Cube Biotech

Application Note

PURIFICATION OF *IN VIVO* BIOTINYLATED AVI-TAG PROTEINS USING STREP-TACTIN®XT

One-step purification with mild elution directly from cell culture supernatants

Introduction

The streptavidin-biotin interaction is a popular choice for applications that require efficient immobilization of proteins for analytical applications, but is not suitable for initial protein purification. The high affinity interaction requires harsh elution conditions such as high concentrations of guanidine HCl (GuHCl) or boiling. Consequently, the functionality of a protein is affected, preventing further downstream applications. Therefore, protein purification is usually done via a second affinity tag.

Here, we demonstrate that a second affinity tag is not needed for purifying *in vivo* biotinylated Avi-tag proteins from cell culture supernatants. By using Strep-Tactin®XT as ligand, a streptavidin mutant with lower affinity to biotin, mild elution and thus easier purification of Avi-tagged proteins could be achieved. As an additional advantage, Strep-Tactin®XT could be regenerated several times, highlighting it as a cost-effective and sustainable option for biotinylated protein purification.

Results

Strep-Tactin®XT yields highly pure *in vivo* biotinylated protein under mild elution conditions

Due to the reversible binding of biotin to Strep-Tactin®XT, we hypothesized that this ligand might be suitable for purifying biotinylated proteins under mild elution conditions. As proof of principle, we used Strep-Tactin®XT 4Flow® high capacity to isolate CD56 antigen, which was *in vivo* biotinylated via Avi-tag, from cell culture supernatants. We observed efficient enrichment of highly pure protein, while only low amounts of protein were found in wash and flow-through fractions **(Figure 1A).** Target protein purity was at least 95% as determined by analytical SEC **(Figure 1B).** This demonstrates that Strep-Tactin®XT is suitable for efficiently purifying biotinylated proteins, while only mild elution with biotin is required.

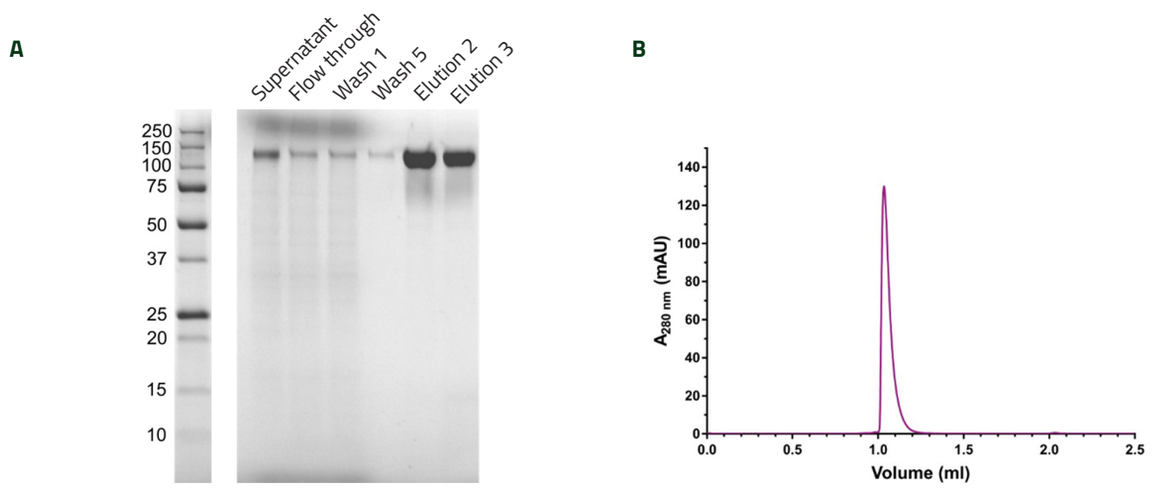

Figure 1: Purification of *in vivo* biotinylated CD56 antigen from cell culture supernatants. CD56 antigen was *in vivo* biotinylated via Avi-tag and purified from 100 ml cell culture supernatant. Samples were collected during every purification step and analyzed via SDS PAGE (A; representative example). Purity was determined by analytical SEC (B; representative example).

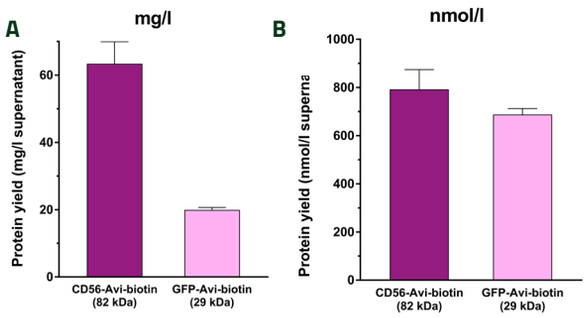

Figure 2: Protein yield for *in vivo* biotinylated CD56 antigen and GFP. CD56 antigen and GFP were *in vivo* biotinylated via Avi-tag (CD56-Avi-biotin and GFP-Avi-biotin, respectively) and purified from 100 ml cell culture supernatants using Strep-Tactin®XT 4Flow® high capacity (n=2 for each protein). Yields were determined with NanoDrop and shown as mg/l **(A)** and nmol/l **(B)**.

Protein yield is independent from protein type

To compare the performance of Strep-Tactin®XT for different types of protein, the yields for biotinylated CD56 antigen (82 kDa) and GFP (29 kDa) were compared. To maximize the yield, protein remaining in flow-through was retrieved using a second gravity flow column. For CD56 antigen, 60 mg/l could be purified, while GFP purification resulted in 20 mg/l protein **(Figure 2A)**. With 700-800 nmol/l, the overall yield for both proteins was similar **(Figure 2B),** indicating efficient purification independent from protein type.

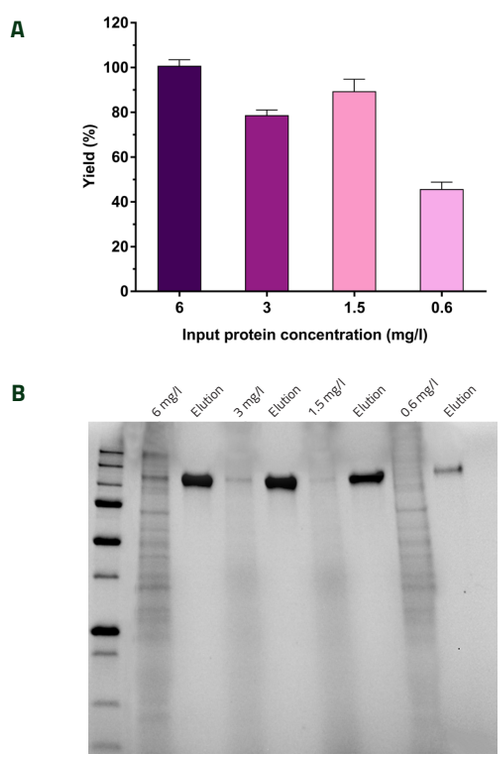

Figure 3: Concentration-dependent protein purification efficiency. Pre-purified biotinylated CD56 antigen was supplemented into cell culture supernatants at different concentrations as indicated. Protein was purified using Strep-Tactin®XT 4Flow® high capacity. Protein yield relative to input was determined for all samples (n=2) **(A)** and purity was evaluated by SDS-PAGE **(B)**.

Protein yield is concentration-dependent

Similar as for the His-tag – nickel (Ni^{2+}) interaction, the affinity of Strep-Tactin®XT to biotin is in a µM range. We tested if this affinity is sufficient for purifying protein from low concentrated samples. To evaluate the yield, pre-purified *in vivo* biotinylated CD56 antigen was supplemented into cell culture supernatant at concentrations ranging from 6 mg/l to 0.6 mg/l, which is much lower than typical expression levels of 140 – 600 mg/l that can be achieved in HEK293 cells[1]. While 1.5 mg/l CD56 antigen still yielded 90% of protein, retrieved protein was significantly less for input concentrations below or equal to 0.6 mg/l **(Figure 3A).** However, high protein purity was observed even for low concentrated samples **(Figure 3B)**. This suggests that purification efficiency of biotinylated proteins with Strep-Tactin®XT 4Flow® high capacity is reduced for very low concentrated samples, but high target protein purity can still be achieved.

Biotin in cell culture media does not affect purification efficiency

Since biotin is the elution agent for Strep-Tactin®XT, we studied if additional biotin in the sample has a negative effect on purification efficiency. Different concentrations of biotin ranging from 300 µg/l (ca. 1 µM) to 2500 µg/l (ca. 10 µM) were supplemented to cell culture medium prior to purification. A control sample without any biotin was included. All samples contained 5 mg of an in vitro biotinylated anti-CD31 Fab fragment. We observed no effect on purification efficiency for all tested biotin concentrations, showing that commercially available cell culture media containing biotin should be compatible with protein purification using Strep-Tactin®XT 4Flow® high

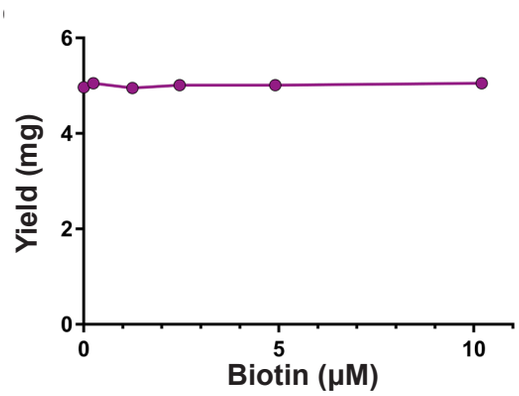

Figure 4: Effect of biotin present in sample on purification efficiency. 50 ml cell culture medium was supplemented with 5 mg biotinylated anti-CD31 Fab fragment and different biotin amounts as indicated. Protein was purified with Strep-Tactin®XT 4Flow® high capacity. Yield was determined with NanoDrop (n=2).

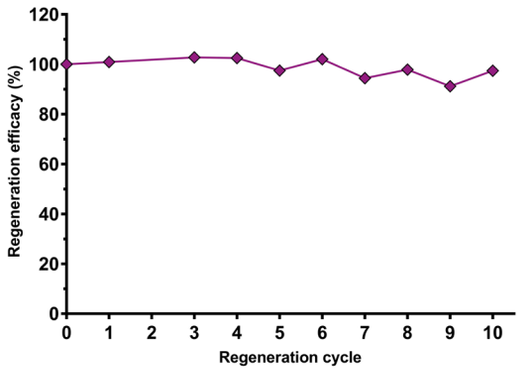

Figure 5: Regeneration efficacy of Strep-Tactin®XT. 3 mg chemically biotinylated BSA was purified from 200 µl Buffer W using Strep-Tactin®XT 4Flow® high capacity in 11 consecutive purification rounds. Regeneration after each round was done with 100 mM NaOH. Regeneration efficacy was determined by comparing relative binding capacities compared to initial purification (n=2, one representative example).

Strep-Tactin®XT can be regenerated at least 10 times

Due to the requirement for harsh elution conditions of conventional streptavidin resins, those resins are suitable for single-use only, which contributes to high costs and a high amount of waste. Since Strep-Tactin®XT reversibly binds to biotin and can be regenerated with NaOH, we tested the efficacy of the regeneration process after purification of biotinylated BSA. The binding capacity of Strep-Tactin®XT 4Flow® high capacity remained stable over at least 10 regeneration cycles, indicating robust reusability and reliability for multiple purification cycles.

Conclusion

Biotinylation is a widely used strategy for protein immobilization. However, the initial purification of biotinylated proteins can be challenging. Strep-Tactin®XT offers an efficient, one-step purification solution that eliminates the need for second affinity tags such as His-tag or the use of harsh elution conditions typically required with streptavidin. Its ability to be reused multiple times not only enhances cost-efficiency but also supports sustainability, all while mild, non-denaturing elution conditions keep biotinylated proteins in their natural state.

Methods

MEXi-293E cells, a derivative of the HEK293 cell line, were transiently transfected with GFP-Avi-tag (29 kDa) or CD56-Avi-tag (82 kDa) and biotin ligase BirA, using PEI (1.5 x 10^6 cells per ml, 75 x 10^6 cells in total) in 50 ml MEXi-TM medium. Afterwards cells were diluted 1:1 with MEXi-CM cell culture medium to a final concentration of 0.75 x 10^6 cells per ml. Cells were cultured for 5-7 days. Cell culture supernatant was collected, centrifuged at 4000 rpm for 40 min at 4°C and subsequently applied to a 1 ml Strep-Tactin®XT 4Flow® high capacity gravity flow column. The WET FRED application aid was used to apply the volume to the column. To maximize yield, flow-through was added to a second Strep-Tactin®XT 4Flow® high capacity gravity flow column where indicated. Washing was done with 1x Buffer W and protein elution was performed with 1x Buffer BXT containing 50 mM biotin. Protein concentration in eluate was determined via absorption at 280 nm using a NanoDrop spectrophotometer. Purity was evaluated by SDS-PAGE and analytical size exclusion chromatography.

To test effective purification from low concentrated samples, different amounts of purified biotinylated CD56 protein were added to 100 ml cell culture supernatants collected from MEXi-293E cells. Final concentrations were 6 mg/l, 3 mg/l, 1.5 mg/l and 0.6 mg/l, respectively.

To evaluate regeneration efficacy of Strep-Tactin®XT 4Flow® high capacity after purification, 3 mg chemically biotinylated BSA was added to 0.2 ml PBS. Protein was purified using a 0.2 ml Strep-Tactin®XT 4Flow® high capacity gravity flow column. After elution with 1x Buffer BXT, columns were regenerated with 100 mM NaOH. Prior to the next round of purification, columns were rinsed with 1.6 ml 1x Buffer W.

References

[1]Jäger et al., High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells. J Vis Exp. 2015 Dec 28;(106):53568. doi: 10.3791/53568

Cube Biotech Creative-Campus-Allee 12 · 40789 Monheim · Germany · Phone +49 2173993730 · contact@cube-biotech.com

Version AN02-38-0001