



HAL
open science

A novel, generic and effective method for the rapid purification of G protein-coupled receptors

Thierry Magnin, Cédric Fiez-Vandal, Noelle Potier-Holler, Aline Coquard, Isabelle Leray, Tania Steffan, Christel Logez, Fatima Alkhalfioui, Francis Pattus, Renaud Wagner

► To cite this version:

Thierry Magnin, Cédric Fiez-Vandal, Noelle Potier-Holler, Aline Coquard, Isabelle Leray, et al.. A novel, generic and effective method for the rapid purification of G protein-coupled receptors. *Protein Expression and Purification*, 2009, 64 (1), pp.1-7. 10.1016/j.pep.2008.09.007 . hal-02979097

HAL Id: hal-02979097

<https://hal.science/hal-02979097>

Submitted on 26 Oct 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

A novel, generic and effective method for the rapid purification of G protein-coupled receptors

Thierry Magnin^{a,*}, Cédric Fiez-Vandal^a, Noëlle Potier^b, Aline Coquard^a, Isabelle Leray^a, Tania Steffan^a, Christel Logez^a, Fatima Alkhalfioui^a, Franc Pattus^a, Renaud Wagner^a

^aLC1-UMR 7175, Institut Gilbert Laustriat, Pôle API, Bd Sébastien Brant, BP 10413, 67412 Illkirch Cedex, France

^bInstitut de Chimie LC3 – CNRS-UMR 7177 ISIS, 8 allée Gaspard Monge, 67083 Strasbourg, France

ARTICLE INFO

Article history:

Received 5 June 2008

and in revised form 1 September 2008

Available online xxxxx

Keywords:

G protein-coupled receptor

Pichia pastoris

Solubilization

Purification

Cannabinoid

Opioid

Ligand

ABSTRACT

G protein-coupled receptors (GPCRs) constitute the largest family of membrane receptors and are of major therapeutic importance. Structure determination of G protein-coupled receptors and other applications require milligram quantities of purified receptor proteins on a regular basis. Recombinant GPCRs fused to a heterologous biotinylation domain were produced in the yeast *Pichia pastoris*. We describe an efficient method for their rapid purification that relies on the capture of these receptors with streptavidin immobilized on agarose beads, and their subsequent release by enzymatic digestion with TEV protease. This method has been applied to several GPCRs belonging to the class A rhodopsin subfamily, leading to high yields of purified proteins; it represents a method of choice for biochemical and biophysical studies when large quantities of purified GPCRs are needed.

© 2008 Elsevier Inc. All rights reserved.

Considerable research is ongoing in the area of membrane protein structure and function, and in recent years many efforts have been, in particular, focused on G protein-coupled receptors (GPCRs).¹ These proteins share a conserved architecture based on seven transmembrane segments; they are the key players of signal transduction and constitute the major group of drug targets for the pharmaceutical industry, with already more than half of modern drugs and almost 25% of the top 200 best-selling drugs targeting them [1]. Despite these crucial biological implications, very little is known about the detailed molecular mechanisms by which these membrane proteins recognize their extracellular stimuli and transmit the associated messages. The speed of discovery of novel GPCR-active drugs is hampered by the lack of structural information about these proteins, which is reflected by the fact that, to date, only rhodopsin, the rod-cell GPCR involved in dim light vision and the β_2 -adrenergic receptor, which plays an important role in cardiovascular physiology, have been crystallized and their structures solved at 2.2 and 2.4 Å, respectively [2,3].

For several protein families, including most of helical membrane proteins and in particular GPCRs, structural studies are still limited by low expression levels in heterologous systems

and/or by difficulties in obtaining pure, homogeneous protein preparations [4].

During the last decade, the methylotrophic yeast *Pichia pastoris* has been increasingly used for heterologous GPCR expression [5–8]. This yeast is particularly suitable for these applications, combining multiple advantages: (i) the relatively low costs related to protein expression; (ii) the availability of protease deficient *P. pastoris* strains; (iii) the presence of a well-defined strong promoter (i.e. the alcohol oxidase promoter) for efficient overexpression; (iv) the eukaryotic nature of post-translational modifications that lack extensive glycosylation; (v) the addressing of proteins to the plasma membrane due to the presence of targeting sequences and (vi) the feasibility of large-scale fermentation culturing when substantial quantities of material are needed.

We are currently using the *P. pastoris* system to express a dozen of GPCRs. Expression of these proteins has been optimized in a previous work [9], but their purification presented several problems: despite the relatively good levels of expression, GPCRs expressed in *P. pastoris* were very difficult to purify to homogeneity and in large quantities when conventional methods such as metal affinity chromatography and/or flag-agarose chromatography were used.

We have developed a rapid, generic purification procedure for GPCRs expressed in *P. pastoris*. This method relies on the presence of a C-terminal biotinylation domain in the recombinant proteins, allowing the high affinity capture of solubilized proteins on streptavidin (SAV) coupled to agarose beads. We also used the highly

* Corresponding author. Fax: +33 390244829.

E-mail address: magnin@esbs.u-strasbg.fr (T. Magnin).

¹ Abbreviations used: GPCRs, G protein-coupled receptors; SAV, streptavidin; TEV, tobacco etch virus; PBS, phosphate buffer saline; CHS, cholesteryl hemisuccinate.

specific tobacco etch virus (TEV) protease for the removal of both N- and C-terminal tags and the concomitant release of the native protein. Scale-up of this purification protocol will allow large quantities of receptors to be purified, thus enabling three-dimensional crystallization attempts and other biophysical approaches.

Materials and methods

Strains and plasmids

Pichia pastoris strains expressing different GPCRs were obtained as previously described [9].

The plasmid for tobacco etch virus (TEV) protease overexpression in *Escherichia coli* was obtained from Gunter Stier (EMBL, Heidelberg).

The plasmid for streptavidin overexpression in *E. coli* was obtained from Prof. Arosio (University of Brescia) [10].

Growth of *P. pastoris* and protein expression

Cells were grown overnight in BMGY (1% yeast extract; 1% peptone; 1.34% yeast nitrogen base; 1% glycerol; 0.00004% biotin; 100 mM potassium phosphate pH 6.0). Next day, the cells were diluted in BMGY and grown to an OD₆₀₀ of 10. The culture was centrifuged and the pellet resuspended in the same volume of BMMY (1% yeast extract; 1% peptone; 1.34% yeast nitrogen base; 0.5% methanol; 0.00004% biotin; 100 mM potassium phosphate pH 6.0; 2.5% DMSO; 0.4 mg/ml histidine) for the induction of protein production through the AOX1 promoter. After 18 h at 23 °C, the cells were spun down, washed with PBS (phosphate buffer saline: 20 mM sodium phosphate pH 7.4; 2 mM KCl; 150 mM NaCl) and either used immediately or frozen at –80 °C.

The induction conditions deviate slightly from standard ones: we showed previously that for most receptors, including DMSO in the induction medium and performing the induction at 23 °C led to an increase in the ratio of active versus total receptor [9].

Preparation of the cell membranes

All procedures were performed on ice. The cells were resuspended in cold lysis buffer (50 mM sodium phosphate pH 7.4; 100 mM NaCl; 5% glycerol; 5 mM EDTA; 1 mM PMSF). Cells were broken with glass beads (0.5 mm; 3 × 20 s), using a MP Biomedicals fast prep device. Cell debris and unbroken cells were removed by centrifugation at 5000g for 10 min and the turbid supernatant containing cell membranes was ultracentrifuged at 150,000g for 45 min. The resulting pellets were resuspended in membrane buffer (50 mM Tris; pH 8.0; 120 mM NaCl; 10% glycerol; 1 mM PMSF) and ultracentrifuged again. The membrane pellets were resuspended in membrane buffer, flash frozen in liquid nitrogen and stored at –80 °C. Protein concentrations were determined by bicinchoninic acid assay (Pierce) using bovine serum albumin as standard.

Western blotting and protein detection

Chemiluminescent Western blotting detection reagents were from Pierce. Mouse monoclonal anti-Flag M2 antibody was from Sigma. Peroxidase-linked anti-mouse IgG was purchased from GE Healthcare. Alkaline phosphatase-coupled streptavidin and the corresponding substrate tablets (NBT/BCIP) were purchased from Sigma.

Proteins were separated by SDS–PAGE (10% gel) and visualized with coomassie blue or transferred overnight at 30 V to a nitrocellulose membrane. The membrane was developed either with the

anti-FLAG M2 antibody or with alkaline phosphatase conjugated streptavidin.

In vitro biotinylation

Membranes (50 µg) were mixed with 10 mM ATP, 10 mM MgOAc, 50 µM biotin, 50 mM bicine buffer (pH 8.3) with or without 12,500 U of biotin–protein ligase (BirA; Genecopoeia). The suspensions were incubated at 30 °C for 40 min, then submitted to SDS–PAGE and Western blotting. Biotinylated proteins were detected as described above.

Solubilization

Detergents were obtained from Anatrache except for CHAPS (Euromedex). Membranes were diluted to obtain a protein concentration of 1 mg/ml in the solubilization buffer (final concentrations: 50 mM sodium phosphate pH 7.4; 10% glycerol; 400 mM NaCl). Then the detergent *n*-dodecyl β-D-maltopyranoside (DDM) was added to a final concentration of 1% and the solution was stirred at room temperature for 15 min. After ultracentrifugation (150,000g, 45 min), the supernatant was directly used for purification.

Purification with streptavidin beads

The supernatant obtained after solubilization was mixed with streptavidin-coated beads (see below) at various ratios. This preparation was kept overnight at 4 °C with orbital shaking; then the suspension was centrifuged at 1000g for 5 min, the supernatant was removed and the beads were incubated for 1 h at 30 °C in solubilization buffer supplemented with 0.1% DDM and endoglycosidase (PNGase F, 1000 U). The beads were then sequentially washed with (i) solubilization buffer supplemented with 1% CHAPS, (ii) solubilization buffer supplemented with 0.1% DDM, (iii) solubilization buffer supplemented with NaCl (1.5 M final concentration) and 0.1% DDM, (iv) solubilization buffer supplemented with 1 M NaSCN and 0.1% DDM, (v) three times with solubilization buffer supplemented with 2 mM DTT and 0.1% DDM (TEV buffer). All washing steps were performed at room temperature with gentle orbital shaking. Immobilized receptor were then cleaved off their tags and therefore released from the beads by addition of TEV protease (1 µg of protease for 20 mg of starting membrane proteins) and shaking at 25 °C for 0.5–16 h.

Preparation of streptavidin, streptavidin-coated agarose beads (SAV beads) and TEV protease

Streptavidin was overproduced in *E. coli* and purified as described [10]. The protein was dialyzed against 0.1 M NaHCO₃ pH 8.3 containing 0.5 M NaCl and then concentrated on a vivaspin concentrator (30 kDa cut-off). The protein (0.8 mg/ml final concentration) was then coupled to cyanogen bromide-activated sepharose 4B (Sigma) according to the manufacturer's instructions, except that the sepharose beads were pre-hydrolyzed for 2 h at pH 8.3 before adding streptavidin. The beads were kept at 4 °C in PBS supplemented with 0.02% sodium azide (four volumes of PBS/azide added to one volume of wet beads), and no decrease in the GPCR capture efficiency was noticed after 6 months of storage. In some experiments, used streptavidin beads were recycled by denaturation with 8 M guanidine–HCl in 50 mM citrate pH 3.0 followed by renaturation with a decreasing guanidine concentration gradient. The capacity of these recycled beads to immobilize receptors was decreased by 40–60%, when compared with unused beads.

TEV protease was overexpressed in *E. coli* BL21(DE3)pLysS (overnight induction in LB at 23 °C with 0.5 mM IPTG) and then

198 purified on an IMAC column (His Trap, GE healthcare) followed by
 199 a desalting step and a cation exchange chromatography at pH 6.1
 200 on a Mono S column (GE healthcare). After elution with a
 201 0–1.0 M NaCl gradient, protease containing fractions, which eluted at
 202 750 mM NaCl, were aliquoted and stored at -80°C .

203 Ligand binding experiments

204 Binding assays were performed as previously described [9],
 205 using 10 nM tritiated ligands CP55940 and rauwolfscine for
 206 CNR2_HUMAN and ADA2B_HUMAN, respectively. The binding
 207 buffer used for the CNR2 receptor contained 50 mM Tris-HCl
 208 pH 7.4, 2.5 mM EDTA, 5 mM MgCl_2 and 0.5 mg/ml bovine serum
 209 albumin.

210 MALDI-TOF mass spectrometry

211 SDS-PAGE separated protein bands were excised from the gel
 212 and the proteins were digested according to the Shevchenko proto-
 213 col [11]. The gel plugs were washed three times with 100 μl of
 214 25 mM ammonium hydrogen carbonate (NH_4HCO_3) and 100 μl of
 215 acetonitrile. The cysteine residues were reduced by 50 μl of
 216 10 mM dithiothreitol at 56°C and alkylated by 50 μl of 55 mM
 217 iodoacetamide. After dehydration with acetonitrile, the proteins
 218 were cleaved with 15 μl of a 12.5 ng/ μl solution of proteomics
 219 grade trypsin (Sigma) in 25 mM NH_4HCO_3 . The digestion was per-
 220 formed overnight at room temperature. The resulting peptides
 221 were extracted with a 65% acetonitrile solution containing 5% acid
 222 formic and 0.05% SDS for 1 h. MALDI-TOF mass measurements were
 223 carried out on an AutoFlex II TOF/TOF (Bruker Daltonik GmbH, Bre-
 224 men, Germany) operating in positive reflectron mode. The samples
 225 were prepared by standard dried droplet preparation on stainless
 226 steel MALDI targets using α -cyano-4-hydroxycinnamic acid as ma-
 227 trix. The samples were washed twice with 5% formic acid. External
 228 calibration of MALDI mass spectra was carried out using singly
 229 charged monoisotopic peaks of a mixture of bradykinin 1–7
 230 ($m/z = 757.400$), human angiotensin II ($m/z = 1046.542$), human
 231 angiotensin I ($m/z = 1296.685$), substance P ($m/z = 1347.735$), bom-
 232 besin ($m/z = 1619.822$), ACTH 1–17 ($m/z = 2093.087$) and ACTH
 233 18–39 ($m/z = 2465.199$). Monoisotopic peptide masses were auto-
 234 matically annotated using Flexanalysis 3.0.

235 Results

236 GPCRs were overexpressed in *P. pastoris* as previously described
 237 [9]. The recombinant proteins comprise an N-terminal α -factor sig-
 238 nal sequence from *Saccharomyces cerevisiae* followed by a Flag tag,
 239 a deca-histidine-tag, a tobacco etch virus (TEV) protease site, the
 240 GPCR open reading frame, a second TEV site and finally the *Propi-*
 241 *onibacterium shermanii* biotinylation domain at the C-terminus.

242 Our earlier purification schemes relied on the presence of the
 243 N-terminal deca-histidine and Flag tags, but this strategy gave
 244 unsatisfactory results because of the poor specificity and efficiency
 245 of these tags. In these recombinant proteins, the C-terminal trans-
 246 carboxylase biotinylation domain had been added mainly to detect
 247 the protein throughout the purification steps and to maintain its
 248 stability [12,13]. As an alternative to the first purification strategy,
 249 we decided to take advantage of the biotinylation domain to effi-
 250 ciently capture the GPCRs on SAV-agarose beads. The protein is
 251 immobilized on the beads through high affinity interaction, and
 252 can undergo subsequent washing treatment and might also be
 253 reconditioned. The final step relies on exploiting the TEV protease
 254 sites flanking the receptor to release the purified protein from the
 255 beads (Fig. 1).

256 *In vivo* biotinylation of recombinant GPCRs expressed in *Pichia* 257 *pastoris*

258 A prerequisite for the development of such a purification
 259 method is that the receptors are efficiently biotinylated *in vivo*.
 260 Several studies conducted on membrane proteins fused with bio-
 261 tinylation tags and expressed in *P. pastoris* have shown that this
 262 yeast indeed possesses a biotin ligase (ortholog of the *E. coli* BirA
 263 protein) which biotinylates not only several low abundance *Pichia*
 264 proteins but also heterologous proteins [14,15].

265 Thus, we first verified that the *Pichia* strains used in this study
 266 effectively contained a biotin ligase activity and that the level of
 267 *in vivo* biotinylation was sufficient to directly undertake purifica-
 268 tion on streptavidin-agarose. For this purpose, yeast cells over-
 269 expressing four different GPCRs or yeast cells transformed with
 270 an empty vector were grown in induction conditions. Then,
 271 membrane proteins were extracted, submitted to SDS-PAGE and
 272 transferred to a nitrocellulose membrane prior to the detection

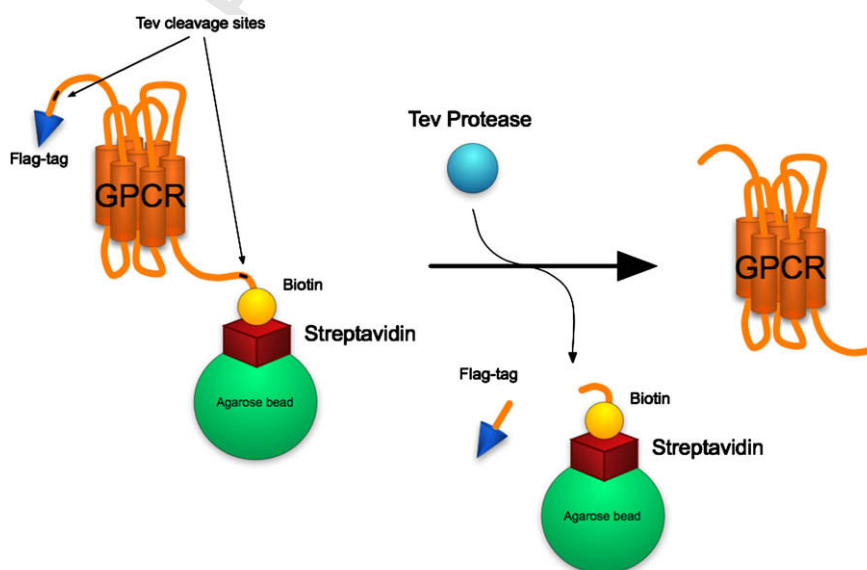


Fig. 1. Schematic representation of the method. A solubilized receptor is captured on immobilized streptavidin. The TEV protease then cleaves the N- and C-terminal tags, thus releasing the receptor and the N-terminal tag. The C-terminal tag remains bound to streptavidin.

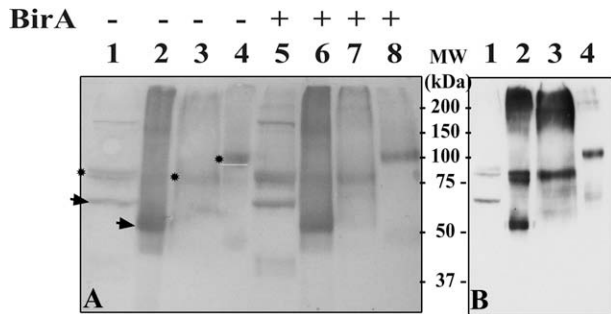


Fig. 2. Detection of four GPCRs expressed in *Pichia pastoris*. Membrane proteins were subjected to SDS-PAGE, and transferred to nitrocellulose. The receptors were detected with (A) Alkaline phosphatase-linked streptavidin or (B) anti-Flag M2 monoclonal antibody. Lanes 1 and 5: ADA2B_HUMAN; lanes 2 and 6: CNR2_HUMAN; lanes 3 and 7: OPRK_HUMAN; lanes 4 and 8: NK3R_HUMAN. Membranes from lanes 5 to 8 were subjected to *in vitro* biotinylation with the BirA protein-biotin ligase prior to SDS-PAGE. Arrows point to receptor bands at the expected size. Asterisks show receptors for which the signal sequence was not processed, leading to molecular masses higher than expected.

Experimental section. The difference is most likely due to the Q1 316 different levels of substitution of the agarose beads with SAV: 317 the pre-hydrolysis of the beads which we included prior to strep- 318 tavidin coupling most probably leads to a lower density of SAV 319 on the beads and thus reduces the steric hindrance problems that 320 could be associated with a higher SAV density. 321

After solubilization, receptors were captured on SAV-coated 322 beads in a batch mode. Surprisingly, very small quantities of the 323 receptors bound to the beads within the first hours, although the 324 streptavidin-biotin interaction is the strongest non-covalent 325 biological interaction ($K_a = 10^{15} \text{ M}^{-1}$) between a protein and its 326 ligand known to date. The bond formation between SAV and biotin 327 is normally rapid and essentially non-reversible, suggesting that 328 most probably, in our case, steric hindrance due to the interaction 329 of the biotinylated C-terminal part of the tagged GPCR with neigh- 330 boring parts of the protein prevented the rapid capture of biotin by 331 SAV. However, the quantity of bound protein increased with time, 332 and an overnight contact at 4 °C proved to be optimum. Longer 333 incubation times, even at higher temperatures (20–37 °C), did 334 not increase the yield (data not shown). The optimal amount of 335 home-made SAV-agarose resin necessary for the purification, 336 starting from 20 mg of membrane proteins, was calculated to be 337 1.2 ml of resin. Increasing the resin quantity did not improve the 338 yield of purified protein but actually slightly increased the amount 339 of contaminants. 340

After capture of CNR2 on SAV, the beads were incubated in 341 presence of an endoglycosidase (PNGase F). They were further 342 extensively washed to remove non-specific contaminants. Several 343 washing buffers were tested, including high and low pH buffers, 344 high detergent and high salt buffers. The best results in terms of 345 final receptor purity consisted in sequential washes with buffers 346 containing (i) 1% CHAPS for removal of contaminants interacting 347 through hydrophobic bonds, (ii) 1.5 M NaCl for contaminants 348 bound through hydrophilic interactions and in some cases and 349 (iii) the chaotropic agent NaSCN (1.0 M) for very strongly bound 350 contaminants. Then, the beads were equilibrated in TEV buffer 351 containing 2 mM DTT which is an essential component to maintain 352 the functionality of the TEV protease over long periods of time. The 353 TEV protease cleaves off the receptors from the SAV-coated beads 354 to which the receptors are bound by their C-terminal biotinylated 355 tag, and also releases the N-terminal tags from the receptors. After 356 addition of the protease, a time-course was performed to estimate 357 the optimal incubation time: Fig. 3 shows that the cleaved receptor 358 can be detected after 1 h of incubation (lane 2). Its quantity 359 increased from 1 to 3 h (lanes 1–4). However, the maximum 360 amount of receptor was obtained either after 3 h (lane 4) or after 361 overnight incubation (lane 5). Two bands can be seen, the main 362 one at 36 kDa and a more hazy band at ~40 kDa. Both were iden- 363 tified as the cannabinoid receptor by mass spectrometry (see 364 below). The 40 kDa band most likely represents a post-translationally 365 modified version of the receptor. 366

Several purified GPCRs 367

The one step purification method described above was tested 368 out on several other GPCRs belonging to the rhodopsin-related 369 class A subfamily. Fig. 4 shows the results obtained with five of 370 these receptors, the human neurokinin-3 receptor (NK3R_HUMAN, 371 theoretical molecular weight 52 kDa), the human purinergic recep- 372 tor type-1 (P2RY1_HUMAN, 42 kDa), the human κ -opioid receptor 373 (OPRK_HUMAN, 43 kDa), the human histamine H_1 receptor 374 (HRH1_HUMAN, 56 kDa), the human α_{2B} -adrenergic receptor 375 (ADA2B_HUMAN, 50 kDa). All of these receptors were obtained in 376 a relatively pure state (Fig. 4). NK3R (lane 1) and ADA2B (lane 5) 377 migrate at the expected molecular weights, but P2RY1 (lane 2), 378 OPRK (lane 3) and HRH1 (lane 4) migrate slightly faster than 379

of biotinylated proteins with alkaline phosphatase-linked strepta- 273 vidin. For yeast transformed with an empty vector, several endog- 274 enous biotinylated proteins were detected in the soluble fractions, 275 but none could be detected in the membrane fractions (results not 276 shown). Fig. 2A (lanes 1–4) shows that all four receptors tested, 277 respectively, ADA2B_HUMAN, CNR2_HUMAN, OPRK_HUMAN and 278 NK3R_HUMAN are biotinylated in *P. pastoris*. The sizes of the 279 detected proteins are in good agreement with those expected for 280 ADA2B (observed Mr: 65 kDa) and CNR2 (55 kDa), taking into 281 account the size contribution of the N- and C-terminal tags. For 282 OPRK (85 kDa) and NK3R (100 kDa), the observed sizes are higher 283 than expected. This is likely to be due to a lack of processing of the 284 N-terminal α -factor signal sequence by internal Kex2 endopepti- 285 dase of *P. pastoris*. The same probably applies to the additional 286 ~85 kDa doublet observed for the ADA2B receptor (Fig. 2, lanes 1 287 and 5). This absence of signal sequence processing was reported 288 for the mouse 5HT_{5A} receptor also expressed in *P. pastoris* [14]. 289

When the membranes were treated with exogenously added 290 biotin-protein ligase (BirA), only a slight increase in the level of 291 biotinylation could be observed (Fig. 2A, compare lanes 1–4 and 292 5–8), indicating that, *in vivo*, the biotinylation of the proteins 293 was almost complete. Fig. 2B shows the same receptors detected 294 with an anti-flag M2 monoclonal antibody; the bands observed 295 are essentially the same as those detected with streptavidin-phos- 296 phatase. Taken together, these results indicate that both the N- and 297 C-terminal parts of the proteins are intact since they are detected 298 by the anti-flag antibody and streptavidin-phosphatase, respec- 299 tively. Dimers and higher order oligomers are also detected, espe- 300 cially for CNR2 and OPRK (Fig. 2A, lanes 2, 3, 6 and 7; Fig. 2B, lanes 301 2 and 3). Such SDS-resistant dimers/higher order oligomers have 302 been reported for an ever-increasing number of GPCRs [16]. 303

These results along with the efficient *in vivo* biotinylation of 304 these receptors prompted us to perform further purification tests. 305 With the aim of developing a generic method (see Fig. 1), the 306 human peripheral cannabinoid receptor (CNR2_HUMAN) was used 307 as a prototypical GPCR. 308

CNR2_HUMAN capture onto streptavidin-coated beads and its release 309 by TEV protease 310

Membrane proteins (1 mg/ml) containing CNR2_HUMAN were 311 solubilized with 1% DDM (*n*-dodecyl β -D-maltopyranoside) and 312 then mixed with SAV-agarose beads. Commercial beads were 313 tested in preliminary experiments, but the results were not as good 314 as with our in-house made SAV beads prepared as described in 315

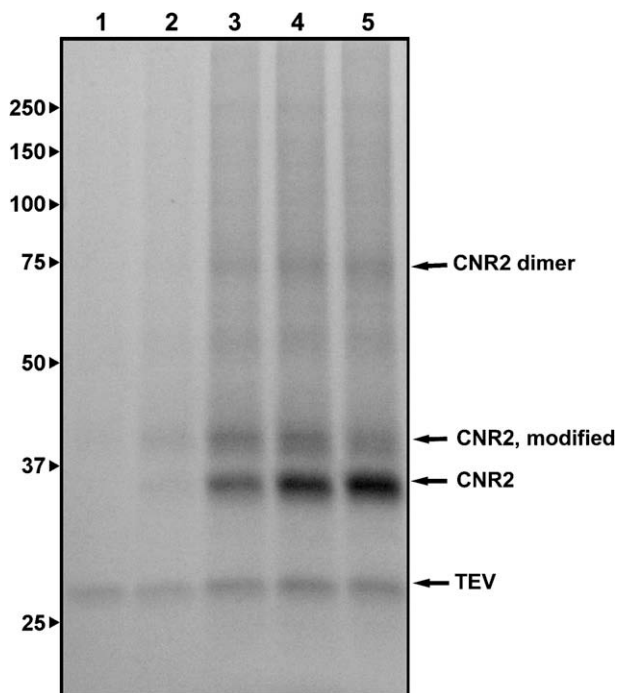


Fig. 3. Time course of CNR2 release by the TEV protease. CNR2 was solubilized and bound to streptavidin-agarose beads; the beads were washed and samples were taken at different time points after addition of the TEV protease and denatured with SDS sample buffer. Lane 1, time 0 after addition of the TEV protease; lane 2, 1 h after addition; lane 3, 2 h; lane 4, 3 h; lane 5, 14 h (overnight).

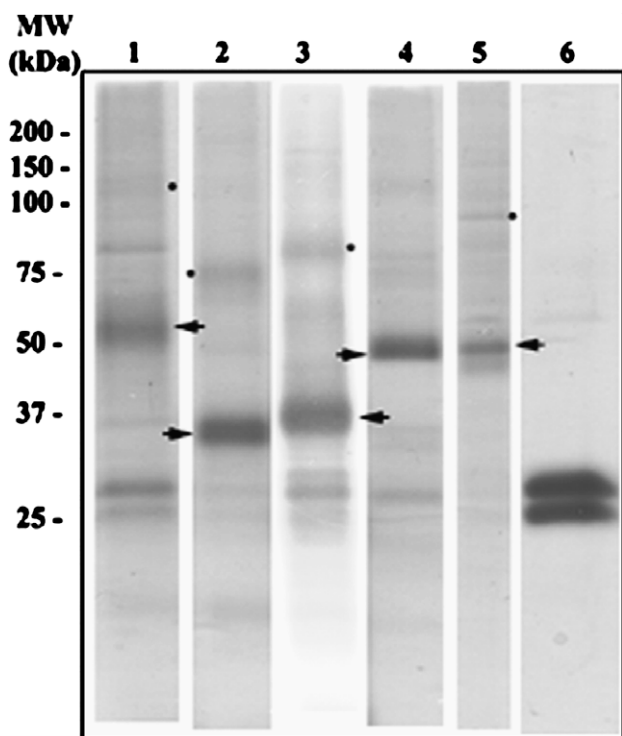


Fig. 4. Several purified GPCRs. Proteins purified with streptavidin beads were separated by SDS-PAGE and stained with coomassie blue. Lane 1, NK3R_HUMAN; lane 2, P2Y1_HUMAN; lane 3, OPRK_HUMAN; lane 4, HRH1_HUMAN; lane 5, ADA2B_HUMAN; lane 6, TEV protease. Arrowheads indicate purified GPCRs and asterisks indicate probable dimers.

Table 1
Tryptic fragments observed by MS

Receptor	Measured masses	Sequence
OPRK	1086.536	157–165
	1176.577	362–371
	1389.618	340–349
	1431.763	360–371
CNR2	758.386	229–235
	971.483	345–352
	1045.551	229–237
	1243.616	322–333
	1333.612	307–317
	1542.740	215–228
	2075.997	334–352
	2109.026	14–32
ADA2B	854.459	58–65
	980.569	23–30
	1055.676	140–150
	1186.689	254–264
	1320.670	163–174
	1342.804	254–265
	1395.967	241–263
	1722.032	175–191
	1822.928	158–174
	2202.289	117–139
	2441.589	126–150

Tryptic fragments were obtained and analyzed by MALDI mass spectrometry, as described in Methods section, for the following receptors: OPRK_HUMAN, CNR2_HUMAN and ADA2B_HUMAN.

among GPCRs [17]. They may bind more SDS than other proteins of similar size, due to a higher level of hydrophobicity and thus migrate faster during SDS-PAGE.

In order to confirm the identities of the purified receptors, the SDS-PAGE bands of purified OPRK, CNR2 and ADA2B, respectively, were excised and submitted to in-gel digestion followed by MALDI-TOF mass fingerprinting. As shown in Table 1, a total of 4, 8 and 11 peptides were identified for OPRK, CNR2 and ADA2B, respectively. Their masses were in concordance with the calculated molecular masses of theoretical tryptic peptides derived either from the cytoplasmic loops or from the N- and C-terminal domains. These analyses provided an unambiguous confirmation of the identities of the purified receptors. In the case of the CNR2 receptor, that gives two bands on SDS-PAGE (Fig. 3), both bands gave a similar pattern, thus confirming that the upper band likely represents a post-translationally modified version of the receptor.

Ligand binding capacity of purified CNR2 and ADA2B receptors

Binding assays were realized for two purified, SAV-agarose bound receptors, CNR2_HUMAN and ADA2B_HUMAN, using specific ³H-labelled ligands (CP 55940 and rauwolscine, respectively). Two purification conditions were tested, the first including 0.1% DDM in all buffers, the second with 0.1% DDM and 0.02% cholesteryl hemisuccinate (CHS), a well-known GPCR stabilizer. For CNR2, the binding level was 9 nmol/mg in the first condition and 10.5 nmol/mg in the second, corresponding, respectively, to 36% and 42% of active receptors in the preparation. For the ADA2B receptor, the values were much lower: 0 and 0.2 nmol/mg in the two conditions tested, respectively, corresponding to only 0% and 1% of active receptors.

Yields of purification

The yields obtained for several receptors are displayed in Table 2. They were mainly determined by two methods. The first consisted of protein measurements made after the removal of the TEV protein and the N-terminal free tags over a gel filtration column (Superose

expected (34, 37 and 50 kDa, respectively). This behavior is relatively frequent with membrane proteins and particularly

Table 2
Purification yields for 10 GPCRs

Receptor	Yield (mg)
CNR2_HUMAN	0.9
NK1R_HUMAN	0.05
NK3R_HUMAN	0.4
ADA2B_HUMAN	0.2
ADA1B_MESAU	0.1
HRH1_HUMAN	0.5
D2DR_HUMAN	0.05
P2Y1_HUMAN	0.5
OPRD_HUMAN	0.7
OPRK_HUMAN	0.8
ADA2C_RAT	<0.02
PAR1_HUMAN	<0.02

The yields are expressed in mg of purified GPCR, obtained from 1 L of induced culture.

12). The second was obtained by comparing the band intensities of purified receptors on a coomassie blue stained gel with known quantities of BSA loaded on the same gels. The yields vary between 0.05 and 0.9 mg of purified receptor per liter of culture. Since several liters of *P. pastoris* culture can be easily handled and processed, especially using the easy purification method we describe here, most of the receptors are available in sufficient quantities for biophysical studies or for other studies requiring large amounts of purified proteins. A few receptors could not be purified, e.g. the human serotonin 5HT_{1B} receptor (5HT1B_HUMAN) or only purified in low amounts, e.g. the human proteinase-activated receptor 1 precursor (PAR1_HUMAN), the mesau α_{1B} -adrenergic receptor (ADA1B_MESAU), the human neurokinin-1 receptor (NK1R_HUMAN) and the rat α_{2C} -adrenergic receptor (ADA2C_RAT). In addition, the human neurokinin-2 receptor (NK2R_HUMAN) could only be purified as a low molecular weight, breakdown product, due to the presence of a cryptic TEV site in its sequence, as was the case for the human neurotensin receptor [18].

Discussion

The GPCRs are the largest protein family known to date, comprised of an estimated 600–1000 members involved in various types of stimulus-response pathways, ranging from intercellular communication pathways to determination of physiological senses. GPCRs are involved in a wide range of disorders, including allergies, cardiovascular dysfunction, depression, obesity, cancer, pain, diabetes and various central nervous system disorders. The GPCR family of receptors is well-established as the premier target family for drug development: about half of the drugs on the market today are targeted towards GPCRs. Thus, taking into consideration the pharmacological importance of GPCRs, it is crucial to be able to produce pure and functional GPCRs on a custom basis to assist in the development of new GPCR targeting drugs.

Methods in use for GPCR purification from mammalian, insect, yeast or bacterial cells usually include numerous, lengthy, high-cost purification steps [4,19]. Recent advances have been made with cell-free translational systems and the results obtained seem to be promising. However, the number of GPCRs expressed in this way is still low and the experiments are still costly and require a lot of development and optimization [20–22].

Purification methods for membrane proteins that use the avidin–biotin interaction have been developed in a few cases [23,24]. Usually, monomeric avidin bound to agarose has been used, but some problems, mainly concerning elution of the proteins, have been frequently reported [24]. The streptavidin–biotin is not generally used because of the very high strength of this interaction, making it virtually impossible to elute the protein of interest in mild conditions. We turned this drawback to our

advantage by actually conserving the N-terminal tags in position on the streptavidin protein and exploiting TEV elution to liberate the protein of interest. The strength of the streptavidin–biotin interaction also allows, when necessary, the use of harsh washing conditions such as very high or low pH, or high concentrations of denaturants such as urea or guanidine.

The method described here is highly efficient and allows the rapid purification of GPCRs: the purification itself can be performed within 18 h, and only 3 days are necessary to obtain the purified protein from a starter culture. Preliminary tests can be performed, when a new GPCR or a mutated GPCR is being studied, with only 20–50 mg of membrane proteins, thus limiting the amount of handwork needed. The costs involved are very low because of the use of the yeast *P. pastoris*, but also because no expensive purification resins or apparatuses are needed, since the method relies on the sole streptavidin–agarose resin and batch method.

The method allows easy exchange of buffers, salts and detergents, as well as addition of stabilizing additives early on in the purification process, or only at the last step involving elution by TEV. In our hands, no strong inhibition of the TEV protease could be observed, whatever the additives (specific ligands and/or CHS) or detergents used. In a previous study, the protease was shown to be inhibited by several detergents, however, the detergent concentrations used were much higher, at least by an order of magnitude, than the concentrations used in our study [25].

For most GPCRs used in this study, substantial amounts of tag-free proteins have been purified in quantities and at purity levels sufficient to initiate structural studies. Among the receptors tested, one could not be purified and a few were obtained only in low amounts. Most of these refractory receptors were nevertheless expressed and biotinylated to levels comparable to receptors purified with good yields, indicating that the problem most probably lies in the organization of the C-terminal part of the proteins, where the biotin moiety could be hindered. A possible remedy would be to add a linker between the GPCR and the biotinylation domain, thus rendering the biotin able to interact with SAV.

Besides structural studies, a possible application of the method would be to use streptavidin–agarose bound receptors to go “fishing” for receptor-interacting proteins in soluble/membrane extracts of various types of cells or tissues, in the presence or absence of added ligands. We recently obtained preliminary results showing that β -arrestin-1, a protein involved in GPCRs internalization, can be retrieved with SAV–agarose bound CNR2 receptor from an *E. coli* extract containing arrestin expressed in low amounts (results not shown). The method described here would allow (i) a direct biochemical confirmation of interactions detected by other methods such as immunoprecipitation, (ii) the identification of novel interacting partners and (iii) the possibility to release the GPCR-interacting partner complexes with the TEV protease and to realize a biochemical study of such complexes.

Acknowledgments

We are grateful to Rim Benmamaar for improving the English of the article. This study was funded by the European Membrane Protein consortium (eMeP).

References

- [1] R. Fredriksson, M.C. Lagerström, L.-G. Lundin, H.B. Schiöth, The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints, *Mol. Pharmacol.* 63 (2003) 1256–1272.
- [2] T. Okada, M. Sugihara, A.N. Bondar, M. Elstner, P. Entel, V. Buss, The retinal conformation and its environment in rhodopsin in light of a new 2.2 Å crystal structure, *J. Mol. Biol.* 342 (2004) 571–583.

- 526 [3] V. Cherezov, D.M. Rosenbaum, M.A. Hanson, S.G. Rasmussen, F.S. Thian, T.S.
527 Kobilka, H.J. Choi, P. Kuhn, W.I. Weiss, B.K. Kobilka, R.C. Stevens, High-
528 resolution crystal structure of an engineered human {beta}2-adrenergic G
529 protein coupled receptor, *Science* 318 (2007) 1258–1265.
- 530 [4] J.J. Lacapère, E. Pebay-Peyroula, J.-M. Neumann, C. Etchebest, Determining
531 membrane protein structures: still a challenge!, *Trends Biochem Sci.* 32 (2007)
532 259–270.
- 533 [5] S. Noguchi, Y. Satow, Purification of human β_2 -adrenergic receptor expressed
534 in methylotrophic yeast *Pichia pastoris*, *J. Biochem.* 14 (2006) 799–804.
- 535 [6] H.M. Weiß, W. Haase, H. Reiländer, Expression of an integral membrane
536 protein, in: *Methods in Molecular Biology, Pichia Protocols*, vol. 103, Humana
537 press, 1998, pp 227–239.
- 538 [7] N.J. Fraser, Expression of a glycosylation deficient version of the human
539 adenosine 2a receptor for structural studies, *Protein Expr. Purif.* 49 (2006)
540 129–137.
- 541 [8] V. Sarramegna, F. Talmont, P. Demange, A. Milon, Heterologous expression of
542 G-protein-coupled receptors: comparison of expression systems from the
543 standpoint of large-scale production and purification, *Cell. Mol. Life Sci.* 60
544 (2003) 1529–1546.
- 545 [9] N. André, N. Cherouati, C. Prual, T. Steffan, G. Zeder-Lutz, T. Magnin, F. Pattus,
546 H. Michel, R. Wagner, C. Reinhart, Enhancing functional production of G
547 protein-coupled receptors in *Pichia pastoris* to levels required for structural
548 studies via a single expression screen, *Protein Sci.* 15 (2006) 1115–1126.
- 549 [10] A. Gallizia, C. de Lalla, E. Nardone, P. Santambrogio, A. Brandazza, A. Sidoli, P.
550 Arosio, Production of a soluble and functional recombinant streptavidin in
551 *Escherichia coli*, *Protein Expr. Purif.* 14 (1998) 192–196.
- 552 [11] O.M. Jensen, M. Wilm, A. Shevchenko, M. Mann, Peptide sequencing of 2-DE
553 gel-isolated proteins by nanoelectrospray tandem mass spectrometry,
554 *Methods Mol. Biol.* 112 (1999) 571–588.
- 555 [12] K. Lundstrom, R. Wagner, C. Reinhart, A. Desmyter, N. Cherouati, T. Magnin, G.
556 Zeder-Lutz, M. Courtot, C. Prual, N. André, G. Hassaine, H. Michel, C. Cambillau, F.
557 Pattus, Structural genomics on membrane proteins: comparison of more than
558 100 GPCRs in 3 expression systems, *J. Struct. Funct. Genomics* 7 (2006) 77–91.
- 559 [13] S. Grünewald, W. Haase, E. Molsberger, H. Michel, H. Reiländer, Production of
560 the human D2S receptor in the methylotrophic yeast *P. Pastoris*, *Receptors*
561 *Channels* 10 (2004) 37–50.
- 562 [14] H.M. Weiß, W. Haase, H. Michel, H. Reiländer, Comparative biochemical and
563 pharmacological characterization of the mouse 5HT_{5A} 5-hydroxytryptamine
receptor and the human β_2 -adrenergic receptor produced in the
methylotrophic yeast *Pichia pastoris*, *Biochem. J.* 330 (1998) 1137–1147.
- 564 [15] M. Julien, S. Kajiji, R.H. Kaback, P. Gros, Simple purification of highly active
565 biotinylated P-glycoprotein: enantiomer-specific modulation of drug-
566 stimulated ATPase activity, *Biochemistry* 39 (2000) 75–85.
- 567 [16] J.A. Javitch, The ants go marching two by two: oligomeric structure of G-
568 protein-coupled receptors, *Mol. Pharmacol.* 66 (2004) 1077–1082.
- 569 [17] L. Gan, J.M. Alexander, A. Wittelsberger, B. Thomas, M. Rosenblatt, Large-scale
570 purification and characterization of human parathyroid hormone-1 receptor
571 stably expressed in HEK293S GnTI⁻ cells, *Protein Expr. Purif.* 47 (2006) 296–
572 302.
- 573 [18] J.F. White, L.B. Trinh, J. Shiloach, R. Grisshammer, Automated large-scale
574 purification of a G protein-coupled receptor for neurotensin, *FEBS Lett.* 564
575 (2004) 289–293.
- 576 [19] V. Sarramegna, I. Muller, A. Milon, F. Talmont, Recombinant G protein-coupled
577 receptors from expression to renaturation: a challenge towards structure, *Cell.*
578 *Mol. Life Sci.* 63 (2006) 1149–1164.
- 579 [20] C. Klammt, A. Srivastava, N. Eifler, F. Junge, M. Beyermann, D. Schwarz, H.
580 Michel, V. Doetsch, F. Bernhard, Functional analysis of cell-free-produced
581 human endothelin B receptor reveals transmembrane segment 1 as an
582 essential area for ET-1 binding and homodimer formation, *FEBS J.* 274
583 (2007) 3257–3269.
- 584 [21] C. Klammt, D. Schwarz, N. Eifler, A. Engel, J. Piehler, W. Haase, S. Hahn, V.
585 Doetsch, F. Bernhard, Reprint of “Cell-free production of G protein-coupled
586 receptors for functional and structural studies”, *J. Struct. Biol.* 158 (2007) 482–
587 493.
- 588 [22] G. Ishihara, M. Goto, M. Saeki, K. Ito, T. Hori, T. Kigawa, M. Shirouzu, S.
589 Yokoyama, Expression of G protein coupled receptors in a cell-free
590 translational system using detergents and thioredoxin-fusion vectors,
591 *Protein Expr. Purif.* 41 (2005) 27–37.
- 592 [23] D. Krepliy, K. Wong, K. Gawrisch, A. Yeliseev, Bacterial expression of
593 functional, biotinylated peripheral cannabinoid receptor CB2, *Protein Expr.*
594 *Purif.* 49 (2006) 60–70.
- 595 [24] M. Jidenko, G. Lenoir, J.M. Fuentes, M. le Maire, C. Jaxel, Expression in yeast and
596 purification of a membrane protein, SERCA1a, using a biotinylated acceptor
597 domain, *Protein Expr. Purif.* 48 (2006) 32–42.
- 598 [25] A.K. Mohanty, C.R. Simmons, M.C. Wiener, Inhibition of tobacco etch virus
599 protease activity by detergents, *Protein Expr. Purif.* 27 (2003) 109–114.
- 600
601
602