# Purification of $His_6$-tagged Proteins

## Metal Chelate Chromatography with Sartobind® Membrane Adsorbers


### Introduction

One of the practical methods to isolate recombinant proteins is to label target proteins and to purify them by affinity chromatography. Protein tagged with six histidine ($His_6$)* residues can be purified by metal chelate chromatography. Metal chelate chromatography is based on the complexing of metal ions as $Ni^{2+}$, $Cu^{2+}$ or $Co^{2+}$ immobilized by chelate formation through imidazole ring of the amino acid histidine. The bound $His_6$-tagged* protein can be eluted from the membrane selectively with imidazole, which competitively binds to the metal complex. Sartobind IDA enables to immobilize suitable metal ions on its membrane and allows subsequent purification. For evaluation the parameters as capacity, effectiveness and handling are used.

### Materials and Methods

#### Proteins

Green fluorescent protein (GFP) and β-glucanase were used, which were fused with $His_6$ residues to the N-terminus.

GFP is commonly used as 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 (stimulation maximums). Therefore it is relatively easy to follow GFP during processing by means of fluorescence light. However, it becomes unstable or looses its fluorescence under certain conditions. GFP is an intracellular protein produced in *E.coli* that has to 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 by the T7 promoter which was induced by IPTG. 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 medium. The isolation and purification of β-glucanase from fermentation medium was carried out after removal of *E. coli* cells.

#### Production of $His_6$-tagged GFP ($His_6$-GFP)

*E.coli* BL21 p$His_6$-GFP was cultivated under the same condition as the glucanase and the cells were separated by centrifugation. The pellet (23.3 g) was suspended in approx. 10 ml of lysis buffer (50 mM $NaH_2PO_4$, 300 ml NaCl, 10 mM imidazole, pH 8.0, lysozyme 1 mg/l), and 25 mg of lysozyme dissolved in 1 ml buffer was added. The cells were disrupted in ice bath using pulsed ultrasound. The lysate was centrifuged. After removal of 5 ml for analysis, the supernatant was passed through a 0.2 µm filter. The filtrate was used for purification.

#### Production of Bgl-His

*E.coli* BL21 (DE3) pET-bgl-$His_6$-sec was 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. One milliliter of the culture was inoculated and incubated overnight at 37°C on the shaker. The induction with IPTG was carried out after 2 hours. The cells were harvested by centrifugation at 11,000 x g for 30 min. A part 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 IDA unit containing 9.3 $cm^2$ adsorption area (3 × 3.1 $cm^2$ membrane disks put into a filter holder) was flushed with 10 ml of deionized water, which was slowly pumped from bottom to top until the unit was vented completely. Then the remained water was allowed to run by gravity. The following solutions were passed through in the same way.

1. 0.5 M $CuSO_4$ (charging buffer)
2. 0.01 M Imidazol in 0.025 M $KPO_4$, pH 8 (equilibration buffer 1)
3. 0.025 M $KPO_4$, pH 8 (equilibration buffer 2)

#### Purification of Proteins

The filtered lysate of $His_6$-GFP (11 ml) and the filtered cell-free supernatant of Bgl-Hg (80 ml) were applied to the Sartobind IDA units. The flow-through was collected. The units were washed twice with 10 ml of washing buffer (50 mM $NaH_2PO_4$, 300 ml NaCl, 20 mM imidazole, pH 8.0). All wash fractions were collected together. The proteins were eluted twice with 3 ml of elution buffer (50 mM $NaH_2PO_4$, 300 ml NaCl, 250 mM imidazole, pH 8.0). Each eluate was collected separately. All obtained fractions were stored at -20°C.

#### Protein Analysis

Polyacrylamide gel electrophoresis (PAGE) and estimation of fluorescence of GFP and enzyme activity of Bgl were carried out.

## Results and Discussion

$His_6$-GFP

Lysate (lane 2, 3) and flow through (lane 4) show plenty of bands of intracellular proteins. Only the bands of the proteins in high concentrations are visible in wash fraction (lane 5). Eluate 1 (lane 6) shows a clear band below 30 kD. This corresponds to the $His_6$-GFP with 27.7 kD. The two other bands are intracellular proteins that were also bound. It is common occurrence at lysate purification. Eluate 2 (lane 7) shows no band what indicates all proteins have been eluted in the first step.

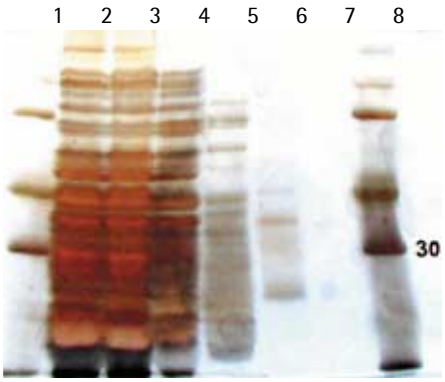


Fig. 1 PAGE of GFP purification steps

1 Size standard
2 Lysate
3 Lysate after filtration
4 Flow-through
5 Wash fraction
6 Eluate 1
7 Eluate 2
8 Size Standard

Bgl-His

The samples of supernatant and flow through were concentrated. These lanes show the clear broad bands of the glucanase. There is still the very broad band in the flow-through what suggests the concentration of the glucanase was too high to isolate entire protein. The wash fraction (lane 5) show no visible band, that means, the target protein binding was stable. Each eluate (lane 6, 7) shows one band of the target protein. This indicates that the Membrane Adsorber unit was really completely saturated with the target protein. The transparent background demonstrates additionally that there were no proteins in remarkable amount which might have bound to the unit.

The protein concentration in eluate 1 and 2 were 232 and 127 µg/ml, respectively. Since 3 ml of each were collected, the total amount is 1077 µg. With assumption that a third eluate would still contain some protein, the minimum capacity is approx. 1.1 mg which represents a binding capacity of 118 µg/$cm^2$ or 4.3 mg/ml (1 ml = 36.4 $cm^2$ membrane).

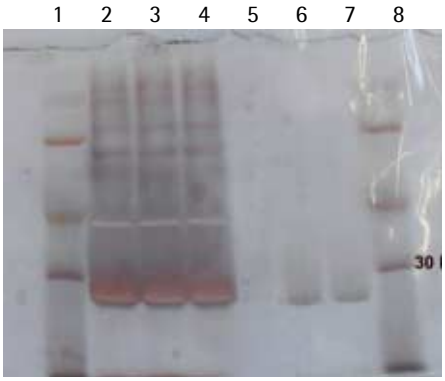


Fig. 2 PAGE of Bgl purification steps

1 Size standard
2 Culture supernatant**
3 Culture supernatant after filtration**
4 Flow-through**
5 Wash fraction
6 Eluate 1
7 Eluate 2
8 Size Standard

## Conclusion

The Sartobind IDA membrane charged with $Cu^{2+}$ are suitable for purification of both intracellular and extracellular proteins.

## References

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