

# Addition of imidazole during binding improves purity of histidine-tagged proteins

The purity of histidine-tagged proteins purified by metal chelate affinity chromatography can often be improved by optimizing the imidazole concentration in the sample and binding buffer to achieve a balance between purity and yield of the protein of interest. We<sup>1</sup> determined a concentration range of imidazole that minimized the nonspecific binding of untagged proteins to Ni Sepharose™ High Performance, thereby greatly improving target protein purity. We demonstrate this approach using histidine-tagged protein kinase G ([His]<sub>6</sub>-PknG) from *Mycobacterium bovis*.

## Introduction

The (histidine)<sub>6</sub> tag is one of the most common tags used to facilitate the purification of recombinant proteins. However, the presence of surface-exposed histidine residues or other complex-forming amino acids can lead to nonspecific binding of untagged host cell proteins to purification media. These untagged proteins elute with the target protein and must be subsequently removed. In general, the binding affinity of these contaminants is lower than that of the tagged recombinant proteins, allowing their separation from the protein of interest by using more stringent conditions.

There are several ways to reduce the binding of contaminating proteins to Ni Sepharose media. On one hand, the amount of Ni Sepharose can be adapted to the expected amount of protein to be purified. On the other hand, imidazole can be used as a competitive agent. In this application note, we demonstrate the importance of imidazole as a useful tool to enhance the purity of (His)<sub>6</sub>-PknG purified with Ni Sepharose High Performance.

<sup>1</sup> See acknowledgement.

## Determination of the optimal imidazole concentration

To determine the optimal imidazole concentration to use during the binding of (His)<sub>6</sub>-PknG to Ni Sepharose High Performance, a cell lysate containing (His)<sub>6</sub>-PknG was loaded on a column with 10 mM imidazole in the binding buffer. After additional washing with binding buffer, (His)<sub>6</sub>-PknG was eluted using a linear gradient of up to 50% elution buffer (250 mM imidazole) within 20 column volumes (CV). By analyzing the collected fractions, it was found that most of the contaminants eluted in the range of about 40 to 70 mM imidazole (data not shown).

## Improved purity

Based on this finding, 45 mM imidazole was used in the sample and binding buffer during the final purification procedure. To achieve a higher protein concentration, the protein was eluted in a two-step gradient (Fig 1). To demonstrate the advantageous effect of imidazole, an additional purification was performed under the same conditions except that imidazole was omitted from the sample and binding buffer (Fig 2). SDS-PAGE of the pooled elution fractions indicated a large improvement in purity when 45 mM imidazole was included in the sample and binding buffer (Fig 3).

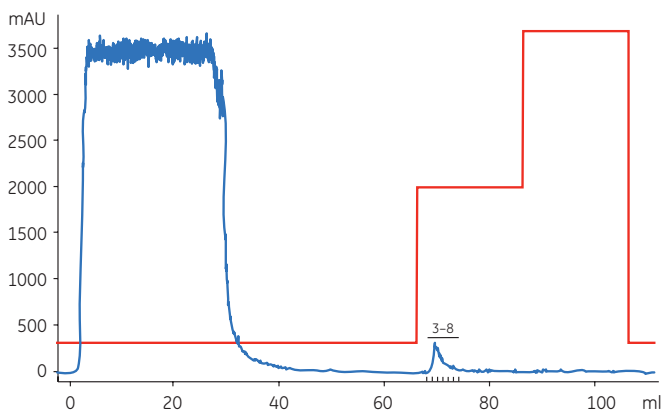
## Conclusion

Using a linear gradient, we determined a concentration range of imidazole that resulted in the removal of a majority of contaminating proteins, while retaining (His)<sub>6</sub>-PknG. The addition of 45 mM imidazole to the sample and binding buffer resulted in much higher purity of the desired protein.



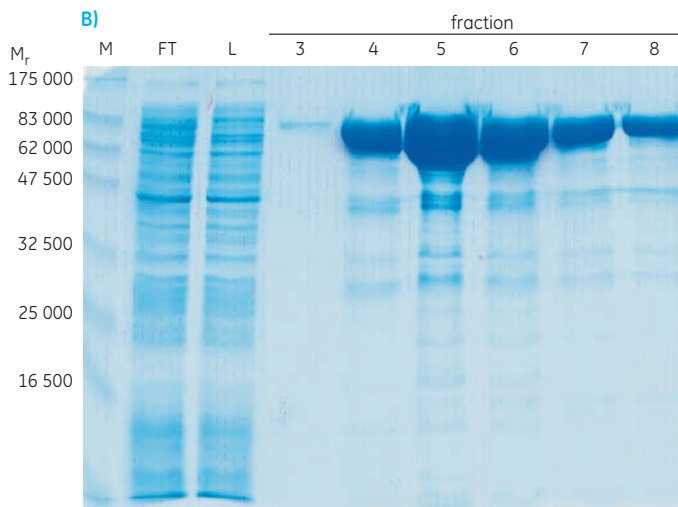
A)

Column: Ni Sepharose High Performance, 2 ml in XK 16/20  
Sample: Histidine-tagged PknG in 26 ml *E. coli* M15 extract  
Binding buffer: 20 mM Tris, pH 8.0, 0.5 M NaCl, 1% Triton™ X-100,  
10% glycerol, 10 mM β-mercaptoethanol, 45 mM imidazole  
Elution buffer: 20 mM Tris, pH 8.0, 0.5 M NaCl, 1% Triton X-100,  
10% glycerol, 10 mM β-mercaptoethanol, 500 mM imidazole  
Gradient: step 50% elution buffer, 20 CV; 100% elution buffer 20 CV  
Flow rate: 1 ml/min  
System: ÄKTApurifier 10



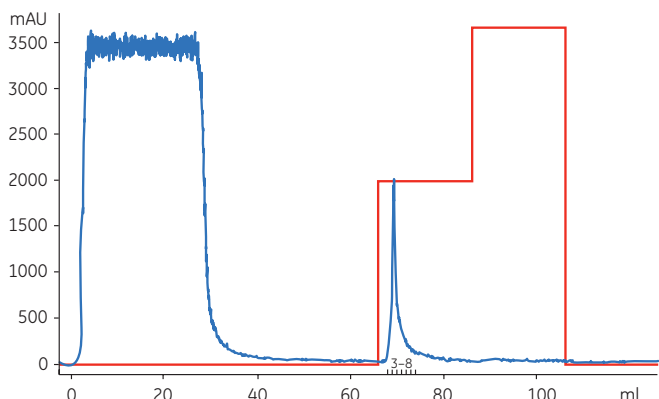
**Fig 1.** Purification of (His)<sub>6</sub>-PknG with 45 mM imidazole in sample and binding buffer. (A) Chromatogram showing the purification of (His)<sub>6</sub>-PknG. The lysate of a 2 l *E. coli* culture (sample volume: 26 ml; filtered through a 0.45-μm syringe filter) was loaded on a 2-ml Ni Sepharose High Performance column (XK 16/20 column) (GE Healthcare) using ÄKTApurifier™. The kinase was eluted in a two-step gradient with 50 and 100% of elution buffer. (B) SDS-PAGE analysis (12% gel) of the purification of (His)<sub>6</sub>-PknG. M: prestained protein marker, broad range (New England Biolabs); L: lysate; FT: flowthrough; (His)<sub>6</sub>-PknG mainly eluted in fractions 4–8 (with an imidazole concentration of 250 mM).

B)



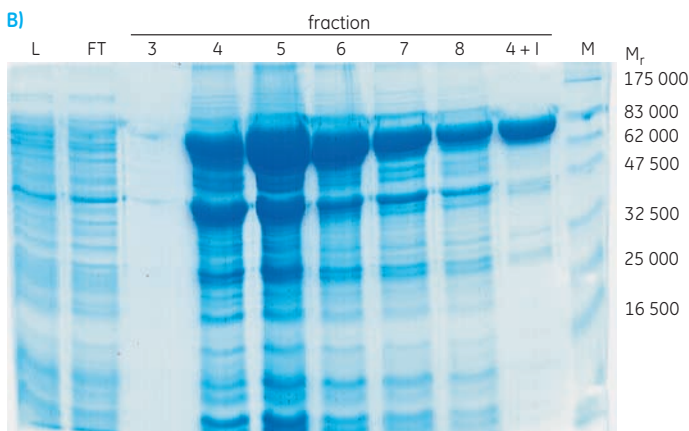
A)

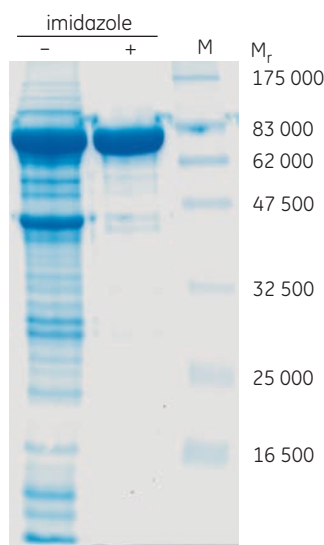
Column: Ni Sepharose High Performance, 2 ml in XK 16/20  
Sample: Histidine-tagged PknG in 26 ml *E. coli* M15 extract  
Binding buffer: 20 mM Tris, pH 8.0, 0.5 M NaCl, 1% Triton X-100,  
10% glycerol, 10 mM β-mercaptoethanol  
Elution buffer: 20 mM Tris, pH 8.0, 0.5 M NaCl, 1% Triton X-100,  
10% glycerol, 10 mM β-mercaptoethanol,  
500 mM imidazole  
Gradient: step 50% elution buffer, 20 CV; 100% elution  
buffer 20 CV  
Flow rate: 1 ml/min  
System: ÄKTApurifier 10



**Fig 2.** Purification of (His)<sub>6</sub>-PknG without imidazole in sample and binding buffer. (A) Chromatogram showing the purification of (His)<sub>6</sub>-PknG. Lysate and column as described in Fig 1. The kinase was eluted in a two-step gradient with 50% and 100% of elution buffer. (B) SDS-PAGE (12% gel) of fractions from the purification of (His)<sub>6</sub>-PknG. M: molecular weight marker as in Fig 1; L: lysate; FT: flowthrough; 4 + I: fraction 4 from the purification with imidazole in sample and binding buffer (see Fig 1). (His)<sub>6</sub>-PknG mainly eluted in fractions 4–8 (with an imidazole concentration of 250 mM).

B)





**Fig 3.** SDS-PAGE (12% gel) of  $(\text{His})_6$ -PknG fractions. Comparison of the pooled  $(\text{His})_6$ -PknG eluates purified without (-) or with (+) imidazole (45 mM) in the binding buffer. M: molecular weight marker as in Fig 1.

## Acknowledgement

This work was performed by K. Hölscher, M. Richter-Roth, and B. Felden de Neumann, GPC Biotech AG, Martinsried, Germany.

## Ordering Information

Products	Quantity	Code No.
Ni Sepharose High Performance	25 ml <sup>†</sup>	17-5268-01
Ni Sepharose High Performance	100 ml <sup>†</sup>	17-5268-02
HisTrap™ HP 1 ml *	5 × 1 ml	17-5247-01
HisTrap HP 1 ml *	100 × 1 ml <sup>‡</sup>	17-5247-05
HisTrap HP 5 ml *	1 × 5 ml	17-5248-01
HisTrap HP 5 ml *	5 × 5 ml	17-5248-02
HisTrap HP 5 ml *	100 × 5 ml <sup>‡</sup>	17-5248-05

\* Includes connectors for easy connection to syringe, pump, or chromatography system.

† Larger quantities are available.

‡ Pack size available by special order.

Related products	Quantity	Code No.
HiTrap™ Desalting 5 ml	5 × 5 ml	17-1408-01
HiTrap Desalting 5 ml	100 × 5 ml*	11-0003-29
PD-10 Desalting Column	30	17-0851-01
HiPrep™ 26/10 Desalting	1 × 53 ml	17-5087-01
HiPrep 26/10 Desalting	4 × 53 ml	17-5087-02

\* Pack size available by special order.

Empty lab-scale columns	Quantity	Code No.
Tricorn™ 5/20 column	1	18-1163-08
Tricorn 5/50 column	1	18-1163-09
Tricorn 10/20 column	1	18-1163-13
Tricorn 10/50 column	1	18-1163-14
Tricorn 10/100 column	1	18-1163-15
XK 16/20 column	1	18-8773-01
XK 16/40 column	1	18-8774-01
XK 26/20 column	1	18-1000-72
XK 26/40 column	1	18-8768-01

Accessories	No. Supplied	Code No.
1/16" male/luer female*	2	18-1112-51
Tubing connector flangeless/M6 female*	2	18-1003-68
Tubing connector flangeless/M6 male*	2	18-1017-98
Union 1/16" female/M6 male*	6	18-1112-57
Union M6 female /1/16" male*	5	18-3858-01
Union luerlock female/M6 female	2	18-1027-12
HiTrap/HiPrep, 1/16" male connector for ÄKTAdesign™	8	28-4010-81
Stop plug female, 1/16" <sup>†</sup>	5	11-0004-64
Fingertight stop plug, 1/16" <sup>‡</sup>	5	11-0003-55

\* One connector included in each HiTrap/HisTrap package.

† Two, five, or seven stop plugs female included in HiTrap/HisTrap packages depending on the product.

‡ One fingertight stop plug is connected to the top of each HiTrap/HisTrap column.

Related literature	Quantity	Code No.
Recombinant Protein Handbook	1	18-1142-75
Affinity Chromatography Handbook	1	18-1022-29
Affinity Chromatography Columns and Media Product Profile	1	18-1121-86
HiTrap Column Guide	1	18-1129-81

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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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