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Use of Tandem Affinity Chromatography for Purification of Cannabinoid Receptor CB₂

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Abstract

Tandem affinity purification has been increasingly applied to isolation of recombinant proteins. It relies on two consecutive chromatographic steps that take advantage of the affinity tags placed at opposing ends of the target protein. This allows for efficient removal of contaminating proteins, including products of proteolytic degradation of the fusion that lack either N- or C-terminal tags. Here, we describe the use of two small affinity tags, a poly-histidine tag and a Strep-tag for expression and purification of the human cannabinoid receptor CB₂, an integral membrane G protein-coupled receptor.

Keywords

His-tag; Strep-tag; Dual affinity tags; Membrane protein; CB₂ receptor; StrepTactin; Ni-NTA

1 Introduction

Affinity tags have become increasingly popular for purification of recombinant proteins.

We describe here the use of a combination of two small affinity tags, the polyhistidine tag [1] and a Strep-tag [2] for expression and purification of human cannabinoid receptor CB₂ [3], a class-A G protein-coupled receptor. The purification is performed in the presence of the nonionic detergent dodecyl maltoside (DDM), the zwitterionic detergent CHAPS as well as a derivative of cholesterol, cholesteryl hemisuccinate, required for stabilization of CB₂ in micelles. The necessity to perform purification in the presence of detergents constitutes a big challenge since they may affect the affinity and specificity of interaction between the tag and the resin by covering the tag and decreasing its accessibility [1].

The polyhistidine, or His-tag, usually contains 5–6 and, in some instances, as many as 10–14 consecutive histidine residues that bind to metal ions such as Ni⁺² or Co⁺² incorporated in different types of resins, allowing for purification of recombinant proteins via immobilized-metal affinity chromatography (IMAC) (Fig. 1a). The procedure described in this chapter is based on the Ni-NTA technology (Qiagen) [4]. The tetradentate chelant nitrilotriacetic acid occupies four valencies of the Ni⁺² ion, with two remaining valencies available for interaction with imidazole rings of histidine residues.

The binding step is usually performed at a pH of 7–8 to ensure that all histidine residues are deprotonated and, consequently, capable of interacting with nickel ions. The elution takes place in the presence of imidazole, which competes with histidine for the metal ions. While the high affinity binding of the His₆-tag to Ni-NTA resin has been reported ($K_D = 14$ nM [5]), both the affinity and specificity of binding can vary dramatically depending on the particular target protein, location of the tag, composition of the binding buffer, accessibility of the tag and the number of chelating residues. To improve the efficiency of interaction with the resin, the tag is usually placed at either N- or C-terminus of the target protein, where (in many cases) it is also less likely to interfere with the biological activity of the recombinant protein.

The second chromatographic step of our protocol involves purification on a StrepTactin resin using the repeat sequence of Strep-tag II (Fig. 1b). Strep-tag II is an 8-amino-acid polypeptide consisting of Trp-Ser-His-Pro-Gln-Phe-Glu-Lys that exhibits affinity (K_D for the binding is 1 μ M) for an engineered streptavidin termed StrepTactin [6, 7]. Because of its small size, the Strep-tag usually does not interfere with the biological activity of its fusion partner. Furthermore, the purification is performed under mild conditions that preserve the bioactivity of most proteins. The elution of the purified recombinant protein from the resin by desthiobiotin, an analog of biotin, is specific since only Strep-tagged proteins are displaced from the interaction with the StrepTactin resin. The resin can be reused after regeneration with the HABA (hydroxy-azophenyl-benzoic acid, pH 8.0) which, when provided in excess, displaces desthiobiotin from the resin.

In this chapter we present protocols for expression of peripheral cannabinoid receptor CB₂ in *E. coli*, its extraction from membranes, the solubilization and stabilization of the recombinant receptor in detergent micelles, its affinity purification using His-tag followed by proteolytic removal of fusion partners by TEV protease, and its final purification via Strep-tag. To achieve efficient purification, a C-terminal His₁₀ as well as two identical Strep-tag sequences bridged by a 4-amino acid linker were attached to the N-terminus of CB₂ to increase the binding affinity to their respective resin [3]. Purification is performed under mild conditions to maintain the functional fold of the receptor. The purity of the resulting CB₂ can be determined by SDS-PAGE followed by Coomassie Blue staining or western blot, by assessing its biophysical properties in micelles through a variety of techniques including circular dichroism spectroscopy and NMR, or by evaluating its functionality by binding of radioligands and measuring its ability to activate cognate G proteins upon reconstitution into proteoliposomes (Fig. 2).

2 Materials

2.1 Expression of CB₂ Fusion Protein and Preparation of Membranes

1. Plasmid pAY-125 for expression of CB₂.
2. BL21(DE3) competent cells.
3. Shaker-incubator with a temperature range capability between 20 and 37 °C.

4. 50 mg/mL Ampicillin stock solution: Dissolve ampicillin in water and filter the solution through a 0.22 μ m filter.
5. 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) stock solution: Dissolve IPTG in water and filter through a 0.22 μ m filter.
6. 20 % (w/v) Glucose stock solution: To 250 mL water slowly add 100 g of glucose while stirring, complete to 500 mL with water and sterilize by autoclaving.
7. Luria-Bertani broth (LB) medium: 10 g/L casein digest, 5 g/L yeast extract, 5 g/L NaCl. Dissolve powder in water and sterilize by autoclaving.
8. Baffled, 125-mL shake flasks. Sterilize by autoclaving.
9. Double-strength YT-medium ($2 \times$ YT): 16 g/L casein digest, 10 g/L yeast extract, 5 g/L NaCl. Autoclave 500 mL medium in 2 L baffled shake flasks.
10. Phosphate-buffered saline (PBS): 10 mM sodium phosphate, 150 mM NaCl, pH 7.4.
11. “Complete” protease inhibitor cocktail tablets (Roche).
12. Membrane storage solution: 10 mL of PBS adjusted to 20 % sucrose (2 g sucrose/10 mL PBS) with 1 tablet of “complete” protease inhibitor cocktail.
13. French press.
14. Handheld glass homogenizer.

2.2 Extraction, Solubilization and Stabilization of CB₂ in Detergent Micelles

1. Tris-buffered saline (TBS): 25 mM Tris-HCl, 130 mM NaCl, 2.7 mM KCl, pH 7.5.
2. EDTA-free protease inhibitor cocktail tablets (Roche).
3. DNase I stock solution: 5 mg/mL DNase I in water, approximately 1,000 U/mL.
4. 1 M MgCl₂ stock solution.
5. Anti foam A (Sigma).
6. $2 \times$ Solubilization buffer: 100 mM Tris-HCl, 400 mM NaCl, 60 % glycerol, pH 7.5.
7. $12 \times$ 3-[(Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)/cholesteryl hemisuccinate (CHS) stock solution: 6 % CHAPS, 1.2 % CHS, store at 4 °C (*see* Note 1).
8. 10 % *n*-dodecyl- β -D-maltoside (DDM) stock solution, store at 4 °C.

¹CHS will not solubilize when resuspended in water directly—it has to be dissolved in a concentrated CHAPS solution. For preparation of 400 mL of the $12 \times$ stock solution of 6 % CHAPS and 1.2 % CHS we recommend the following protocol:

- Take 4.8 g CHS and resuspend in 200 mL of water in a 250-mL beaker under vigorous stirring. Continue stirring through the entire solubilization procedure.
- Dissolve 24 g CHAPS in 150 mL of water with stirring.
- Add CHS suspension drop by drop to the CHAPS solution while stirring, until the solution is clear.
- Adjust volume to 400 mL with water. Solution can be stored at 4 °C for at least 2–3 months.

9. 20 mM CP-55,940 stock solution: Dissolve CP-55,940 in ethanol. Store at -20°C .
10. Cell homogenizer (Avestin) or a sonicator.

2.3 Purification

1. Ni-NTA agarose (Qiagen).
2. Buffer A: 50 mM Tris-HCl, 200 mM NaCl, 0.5 % [w/v] CHAPS, 0.1 % [w/v] CHS, 1 % [w/v] DDM, and 30 % glycerol, pH 7.5.
3. Buffer B: 50 mM Tris-HCl, 200 mM NaCl, 0.5 % [w/v] CHAPS, 0.1 % [w/v] CHS, 1 % [w/v] DDM, and 30 % glycerol, 250 mM imidazole, pH 7.5.
4. Buffer C: 50 mM Tris-HCl, 100 mM NaCl, 15 % glycerol, 0.5 % [w/v] CHAPS, 0.1 % [w/v] CHS, 1 % [w/v] DDM, pH 7.5.
5. TEV protease: Either commercially available or in-house-prepared recombinant tobacco etch virus (TEV) protease can be used. We express and purify the recombinant poly-His-poly-Arg-tagged protease according to established protocols [8 – 10]. Expression in BL21(DE3) harboring the expression plasmid and purification via Ni-NTA affinity chromatography followed by cation exchange chromatography are described in [11].
6. StrepTactin Poros column (EMD Biosciences).
7. 5 μM Desthiobiotin in Buffer A: Weigh the required amount of powder desthiobiotin (EMD Biosciences) in a small Eppendorf tube and carefully dissolve in 100–200 μL of 0.1 N NaOH. Mix with buffer A to achieve a final concentration of 5 μM (*see* Note 2).
8. Centrifugal spin concentrators with a 30–70 kDa molecular mass cutoff.

3 Methods

3.1 Expression of Fusion CB₂ in Shaker Flasks

The expression and purification protocols described here were developed for purification of the peripheral cannabinoid receptor CB₂, expressed as a fusion with several tags [3, 12] (*see* Note 3). The corresponding DNA construct, CB₂-125 is represented below (Fig. 3).

1. Inoculate 25 mL of LB medium supplemented with 25 μL of 50 mg/mL ampicillin in 125-mL baffled flask with *E. coli* BL21(DE3) cells harboring the expression plasmid (*see* Note 4).
2. Incubate overnight (ON) at 37°C and 230 rpm agitation.
3. Prepare expression medium by adding 5 mL glucose solution and 500 μL ampicillin solution to the 500 mL of double-strength YT-medium flask immediately before inoculation. Use an appropriate number of flasks assuming the yield of ~400 μg of purified CB₂ protein from each flask.

²15–20 mL of elution buffer is usually enough to accomplish a single StrepTactin purification.

4. Add 1–2 mL of the ON culture to each 2 L flask and incubate at 37 °C under agitation for 3–4 h until the optical density of the culture at 600 nm reached 0.4–0.5 U.
5. Lower the temperature of incubation to 20 °C (*see* Note 5).
6. Add 500 µL of IPTG solution to induce synthesis of the recombinant protein.
7. Add 62.5 µL of a solution of CP-55,940 ligand (20 mM in Ethanol, *see* Note 6).
8. Incubate for additional 38–40 h (*see* Note 5).
9. Collect cells by centrifugation at $4,000 \times g$ for 30 min at 4 °C.
10. Resuspend and wash cells once in ice-cold PBS.
11. Collect cells by centrifugation as above and store pellet at –80 °C.

3.2 Preparation of Membranes

We recommend testing the expression levels of the target protein in cell-membrane preparations obtained from small-scale cultures before proceeding with the large scale fermentation and purification. The following protocol is based on a previously published method for CB₂ expression [13]. Carry out all steps of membrane preparation on ice or at 4 °C.

1. Perform expression as described in Subheading 3.1, and collect cells from 25 to 50 mL of culture in a conical tube by centrifugation at $4,000 \times g$ for 10 min. Alternatively, perform expression in a smaller scale format using 250 mL flasks and 50 mL of culture medium.
2. To 20 mL of cold PBS add 1 tablet of “complete” protease inhibitor cocktail.
3. Resuspended cells in a small volume (3–5 mL) of the cold PBS prepared in step 2.
4. Disrupt cells by passing the cell paste twice through a French Press.

³CB₂-125 includes the following fusion partners:

- N-terminal Maltose-binding protein (MBP): facilitates proper folding of CB₂ as well as the insertion of the hydrophobic polypeptide into the cytoplasmic membrane of *E. coli*.
- Tobacco etch virus protease recognition site (TEV): is a sequence recognized by TEV protease and is required for cleavage of the fusion protein. We use the recognition sequence E-N-L-Y-F-Q-S which is cleaved in between the Q and S residues, leaving the following non-native sequences attached to CB₂: S (the remainder of the TEV site) followed by WSHPOFEK (Strep-tag), GSGGAS (linker) followed by WSHPOFEK (2nd Strep-tag) followed by GGGS (linker) upstream of CB₂, and A₃ N₅ G₃ S (linker) followed by ENLYFQ (remainder of TEV site) downstream of CB₂.
- Strep-tag (Strep): required for affinity chromatography on a StrepTactin-agarose (final chromatographic step in our protocol).
- Thioredoxin A (Trx A): enhances the expression level of functional CB₂ receptor in the plasma membrane of *E. coli*.
- Polyhistidine tag (His₁₀): required for affinity purification of the recombinant protein on a Ni-NTA resin (first chromatographic step in our protocol).

⁴Work under sterile conditions and wear gloves on all occasions where cultivation of cells or handling of potentially harmful chemicals is involved.

⁵Expression conditions were optimized previously [12]. While cells can be grown at 37 °C until they reach OD₆₀₀ of 0.5, expression of the recombinant protein is performed at 20 °C to facilitate production of functional receptor. Incubation proceeds for 40 h after induction because the accumulation of cellular biomass reaches a maximum at this time.

⁶The addition of CP-55,940 to the growth medium enhances levels of expression and stabilizes functional CB₂ receptor [14].

5. Remove unbroken cells and cell debris by centrifugation at $\sim 20,000 \times g$ for 30 min at 4 °C.
6. Subject supernatant to a high-speed centrifugation at $\sim 250,000 \times g$ for 1 h at 4 °C.
7. Wash the resulting membrane pellet with cold PBS and resuspended in a small volume of membrane storage solution using a handheld glass homogenizer (*see* Note 7).
8. Flash-freeze small aliquots of the membrane suspension in liquid nitrogen and store at -80 °C until needed.
9. Proceed with evaluation of expression levels and functional activity of the target protein (*see* Note 8).

3.3 Extraction, Solubilization, and Stabilization of CB₂ from E. coli Cells

Starting from 10 L of cell culture (~ 80 g of wet biomass), typically 600 mL of crude extract can be obtained. Volumes of added solutions were calculated based on that number—adjust accordingly if necessary. Perform all the following procedures on ice or at 4 °C.

1. Transfer cell pellet (80 g) with a large spatula to a 1 L blender (*see* Note 9).
2. Add 100 mL of ice-cold TBS buffer into the blender.
3. Add 2–3 tablets of EDTA-free protease inhibitor cocktail solubilized in 10 mL water.
4. Homogenize cell suspension in blender for 20–30 s.
5. Transfer the suspension on ice and start stirring.
6. Add 50 μ L of DNase I solution.
7. Add 3 mL of 1 M MgCl₂ to achieve a final concentration of 5 mM.
8. Add Anti-foam A until the foam disappears (20–40 μ L).
9. Pass the suspension two times through a cell homogenizer (*see* Note 10).
10. Place the homogenized suspension in a 1,000-mL beaker on ice and start stirring.

⁷Use a volume of 400–600 μ L to resuspend the pellet. Transfer the partially resuspended pellet to a handheld glass homogenizer with an appropriate transfer pipette. Homogenize and transfer to an Eppendorf tube. Repeat the process with half of the original volume. Combine both fractions. Avoid formation of air bubbles to prevent losing membranes.

⁸We recommend testing effects of tags on expression levels and functional state of the target protein before proceeding with a large-scale expression and purification. For CB₂, the expression levels of various constructs and functionality of the resulting recombinant fusion CB₂ are typically evaluated by western blot analysis, ligand binding assays, and G-protein activation assay performed on membrane preparations [12]. Incorporation of the N-terminal Strep-tag and C-terminal His₁₀ tag does not significantly affect the expression levels of the CB₂ as shown by western blot analysis using anti-CB₂, anti-His, and anti-Strep-tag antibodies [3, 12]. The ability to detect small affinity tags on fusion CB₂ in membrane preparations subjected to SDS-PAGE and western blot, by assessing interaction with their respective antibodies does not guarantee that these same tags will be accessible for interaction with the chromatographic resin. However, we observe that the western blot analysis is usually an accurate predictor of the accessibility of a tag (including poly-histidine- and Strep-tag) for interaction with the respective resin, for subsequent chromatographic purification of CB₂ (unpublished observations).

⁹Some pre-warming of the cell pellet (at room temperature for 20–30 min) may be required to facilitate transfer to the blender.

¹⁰Alternatively, if a cell homogenizer is not available, the mixture can be subjected to sonication for 15 min (Branson Sonifier 250, 1/2-in. flat tip, output 6, duty cycle 50 %) [12]. Make sure that the solution is not overheated, and place it on ice and stir during sonication.

11. Add 300 mL of 2× solubilization buffer (*see* Note 11).
12. Add 50 mL CHAPS/CHS 12× solution (*see* Note 11).
13. Add 60 mL of a 10 % DDM solution (*see* Note 11).
14. Add 300 µL of 20 mM ligand CP-55,940 solution (*see* Note 11).
15. Continue stirring for 40–60 min. Ensure that the final composition of the buffer in which CB₂ is solubilized is 50 mM Tris–HCl at pH 7.5, 200 mM NaCl, 0.5 % [w/v] CHAPS, 0.1 % [w/v] CHS, 1 % [w/v] DDM, 10 µM CP-55,940, 5 mM MgCl₂, and 30 % glycerol, supplemented with DNase I (0.5 µg/mL) and EDTA-free protease-inhibitor cocktail.
16. Remove cell debris by centrifugation at a $\sim 200,000 \times g$ for 1 h at 4 °C.
17. Pass supernatant through a 0.45-µm filter and proceed with purification.

3.4 Ni-NTA Chromatography

All buffers used for purification should be supplemented with CP-55,940 at a final concentration of 10 µM. All procedures should be performed on ice or at 4 °C. Remember to take samples at each step to follow the purification process by SDS-PAGE and western blot (*see* Note 12).

1. Pack 8 mL of Ni-NTA resin to a suitable column compatible with an available chromatography work station (we use AKTA Purifier 100 [GE Healthcare]) allowing gradient-based chromatography and automated fraction collection.
2. Equilibrate the resin using at least 5 column volumes (CV) of buffer A + CP-55,940.
3. Load the filtered CB₂ solution at a flow rate of 0.5 µL/min. Maintain this flow rate for the entire purification.
4. Wash the resin with 20 CV of buffer A + CP-55,940 supplemented with 40 mM imidazole.
5. Elute protein with 10 CV of buffer B + CP-55,940 and collect 4 mL fractions (*see* Note 13).

¹¹In order to stabilize the CB₂ and minimize losses of active protein during purification, several parameters are critical [14]. Temperature has to be maintained at 4 °C, and the entire purification procedure should take no longer than 3 days to achieve the yield of functional protein 90 %. Solubilization of the fusion protein is performed in a mixture of CHAPS (0.5 %), DDM (1 %), and CHS (0.1 %) in the buffer (triple detergent buffer, TD), as described previously [12]. The presence of 0.1 % CHS during solubilization and purification is critical to maintain the functional structure of CB₂ in detergent micelles. In addition, the buffer contains 30 % glycerol. Supplementation with the high affinity ligand such as CP-55,940 during purification was also shown to significantly increase the recovery of active protein. We recommend 10 µM CP-55,940 in all purification buffers.

¹²Take samples from load, flowthrough, washes, elution fractions (separated or combined) for each chromatographic step as well as before and after TEV cleavage. In order to prepare CB₂ for SDS-PAGE it is important not to subject the sample to boiling or even high-temperature treatment since this will result in aggregation of the protein. We recommend mixing the protein sample with equal volume of the 2× Laemmli sample buffer and incubating at 37 °C for 30 min. Detection with several antibodies is recommended, as some (such as anti-CB₂ antibody) will allow visualization of CB₂ in all steps whereas others (such as anti MBP or anti His-tag antibodies) will detect fusion or cleavage products before or after TEV treatment respectively.

¹³For regeneration of the Ni-NTA resin, wash extensively with imidazole buffer and remove imidazole by extensive washing with binding buffer before use, according to the manufacturer's instructions.

6. Select the CB₂-containing fractions by SDS-PAGE followed by Coomassie Blue staining or by dot blot using anti CB₂ mAb or anti His-tag Ab (*see* Note 14).

3.5 Removal of Fusion Partners by Tobacco Etch Virus (TEV) Protease

1. Combine CB₂-containing fractions (typically 20 mL) and concentrate ~2.5-fold in centrifugal spin concentrators with a 30 kDa molecular mass cutoff (*see* Note 12).
2. Dialyze the concentrated sample against two exchanges of buffer C + CP-55,940 at 4 °C (*see* Note 12).
3. Add 0.5–1 mg of purified TEV protease to the dialyzed sample (5:1, CB₂: TEV, mol/mol) and allow digestion for at least 4 h or overnight at 4 °C (*see* Note 15).
4. Proceed with final step of purification or perform an intermediate purification step (*see* Note 16) to increase purity of the final product.

3.6 Purification via StrepTactin Affinity Tag

1. Pack a 3 mL-StrepTactin Poros column and operate it by gravity.
2. Equilibrate the resin with 5 CV of buffer C + CP-55,940.
3. Load the products of the TEV protease cleavage reaction (8 mL) onto the StrepTactin column.
4. Wash with 4 CV of buffer C + CP-55,940.
5. Elute CB₂ with 3 CV of buffer C + CP-55,940 supplemented with 5 μM desthiobiotin (*see* Note 17).
6. Combine elution fractions and concentrate in centrifugal spin concentrators with a 30 kDa molecular mass cutoff.
7. Dialyze against buffer A + CP-55,940 at 4 °C overnight.
8. Determine protein concentration with the Bio-Rad DC kit (*see* Note 18).
9. Aliquot the concentrated protein into Eppendorf tubes.
10. Freeze aliquots in liquid nitrogen and store at –80 °C until needed.

¹⁴This step can be omitted once the pattern of elution of the target protein is determined. We typically observe elution of CB₂ in fractions 3–7.

¹⁵Conditions for efficient cleavage by TEV protease were established previously [3]. Since detergents, salts, imidazole and glycerol at concentrations present in buffer B inhibit TEV protease cleavage, we recommend concentrating the sample no more than 2 to 2.5-fold and dialyzing for 2 h against buffer C prior to TEV cleavage.

¹⁶In order to increase the purity of the final product, removal of His-tagged TEV and His-tagged cleavage product can be performed by Ni-NTA chromatography as an intermediate step.

- Pack a column with 1 mL Ni-NTA agarose and equilibrate it with 5 CV of buffer C containing 20 mM imidazole.
- Pass the reaction mixture after TEV cleavage through the small Ni-NTA column by gravity to retain His-tagged products of cleavage as well as TEV protease, and proceed with purification of the flowthrough.

¹⁷To regenerate the StrepTactin resin, use the HABA reagent (EMD Biosciences) according to manufacturer's instructions.

¹⁸Concentrating the protein solution fourfold to fivefold on a centrifugal spin-concentrator results in a proportional co-concentration of detergents. Typically we obtain a concentrated 1.5–2 mg/mL solution of CB₂ that also contains ~0.5 % DDM, 2.5 % CHAPS, and 0.5 % CHS. Determination of protein concentration can be performed by Bio-Rad DC assay kit or similar system suitable for measurement of protein concentration in the presence of detergents.

11. Evaluate purity of the preparation (*see* Note 19).
12. Characterize functional state of the purified protein (*see* Note 20).
13. The purified protein can be analyzed by a range of biophysical techniques (*see* Note 21).

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¹⁹The purity of the final preparation can be evaluated by Coomassie Blue staining (Fig. 4). Be aware of the fact that the efficiency of staining of highly hydrophobic membrane proteins (like CB₂) may be much lower than that of soluble proteins, resulting in a weaker than expected signal in Coomassie-stained gels.

²⁰To evaluate the functional state of the purified CB₂ protein, reconstitution in liposomes is required [14, 15]. Then, a G-protein activation assay with proteoliposomes can be performed [14]. Reaction conditions were optimized for 3–10 ng of CB₂ per reaction to ensure that less than 30 % of the available [³⁵S] GTPγS is consumed.

²¹Purified CB₂ in detergent micelles is suitable for characterization by circular dichroism spectroscopy [3, 16], high-resolution NMR (ligand binding studies [12] and diffusion experiments [16]) or other biophysical techniques.

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Fig. 1.
Ni-NTA affinity chromatography (**a**) and StrepTactin affinity chromatography (**b**).
Chemical formulas of compounds required for chromatographic purification are shown

Fig. 2.
Flow chart of procedures for expression, purification and characterization of CB₂

Fig. 3.
Fusion protein CB₂-125

Fig. 4.

Purification of CB₂-125. Coomassie Blue staining of a typical gel is presented. *Lane 1*, crude extract before 1st Ni-NTA chromatography; *lane 2*, concentrated fractions after 1st Ni-NTA chromatography; *lane 3*, fractions after dialysis against buffer B; *lane 4*, proteins after TEV protease digest; *lane 5*, combined fractions (flowthrough and wash) from the 2nd Ni-NTA column; *lane 6*, flowthrough from the StrepTactin column; *lane 7*, wash fraction from the StrepTactin column; *lanes 8 and 9*, eluate fractions from StrepTactin column, after concentration (5 and 2.5 µg of protein per lane, respectively)