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Expression and preparation of a G protein-coupled cannabinoid receptor CB₂ for NMR structural studies

Alexei Yeliseev

National Institute on Alcoholism and Alcohol Abuse, NIH 5625, Fishers Lane, Room 3N11, Rockville, MD 20815, USA

Abstract

Cannabinoid receptor type II, CB₂ is an integral membrane protein that belongs to a large class of G protein-coupled receptors (GPCR). CB₂ is a part of the endocannabinoid system that plays an important role in regulation of immune response, inflammation, and pain. Information about the structure and function of CB₂ is essential for development of specific ligands targeting this receptor. We present here a methodology for recombinant expression of CB₂, stable isotope labeling, purification, and reconstitution into liposomes, in preparation for characterization of this protein by methods of nuclear magnetic resonance (NMR). A correctly folded, functional CB₂ labeled with ¹³C, ¹⁵N amino acid (tryptophan) or uniformly labeled with ¹³C, ¹⁵N is expressed in a medium of a defined composition, under controlled aeration, pH and temperature. The receptor is purified by affinity chromatography and reconstituted into lipid bilayers in a form of proteoliposomes suitable for analysis by NMR spectroscopy.

Keywords

G protein-coupled receptor; CB₂ cannabinoid receptor; NMR; integral membrane protein; expression; purification

Introduction

The cannabinoid receptor CB₂, a G protein-coupled receptor plays an important role in inflammation processes in various tissues including kidney, liver, and the gastrointestinal system. It is an integral membrane protein primarily located in cells of immune and hematopoietic systems as well as in neuronal microglia (Cabral and Griffin-Thomas 2009). To study the structure and function of CB₂ in membranes by nuclear magnetic resonance (NMR), the expression and purification of a functional, stable isotope labeled receptor in milligram quantities is required (Yeliseev et al. 2007; Yeliseev and Vukoti 2011; Kimura et al. 2014).

The stable isotope-labeled protein is produced by fermentation of the *Escherichia coli* cells expressing CB₂ in a medium of a defined composition (minimal salt medium, MSM) (Berger et al. 2010). The bacterial culture is grown in a fermenter in MSM supplemented

with either stable isotope labeled amino acid(s) or precursors of amino acids (glucose, ammonium salts). A stable isotope labeling scheme is selected to satisfy the requirements of NMR experiment, taking into consideration the properties of the expression host, an availability, properties and the cost of stable isotope-labeled nutrients. Labeling can be performed by supplementing the expression medium with either one amino acid at a time or by supplying two or more labeled amino acids. Alternatively, rather than amino acids, other labeled biosynthetic precursor(s) such as glucose and ammonium salts can be used. It is imperative though that the labeled amino acid is incorporated into the target protein with high efficiency, and does not get metabolized by the host cell. In the present work we describe the use of the amino acid tryptophan for production of labeled CB₂ receptor. An exogenously supplied ¹³C, ¹⁵N-labeled tryptophan is efficiently taken up from the cultivation medium by *E. coli* cells, and utilized for the synthesis of cellular proteins including CB₂ receptor. This strategy allows us to use the commercially available *E. coli* production strains such as BL21 (DE3) and renders the use of specialized auxotrophic strains unnecessary.

The description of expression of uniformly ¹³C, ¹⁵N-labeled cannabinoid receptor is provided in Basic Protocol 1, whereas the labeling of the protein with individual amino acid (tryptophan) is described in Alternate Protocol 1. CB₂ receptor is expressed as a fusion with the N-terminal maltose binding protein (MBP) of *E. coli* that facilitates the correct folding of the receptor and its accumulation in bacterial cytoplasmic membrane (Yeliseev et al. 2005). The production of the recombinant receptor is performed as a controlled fed batch process that allows fine regulation of the consumption of nutrients and cell growth conditions. The *E. coli* cells harboring the expression plasmid are adapted to growth in a minimal salt medium supplemented with glucose and ammonium. Cells are grown as a batch at 37 °C in a fermenter under controlled pH and aeration, until the density of biomass is sufficiently high, and the expression of the target protein is induced by addition of IPTG. The induction is performed at 20 °C, and the labeled nutrients are added as needed. The fed batch fermentation results in the accumulation of a significant amount of biomass in a relatively small volume of culture, thus allowing an efficient utilization of costly stable isotope-labeled amino acids or precursors.

The MBP-CB₂ fusion protein is solubilized from membranes in a mixture of detergents and further purified by affinity chromatography, taking advantage of two small affinity tags that flank the sequence of the receptor (Yeliseev et al. 2007; Yeliseev et al. 2016) as described in Basic Protocol 2. The purified protein is then reconstituted into lipid bilayers in the form of proteoliposomes as described in Basic Protocol 3. The lipid-stabilized receptor preparations are suitable for characterization by NMR (Kimura et al. 2012; Kimura et al. 2014). The homogeneity of the proteoliposome preparation can be assessed by following the Basic Protocol 4 (Kimura et al. 2012). The expression and purification protocols described here can be applicable to other recombinant integral membrane GPCR expressed in *E. coli*.

Basic Protocol 1

Preparation of uniformly ^{13}C , ^{15}N labeled CB_2 receptor

This protocol describes the basic procedures in preparation of uniformly ^{13}C , ^{15}N -labeled cannabinoid receptor CB_2 by bacterial fermentation. The method comprises the adaptation of the bacterial culture to growth in minimal medium, preparation of the fermenter, sterilization of media components, and the fed-batch fermentation. After the biomass is collected, it can then be stored at -80°C until purification of the accumulated protein is performed. The protocol is written for 1 liter of bacterial culture. The method can be scaled up or down depending on the yield of protein and the amount of receptor needed in downstream applications.

Materials—Luria broth (LB) (see recipe)

Minimal salt medium (MSM) (see recipe)

Ampicillin stock solution (see recipe) or other appropriate antibiotic to select the plasmid encoding the target GPCR

Frozen stock of *E. coli* expression strain harboring the expression plasmid. Competent cells BL21(DE3) can be obtained from available from New England Biolabs (C2527) or other commercial sources. Plasmid pAY-130 was constructed in this laboratory based on a vector pMALTM-p5X *New England Biolabs) specially for expression of CB_2 receptor in *E. coli*.

4 × 125 mL sterile culture flasks

1 x SDS sample buffer (Bio-Rad, cat no. 161-0737)

1 M IPTG (RPI, cat. Bo. 156000)

Potassium phosphate dibasic (Sigma Aldrich, cat. No. 60353)

Sodium phosphate monobasic dihydrate (Sigma Aldrich cat. No. 71505)

NaOH, 10 N (VWR, cat. No. VW3247-1)

Phosphoric acid, 85% (Sigma, cat. No. P5811)

50% D-glucose (Sigma, cat. No. G-7528)

27.4% NH_4Cl (Sigma, cat.no A9434)

50% $^{13}\text{C}_6$ - D-glucose (Cambridge Isotopes, cat. No. CLM-1396)

27.4% $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotopes, cat. No. NLM—467)

Test tubes, 10-mL (sterile)

4 × 500 mL flasks (autoclaved)

Shakers at 37 °C

7.5 L BioFlo 110 Bench-Top Fermenter (New Brunswick Sci. Co., Edison, NJ, product number M1273–0054)

Assure Blood Glucose Monitoring System Arkray USA 500001 (Arkray, USA)

Ammonia electrode with BNC connector (Orion™ High-Performance Ammonia Electrode 512HPBNWP, Thermo Scientific)

Cell density meter (WPA Biowave CO8000, Biochrom Ltd)

1.5 mL microcentrifuge tubes

0.45 µm syringe filter (Acrodisc 32 mm Syringe Filter, Pall Gelman Laboratory)

0.45 µm sterile filter (Nalgene, 50 mm, for 150 or 250 mL bottles, Thermo Scientific).

Sterile disposable plastic syringes with Luer lock tip or similar (1 mL, 5 mL, 20 mL, 50 mL; Kendall Monoject, Tyco Healthcare Group, USA)

2 mL cryovials (Cryo.s Freezing tubes, Greiner Bio-one, Frickenhausen, Germany, cat # 126280)

1.5 mL Disposable plastic cuvettes (Fisherbrand™ Disposable Cuvettes, Standard; Polystyrene, 1.5 mL, Fisher, USA, cat #14–955-12)

Microcentrifuge (Eppendorf, cat. No. 5417)

BioWave cell density counter (WPA)

Preparation of sterile stock solutions of amino acids, antibiotics, stable isotope labeled glucose and ligands

1. Ampicillin: prepare stock solution of 50 mg/mL ampicillin in water. Solution is sterilized by passing it through 0.45 µm syringe filter (Acrodisc 32 mm Syringe Filter, Pall Gelman Laboratory). Aliquot 0.5 mL stock solution into sterile 1.5 mL Eppendorf tubes and store at –80 °C for up to 3–4 weeks.
2. ¹³C-glucose: prepare stock solution of ¹³C glucose at a concentration of 20% (w/v) in water and filter-sterilize it through 0.45 µm sterile filter (Nalgene, 50 mm, for 150 or 250 mL bottles, Thermo Scientific). Solution can be stored at room temperature for several days.
3. Prepare stock solution of 0.5 M IPTG in water and filter-sterilize it through 0.45 µm syringe filter. Solution can be prepared on a day of fermentation or stored for several days at –20 °C.

4. Prepare stock solution of $^{15}\text{NH}_4\text{Cl}$ at a concentration of 27.4% (w/v) in water and filter-sterilize it through 0.45 μm sterile filter. The solution can be kept at room temperature for several days.
5. Prepare stock solution of ligand CP-55,940 by dissolving it in ethanol at a concentration of 10 mM. Ligand does not need to be sterilized. The aliquots of the stock solution in 1.5 mL Eppendorf tubes can be kept at -80°C for several months.

Adaptation of bacterial cultures to minimal salt medium

8. Prepare two 15 mL culture tubes containing 5 mL LB with 100 $\mu\text{g}/\text{mL}$ ampicillin (10 μL of 50 mg/mL ampicillin stock solution). Inoculate a culture with a single *E.coli* colony from a Petri dish or from a frozen stock. Incubate the cultures for 16 hours on a shaker at 230 rpm at 37°C .
9. Next morning, transfer 50 μL of overnight culture to a sterile 125 mL flask containing 10 mL of MSM with 100 $\mu\text{g}/\text{mL}$ ampicillin and incubate on a shaker at 230 rpm, 37°C . In the late afternoon, take 50 μL of culture and inoculate a fresh sterile 125 mL flask with 10 mL MSM/ 100 $\mu\text{g}/\text{mL}$ ampicillin. Incubate the culture on a shaker overnight at 37°C .
10. The following morning take 50 μL of overnight culture and inoculate a fresh sterile 125 mL flask with 10 mL MSM/ 100 $\mu\text{g}/\text{mL}$ ampicillin, and grow on a shaker at 37°C . By the late afternoon, the culture is adapted to MSM (3 rounds of adaptation). Take 1 mL of culture and mix with 1 mL of sterile 50% glycerol (v/v) in a cryovial. These vials can be stored at -80°C until needed.

Preparation of fermenter

11. Sterilize 3 L glass vessel filled with 500 mL of water.

If large quantities of receptor are required, the fermentation volume can be adjusted accordingly. We also performed fermentations in 7.5 L vessel filled with up to 5 L medium.

12. Add salts, microelements, vitamins, glucose, and ammonium on the day of the fermentation.

(see recipe for salt preparation)

13. Adjust the volume to 800 mL, to allow for additional 200 mL of seed culture for a total volume of 1 L.
14. Adjust pH to 7.0. The typical starting pH is between 6 and 8. pH is adjusted in an automated mode by setting pH = 7.0 as a target, using “acid” pump primed with 10% (v/v) of H_3PO_4 and “base” pump primed with 10% (v/v) of NaOH. Stirrer should be on at a speed of between 100–200 rpm.
15. Set temperature of fermenter to 37°C . Calibrate the oxygen electrode by flowing the manufacturer’s instructions (BioFlo 100). Briefly, the electrode is disconnected from the control module, and setting the “dissolved oxygen” value at 0%. The electrode is then connected back to the control module, the stirrer is

set at the maximum rotation speed (1250 rpm for BioFlo 100), and the air flow is set at 10 L/min. After the medium is saturated with oxygen (typically 5–10 min) and the oxygen concentration reading stabilized, the oxygen concentration is set as 100%. Then the mixer speed is reduced to 250 rpm and the air flow is reduced to 3 L/min. The targeted concentration of dissolved oxygen is set at 40% of saturating concentration. Set the control of dissolved oxygen by a cascade of stirring speed and air flow. During the fermentation the stirring speed and air flow is controlled automatically. The starting stirring speed is 250 rpm, the maximal value being 1250 rpm. The starting air flow is 3 L/min, the maximal value being 10 L/min.

16. Calibrate the Assure 3 Blood Glucose Monitoring system (Arkay, USA) using standard glucose solutions using manufacturer's instructions: http://arkayusa.com/diabetes-management/sites/arkayusa.com.diabetes-management/files/Vital%20User%20Manual_English%201850-03.pdf

17. Calibrate the ammonia electrode (Thermo Scientific) according to the manufacturer's instructions <https://assets.fishersci.com/TFS-Assets/LSG/manuals/D17171~.pdf> using standard solutions of NH_4Cl from 1 to 5 g/L.

The morning the day before fermentation, inoculate 50 μL of glycerol stock into 25 mL MSM supplemented with ampicillin in 125 mL flask. Grow cells at 37 °C on a shaker at 230 rpm. We typically use 100 $\mu\text{g/mL}$ concentration of ampicillin although concentration 50 $\mu\text{g/mL}$ can also be used. The 50 mg/mL stock solution of antibiotic can be prepared in advance, filter-sterilized and kept frozen at –20 °C for 3–4 weeks.

18. In the afternoon, transfer 1–5 mL of the grown culture into 500 mL MSM supplemented with ampicillin in 2 L flasks. Grow overnight at 37°C on a shaker at 230 rpm to $\text{OD}_{600} = 3.0\text{--}3.5$.

Growing cells to higher cell density should be avoided since it will result in a significant lag phase. If that happens the total duration of the fermentation may be unnecessarily extended by 2–4 hours. Similarly, collecting cells grown at lower density will result in lower OD_{600} of culture at the start of fermentation, and will unnecessarily prolong the fermentation.

It is important that the glycerol stock of the expression strain is stored at –80 °C. We recommend preparing several identical vials of glycerol stock and using one vial per experiment. The vial can be thawed and re-frozen a few times, but the cell viability decreases after each round of freeze/thaw.

Fermentation in minimal salt medium

19. Next morning, collect cells by centrifugation at 3000 x g for 20 min in sterile 250 mL bottles.
20. Resuspend cell pellet in 200 mL of sterilized, cold tap water.

Resuspending can be performed by vortexing the bottle filled with small volume of sterile tap water. Once cells are properly re-suspended, more water can be added.

- 21.** Inject cells into the fermenter so that the optical density at 600 nm (OD_{600}) of the culture is about 1.0. The optical density can be measured by withdrawing 1–2 mL sample of the culture (should be done by using a 5 mL disposable plastic syringe attached to a dedicated port in a fermenter), placing it in a disposable plastic cuvette and measuring the absorbance on a spectrophotometer (we use a BioWave cell density counter, WPA). If the optical density of the culture is greater than 2 we recommend diluting the sample with the medium. The measurements are performed against a blank (MSM medium).

Cell density lower than 1 are also acceptable. However, the lag phase for cell growth in fermenter may become much longer.

- 22.** Perform the fermentation as a fed-batch process. Cells are cultivated in 1L of medium under controlled aeration, temperature and pH, and nutrients (glucose and ammonium) are added as needed during fermentation.
- 23.** Monitor the concentration of glucose with the Assure 3 Blood Glucose Monitoring system. Adjust the concentration of glucose to 10 g/L as needed by addition of sterile 50% glucose solution.

At the beginning of fermentation, the adjustment of the concentration of glucose and ammonium is needed after 1.5–2 hours. As the density of cells increases, the concentration of glucose and ammonium need to be measured every 30–60 min.

- 24.** Monitor the concentration of ammonium with Ammonia electrode with BNC connector. Adjust the concentration of ammonium chloride to 2.7 g/L as needed by addition of sterilized ammonium chloride stock solution.
- 25.** Adjust the pH of the medium to 7.0 by the controlled addition of 10% (v/v) NaOH and 10% (v/v) H_3PO_4 .
- 26.** When the OD_{600} of the culture exceeds 10, temporarily halt the addition of ammonium until the concentration of dissolved oxygen started to raise that indicates that the available ammonium is consumed. Add sterile $^{15}NH_4Cl$ to a final concentration of 2.74 g/L and continue adding it as needed until the end of fermentation.

Sterile stock solution of $^{15}NH_4Cl$ can be prepared by filter-sterilizing and delivered into fermentation vessel by injecting with a syringe.

- 27.** Temporarily halt the addition of glucose until the concentration of dissolved oxygen starts to increase.

A sudden increase in dissolved oxygen concentration will indicate that the glucose in the medium has been consumed.

- 28.** Immediately add sterile $^{13}C_6$ -glucose to final concentration of 10 g/L. Monitor glucose levels periodically and add more $^{13}C_6$ -glucose as needed until the end of fermentation.

Sterile stock solution of $^{13}C_6$ -glucose can be prepared by filter-sterilizing and delivered into fermentation vessel by injecting with a syringe.

29. When OD₆₀₀ of cells approaches 20, turn the temperature down to 20 °C.

30. Add the stock solution of CP-55,940 to the final concentration of 5 µM.

(Other high affinity cannabinoid ligand(s) can be added instead of CP-55,940)

31. Induce production of the recombinant protein by addition of IPTG to the final concentration of 0.5 mM.

32. After 2 h of induction, add another portion of IPTG to the final concentration of 1 mM.

33. Continue fermentation for another 5–6 hours.

The concentration of glucose and ammonium at this stage should be measured every hour or as appropriate according to the rate of cell growth. To minimize the cost of stable isotope labeled precursors, both glucose and ammonium chloride solutions should be added in small portions, so that to match their concentrations set at the beginning of fermentation.

34. Collect cells by centrifugation at 5000 x g for 30 min at 4 °C. Wash the cell pellet with ice-cold PBS buffer. Collect the pellet by centrifugation at 5000 x g for 30 min and store at –80 °C.

Alternate Protocol 1

Preparation of CB₂ uniformly labeled with ¹⁵N and selectively labeled with ¹³C, ¹⁵N-L-Tryptophan

The Alternate Protocol 1 describes the preparation of CB₂ receptor selectively labeled with ¹³C-amino acid tryptophan and uniformly labeled with ¹⁵N. The protein is produced by *E. coli* fermentation in MSM, similar to the Basic Protocol 1. MSM is supplemented with labeled amino acid tryptophan and ¹⁵N-labeled ammonium salt. If needed, this protocol can be modified, and labeling with other amino acid can be performed similar way, assuming that the use of the amino acid of interest does not result in isotope dilution and scrambling. Likewise, the ammonium salt with a natural abundance of nitrogen isotopes can be used if no ¹⁵N enrichment of protein is desired. The protocol has been developed for 1 L of culture medium; it can be scaled up or down depending on the needs of downstream experiments.

Materials—The materials used are the same as in Basic Protocol 1, except for ¹³C-labeled glucose. In addition, the following materials are used:

¹³C₁₁, ¹⁵N₂-L-Tryptophan (Sigma Aldrich, cat # 574597)

¹⁵N-ammonium chloride (99%) Cambridge Isotope Laboratories, Cat # NLM-467–50

¹⁵N-L-asparagine (Sigma, cat # 641960)

1. Prepare stock solution of ¹³C, ¹⁵N-L-Trp at a concentration of 2% (w/v) by dissolving it first in 0.1 N NaOH at stirring and then carefully adjust the pH of the solution to 8.0 by adding 0.1 N HCl. The solution is then filter-sterilized through 0.45 µm syringe filter.

2. Prepare stock solution of 100 mM ^{15}N -asparagine by dissolving it in water and carefully adjusting the pH to 7.0 by addition of 0.1 N NaOH. Filter sterilize it through 0.45 μm syringe filter. The solution can be kept at room temperature for several days.
3. Proceed with fermentation according to **Basic Protocol 1 until pp.18**
4. Continue with fermentation periodically supplementing the medium with $^{15}\text{NH}_4$ and glucose (natural abundance of ^{13}C) as needed, until cells reach $\text{OD}_{600} \sim 20$.
5. Add ^{13}C , ^{15}N -L-Tryptophan to the final concentration of 1 mM.
6. Add ^{15}N -L-asparagine to the final concentration of 5 mM (this step is optional, but it increases the yield of the recombinant receptor).

The addition of asparagine boosts production of the recombinant receptor. The addition of ^{15}N -L-asparagine ensures uniform ^{15}N isotope incorporation into CB₂ receptor.

7. Add stock solution of CP-55,940 to the medium to final concentration of ligand of 5 μM .

Other high affinity cannabinoid ligand can be added instead.

8. Lower the temperature of fermenter to 20 $^{\circ}\text{C}$.
9. Add IPTG to the final concentration of 0.5 mM.
10. Continue fermentation for another 2 hours. Add another portion of ^{15}N -L-asparagine (to concentration of 5 mM) and IPTG (0.5 mM).
11. Continue fermentation for another 5–6 hours, collect cells, wash and store frozen as described in pp. 28 of the basic Protocol 1.

Basic Protocol 2

Purification of recombinant CB₂ receptor

The Basic Protocol 2 describes the extraction of the MBP-CB₂ receptor fusion from *E. coli* cell membranes by the action of detergents, purification of the receptor by two rounds of affinity chromatography on Ni-NTA resin and StrepTactin resin, removal of the MBP fusion partner by treatment with specific TEV protease, and analysis of the purified protein by SDS-PAGE, Western blot and protein concentration assay. The purified CB₂ protein can be studied in detergent micelles or reconstituted into proteoliposomes (as described in Basic Protocol 3), in which form it is more stable for analysis by various biophysical techniques.

Materials—Cell suspension buffer, TBS (Tris-buffered saline, 25 mM Tris base, 2.7 mM KCl, 137 mM NaCl). 10x solution (RPI research products, cat. No. T60075)

Solubilization buffer (10x) (see recipe)

MgCl₂, 1M solution (Quality Biological, cat. No. 351–033-721)

NaCl, 5 M solution (Quality Biological, cat. No. 351–036-101)

Deoxyribonuclease I (Sigma, cat. No. D4527)

Complete EDTA-free protease inhibitor cocktail (Roche, cat. No 05056489001).

DDM, n-dodecyl- β -D-maltopyranoside (Anatrace, cat. No D310)

CHS, cholesteryl hemiscuccinate Tris salt (Anatrace cat. No CH210)

CHAPS, 3-[(3-Cholamidopropyl)-Dimethylammonio]-1-Propane Sulfonate] • N,N-Dimethyl-3-Sulfo-N-[3-[[3 α ,5 β ,7 α ,12 α)-3,7,12-Trihydroxy-24-Oxocholan-24-yl]Amino]propyl]-1-Propanaminium Hydroxide, Inner Salt (Anatrace, cat. No. C316)

Ni-NTA Superflow affinity resin (Qiagen, cat. No. 30430)

IMAC binding buffer (buffer A) (see recipe)

IMAC elution buffer (buffer B) (see recipe)

Dialysis buffer (buffer C) (see recipe)

TEV protease (Sigma Aldrich, cat. No. T4455)

StrepTactin XT Supeflow (IBA, cat. No. 2–240-025)

StrepTactin elution buffer with biotin (see recipe)

Dialyzer Mega tube (20 mL capacity, MWCO 6–8 kDa) (EMD Biosciences, cat. No. 71746–3)

DC protein assay kit (BioRad, cat. No. 5000113)

Instant Blue staining solution for protein gels (Expedeon, cat. No. ISB1L)

Large-scale centrifuge that holds 1 L centrifuge bottles (Beckman Coulter, rotor JLA 8.1000)

1L centrifuge bottles

Cell homogenizer (Avestin Emulsiflex C3 or similar)

Ultracentrifuge (Beckman Coulter Optima XE-90)

Ultracentrifuge rotor 45 Ti

Ultracentrifuge rotor 70 Ti

Ultracentrifuge rotor TLA 100.3

Ultracentrifuge tubes capacity 70 mL with caps (Beckman Coulter, cat. No. 355655)

Ultracentrifuge tubes capacity 25 mL with caps. (Beckman Coulter, cat. No. 355654)

Ultracentrifuge tubes capacity 3.8 mL (Beckman Coulter, cat. No. 349622)

BenchMark Prestained Protein Ladder (Invitrogen, cat. No. 10748–1010)

Anti-6x-His monoclonal antibody (Thermo Fisher Scientific, cat. No. MA1–21315)

Anti-mouse IgG horseradish peroxidase-linked antibody (Amersham, cat no. NA931)

SuperSignal West Pico Chemoluminescent Substrate (Pierce, cat. No. 34080)

Nitrocellulose/ Filter Paper Sandwiches, 0.45 µm (Bio-Rad, cat. No. 1620215)

Additional reagents and equipment for SDS gel electrophoresis of proteins:

–4–20% gradient polyacrylamide SDS-PAGE gels (Mini-PROTEAN TGX, cat # 456–1095, BioRad)

–SDS-PAGE running buffer, (10x Tris/Glycine/SDS buffer, cat #161–0772, Bio-Rad) – to be diluted 1:10 with double distilled water

–protein molecular weight markers (BioRad, cat. No. 161–0375)

Purification of recombinant CB₂ receptor—The following protocol describes the processing of 100 g of wet biomass. Depending on the size of the cell pellet adjust the quantities of reagents accordingly.

1. Defrost frozen cell biomass on ice and re-suspend in 150 mL of cold TBS buffer. Perform all subsequent procedures at 4°C or on ice.
2. Supplement the cell suspension with 3.25 mL of 1M MgCl₂, 1500 U of Deoxyribonuclease I (Sigma, cat. No. D4527) and 4 tablets of Complete EDTA-free protease inhibitor cocktail dissolved in 10 mL of water just prior to use (Roche, cat. No 05056489001).

The addition of DNase I and magnesium will help to digest the released DNA and decrease the viscosity of the extract. The protease inhibitor cocktail is needed to prevent the proteolytic degradation of the expressed protein.

3. Homogenize the cell suspension using 200 mL PKontes brand Potter-Elvehjem Pyrex brand tissue grinder with Teflon pestle (Corning, cat.no. 7725T).

Homogenization of the cell paste prior to cell disruption is necessary to increase the speed of the preparation of cell-free extract.

4. Pass the cell suspension twice through EmulsiFlex-C3 cell homogenizer (Avestin, Canada) at 15,000 psi pressure setting.
5. Under continuous stirring, add 122 µL of 26.6 mM stock solution of CP-55,940 in methanol (Cayman, cat. No. 90084), 325 mL of double-concentrated solubilization buffer (see Recipe) and 65 mL of 10x solution of detergents CHAPS, CHS and DDM (concentration 5%, 1% and 10% w/v, respectively, see Recipe). Continue stirring on ice for 1 h.

1 h solubilization is sufficient to extract most of the recombinant protein from cell membranes. Solubilization can be extended to several hours if needed. The detergents CHAPS, DDM and a stabilizing cholesterol derivative CHS should be of a purity of 99% or better. Detergent stock solutions can be prepared several days in advance, filtered and stored at 4°C.

6. Centrifuge the solution in type 45Ti rotor (Beckman) at 43,000 rpm for 1 h. Collect the supernatant.
7. Wash 65 g of Dowex 1×4 chloride form (Sigma, cat. No. 428612) with 1 L of distilled water in a glass beaker. Decant water and repeat wash one more time. Wash the resin with 200 mL of buffer A. Decant supernatant. Add the resin to the centrifuged cell extract and incubate on a shaker at 4°C for 1 h.

Treatment of the cell extract with Dowex resin clears the extract and assists better binding of the His-tagged protein to chromatography resin.

8. Filter suspension through 0.45 µm filter.

Filtration is necessary to remove large particles that may clog the chromatography column.

9. Apply the filtered solution at a flow rate of 0.5 mL/ min onto 4 mL Complete His resin packed into 0.6 cm (inner diameter) x 10 cm (length) glass column (Spectra Chrom, cat. No. 123903), pre-equilibrated with buffer A, on AKTA Purifier system or similar chromatography system. Monitor the absorbance of the eluate at 280 nm.
10. Wash the resin with 20 column volumes [Author: specify the column volume] of a mixture of 97% of buffer A and 3% of buffer B (concentration of imidazole in wash buffer is 7.5 mM). Typical column volume is 4–5 mL, which translates into 80–100 mL of wash solution.

If other than Complete His chromatography resin is used, adjust concentration of imidazole in wash buffer according to manufacturer's instructions.

11. Elute protein by applying 100% of buffer B over 7 column volumes (column volume = 4 mL) at a flow rate of 0.2 mL/min. Collect 4 mL fractions.

Elution needs to be performed at slow flow rate to ensure that the recombinant protein is released from the resin in reasonably small volume. We typically observe the bulk of protein released in the first three 4-mL fractions.

12. Combine fractions containing protein in Dialyzer Mega tube (20 mL capacity, MWCO 6–8 kDa) (EMD Biosciences, cat. No. 71746–3) and dialyze against 200 mL of buffer C for 1 h at 4°C. The instructions for Dialyzer Mega tubes can be found here: http://www.emdmillipore.com/US/en/product/D-Tube-Dialyzer-Maxi-MWCO-6-8-kDa,EMD_BIO-71509#anchor_USP. Briefly, the dialysis tubes are pre-hydrated for several minutes, the protein solution is loaded into the tube, and placed on a floating support in a dialysis buffer in 200 mL glass beaker.
13. Transfer the dialyzed protein solution to a 50-mL Falcon tube. Add 2 mg of TEV protease and incubate for 4 h.

14. Apply the dialyzed protein solution on 4 mL StrepTactin XT resin (IBA Biosciences) packed in a disposable Econo-Pac column (Bio-Rad, cat. No. 732–1010) by gravity, pre-washed with 5×4 mL of buffer A supplemented with 10 μ M of CP-55,940. Collect the protein flow-through and reapply to the column to ensure complete binding.
15. Wash the column with 80 mL of buffer A supplemented with 10 μ M of CP-55,940.
16. Elute the protein with 5×4 mL of StrepTactin elution buffer. Close the column after addition of every portion of elution buffer and incubate for 10 min.

The release kinetics of the recombinant protein from StrepTactin resin is relatively slow. Therefore, it is recommended to close the column after each addition of the elution buffer and incubate for several minutes before collecting eluate.

17. Combine elution fractions and concentrate on the Amicon Ultracel-30K spin concentrator to the final volume of 1–2 mL.

We recommend using spin concentrator with 30 kDa molecular weight cutoff membranes since it allows reasonably fast concentration of protein sample. The use of smaller size pore concentrators will result in an extended time of concentration. The use of filters made of regenerated cellulose is recommended since such filters have low non-specific protein-retention properties.

Analyze the yield and purity of protein

18. Measure the protein concentration in the sample by a Bio-Rad DC protein assay kit. Follow the manufacturer's directions.

The Bio-Rad DC protein assay is a colorimetric assay for protein concentration following detergent solubilization. The reaction is based on the well-documented Lowry assay. For accurate measurements the concentration of protein in the sample should be between 0.5–1.5 mg/mL. If concentration of the protein exceeds 2 mg/mL take an aliquot of the sample and dilute with elution buffer.

19. Aliquot the protein into 1.5 mL Eppendorf tubes and snap-freeze in liquid nitrogen. Store at -80°C until use.
20. Analyze the purity of the sample on a 10% SDS-PAGE gel. Mix 10 μ L of the protein solution with 10 μ L of 2x sample buffer. Incubate at 37°C for 30 min.

Do not use temperature higher than 37°C since the protein may aggregate.

21. Load 15 mL of the sample prepared in step 20 into the gel. In a separate well load a molecular weight marker (BenchMark Prestained Protein Ladder, Invitrogen, cat. No. 10748–1010). Run the gel at 100 V constant for approximately 1 h 20 min. Place the gel in 20 mL of Instant Blue (Expedeon, cat no. ISB1L) for 30 min and incubate for 30 min with gentle rocking. Decant the staining solution, add dd H_2O and incubate with gentle rocking for 10–15 min. Repeat washing procedure 2–3 times until the clear background on gel is obtained.

22. The protein sample can be analyzed by immunoblot. Run the protein on an SDS-PAGE as described in pp. 66. After the run is completed, transfer the protein to the Nitrocellulose membrane (Bio-Rad Nitrocellulose/ Filter paper Sandwiches, 0.45 μ m) using BioRad Western blot transfer unit (or similar device) according to manufacturer's instructions, for 1 h at 80 v.
23. Block the membrane in 3% (w/v) BSA solution in TBST for 1 h at room temperature on a rocking shaker. Discard the blocking solution and add 7 mL of TBST supplemented with 3% BSA and anti-6x-His monoclonal antibody (Thermo Fisher Scientific, cat. No. MA1-21315) diluted 1:2,000. Incubate for 2 hours on a shaker at room temperature. Discard the solution and wash the membrane with 3 $\times$ 20 mL of TBST, 5 min each, on a shaker. Incubate the membrane for 1 h in 7 mL of TBST supplemented with 3% BSA and anti-mouse IgG horseradish peroxidase-linked antibody (Amersham, cat no. NA931)
24. Discard the antibody solution and wash the membrane 3 times with 15–20 mL of TBST, 5 min each time. Develop the membrane with 1–1.5 mL of SuperSignal West Pico Chemoluminescent Substrate (Pierce, cat. No. 34080) according to manufacturer's instructions as described here: <https://www.thermofisher.com/order/catalog/product/34580>. Detect chemoluminescent signal on Kodak imaging station or similar imaging device. Briefly, mix two components of the chemoluminescent substrate (1 mL each) and overlay the nitrocellulose membrane for 1–2 minutes. Remove an excess of reagent by blotting with filter paper, and place membrane on a Saran wrap. Make sure that no air bubbles are visible. Place the membrane onto a holder of the Kodak imager, make sure that resolution of the image on a computer screen is of suitable quality, and acquire the image for a suitable period of time (typically 1–2 minutes).

Basic Protocol 3

Liposome-reconstitution of purified CB₂ receptor

This protocol is designed to prepare proteoliposomes from CB₂ protein purified using Basic protocol 2. The receptor reconstituted into proteoliposomes is more stable than in detergent micelles and is suitable for characterization by a range of various biophysical techniques. The homogeneity of proteoliposomes is further assessed by sucrose gradient centrifugation as described in Basic Protocol 4.

Materials—Pierce Detergent Removal Spin Columns (Thermo Scientific, cat. No. 87780)

1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) (Avanti Polar Lipids, cat. No. 850457)

1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG) (Avanti Polar Lipids, cat. No. 840457)

Dil Stain (1,1'-Diocadecyl-3,3',3'-Tetramethylindocarbocyanine Perchlorate ('DiI'; DiIC₁₈(3))) (Thermo Fisher, cat.no. D282)

CHAPS, 3-[(3-Cholamidopropyl)-Dimethylammonio]-1-Propane Sulfonate] • N,N-Dimethyl-3-Sulfo-N-[3-[[3 α ,5 β ,7 α ,12 α)-3,7,12-Trihydroxy-24-Oxocholan-24-yl]Amino]propyl]-1-Propanaminium Hydroxide, Inner Salt (Anatrace, cat. No. C316)

DC protein assay kit (BioRad, cat. No. 5000113)

1. Prepare four 4-mL Pierce Detergent removal columns by equilibrating in phosphate buffered saline (PBS) according to Manufacturer's instructions. Briefly, remove bottom closure and loosen cap. Place column into a 50 mL Falcon collection tube and centrifuge in swinging basket rotor at 1000 x g for 2 min. Discard the flowthrough. Add 4 mL of PBS and centrifuge again for 2 min. Repeat wash procedure two more times. Transfer the column into a fresh collection tube.

The equilibrated columns can be kept at room temperature for 1–2 hours until ready to use.

2. Prepare 30 mg of a mixture of POPC, POPG and Dil C18 (4:1:0.01 mol/mol/mol) by mixing appropriate volumes of stock solutions of these lipids in organic solvent (methanol) in a glass test tube. Evaporate organic solvents under a stream of nitrogen or argon gas.

A mixture of lipids in a glass tube can be prepared in advance, and after evaporation of organic solvent can be stored at –80 °C.

3. Dissolve lipids in 2 mL of 1% (w/v) CHAPS.
4. Thaw on ice and mix 3 mg of CB₂ protein with 30 mg of lipids dissolved in CHAPS.

At this point a mixture of protein, lipids and detergents can be incubated on ice for 30–60 min before proceeding to the next step.

5. Apply the protein-lipid-detergent mixture onto detergent removal columns (between 500–1000 μ L of sample per column). Incubate for 2 minutes at room temperature.
6. Centrifuge columns at 1000 x g for 2 min to collect detergent-free proteoliposome sample
7. The concentration of protein in proteoliposome preparation can be determined by using DC protein assay kit or similar as described in p. 64. If desired, the functional activity of the protein can also be determined at this point by performing a G protein activation test as described in (Vukoti et al. 2012).
8. Proteoliposomes can now be collected as described in this section below. The homogeneity of preparation can be studied by ultracentrifugation in sucrose gradient as described in **Basic Protocol 4**.
9. To remove residual detergents, combine flowthrough fractions containing liposomes and transfer them into 25 mL centrifuge tube for 70 Ti rotor. Add cold PBS to fill the tube almost to the top. Centrifuge at 60,000 rpm at 4°C overnight.

10. Aspirate the supernatant and re-suspend the pellet in 1–2 ml of cold PBS. Fill the tube with more PBS and centrifuge for 2 h at 60,000 rpm at 4°C.
11. Aspirate the supernatant. Re-suspend the proteoliposome pellet in 1–2 mL of cold PBS and transfer into a 3.8 mL tube for TLA 100.3 rotor. Carefully fill the tube with PBS to the level of 3.5 mL and centrifuge for 2 h at 90,000 rpm at 4°C.
12. Remove the supernatant. Carefully blow nitrogen gas over the pellet to remove residual liquid.
13. Transfer the liposome pellet into NMR rotor or store it at –80°C until ready for measurements.

Basic Protocol 4

Isopycnic Ultracentrifugation of proteoliposomes

This protocol describes the characterization of homogeneity of proteoliposome preparation by isopycnic centrifugation in a transparent plastic tube filled with a solution of sucrose that forms a gradient of density. Upon centrifugation, the proteoliposomes will form a distinct band that can be isolated. If several species with different densities are present in proteoliposome preparations, several bands can be visible upon centrifugation.

Materials: Phosphate-buffered saline (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.8; PBS). 10 x stock solution (Bio-Rad, cat. No. 161–0780)

Sucrose (Sigma, cat # S0389)

Fluorescence plate reader (Synergy HTX multimode reader, BioTek) or similar

Refractometer VISTA C10 Abbe (Nova Tech International,) or similar

Ultracentrifuge (Beckman Coulter Optima XE-90)

SW41 rotor (Beckman Coulter)

12 L centrifuge tubes (Ultra-Clear, Beckman cat # 344059)

1. Prepare 5.5 mL solutions of 3% sucrose (w/v) (density 1.010 g/cm³) and 20% sucrose (w/v) (1.081 g/cm³).
2. Prepare linear sucrose density gradient from 3% to 20% in 12-mL centrifuge tubes (Beckman, cat no. 344059) using gradient maker (Hoefer, cat. No. SG100) or similar.
3. Place proteoliposome suspension (no more than 500 µL) on top of a sucrose solution.

The centrifuge tubes should be handled extremely gently not to disturb the sucrose gradient.

4. Centrifuge at 180,000 x g at 4°C for 42 h on a XL-90 ultracentrifuge (Beckman) with SW41 rotor (Beckman Coulter).
5. Aliquot sucrose solution into approximately 120 fractions, 100 µL (microliters) each into micro-well plate.
6. Determine the location of the lipid band by measuring the fluorescence of fractions on a fluorescence microplate reader (Synergy or similar) using 530 nm excitation and 590 nm emission filters as described in (Kimura et al. 2012). DiIC18 dye is introduced into proteoliposomes during reconstitution of the protein (see Basic Protocol 3, step 68) and is used for fluorescent tracking.
7. Determine the density of the sucrose solution at the location of proteoliposome band by measuring the refractive index on a Vista C10 Abbe Refractometer or similar instrument, following manufacturer's instructions.

Reagents and Solutions

Use Milli-Q-purified water or equivalent in all recipes and protocols.

IPTG, isopropyl-b-D-thiogalactopyranoside, stock solution, 1 M

Dissolve 3.6 g IPTG (Thermo Scientific, cat. No. R0392) in 15 mL H₂O. Filter sterilize and aliquot. Store at –20 °C for several months.

Ampicillin stock solution

Prepare 50 mg/mL ampicillin (RPI, cat. No. A40040) in 10 mL dH₂O. Filter sterilize, prepare 1-mL aliquots in screw cap microcentrifuge tubes, and store at –20 °C for up to several months.

Luria Broth (LB)

Stir to suspend 20 g powder LB Broth (Lennox) (Sigma, cat. No. L7658) containing:

10 g tryptone

5 g yeast extract

5 g NaCl

Dissolve in 1 L dH₂O

Sterilize by autoclaving for 15 min at 121 °C.

Allow to cool before making additions, such as antibiotics

Store at room temperature for several months

LB Broth with agar (Lennox)

Dissolve 8 tablets of LB Broth with agar (Sigma L7025) in 1L of dH₂O.

Autoclave.

Store at room temperature for several months.

Minimal Salt Medium (MSM)

MSM medium contains:

4.65 g/L Na_2SO_4

14.6 g/L K_2HPO_4

4.07 g/L $\text{NaH}_2\text{PO}_4 \times 2\text{H}_2\text{O}$

1.2 g/L $\text{MgSO}_4 \times 7\text{H}_2\text{O}$

3.32 mg/L $\text{CaCl}_2 \times 2\text{H}_2\text{O}$

0.72 mg/L $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$

0.4 mg/L $\text{MnSO}_4 \times \text{H}_2\text{O}$

69.48 mg/L ethylenediaminetetraacetic acid (EDTA)

40.1 mg/L FeCl_3

0.236 mg/L $\text{CuSO}_4 \times 5\text{H}_2\text{O}$

0.84 mg/L $\text{CoCl}_2 \times 6\text{H}_2\text{O}$

Media is sterilized and supplemented with:

10 g/L glucose (sterilized separately)

2.74 g/L NH_4Cl (sterilized separately)

100 mg/L thiamine hydrochloride (sterilized separately)

100 mg/L ampicillin

Sterilize phosphate salts, magnesium salt, calcium salt, microelements, vitamins, glucose and ammonium separately and mix in fermenter vessel at room temperature.

Detergent stock solutions

DDM, DDM, n-dodecyl- β -D-maltopyranoside (Anatrace, cat. No D310) (Anatrace, cat. No. D310), prepare 10% stock solution, store at 4°C for several weeks. We recommend using the DDM of 99% purity or better, preferably from Anatrace or comparable source.

CHAPS/ CHS stock solution: prepare 6% (w/v) CHAPS/ 1.2% (w/v) CHS stock solution by using the following procedure:

Dissolve CHAPS (Anatrace, cat. No. C316) at a concentration of 12% in 200 mL H₂O under stirring

Re-suspend 4.8 g CHS (Anatrace, cat. No. CH210) in 150 mL H₂O under stirring

Add the suspension of CHS dropwise to the concentrated solution of CHAPS under stirring. Continue to stir until the solution becomes clear. Adjust the volume to 400 mL. Filter and keep the stock solution at 4°C. The stock solution can be stored for 2 months without any noticeable degradation.

Receptor Solubilization buffer

50 mM Tris-HCl buffer pH 7.5

100 mM NaCl

30% (v/v) glycerol

1% (w/v) DDM

0.5% (w/v) CHAPS

0.1% (w/v) CHS

10 µM CP-55,940

The solubilization buffer can be stored at 4 °C for 2 months.

Chromatography wash buffer (buffer A)

50 mM Tris-HCl buffer pH 7.5

100 mM NaCl

30% (v/v) glycerol

0.1% (w/v) DDM

0.5% (w/v) CHAPS

0.1% (w/v) CHS

10 µM CP-55,940

Buffer A can be stored at 4 °C for two months.

IMAC elution buffer (buffer B)

50 mM Tris-HCl buffer pH 7.5

100 mM NaCl

30% (v/v) glycerol

0.1% (w/v) DDM

0.5% (w/v) CHAPS

0.1% (w/v) CHS

250 mM imidazole

10 μ M CP-55,940

Buffer B can be stored at 4 °C for two months.

Dialysis buffer (buffer C)

50 mM Tris-HCl buffer pH 7.5

100 mM NaCl

10% (v/v) glycerol

0.1% (w/v) DDM

0.5% (w/v) CHAPS

0.1% (w/v) CHS

10 μ M CP-55,940

Buffer C can be stored at 4 °C for two months.

StrepTactin elution buffer

50 mM Tris-HCl buffer pH 7.5

500 mM NaCl

30% (v/v) glycerol

0.1% (w/v) DDM

0.5% (w/v) CHAPS

0.1% (w/v) CHS

50 mM biotin. Dissolve powder biotin sufficient for 20 mL elution buffer in 100–200 μ L 0.1 N NaOH. Add other components of the buffer and adjust pH to 7.5.

10 μ M CP-55,940

The Streptactin elution buffer can be stored at 4 °C for two months.

TBST

Add Tween 20 to 2L of TBS buffer to the final concentration of 0.05% (v/v)

Nitrocellulose membrane blocking solution

Dissolve BSA in 40 mL of TBST to the final concentration of 3% (w/v). We recommend preparing the BSA blocking buffer just before use. It can be stored at 4 °C overnight.

Commentary

Background information

GPCR represent the largest protein family encoded in the human genome (Takeda et al. 2002). The development of drugs that act upon GPCR-mediated pathways is hindered by insufficient information about three-dimensional structures of these proteins (Lefkowitz 2013). However, GPCR are large, hydrophobic integral membrane proteins that reside in the lipid bilayer of cell membrane, and their structure determination is very challenging due to problems involving in their low expression levels, instability, poor solubility, and difficulty in obtaining well-diffracting crystals (Sun et al. 2017) (Yeliseev and Vukoti 2011). Although a growing number of crystal structures of GPCR has been reported during the last decade, many of them was obtained through modifications of target proteins, removal of flexible regions, thermostabilization, use of high affinity ligands and lipidic monolayer environments. (Park et al. 2012; Thal et al. 2016; Sun et al. 2017). On the other hand, the advantage of nuclear magnetic resonance (NMR) for structural studies of GPCR is that it enables (potentially) wild type receptors to be analyzed in their native environment of planar hydrated lipid bilayers under physiologically relevant conditions (Park et al. 2012). However, NMR requires substantial quantities of highly purified proteins, labeled with stable isotopes. Consequently, here we describe our research on expression in a stable isotope-labeled form, purification and lipid-reconstitution of a class A GPCR, cannabinoid receptor CB₂. The expression is performed in *E. coli* cells cultivated in minimal salt medium, that allows supplementation with stable isotope-labeled amino acid and their precursors.

Critical Parameters

Expression of GPCR in *E. coli* cells—Attempts to express GPCR in *E. coli* often result in misfolding of the target protein and formation of inclusion bodies (Schmidt et al. 2017). While the expression levels of such membrane proteins may be quite high (several milligrams per liter of culture), the target protein then needs to be isolated and re-folded. The success of refolding procedure is difficult to predict, and it has almost never 100% efficiency (Schmidt et al. 2010). Thus, an additional step of separating a correctly folded from misfolded protein will still be required (Berger et al. 2013).

We present here an efficient expression protocol that allows preparation of correctly folded receptor imbedded in bacterial cytoplasmic membrane (Calandra et al. 1997; Yeliseev et al. 2005; Krepiy et al. 2006). This is achieved by using low expression temperature (20 °C), low copy number expression vector (pMalE-based plasmid) and expressing the target CB₂

protein as a fusion with N-terminal maltose-binding protein (MBP). The use of MBP as an N-terminal fusion partner, that is targeted to periplasmic space of *E. coli*, allows efficient folding and insertion of CB₂ protein into cytoplasmic membranes. Moreover, the resulting topology of CB₂ in bacterial membrane (N-terminus in periplasm, C-terminus in cytoplasm) ensures the formation of essential disulfide bridge in extracellular loop 2 of CB₂ receptor (Krepkiy et al. 2006). Furthermore, in the expression constructs used in our laboratory, the receptor is flanked by two small affinity tags (His₁₀ and twin-Strep-tag) that allow efficient purification of this protein based on affinity chromatography (Yeliseev et al. 2016). Importantly, a high affinity ligand (CP-55,940) should be added to cultivation medium to ensure the stability of the receptor (Vukoti et al. 2012). We recommend the use of a high affinity ligand such as agonist CP-55,940 or inverse agonist SR-144,528 that efficiently stabilize the recombinant protein in bacterial membranes as well as upon solubilization in detergent micelles. However, other synthetic ligands with suitable properties can be tested. It is also possible to perform ligand exchange during purification of receptor (Basic Protocol 2), if desired (Vukoti et al. 2012).

The CB₂ receptor produced by bacterial fermentation lacks such posttranslational modifications as glycosylation and palmitoylation. However, the bacterially produced receptor is homogenous and stable enough to satisfy conditions of various biophysical methods. Importantly, the *E. coli*-produced receptor is fully functional as assessed by the ligand binding and G protein activation assay (Yeliseev et al. 2005; Yeliseev et al. 2016).

To achieve reasonably high levels of expression of the target protein (2 mg/L or more) we employ fermentation in minimal salt medium (MSM) under controlled aeration, pH and nutrient supply. The use of ¹⁵N and ¹³C-labeled amino acid (Trp) allows incorporation of these isotopes in selective positions in the protein, while the use of uniformly ¹³C-labeled glucose and ¹⁵N-ammonium as sole sources of carbon and nitrogen respectively, results in production of uniformly ¹³C, ¹⁵N-labeled protein (Kimura et al. 2014).

Prior to the fermentation, the expression strain needs to be adapted to growth in MSM. We found that at least three successive rounds of adaptation need to be performed in order to achieve optimal results (Berger et al. 2010). The MSM-adapted cells can be stored as glycerol stocks at –80 °C for several years without loss of performance.

Sufficient quantity of freshly grown cells are required to seed the fermenter. Cells should not be overgrown prior, so the culture needs to be timed so that OD₆₀₀ will be 3–3.5 at harvest. Once in a fermenter vessel, cells are grown to a high optical density (OD₆₀₀ = 10–20) at 37°C and high aeration, and the protein production is initiated after changing the fermentation temperature to 20 °C, that slows the cell growth significantly. It is essential at this point to ensure adequate levels of glucose and ammonium in fermentation medium so that the cell culture growth continues uninterrupted until ready to be harvested (typically 6–8 hours post-induction). Cell pellet needs to be washed with cold buffer to remove excess of salts and can be stored at –80°C for several months.

Solubilization and purification of CB₂ receptor—Solubilization of the receptor is performed in a mixture of nonionic (DDM) and zwitterionic (CHAPS) detergents

supplemented with a derivative of cholesterol (CHS) that stabilizes the receptor. It is essential that a high affinity ligand is present in detergent solubilization buffer to ensure that receptor is stabilized in a functional conformation. Likewise, for stability of CB₂ it is essential that all procedures are performed at low temperature. While 1% (w/v) DDM is used for solubilization of CB₂ from cell membranes, once the receptor is extracted from membranes, the concentration of DDM can be reduced to 0.1% w/v. To increase the binding capacity of the affinity resin it is advisable to treat the solubilized cell extract with Dowex resin prior to applying it to affinity columns.

Purification is performed using two successive steps of affinity chromatography. The binding of the His-tagged (C-terminally) recombinant protein to the Ni resin should be performed by passing the protein extract through the resin packed in a long, narrow column. This increases the time of interaction between the His-tag on a protein and the resin and ensures higher binding of the protein. For the same reason, the flow rate should not exceed 0.5–0.7 ml/min. Batch purification is not recommended as the impurities present in crude protein extract tend to block the matrix of the resin, and the binding of the target protein decreases significantly (Vukoti et al. 2012).

The MBP fusion partner can be removed at this point. Protein eluted from Ni resin should be dialyzed against a buffer with lower salt and glycerol content. This is necessary since TEV protease is inhibited by high salt and glycerol concentrations (Yeliseev et al. 2007). The removal of the MBP N-terminal part of the fusion at the same time allows access to the twin-Strep-tag that is located immediately upstream of CB₂ in these expression constructs. This tag can now be effectively used for binding of the target protein to Strep-Tactin resin. It is recommended that a new generation of the resin known as StrepTactin XT is used since it has a much higher affinity for Strep-tag compared to the early version of this resin (StrepTactin) (Schmidt et al. 2013). This is especially important since the purification is performed from dilute solutions of the recombinant protein, in the presence of detergents that weaken the interaction between the resin and the tag on a receptor. It is also advisable to use a twin-Strep-tag (Yeliseev et al. 2016) rather than a single repeat of the tag is used since it improves the yield and the purity of the recombinant protein significantly.

Reconstitution of the receptor in lipid bilayers—Purified receptor can be reconstituted from detergent micelles into lipid bilayers of proteoliposomes (Kimura et al. 2014). This significantly stabilizes CB₂ and allows the protein sample to be studied by solid state NMR techniques (Kimura et al. 2014). Different protocols can be used to remove detergents and to ensure the incorporation of the protein in liposomes. We recommend the use of detergent-absorbing resin since they are simple, very reproducible and allow preparation of reasonably concentrated proteoliposome samples. The process can be easily scaled up or down by using large columns or using multiple columns. The residual detergents are efficiently removed by spinning down the liposomes in ultracentrifuge. The recovery of the sample is high (typically more than 80%) and highly reproducible.

Troubleshooting

Choice of expression system—While we typically use BL21 (DE3) as an expression host, other *E. coli* strains may be suitable for expression of CB₂ as well. For example, strains C43 and Rosetta Gami appear to perform well at these conditions. For incorporation of stable isotope-labeled tryptophan we successfully employ the BL21 (DE3) expression host. However, it is possible that labeling with other amino acids the use of auxotrophic strains of *E. coli* is warranted, depending on an amino acid of interest. One needs to keep in mind though that the auxotrophic strains of *E. coli* grow poorly on minimal medium as a rule, so that the target protein yield in such system may be compromised.

Expression plasmid—We recommend using a low copy number plasmid such as pMal (Berger et al. 2010)E (New England Biolabs) or similar. High copy number plasmids may result in production of large quantities of misfolded protein.

Poor growth of cells during cultivation—Poor growth of cells in minimal medium can be frequently observed. Therefore, it is essential to perform culture adaptation to minimal salt medium. We recommend at least three rounds of adaptation, after which the glycerol stock of the culture can be prepared and stored in deep freezer for several years without noticeable loss of viability.

Poor yield of target protein during fermentation—This problem can only be detected after the fermentation is completed. There may be multiple issues that result in the low yield of the target protein. In our experience, it is important not to overgrow cells prior to induction (OD₆₀₀ = 10–20 seem to be optimal). Also, we found that intermittent addition of asparagine during the induction phase may boost the production of the receptor. It is also important to keep the induction temperature at 20 °C, and to supplement the culture with the stabilizing ligand.

Poor yield of protein at purification—Retain all fractions during purification and examine them on Western blot. If protein is found in flowthrough fractions from Ni resin, attempt to use low flow rate at loading. If the target protein is present in wash fractions from Ni resin, use lower concentration of imidazole. If CB₂ protein is present in flowthrough fractions from StrepTactin resin, decreasing the loading flow rate or increasing the amount of resin packed in the column should improve the outcome.

Poor purity of the target protein—This can be a result of inadequate washing during chromatographic purification. Since StrepTactin XT binds the target protein very tightly, it is possible to increase the length of the wash step and introduce high salt concentration in the wash buffer to improve the efficiency of purification. Typically, very little target protein is lost during the wash step on StrepTactin XT column.

Inadequate labeling with stable isotopes.—The problem of inadequate labeling is unlikely to occur during the preparation of uniformly ¹³C, ¹⁵N labeled CB₂. ¹³C-uniformly labeled glucose serves as a sole source of carbon in this system, and ¹⁵NH₄Cl serves as a

sole source of nitrogen. Therefore, as long as the target protein is expressed, it is likely that its labeling pattern will be close to optimal.

However, there may be potential problems while selectively labeling with individual amino acids. There may be some isotope dilution if the exogenous labeled amino acid is utilized along with unlabeled amino acid the *de novo* synthesized by the cells in the course of fermentation. Another possible scenario is that the labeled amino acid is metabolized by the cells and is used in metabolic pathways leading to labeling of other amino acids. Therefore, it is recommended to select for labeling those amino acids that repress the *de novo* synthesis in cells, and whose utilization in other amino acid biosynthetic pathways is minimal. L-tryptophan satisfies these requirements. If labeling with other types of amino acids is desired, an optimization of the labeling protocol is likely to be required.

Anticipated Results

The amount of protein produced in fermentation will depend significantly on conditions of fermentation. If the recommendations of Basic Protocol 1 are used, we anticipate the expression level on the order 2–2.5 mg of CB₂ protein from 1 L of culture. Higher or lower levels can be expected depending on expression constructs and composition of fermentation medium. The addition of the high affinity ligand during the induction stage typically increases the accumulation of the target receptor.

The purity of the CB₂ after two successive steps of affinity chromatography is typically at least 90% or better. The purified protein in detergent micelles is extremely sensitive to temperature and should be kept either at 4 °C for a few days or at –80 °C for longer storage. The addition of CHS and a high affinity ligand is strongly recommended to prevent inactivation of the receptor. The stability of protein in lipid bilayers is somewhat better, and CB₂ reconstituted in lipid bilayers can be stored for at least a week at 4°C without significant loss of activity.

Potential negative results may include poor yield of the labeled protein or inadequate labeling. Poor yield of the labeled protein sometimes occurs if the induction of protein production is performed during suboptimal phase of cell growth. It is therefore imperative that the addition of the inducer of protein synthesis (IPTG) and the labeled amino acid (Trp) is timed to coincide with the cell culture attaining adequate cell density (OD₆₀₀ between 10 and 20), and the temperature shift from 37 °C to 20 °C is performed prior to induction.

It is possible that the produced protein may be only partially functional as determined by the ligand binding test. This may occur if the protein synthesis is performed at higher temperature rather than recommended 20 °C. It is also important to maintain reasonably high concentration of a specific ligand in cultivation medium, to ensure that the *de-novo* produced receptor is stabilized in a ligand-bound conformation.

Time Considerations

The time required for adaptation of the expression to MSM is approximately 3–4 days. The preparation of the fermenter, growth of cells prior to fermentation, fermentation and

collecting of biomass should take 3–4 days. The solubilization and purification protocol typically takes 4 days. The reconstitution with subsequent centrifugation steps takes 2–3 days.

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