

January 01, 2004

Keywords or phrases:

His-Tagged Proteins, GFP, β -glucanase, Metal Affinity Chromatography, Membrane Adsorbers, Sartobind® Lab

Purification of His-Tagged Proteins by Metal Affinity Chromatography with Sartobind® Lab IDA

Dr. Ricarda Busse¹, Dr. Miyako Hirai¹, Dr. Carsten Voss¹, Dr. John Cashman^{2*}

1. Sartorius Stedim Biotech GmbH, August-Spindler-Straße 11, 37079 Göttingen, Germany

2. Sartorius UK Ltd., Blenheim Road, Epsom, KT19 9QQ, United Kingdom

* Correspondence

Email: john.cashman@sartorius.com

Abstract

One of the practical methods to isolate recombinant proteins is to label them for purification by affinity chromatography. A common protein tag for this purpose is a peptide of six to eight histidine residues (His₆ or His₈) cloned at the N- or C-terminus, which enables purification by metal chelate chromatography. This modality is based on the complexing of metal ions such as Ni²⁺, Cu²⁺ or Co²⁺, immobilized on a chromatography matrix, by chelate formation through the imidazole ring of the amino acid histidine. The bound His₆-tagged protein can subsequently be eluted from the matrix selectively with imidazole, which competitively binds to the metal complex. Sartobind® IDA utilizes a cellulosic membrane matrix, functionalized with iminodiacetic acid (IDA). These devices therefore enable the immobilization of any suitable metal ion to the membrane for metal affinity purification. In this study, Sartobind® Lab IDA devices were evaluated for binding capacity, effectiveness, and handling in the purification of two hexa-histidine tagged proteins.

Introduction

GFP is commonly used as a reporter protein. During the autocatalytic process, GFP forms a structure that emits green light at 508 nm if stimulated by light with wavelength of between 396 and 475 nm (absorbance maxima). Therefore, it is relatively easy to follow GFP during processing by means of fluorescence light. However, it becomes unstable or loses its fluorescent under certain conditions. GFP may be intracellular when produced in *E. coli* and therefore cells must be disrupted prior to separation.

The gene of β -glucanase is a fusion of glucanase genes from *Bacillus macerans* and *Bacillus amyloliquefaciens* [Borris *et al.*, 1990]. This fusion was cloned into a pET20b+ vector, while the signal sequence of β -glucanase was preserved. Transcription in *E. coli* was carried out under the IPTG-inducible T7 promoter. A secretion cassette is located on the plasmid. It consists of the gene for the bacterial release protein (kil) under the control of the weak stationary phase promoter *fic* [Miksch *et al.*, 1997]. The moderate expression of *kil* allows the secretion of the glucanase from the periplasm into the culture medium. The isolation and purification of β -glucanase can therefore be carried out after removal of *E. coli* from the fermentation medium.

Sartobind® Lab IDA devices offer the possibility to purify virtually any his-tagged target protein by affinity purification. Supplied uncharged, the user is free to choose the metal ion of their choice, to achieve optimum purification conditions. Furthermore, the integral Luer connectors enable convenient use with syringes, while UNF 10-32 adapters included with the devices provide flexibility for operation by peristaltic pump or FPLC systems. In this study, we demonstrate the performance of Sartobind® Lab IDA devices (Figure 1) for metal affinity purification, using Cu^{2+} to isolate both GFP and β -glucanase carrying His₆ tags at their N-termini.



Figure 1: Sartobind® Lab IDA Membrane Adsorber Device

Materials and Methods

Production of His₆-GFP

BL21 *E. coli* were transfected with pHis₆-GFP. Cells were cultivated in 300 mL medium (10 g/L peptone, 10 g/L yeast extract, 20 g/L glycerol, 10 g/L NaCl, 100 mg/L ampicillin) in a 1 L baffled flask, inoculated with 1 mL of starter culture. The culture was incubated with shaking at 37 °C overnight, with gene expression induced by IPTG after 2 hours. The cells were isolated by centrifugation, the resulting pellet (23.3 g) suspended in approximately 10 mL of lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8.0) and 25 mg lysozyme dissolved in 1 mL buffer was added (final concentration = 1 mg/mL). Cells were disrupted in an ice bath using pulsed ultrasound and the resulting lysate centrifuged. After removal of 5 mL for analysis, the supernatant was passed through a 0.2 μm filter and the filtrate was taken forward for purification.

Production of His₆- β -glucanase (His₆-bgl)

BL21 (DE3) *E. coli* cells transfected with pET-bgl-His₆-sec were cultivated under the same conditions as for His₆-GFP production. The cells were harvested by centrifugation at 11,000 g for 30 min. A portion of the supernatant was passed through a 0.2 μm filter and the filtrate was used for purification.

Preparation of Sartobind® IDA Membrane Adsorber

Each Sartobind® Lab IDA device containing 9.3 cm² adsorption area (3x layers of 3.1 cm² membrane discs) was flushed with 10 mL of deionized water, which was slowly pumped from bottom to top until the unit was vented completely. Then the remaining water was applied to the top of the device and allowed to flow through by gravity. The following solutions were passed through the device in the same way:

1. 0.5 M CuSO_4
(charging buffer)
2. 10 mM imidazole in 25 mM potassium phosphate buffer, pH 8 (equilibration buffer 1)
3. 25 mM potassium phosphate buffer, pH 8
(equilibration buffer 2)

Protein Purification

The filtered lysate of His₆-GFP (11 mL) and cell-free supernatant of His₆-bgl (80 mL) were applied to the Sartobind® Lab IDA units and the flow through fractions collected. The units were washed twice with 10 mL of washing buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole, pH 8.0). Both fractions were collected and pooled. The proteins were eluted by applying 2x 3 mL of elution buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 250 mM imidazole, pH 8.0). Each eluate fraction was retained separately and all samples were stored at -20 °C until analysis.

Protein Analysis

Lysate | cell-free samples, and flow through, wash and eluate fractions were analyzed by polyacrylamide gel electrophoresis (PAGE) to assess purity. Protein activity was also assessed by fluorescence estimation for GFP or enzymatic activity of β -glucanase.

Results and Discussion

His₆-GFP

Cell lysate (Figure 2, lanes 2 and 3) and Sartobind® Lab IDA flow through fraction (lane 4) show a broad range of intracellular proteins. Only the bands of abundant proteins are visible in the wash fractions (lane 5). Eluate 1 (lane 6) shows a clear band below 30 kDa, corresponding to His₆-GFP with an expected molecular weight of 27.7 kDa. The two other bands are intracellular proteins which were also bound by the membrane adsorber – a common occurrence in purifications from whole cell lysates. Eluate 2 (lane 7) showed no detectable proteins, indicating that all bound proteins were recovered in the first elution step.

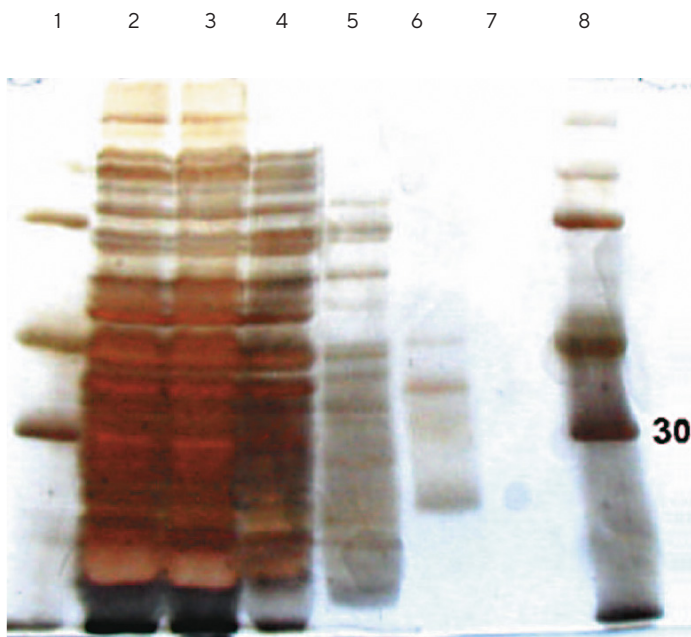


Figure 2: Polyacrylamide Gel Analysis of His₆-GFP Purification Samples

Lane 1: Molecular weight standards
Lane 2: Lysate
Lane 3: Filtered lysate
Lane 4: Flow through
Lane 5: Wash fractions (pooled)
Lane 6: Eluate 1
Lane 7: Eluate 2
Lane 8: Molecular weight standards

His₆- β -glucanase

The samples of cell culture supernatant and Sartobind® Lab IDA flow through were concentrated prior to PAGE. These samples show a distinct, broad band migrating to <30 kDa (Figure 3, lanes 2-4), which corresponds to a high quantity of β -glucanase. The high quantity of this target protein in the flow through suggests that the amount of β -glucanase exceeded the binding capacity of the Sartobind® Lab IDA device. The pooled wash fractions (lane 5) show no detectable protein, indicative of stable binding of the target molecule to the membrane adsorber. Each eluate (lanes 6 and 7) displays a single band of the target protein. This indicates that the membrane adsorber unit was completely saturated with the target protein, in keeping with the previously mentioned detection of a high quantity of β -glucanase in the flow through. No additional proteins were detected in the eluates.

The protein concentration in eluates 1 and 2 was 232 and 127 μ g/mL, respectively, corresponding to a total protein content of 1,077 μ g in the 2x 3 mL fractions. This places the binding capacity of Sartobind® Lab IDA for β -glucanase at approximately 1.1 mg, although we assume a third eluate would have yielded further protein and therefore this represents the minimum capacity of the device (118 μ g/cm² or 4.3 mg/mL, where 1 mL membrane = 36.4 cm²).

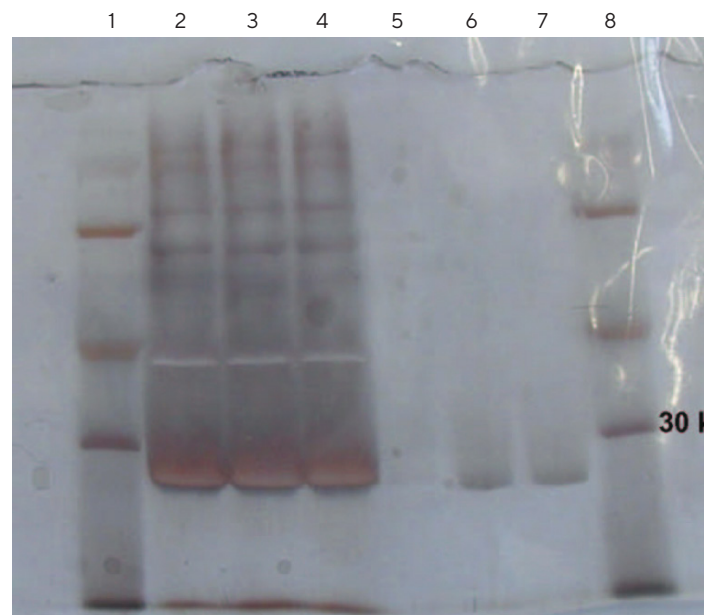


Figure 3: Polyacrylamide Gel Analysis of His₆- β gl Purification Samples

Lane 1: Molecular weight standards
Lane 2: Lysate
Lane 3: Filtered lysate
Lane 4: Flow through
Lane 5: Wash fractions (pooled)
Lane 6: Eluate 1
Lane 7: Eluate 2
Lane 8: Molecular weight standards

Conclusion

Our results show that, when charged with Cu^{2+} , Sartobind® Lab IDA devices are suitable for the purification of both intra- and extra-cellular proteins. The devices exhibited high binding capacities for both His₆-GFP and His₆- β -glucanase, and both proteins were eluted at high purity.

References

Borris, R., Buettner, K. and Maentsaelae, P. (1990). Structure of the beta-1,3-1,4-glucanase gene of *Bacillus macerans*: homologies to other beta-glucanases. *Molecular & general genetics* 222 (2-3), 278-283.

Miksch, G., Fiedler, E., Dobrowolski, P., Fries, K. (1997). The *kil* gene of the ColE1 plasmid of *Escherichia coli* controlled by a growth-phase-dependent promoter mediates the secretion of a heterologous periplasmic protein during the stationary phase. *Arch. Microbiol.* 167 (2+3), 143-150.

This Application Note is made available courtesy of Friehs K., Bielefeld University: Isolierung rekombinanter Proteine mit Membranmodulen. Bericht.

Note


Literature published up to c.2022 may reference the use of Sartobind® MA, which is a name previously used for the Sartobind® Lab membrane adsorbers. When these devices were renamed, there was no change made to fit, form or function. Therefore, results collected and methods established using Sartobind® MA devices remain valid also for Sartobind® Lab.

Germany

Sartorius Lab Instruments GmbH & Co. KG
Otto-Brenner-Straße 20
37079 Göttingen
Phone +49 551 308 0

USA

Sartorius Corporation
565 Johnson Avenue
Bohemia, NY 11716
Phone +1 631 254 4249
Toll-free +1 800 635 2906

 For further information, visit
www.sartorius.com