the chelating column, and the position of the histidine tag on the protein—were investigated for their effect on purification of histidine-tagged proteins by immobilized metal affinity chromatography.

In the first set of experiments, six different (His)<sub>6</sub>-tagged proteins were purified under native or denaturing conditions using HiTrap™ Chelating HP columns charged with different metal ions, Fig 1. Although the results obtained with a specific protein varied somewhat depending on the ion used, several different ions were effective for purifying each protein. When the proteins were characterized by SDS-polyacrylamide gel electrophoresis, a similar level of purity was observed for each protein regardless of the ion used. Results indicated that the strength of binding depends on both the metal ion used and the nature of the (His)<sub>6</sub>-tagged protein itself. Both factors must therefore be considered during optimization of a purification protocol and especially when a "difficult" protein must be purified.

Proteins can be labelled with histidine residues at either their N or C terminus. However, the position of the tag may affect protein behavior and, ultimately, its binding to metal ions. To investigate this possible effect, Maltose Binding Protein was labelled with (His)<sub>6</sub> at its N-terminus, C-terminus, or both termini, and then purified using HiTrap Chelating HP columns charged with different metal ions. Results suggested that the position of the tag on the protein may indeed influence the strength with which a protein binds to a specific metal ion and consequently its purification. This factor should thus be considered when developing and optimizing a purification strategy using immobilized metal affinity chromatography.



**Fig 1.** HiTrap Chelating HP 1 ml columns charged with Cu<sup>2+</sup>, Co<sup>2+</sup>, Ca<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup> and Fe<sup>3+</sup>.

## Introduction

Immobilized metal affinity chromatography (IMAC) is a very useful method for purifying histidine-tagged fusion proteins (1). HiTrap Chelating HP columns, prepacked with Chelating Sepharose™ High Performance, are effective and convenient tools for this application. The medium, supplied free of metal ions, is first charged with transition metal ions that bind strongly to the iminodiacetic acid (IDA) ligand on the Sepharose to form a chelate. Proteins with surface-exposed amino acid residues such as histidine, will bind to the ions, thus forming a complex. The strength of binding between a protein and a metal ion is affected by several factors, including the length of the affinity tag on the protein, the type of ion used, and the pH of buffers. Because the metal ions are strongly chelated by the IDA ligand, bound protein can be eluted either competitively with a strong chelating agent such as imidazole, or by simply lowering the pH. These properties make IMAC especially useful for purifying membrane proteins and protein aggregates where detergents or high ionic strength buffers are required.

His-tagged proteins that contain a stretch of histidine residues (most common is six residues, but any number from two to ten have been used) at the carboxyl or amino terminus will bind to the immobilized divalent/trivalent metal ions. Although exposed histidine groups will form complexes with many transition metal ions, binding effects vary depending on the ion. Therefore, it is not always possible to predict which ion will be most appropriate for purifying a given protein.

## 1. Purification of six different (His)<sub>6</sub>-tagged proteins using different metal ions

### Protein samples

In this study, six different (His)<sub>6</sub>-tagged proteins were purified by IMAC on HiTrap Chelating HP columns. The affinity tag was either positioned at the N or C terminus. (Table 1). All of these proteins were expressed in *E. coli*— five as soluble proteins and one, (His)<sub>6</sub>-scFv fragment, as inclusion bodies that required denaturing conditions for purification. Each protein was purified in a single step using HiTrap Chelating HP columns pre-charged with either Ni<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, Zn<sup>2+</sup>, Ca<sup>2+</sup> or Fe<sup>3+</sup>. The same purification protocol was used for all six proteins to facilitate comparison of results. The purity of each protein after this one-step purification was visually assessed using silver-stained SDS-polyacrylamide gels.

**Table 1.** Proteins purified using HiTrap Chelating HP columns charged with different metal ions.

Protein	Molecular weight (M <sub>r</sub> × 10 <sup>3</sup> )	Tag position	Results
GFP-(His) <sub>6</sub>	28	C terminus	Figure 2
(His) <sub>6</sub> -ribokinase	32	N terminus	Figure 3
(His) <sub>6</sub> -REX	40	N terminus	Figure 3
Lina-(His) <sub>6</sub>	44	C terminus	*
(His) <sub>6</sub> -R5P isomerase	25	N terminus	*
(His) <sub>6</sub> -scFv fragment (inclusion bodies)	38	N terminus	Figure 5

\* Data not shown

### Disruption of cells

Cells of *E. coli* expressing GFP-(His)<sub>6</sub> were homogenized by french press in 20 mM Tris buffer, pH 7, containing 0.5 M NaCl and 5 mM imidazole. Cells expressing (His)<sub>6</sub>-REX, (His)<sub>6</sub>-ribokinase and Lina-(His)<sub>6</sub> were freeze-thawed three times in 20 mM Tris buffer, pH 7, containing 0.5 M NaCl, 5 mM imidazole, 1 mM PMSF, 1 mM DTT, 1 mM MgCl<sub>2</sub>, lysozyme (1 mg/ml), and Benzonase® (10 U/ml; Merck).

Cells expressing (His)<sub>6</sub>-scFv fragment as inclusion bodies

were freeze-thawed three times in 20 mM Tris buffer, pH 7, containing 0.5 M NaCl, 5 mM imidazole, and 8 M urea. For each preparation, a ratio of 1 g of wet cells/5 ml of buffer was used. Cells expressing (His)<sub>6</sub>-ribose-5-phosphate isomerase ((His)<sub>6</sub>-R5P isomerase) were lysed using BugBuster™ (Novagen) according to the manufacturer's instructions.

### Column preparation

HiTrap Chelating HP 1 ml columns were used throughout this study, with a separate column prepared for each ion solution. Prior to use, the columns were pre-washed with 5 column volumes (CV) of distilled water using a syringe. For charging with ion, the columns were loaded with 1 CV of 0.1 M solutions (in water) of either NiSO<sub>4</sub>, CuSO<sub>4</sub>, CoCl<sub>2</sub>, ZnCl<sub>2</sub>, CaCl<sub>2</sub>, or FeCl<sub>3</sub>. The columns were then washed a second time with 5 CV of distilled water to remove any unbound metal ions. The columns were placed in the ÄKTA™explorer 10 system and a blank run was made using binding buffer (20 mM Tris buffer, pH 7, containing 0.5 M NaCl and 5 mM imidazole), followed by elution buffer (20 mM Tris buffer, pH 7, containing 0.5 M NaCl and 0.5 M imidazole), and concluding with binding buffer. Five column volumes of buffer was used in each step.

After each purification, the columns were stripped using 5 CV of binding buffer containing 50 mM EDTA. Each column was then washed with 5 CV of distilled water prior to ion charging, washing, and loading of a new, different protein. Columns that changed color after stripping were replaced. This happened with Fe<sup>3+</sup> and occasionally with Co<sup>2+</sup>.

### Protein purification

Using a scouting method on ÄKTA explorer 10, protein samples were loaded onto the six different ion-charged columns. For each individual protein, the same volume of sample was loaded onto each column. After sample loading, columns were washed with 10 CV of binding buffer. Proteins were eluted using a 25 CV linear gradient going from 0 to 60% elution buffer, followed by a 5 CV linear gradient up to 100% elution buffer. Columns were then washed with 4 CV of 100% elution buffer and finally equilibrated with 5 CV of binding buffer. After purification, the purity of each protein was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using reduced samples and silver stained PhastGel™ Gradient 8–25% on a PhastSystem™.

### Results and discussion

For the six (His)<sub>6</sub>-tagged proteins used in the study, Ni<sup>2+</sup>-charged HiTrap Chelating HP columns consistently yielded the best separation of the target protein from contaminating cellular proteins (Figs. 2–5). Cu<sup>2+</sup>, Co<sup>2+</sup> and Zn<sup>2+</sup> were also effective for purifying most of the proteins, but all six proteins failed to bind to Ca<sup>2+</sup>, and only two—(His)<sub>6</sub>-ribokinase and (His)<sub>6</sub>-REX—bound to Fe<sup>3+</sup>-charged columns. For each

individual protein, the amount and purity of the proteins recovered from the various columns were similar, as shown in the chromatograms and stained SDS-PAGE gels.

HiTrap Chelating HP columns charged with Ni<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup> and Zn<sup>2+</sup> ions were all effective for one-step purification of GFP-(His)<sub>6</sub> (Fig 2) and (His)<sub>6</sub>-ribokinase (Fig 3). For GFP-(His)<sub>6</sub>, the protein eluted from each column in sharp peaks that were well-separated from contaminating cellular material. Equivalent amounts of protein were recovered from each column. Ca<sup>2+</sup>- and Fe<sup>3+</sup>-charged columns failed to bind the protein.

With (His)<sub>6</sub>-ribokinase, binding occurred with the Fe<sup>3+</sup>-charged column, but separation was poor, so the target protein was contaminated with other cellular material (Fig 3B, Lane 5). Ni<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup> and Zn<sup>2+</sup> columns yielded protein with similar levels of purity, although the peak fraction eluted from the Ni<sup>2+</sup> column was separated better from cellular contaminants (Fig 3A).

(His)<sub>6</sub>-REX did not bind at all to Ca<sup>2+</sup> and only very poorly to Cu<sup>2+</sup>. While the purification looked similar regardless of the ion used, the protein was not very pure, as indicated by the high level of other cellular materials in the peak fractions (Fig 4B). This protein in particular would need further purification steps for increasing the purity.

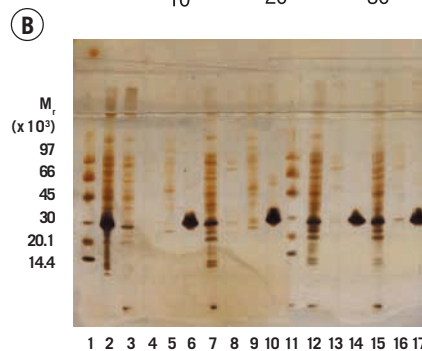
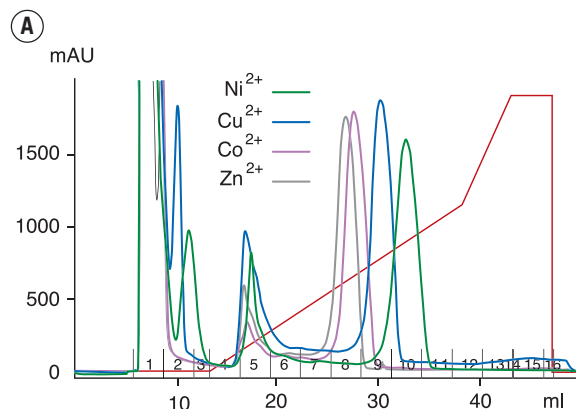
During gradient elution of Lina-(His)<sub>6</sub> (data not shown), the protein split into three peaks with slight variations in height. When analyzed by SDS-PAGE, each of the three peaks contained protein of the same molecular weight. A very low level of contaminating cellular proteins was observed in peak one with each metal ion-charged column. Although Ni<sup>2+</sup>-charged columns produced the best separation, the level of protein purity was still equivalent regardless of the ion used.

The separation achieved for (His)<sub>6</sub>-R5P isomerase using Ni<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup> or Cu<sup>2+</sup> was adequate (data not shown), but too little protein was loaded onto the columns so the peaks were somewhat broad and low in height. However, all the columns yielded protein with a similar level of purity.

(His)<sub>6</sub>-scFv fragment was purified as inclusion bodies from *E. coli*. All buffers therefore contained 8 M urea to maintain denaturing conditions. Although separation was achieved, peaks were rather broad (Fig 5A). While all peak fractions contained some impurities, Ni<sup>2+</sup>-charged columns appeared to yield a purer protein band (Fig 5B, lane 4) than seen with Cu<sup>2+</sup>, Co<sup>2+</sup>, or Zn<sup>2+</sup> columns.

### GFP-(His)<sub>6</sub>

**Column:** HiTrap Chelating HP 1 ml  
**Sample:** GFP-(His)<sub>6</sub>  
**Sample load:** 0.5 ml  
**Binding buffer (A):** 20 mM TrisHCl, pH 7, 0.5 M NaCl, 5 mM imidazole  
**Elution buffer (B):** 20 mM Tris-HCl, pH 7, 0.5 M NaCl, 0.5 M imidazole  
**Flow rate:** 0.5 ml/min (78 cm/h)  
**Fractions:** 3 ml  
**Gradient:** 25 CV 0–60% elution buffer, 5 CV 60–100% elution buffer  
**System:** ÄKTAE explorer 10



Lane 1. LMW (Low Molecular Weight Kit, Amersham Biosciences)  
 Lane 2. Loaded GFP-(His)<sub>6</sub> sample

#### Lanes 3–6. Ni<sup>2+</sup>-charged HiTrap Chelating HP 1 ml

Lane 3. Fr. 1 (flow-through)  
 Lane 4. Fr. 2 (flow-through)  
 Lane 5. Fr. 5 (first elution peak)  
 Lane 6. Fr. 10 (second elution peak)

#### Lanes 7–10. Cu<sup>2+</sup>-charged HiTrap Chelating HP 1 ml

Lane 7. Fr. 1 (flow-through)  
 Lane 8. Fr. 2 (flow-through)  
 Lane 9. Fr. 5 (first elution peak)  
 Lane 10. Fr. 9 (second elution peak)  
 Lane 11. LMW

#### Lanes 12–14. Co<sup>2+</sup>-charged HiTrap Chelating HP 1 ml

Lane 12. Fr. 1 (flow-through)  
 Lane 13. Fr. 5 (first elution peak)  
 Lane 14. Fr. 8 (second elution peak)

#### Lanes 15–17. Zn<sup>2+</sup>-charged HiTrap Chelating HP 1 ml

Lane 15. Fr. 1 (flow-through)  
 Lane 16. Fr. 5 (first elution peak)  
 Lane 17. Fr. 8 (second elution peak)

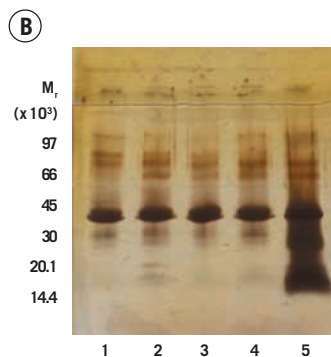
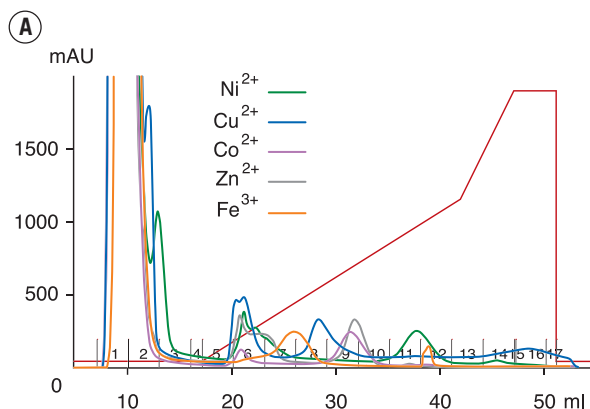
**Fig 2, A-B.** Purification of GFP-(His)<sub>6</sub> on HiTrap Chelating HP 1 ml columns charged with various metal ions.

**A.** Chromatogram showing separation of the proteins on Ni<sup>2+</sup> (green), Cu<sup>2+</sup> (blue), Co<sup>2+</sup> (purple) and Zn<sup>2+</sup> (gray).

**B.** SDS-PAGE analysis of peak fractions for purity.

**(His)<sub>6</sub>-ribokinase**

**Column:** HiTrap Chelating HP 1 ml  
**Sample:** (His)<sub>6</sub>-ribokinase  
**Sample load:** 2.0 ml  
**Binding buffer (A):** 20 mM Tris-HCl, pH 7, 0.5 M NaCl, 5 mM imidazole  
**Elution buffer (B):** 20 mM Tris-HCl, pH 7, 0.5 M NaCl, 0.5 M imidazole  
**Flow rate:** 0.5 ml/min (78 cm/h)  
**Fractions:** 3 ml  
**Gradient:** 25 CV 0–60% elution buffer, 5 CV 60–100% elution buffer  
**System:** ÄKTAexplorer 10



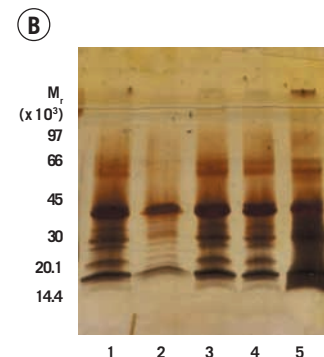
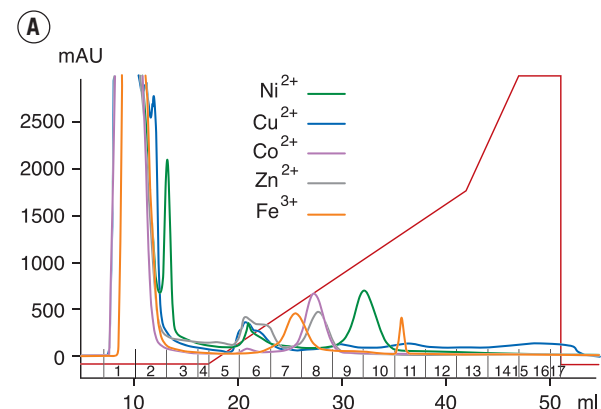
Lane 1. Ni<sup>2+</sup>-charged column: Fr. 11 (second elution peak)  
 Lane 2. Cu<sup>2+</sup>-charged column: Fr. 8 (second elution peak)  
 Lane 3. Co<sup>2+</sup>-charged column: Fr. 9 (second elution peak)  
 Lane 4. Zn<sup>2+</sup>-charged column: Fr. 9 (second elution peak)  
 Lane 5. Fe<sup>3+</sup>-charged column: Fr. 7 (elution peak)

**Fig 3, A-B.** Purification of (His)<sub>6</sub>-ribokinase on HiTrap Chelating HP 1 ml columns charged with various metal ions.

- A.** Chromatogram showing separation of the proteins on Ni<sup>2+</sup> (green), Cu<sup>2+</sup> (blue), Co<sup>2+</sup> (purple), Zn<sup>2+</sup> (gray), and Fe<sup>3+</sup> (orange).
- B.** SDS-PAGE analysis of peak fractions.

**(His)<sub>6</sub>-REX**

**Column:** HiTrap Chelating HP 1 ml  
**Sample:** (His)<sub>6</sub>-REX  
**Sample load:** 2.0 ml  
**Binding buffer (A):** 20 mM Tris-HCl, pH 7, 0.5 M NaCl, 5 mM imidazole  
**Elution buffer (B):** 20 mM Tris-HCl, pH 7, 0.5 M NaCl, 0.5 M imidazole  
**Flow rate:** 0.5 ml/min (78 cm/h)  
**Fractions:** 3 ml  
**Gradient:** 25 CV 0–60% elution buffer, 5 CV 60–100% elution buffer  
**System:** ÄKTAexplorer 10



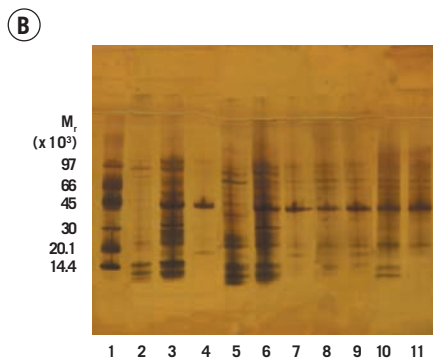
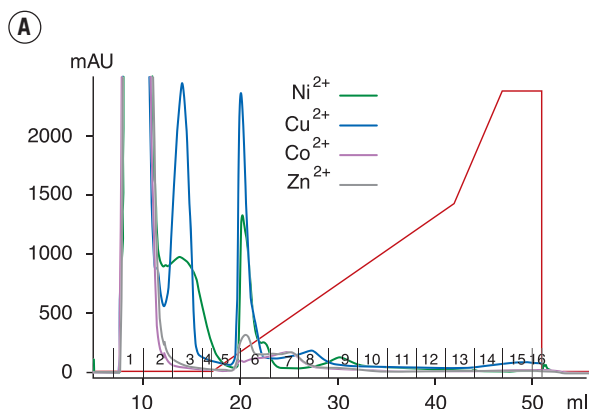
Lane 1. Ni<sup>2+</sup>-charged column: Fr. 10 (second elution peak)  
 Lane 2. Cu<sup>2+</sup>-charged column: Fr. 9 (elution peak)  
 Lane 3. Co<sup>2+</sup>-charged column: Fr. 8 (second elution peak)  
 Lane 4. Zn<sup>2+</sup>-charged column: Fr. 8 (second elution peak)  
 Lane 5. Fe<sup>3+</sup>-charged column: Fr. 7 (first elution peak)

**Fig 4, A-B.** Purification of (His)<sub>6</sub>-REX on HiTrap Chelating HP 1 ml columns charged with various metal ions.

- A.** Chromatogram showing separation of the proteins on Ni<sup>2+</sup> (green), Cu<sup>2+</sup> (blue), Co<sup>2+</sup> (purple), Zn<sup>2+</sup> (gray) and Fe<sup>3+</sup> (orange).
- B.** SDS-PAGE analysis of peak fractions. A smaller amount of protein was loaded onto the gel for fraction 9 from the Cu<sup>2+</sup>-charged column.

**(His)<sub>6</sub>-scFv fragment (inclusion bodies)**

**Column:** HiTrap Chelating HP 1 ml  
**Sample:** (His)<sub>6</sub>-scFv fragment  
**Sample load:** 2.0 ml  
**Binding buffer (A):** 20 mM Tris-HCl, pH 7, 8 M urea, 0.5 M NaCl, 5 mM imidazole  
**Elution buffer (B):** 20 mM Tris-HCl, pH 7, 8 M urea, 0.5 M NaCl, 0.5 M imidazole  
**Flow rate:** 0.5 ml/min (78 cm/h)  
**Fractions:** 3 ml  
**Gradient:** 25 CV 0–60% elution buffer, 5 CV 60–100% elution buffer  
**System:** ÄKTA explorer 10



Lane 1. LMW (Low Molecular Weight Kit, Amersham Biosciences)

**Lanes 2–4. Ni<sup>2+</sup>-charged HiTrap Chelating HP 1 ml**

Lane 2. Fr. 3 (wash)  
 Lane 3. Fr. 6 (first elution peak)  
 Lane 4. Fr. 9 (second elution peak)

**Lanes 5–7. Cu<sup>2+</sup>-charged HiTrap Chelating HP 1 ml**

Lane 5. Fr. 3 (wash)  
 Lane 6. Fr. 6 (first elution peak)  
 Lane 7. Fr. 8 (second elution peak)

**Lanes 8–9. Co<sup>2+</sup>-charged HiTrap Chelating HP 1 ml**

Lane 8. Fr. 6 (elution peak)  
 Lane 9. Fr. 7 (elution peak)

**Lanes 10–11. Zn<sup>2+</sup>-charged HiTrap Chelating HP 1 ml**

Lane 10. Fr. 6 (elution peak)  
 Lane 11. Fr. 7 (elution peak)

**Fig 5, A-B.** Purification of (His)<sub>6</sub>-scFv under denaturing conditions on HiTrap Chelating HP 1 ml columns charged with various metal ions.

- A.** Chromatogram showing separation of the proteins on Ni<sup>2+</sup> (green), Cu<sup>2+</sup> (blue), Co<sup>2+</sup> (purple) and Zn<sup>2+</sup> (gray).  
**B.** SDS-PAGE analysis of peak fractions.

## 2. Purification of a protein tagged with (His)<sub>6</sub> at its N-terminus, C-terminus and both N- and C-termini using different metal ions

To determine if the position of a histidine tag can affect protein purification by IMAC, three variants of Maltose Binding Protein (MBP), M<sub>r</sub> 43 000 were constructed. These variants contained either a single (His)<sub>6</sub> tag at the N-terminus, (His)<sub>6</sub>-MBP or C-terminus, MBP-(His)<sub>6</sub> or two (His)<sub>6</sub> tags in both the N- and C-termini positions, (His)<sub>6</sub>-MBP-(His)<sub>6</sub>. The MBP coding sequence was derived from vector pMAL-c2 (New England Biolabs). All three constructs used the vector pET-23a(+) (Novagen), which performs transcription under the control of the T7 promoter. The three MBP variants were expressed as soluble proteins in *E. coli* BL21, where T7 RNA polymerase itself is induced by a temperature shift from 30 to 42 °C. Each of the three proteins was purified using the same protocol on HiTrap Chelating HP 1 ml columns charged with different metal ions (Ni<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, or Zn<sup>2+</sup>). The purity of each protein was visually assessed by SDS-PAGE and silver-staining.

### Disruption of cells

*E. coli* cells expressing (His)<sub>6</sub>-MBP, MBP-(His)<sub>6</sub> and (His)<sub>6</sub>-MBP-(His)<sub>6</sub> were suspended 1:2 (w/v) in 20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and disrupted by sonication (3 x 20 s on ice). The cell lysates were centrifuged for 30 min at 15 000 rpm (Sorvall SS-34 rotor), and the supernatants were used for purification of the proteins.

### Column preparation

A separate HiTrap Chelating HP 1 ml column was prepared for each metal ion solution. Prior to use, the columns were washed using a syringe with 10 column volumes (CV) of distilled water to remove the storage solution (20% ethanol), then charged with ion by loading with 0.5 CV of 0.1 M solutions (in water) of either NiSO<sub>4</sub>, CuSO<sub>4</sub>, CoCl<sub>2</sub>, or ZnCl<sub>2</sub>. Unbound metal ions were removed by washing with 10 CV of distilled water.

After each purification, the columns were stripped using 6 CV of 20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and 50 mM EDTA. Each column was then washed with 10 CV of distilled water prior to ion charging, washing, and loading of a new different protein.

### Protein purification

Prior to loading of sample on the column, each sample was filtered through a 0.45 μm filter. Columns were then loaded with 2 ml of sample and washed with 10 CV of binding buffer (20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and 20 mM imidazole) prior to elution. Proteins were eluted using a 10 CV linear gradient from 0 to 100% elution buffer

(20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and 0.5 M imidazole), followed by 5 CV 100% elution buffer. Columns were finally washed with 2 CV of binding buffer before stripping and re-charging with a new metal ion.

Due to slight variations in the expression levels of the three MBP proteins, the amounts of sample loaded on the columns varied between the runs. To assess the purity of each protein, 1  $\mu$ l samples (non-reduced) were checked by SDS-PAGE using PhastSystem and silver-stained PhastGel Gradient 10–15%.

## Results and discussion

The separation profiles in Figs. 6–8 and the summarized elution conditions in Table 2 indicate that the location of the (His)<sub>6</sub>-tag on the protein N-terminal, C-terminal, or both does affect the strength with which the protein binds to different metal ions. While all three MBP variants bound most strongly to the Ni<sup>2+</sup>-charged column, some differences were noted with the other metal ions, depending on the position of the tag. When the (His)<sub>6</sub>-tag was attached to the N-terminus of the protein, binding to Zn<sup>2+</sup>, Cu<sup>2+</sup>, and Co<sup>2+</sup> columns was very similar, but weaker than that observed with nickel (Fig 6). Protein tagged at its C-terminus bound equally well to Zn<sup>2+</sup> and Co<sup>2+</sup>, but the strength of binding to these ions was weaker compared with Ni<sup>2+</sup> and Cu<sup>2+</sup>.

Columns charged with Ni<sup>2+</sup> and Cu<sup>2+</sup> gave similar binding, and both gave two peaks (Fig 7). The reason for the two peaks on the Ni<sup>2+</sup>- and Cu<sup>2+</sup>-charged HiTrap Chelating HP has not been investigated.

The binding strength for MBP labelled with (His)<sub>6</sub>-tags at both its N- and C-termini also varied. Ni<sup>2+</sup> bound most strongly, followed by Cu<sup>2+</sup>, Zn<sup>2+</sup> and Co<sup>2+</sup> (Fig 8).

For each individual protein, the amount and purity of the proteins recovered from the various purifications were similar (data not shown).

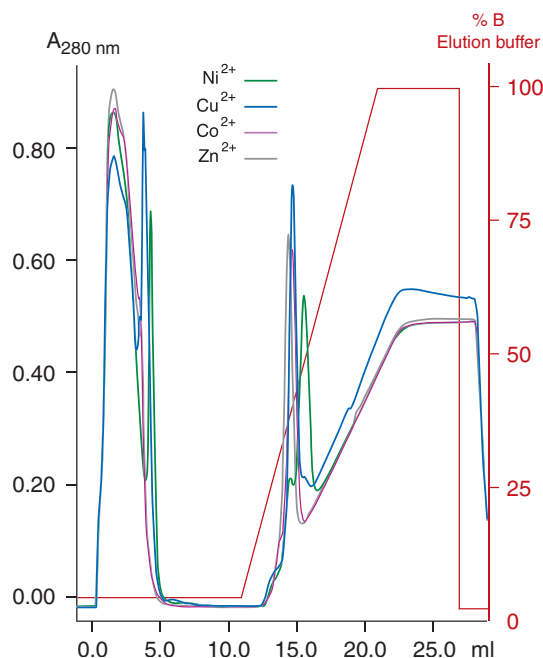
When the purified variants of MBP were checked for their binding strength using BIACORE™ (Fig 9), the MBP variant with two (His)<sub>6</sub>-tags exhibited the most stable binding to the Ni<sup>2+</sup>-charged chip surface, while both of the mono-(His)<sub>6</sub>-tagged MBP variants dissociated rapidly (2). This is an expected result as a double tag will give increased binding strength.

**Table 2.** Summary of elution conditions of (His)<sub>6</sub>-MBP, MBP-(His)<sub>6</sub> and (His)<sub>6</sub>-MBP-(His)<sub>6</sub> on different metal ions. The higher % elution buffer (B) needed for elution the stronger binding of the protein.

	Elution buffer (B), %		
	(His) <sub>6</sub> -MBP	MBP-(His) <sub>6</sub>	(His) <sub>6</sub> -MBP-(His) <sub>6</sub>
Ni <sup>2+</sup>	46%	24%, 48%	59%
Zn <sup>2+</sup>	35%	33%	43%
Co <sup>2+</sup>	38%	34%	33%
Cu <sup>2+</sup>	38%	23%, 46%	46%

### (His)<sub>6</sub>-MBP

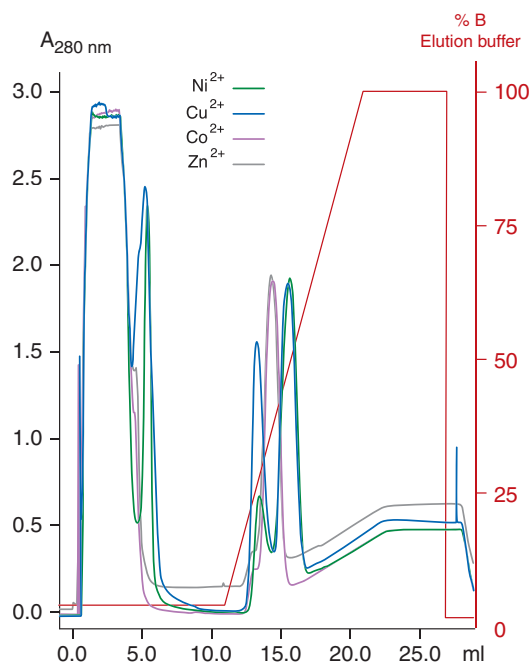
<b>Column:</b>	Zn <sup>2+</sup> -, Cu <sup>2+</sup> -, Co <sup>2+</sup> - or Ni <sup>2+</sup> -charged HiTrap Chelating HP 1 ml
<b>Sample:</b>	(His) <sub>6</sub> -MBP
<b>Sample load:</b>	2 ml
<b>Binding buffer (A):</b>	20 mM sodium phosphate, pH 7.4, 0.5 M NaCl
<b>Wash buffer:</b>	20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 20 mM imidazole
<b>Elution buffer (B):</b>	20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 0.5 M imidazole
<b>Flow rate:</b>	1 ml/min (156 cm/h)
<b>Fractions:</b>	1 ml
<b>Gradient:</b>	Linear, 10 CV 0–100% elution buffer, 5 CV 100% elution buffer



**Fig 6.** Purification of (His)<sub>6</sub>-MBP, (protein is tagged at its N-terminus).

**MBP-(His)<sub>6</sub>**

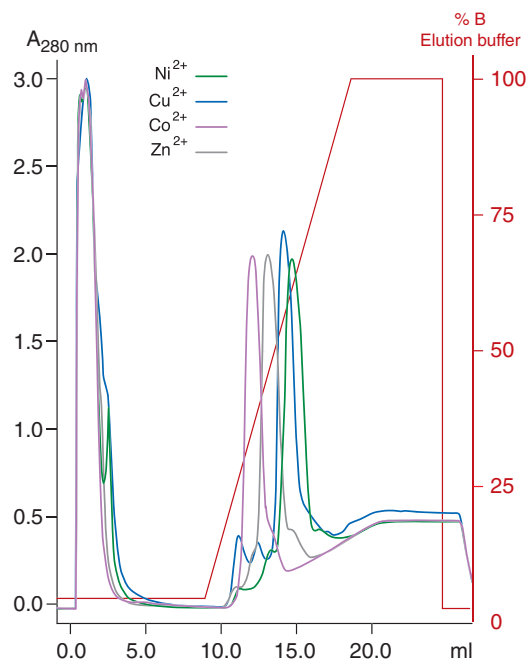
**Column:** Zn<sup>2+</sup>-, Cu<sup>2+</sup>-, Co<sup>2+</sup>- or Ni<sup>2+</sup>-charged HiTrap Chelating HP 1 ml  
**Sample:** MBP-(His)<sub>6</sub>  
**Sample load:** 2 ml  
**Binding buffer (A):** 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl  
**Wash buffer:** 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 20 mM imidazole  
**Elution buffer (B):** 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 0.5 M imidazole  
**Flow rate:** 1 ml/min (156 cm/h)  
**Fractions:** 1 ml  
**Gradient:** Linear, 10 CV 0–100% elution buffer, 5 CV 100% elution buffer



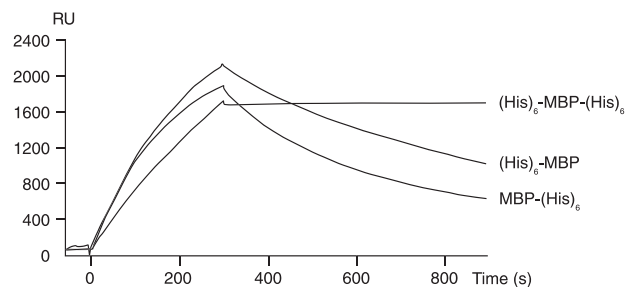
**Fig 7.** Purification of MBP-(His)<sub>6</sub>, (protein is tagged at its C-terminus).

**(His)<sub>6</sub>-MBP-(His)<sub>6</sub>**

**Column:** Zn<sup>2+</sup>-, Cu<sup>2+</sup>-, Co<sup>2+</sup>- or Ni<sup>2+</sup>-charged HiTrap Chelating HP 1 ml  
**Sample:** (His)<sub>6</sub>-MBP-(His)<sub>6</sub>  
**Sample load:** 2 ml  
**Binding buffer (A):** 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl  
**Wash buffer:** 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 20 mM imidazole  
**Elution buffer (B):** 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 0.5 M imidazole  
**Flow rate:** 1 ml/min (156 cm/h)  
**Fractions:** 1 ml  
**Gradient:** Linear, 10 CV 0–100% elution buffer, 5 CV 100% elution buffer



**Fig 8.** Purification of (His)<sub>6</sub>-MBP-(His)<sub>6</sub>, (protein is tagged at its N- and C-termini).



**Fig 9.** Overlay plot showing binding of MBP tagged with (His)<sub>6</sub> at its N-terminus, C-terminus or both N- and C-termini to a Ni<sup>2+</sup>-charged chip using BIACORE. The proteins were all injected for the same length of time and at the same concentration (120 nM). The figure is used with kind permission from Elsevier Science (2).

## Conclusions

The results are summarized in Table 3.

Nickel is the first choice for metal ion according to the results in this investigation using these proteins, but to achieve optimal separation of (His)<sub>6</sub>-tagged proteins from cellular contaminants, it may be necessary to test several metal ions for their ability to bind the tagged proteins. Some ions may be completely ineffective, as was observed with calcium for the six proteins purified in the first section of this study.

As seen in this study, the strength of binding between (His)<sub>6</sub>-tagged proteins and metal ions varies depending on the nature of the protein as well as the ion. Therefore, it is important to keep these factors in mind when making constructs and devising purification schemes, especially those involving proteins that may be difficult to purify or for proteins that should not interact with certain metal ions due to further use.

It is shown that for Maltose Binding Protein (MBP), the position of the (His)<sub>6</sub>-tag can influence how well the protein binds to certain metal ions, and ultimately, how effective the purification is. Although we cannot conclude that all proteins exhibit similar behavior, changing the location of the tag may be an important factor to consider during optimization of an IMAC purification protocol.

**Table 3.** The general patterns observed for the metal ions used to purify the six (His)<sub>6</sub>-tagged proteins and the three variants of MBP used in this study are as follows:

Ni > Cu > Co > Zn	True for GFP-(His) <sub>6</sub> , Lina-(His) <sub>6</sub> , (His) <sub>6</sub> -scFv fragment (inclusion bodies)
Ni > Cu = Co > Zn	(His) <sub>6</sub> -MBP (N-terminus tag)
Ni = Cu > Co = Zn	MBP-(His) <sub>6</sub> (C-terminus tag)
Ni > Cu > Zn > Co	(His) <sub>6</sub> -MBP-(His) <sub>6</sub> (N- and C-termini tags)
Ni > Co = Zn > Cu > Fe	True for (His) <sub>6</sub> -ribokinase, (His) <sub>6</sub> -R5P isomerase (Fe <sup>3+</sup> did not bind)
Ni > Co = Zn > Fe >> Cu	True for (His) <sub>6</sub> -REX

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Linda Öster, Molecular Biology, Swedish University of Agricultural Sciences, Sweden [(His)<sub>6</sub>-REX].

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Jan-Christer Jansson, Center for Surface Biotechnology, Uppsala University, Sweden [(His)<sub>6</sub>-scFv fragment].

Jonas Lidholm, Pharmacia Diagnostics, Uppsala, Sweden [different (His)<sub>6</sub>-tagged variants of MBP].

Åsa Frostell Karlsson, Biacore AB, Uppsala, Sweden [Biacore runs].

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2. Nieba, L. *et al. Analytical Biochemistry* **252**, 217–228 (1997).

## Ordering information

Product	Quantity	Code No.
HiTrap Chelating HP	5 x 1 ml	17-0408-01
HiTrap Chelating HP	1 x 5 ml	17-0409-01
Chelating Sepharose Fast Flow	50 ml	17-0574-01
HiTrap Desalting	5 x 5 ml	17-1408-01
HiPrep™ 26/10 Desalting	1 (20 ml)	17-5087-01
Recombinant Protein Handbook, Protein Amplification and Simple Purification	1	18-1142-75
Affinity Chromatography Handbook, Principles and Methods	1	18-1022-29
Affinity Chromatography, Columns and Media Guide	1	18-1121-86
HiTrap Column Guide	1	18-1129-81

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