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Efficient purification of GFP-tagged membrane proteins

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Summary

Green Fluorescent Protein (GFP) is a protein tag widely used for the analysis and visualization of membrane proteins. Here, we demonstrate the use of the affinity resin ChromoTek GFP-Trap® for the purification of a GFP-tagged membrane protein, the Class A G protein-coupled receptor (GPCR) cannabinoid receptor CB_1. Using the GFP-Trap®, we produce pure, tag-free, and monodisperse CB_1 in a single day. We anticipate that the GFP-Trap® will enable the streamlining of membrane protein purification for many downstream applications such as pharmacological or cryo-electron microscopy (cryo-EM) structural analyses.

Introduction

GFP is arguably the most highly decorated protein tag since the 2008 Nobel Prize in Chemistry was dedicated to its discovery and development. The use of the GFP-tag and its many derivatives is ubiquitous in chemical, biological, and medical research from single-molecule to whole-animal studies.

An increasingly important application of the GFP-tag is the purification of membrane proteins. These pose a notorious challenge owing to their inherent hydrophobicity and thus require detergents and other additives for stabilization in aqueous solution. In answer to these issues, Drew and others (Drew et al., 2006; Drew et al., 2008; Drew et al., 2005; Kawate and Gouaux, 2006; Newstead et al., 2007) established GFP as one of the most widely used fusion tags for the detection and analysis of a membrane protein of interest. The inherent fluorescence of the GFP-tag enables a researcher, for example, to quantify expression levels and solubility, check for the correct subcellular localization, and determine monodispersity using fluorescent size-exclusion chromatography (FSEC). For the purification process, however, other tags such as the His_6-tag were traditionally preferred and fused to the membrane protein.

In this case study, we will demonstrate how the use of the affinity resin ChromoTek GFP-Trap® enables the fast, specific, and efficient purification of GFP-tagged membrane proteins. The ChromoTek GFP-Trap® consists of a recombinant anti-GFP Nanobody. This camelid single-domain antibody is covalently coupled to a cross-linked agarose resin, which is compatible

Figure 1 Crystal structure of the human cannabinoid receptor CB_1 (PDB ID 5U09, Shao et al., Nature 2016). The structure is colored from blue (N-terminus) to red (C-terminus). A co-crystallized inverse agonist, taranabant, is shown in gray. The construct used for crystallization comprised an internal fusion to a Pyrococcus abyssi glycogen synthase domain, which was omitted from this model for clarity.

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with batch, gravity-flow, and FPLC-based affinity purification protocols. Owing to the high stability of the anti-GFP Nanobody, the GFP-Trap® is compatible with a broad range of harsh buffers including a variety of detergents such as n-dodecyl ß-D-maltoside (DDM) and reducing agents such as dithiothreitol (DTT). Furthermore, the GFP-Trap® has a very high affinity, facilitating selective and complete capture even in the face of very low expression.

Here, we will use the GFP-Trap® to purify the membrane protein cannabinoid receptor 1 (CB_1, gene CNR1). This seven-transmembrane helix protein is one of the most abundant GPCRs in the central nervous system (Kendall and Yudowski, 2016). As its name suggests, it is the main receptor of the active ingredient of *cannabis sativa*, Δ^9-tetrahydrocannabinol. The purification of CB_1 is based on seminal work by the Rosenbaum laboratory at the University of Texas Southwestern Medical Center (Shao et al., 2016). They designed a construct of CB_1 suitable for expression in insect cells as well as crystallization (see Figure 1 for the CB_1 crystal structure). To this end, they truncated, among other tweaks, the N- and C-terminal ends and inserted a solubilization domain into intracellular loop 3 (*Pyrococcus abyssi* glycogen synthase domain; here, we used thermostabilized apocytochrome b562 from *Escherichia coli* [BRIL]), which serves to mediate crystallization contacts. Furthermore, the authors established a purification protocol, which, e.g., includes the addition of taranabant as a synthetic ligand for CB_1 throughout the process. This inverse agonist stabilizes the structure of CB_1 in an inactive conformation.

The original purification protocol by Shao et al. comprises two affinity chromatography (AC) steps followed by a polishing step using SEC. Using the ChromoTek GFP-Trap®, we were able to reduce the number of AC steps and thus speed up the purification process significantly.

Material and Methods

Material

- Expression plasmid for cannabinoid receptor 1 (CB_1) fused to a C-terminal GFP-tag via an HRV-3C protease recognition site, expressed in Sf21 cells
- ChromoTek GFP-Trap® Agarose High Capacity version was used for cost-efficient and high-yield purification. Please inquire about GFP-Trap® Agarose High Capacity via email or **www.ptglab.com**
- Lysis buffer: 50 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 10% glycerol, 1 µM taranabant; supplemented with protease inhibitors such as cOmplete Protease Inhibitor Cocktail tablets (Roche, 1 tablet per 50 mL)
- Solubilization buffer: 50 mM HEPES-NaOH pH 7.5, 500 mM NaCl,1% lauryl maltose neopentyl glycol (LMNG), 0.1% cholesteryl hemisuccinate (CHS), 10% glycerol, 1 µM taranabant supplemented with protease inhibitors such as cOmplete Protease Inhibitor Cocktail tablets (Roche, 1 tablet per 50 mL)
- Wash buffer: 50 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 0.05% LMNG, 0.005% CHS, 10% glycerol, 1 µM taranabant
- Storage buffer: 20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 1 µM taranabant, 0.02% LMNG, 0.002% CHS
- Gravity flow column, e.g., Poly-Prep Chromatography columns, from BioRad
- Amicon® Ultra Centrifugal Filter 100 kDA molecular weight cut-off
- Cytiva Superdex 200 Increase 10/300 in an Äkta Pure FPLC system

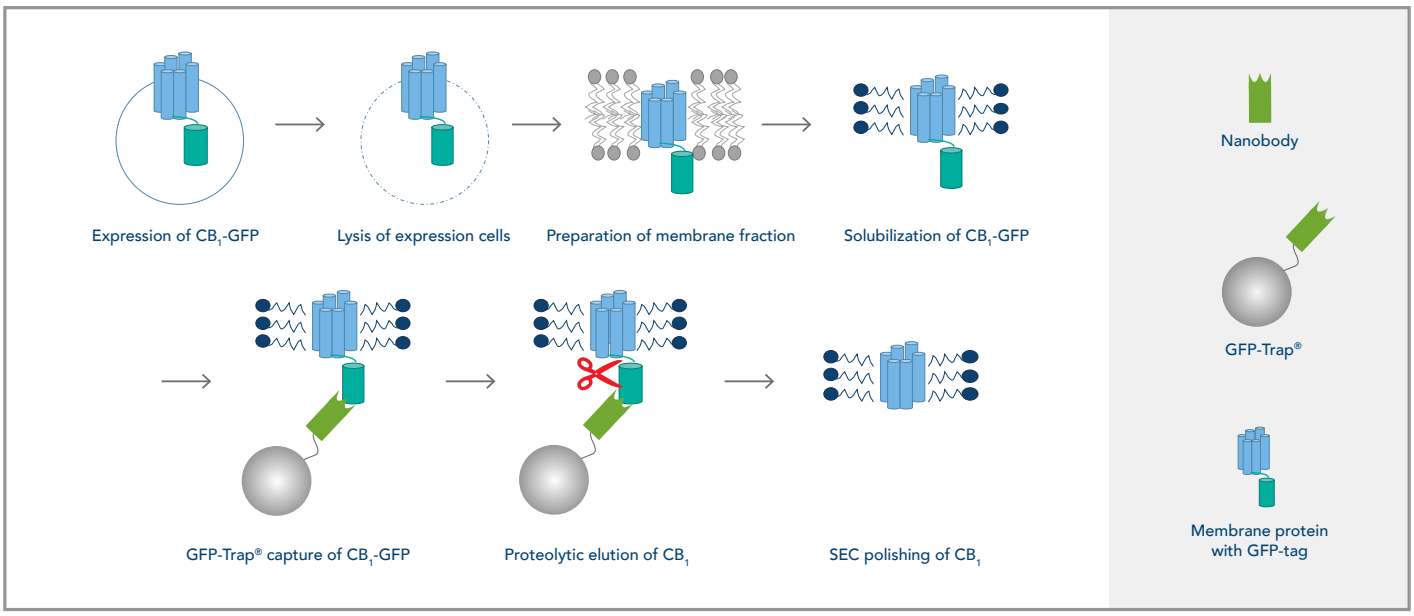

Figure 2 Flowchart for the purification of CB_1 using the GFP-Trap®.

Method

A) Preparation of solubilized CB_1

1. The frozen Sf21 expression cell pellet was resuspended in Lysis buffer (5 mL per 1 g of cells) using a tissue homogenizer.

2. The lysate was clarified by centrifugation to pellet the membrane fraction (47,900 g, 30 min, +4°C). The supernatant was carefully removed and discarded.

3. The membrane pellet was resuspended and incubated in Solubilization buffer (5 mL per 1 g of cells) for 1.5 h to solubilize the CB_1 protein.

4. The solubilized lysate was clarified by centrifugation (47,900 g, 30 min, +4°C) and the supernatant was collected.

B) Equilibration of GFP-Trap® Agarose High Capacity

5. GFP-Trap® Agarose High Capacity beads (5 mL of 50 % slurry, 2.5 mL bed volume [BV]) were transferred to a falcon tube and sedimented using centrifugation (2,500 g, 5 min). The supernatant was carefully removed and discarded.

6. The beads were equilibrated with Wash buffer (10 BV, 25 mL).

7. The beads were sedimented by centrifugation (2,500 g, 5 min) and the supernatant was discarded.

C) Capture of solubilized CB_1

8. The cleared, solubilized lysate was added to the equilibrated GFP-Trap® beads and incubated for 1 h with mild agitation on a rotating wheel.

9. The beads were transferred to a gravity flow column.

10. Unbound protein was released by draining the flow column ("Flow-through").

11. The beads were washed three times with 10 mL of Wash buffer (12 BV in total) for each step.

D) Proteolytic elution of CB_1

12. 125 µg HRV-3C protease and 1 mM DTT were added to the beads and incubated for 3 h.

13. The released protein was eluted from the beads by draining the gravity flow column (eluate).

14. The beads were washed three times with 2.5 mL Wash buffer to elute the remaining released CB_1.

15. The eluate was concentrated to a volume of ca. 1.5 mL using an Amicon® Ultra Centrifugal Filter.

E) Polishing of CB_1

16. An SEC column was equilibrated with Storage buffer.

17. The concentrated CB_1 eluate was subjected to preparative SEC.

18. The eluted CB_1 was injected at a flow rate of 0.5 mL/min to the SEC column. Eluted protein was collected in 0.5 mL fractions.

Results

CB_1, a GPCR membrane protein, was successfully purified using the ChromoTek GFP-Trap® Agarose High Capacity (see Figure 2 for a summary of the process). A cleavable fusion of CB_1 with GFP was designed based on literature (Shao et al., 2016) and expressed in Sf21 insect cells. After cell lysis in a detergent-free buffer (see Figure 3 for an SDS-PAGE analysis), the membrane fraction was solubilized using LMNG and CHS. The solubilized CB_1 was successfully purified by the GFP-Trap® (see the lane for "resuspended resin" in Figure 3). Bound protein was eluted via HRV-3C protease, concomitantly removing the GFP-tag from the target protein CB_1.

The eluted protein was finally separated from HRV-3C protease using SEC. CB_1 was eluted as a single peak (Figure 4A), indicating that this protein is monodisperse and free of aggregation. The resulting CB_1 protein is >90 % pure, as suggested by SDS-PAGE analysis of the SEC fractions (Figure 4B). Peak fractions containing CB_1 were pooled such that the final yield was 0.8 mg of CB_1 at a concentration of 2 mg/ml. The identity of this protein was confirmed using peptide mass fingerprinting.

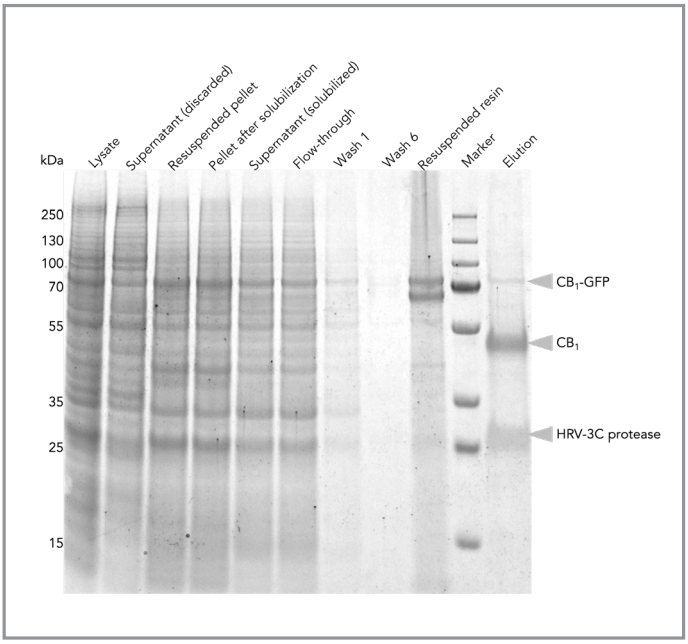

Figure 3 Purification of CB_1 using the GFP-Trap®. SDS-PAGE analysis of the solubilization and capture of CB_1-GFP followed by its proteolytic release.

Discussion

In this case study, we used the ChromoTek GFP-Trap® Agarose High Capacity to purify the GPCR membrane protein CB_1. The use of the GFP-Trap® allowed us to reduce the number of AC steps to one, compared to two in Shao et al.'s original protocol (Shao et al., 2016). In addition, we combined CB_1 elution with GFP-tag removal by performing an on-bead digest using the efficient protease HRV-3C.

Thus, by using the GFP-Trap®, we were able to condense the purification protocol for CB_1 to a one-day process. Despite the significant reduction in processing time, the use of the GFP-Trap® for affinity purification provided comparable protein quality in terms of purity and monodispersity. Furthermore, the purified CB_1 is automatically tag-free, abolishing any adverse effects of the GFP-tag in downstream applications. Most importantly, the GFP-Trap® allowed us to adapt the buffer and elution conditions to fit the needs of the CB_1 protein (and not the other way round as with some affinity media). Furthermore, proteolytic elution avoids dramatic shifts in buffer composition associated with other affinity media such as high imidazole concentrations, which may be deleterious to sensitive proteins.

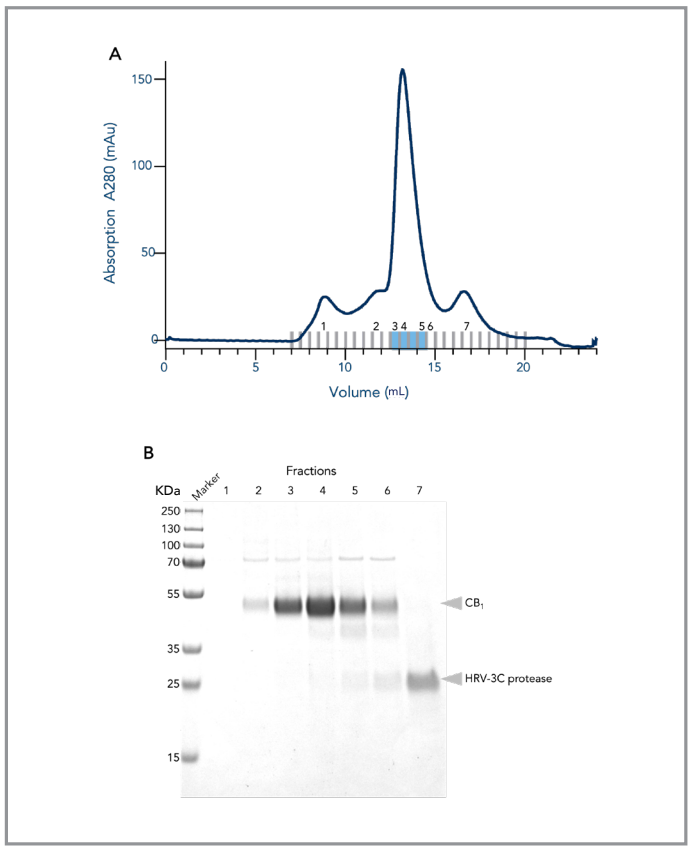

Figure 4 Purification of CB_1 using SEC polishing step. (A) Chromatogram of the SEC polishing step; gray bars indicate fractions. Numbered fractions were subjected to SDS-PAGE analysis in (B). Fractions highlighted in blue were pooled.

In summary, membrane protein purification using GFP-Trap® yields material of sufficient quantity and purity for biochemical, pharmacological, and structural analyses within a day. We foresee that the use of the GFP-Trap® will particularly benefit research into GPCRs, but also other delicate proteins and protein complexes, as it minimizes purification time and thus potential degradation as well as loss of ligands or binding partners.

Both academic research and industrial structure-based drug design have recently been ushered into a new era of structural biology by the "resolution revolution" in the field of cryo electron microscopy (cryo-EM; Kühlbrand, 2014). Indeed, the determination of GPCR structures is now deemed "routine" work thanks to these developments (Danev et al., 2021). Thus, the bottleneck is shifting from structure elucidation to GPCR production. We are confident that the GFP-Trap® will further accelerate this process by removing the production bottleneck and streamlining the purification of GPCRs as well as other membrane proteins.

Literature

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