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ORIGINAL RESEARCH PAPER

Monitoring the human β1, β2, β3 adrenergic receptors expression and purification in *Pichia pastoris* using the fluorescence properties of the enhanced green fluorescent protein

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Abstract The three beta adrenergic receptor subtypes, β1-, β2- and β3-, were expressed in the methylotrophic yeast *Pichia pastoris*. These receptors were *N*-terminally fused to the enhanced green fluorescent protein (EGFP) and the fluorescent properties of EGFP were used: (1) to select the recombinant strains, (2) to monitor the expression of the fluorescent receptors, and (3) to monitor the purification of the receptors by immobilized metal affinity chromatography. We demonstrate here that *Pichia pastoris* can be an alternative host to express and purify milligram amounts of human beta adrenergic receptors.

Keywords Beta adrenergic receptor · Green fluorescent protein · *Pichia pastoris* · Purification

Introduction

Beta-adrenergic receptors (β-AR) belong to the super family of G-protein coupled receptors (GPCRs), which bind a huge number of pharmacological substances, and hence, represent major pharmaceutical targets. Three subtypes of β-AR, β1-AR, β2-AR, β3-AR, have been characterized upon their different tissue localization and distribution and upon their capability to bind a set of different agonists and antagonists. All of these β-AR subtypes are positively coupled to adenylate cyclase via the stimulatory Gs protein (Duport et al. 2003). β1-AR is found in a variety of tissues but is particularly highly expressed in the heart, where it mediates the bulk of the effects of epinephrine on cardiac function, and in the brain, where it plays a key role in regulating synaptic plasticity and memory formation (He et al. 2002). The β2-AR, which is the predominant subtype in most vascular and bronchia smooth muscle, is highly important in pharmaceutical targeting on pulmonary and cardiovascular diseases (Hoffmann et al. 2004). The β3-AR subtype is primarily found in white and brown adipose tissues and increased attention is focused on this subtype as a therapeutic target since selective β3-AR agonists were shown to control fat accumulation (Duport et al. 2003). The human β3-AR was shown to have 49% and 51% homology at the amino acid sequence to human β2-AR and β1-AR, respectively. The β2-AR was the most studied and thus is the best characterized. Very recently, high resolution crystallographic structures of the human β2 and turkey β1 adrenergic receptors were determined (Cherezov et al. 2007; Rasmussen et al. 2007; Rosenbaum et al. 2007; Warne et al. 2008). Many years of hard work were necessary to obtain these successes. These success stories point out that in order to gain insights into the 3D structure of GPCRs, it is essential to develop very efficient heterologous

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expression systems (Sarramegna et al. 2003) and to set up appropriate solubilisation, purification, refolding and mutagenesis strategies (Sarramegna et al. 2006). In the present study, we designed recombinant receptors composed of enhanced green fluorescent protein (EGFP) fused to the *N*-terminus of the three subtypes of β-AR. The fluorescent properties of EGFP were used to follow the expression and purification of β-AR from *Pichia pastoris* cells. Only two other GPCRs have been expressed as EGFP fusion proteins in *Pichia pastoris*: the human ET_B endothelin receptor and the human mu-opioid receptor (Sarramegna et al. 2006). We demonstrate, here, that *Pichia pastoris* can be an alternative host to express and purify high amounts of human beta adrenergic receptors fused to EGFP. Thus, the availability of pure receptors in large quantities is essential for systematic refolding studies and biophysical characterization such as circular dichroism (Muller et al. 2008).

Materials and methods

Plasmid constructs

Human β-AR coding sequences were modified by PCR at the 5′ end and the 3′ end by introducing KpnI and XbaI sites, respectively. Primers used for β1-AR were 5′-CCGCAGGGTACCATGGGCGCGGGGG TGCTCGTCCTG-3′ (forward) and 5′-GGGTCT AGAACCTTGGATTCCGAGGCGAAGCCGGG-3′ (reverse), 5′-GACTGGGTACCATGGGGCAACCC GGGAACGGCAGC-3′ (forward) and 5′-AACTGT CTAGAAGCAGTGAGTCATTTGTACTACAA-3′ (reverse) for β2-AR, 5′-GCGACGGTACCATGGCT CCGTGGCCTCACGAGAAC-3′ (forward) and 5′-G TTTCTAGACCGTCGAGCCGTTGGCAAAGCCT GGG-3′ (reverse) for β3-AR. Modified cDNAs were cloned into TOPO TA cloning vectors (Invitrogen). After digestion with KpnI and XbaI, cDNAs were introduced in a pPIC-GFP-HuMOR-cmyc-his (Sarramegna et al. 2005) digested with KpnI and XbaI leading to pPIC-GFP-β1-AR-cmyc-his, pPIC-GFP-β2-AR-cmyc-his, pPIC-GFP-β3-AR-cmyc-his vectors.

Strains and expression

Escherichia coli strain Top10F' was used for the propagation of recombinant plasmids. *E. coli* transformants were selected on low salt LB plates pH 7.5 (0.5% w/v yeast extract, 1% w/v tryptone, 0.5% w/v NaCl, 1.5% w/v bacteriological agar) supplemented with 25 μg zeocin/ml. *P. pastoris* SMD1163 (his4, pep4, prB1) strain was used for receptor expression. *P. pastoris* transformants were selected on YPDS plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose, 1 M sorbitol, and 1.5% w/v bacteriological agar) with 100 μg zeocin/ml. Zeocin-resistant cells were laid onto minimum methanol plates (1.34% (w/v) yeast nitrogen base, 4×10^{-5}% biotin, 0.5% methanol), supplemented with 0.004% histidine (w/v) and selected for their green fluorescence. *P. pastoris* growth and induction media were BMGY (1% w/v yeast extract, 2% w/v peptone, 0.1 M phosphate buffer pH 7.5, 1% v/v glycerol) and BMMY (same as BMGY except that glycerol was replaced by 0.5% v/v methanol) respectively. Induction of expression was realized at 30°C in shacked flasks.

Crude extract preparation

All operations were carried out at 4°C. After induction of expression, cells that express the GFP-tagged beta adrenergic receptors were harvested and broken during 30 min with glass beads in a breaking buffer (Tris/HCl 10 mM, pH 8) supplemented with protease inhibitors (Sarramegna et al. 2005). The cell lysate was then centrifuged at 1000*g* for 15 min to remove unbroken cells and particulate matter. The supernatant was further centrifuged at 10,000*g* for 30 min to harvest a crude fraction. The resulting pellets were then stored at −80°C in the breaking buffer.

Fluorescence measurements

Expression, solubilisation and purification of β-ARs were quantified from their fluorescence using a spectrofluorimeter with excitation at 470 nm and emission at 508 nm. rEGFP (Clontech) was used, as a standard, to determine receptor concentration.

Solubilisation

Crude 10,000 g enriched fractions prepared from *Pichia pastoris* were initially washed 3 times with an ice-cold solubilisation buffer (SB) without detergent

(100 mM NaH_2PO_4, 10 mM Tris/HCl, 20 mM β-mercapto-ethanol, pH 8). The insoluble pellet was then dispersed in SB containing 8 M urea and 0.1% (w/v) SDS. The solubilisation was performed, on a wheel, for 1 h at room temperature. After this step, the fluorescence of the samples was measured on a small aliquot before and after ultracentrifugation at 100,000*g* (30 min, 4°C). The fluorescence ratio (at 508 nm) between the supernatant resulting from the ultracentrifugation step (containing the solubilised proteins) and the initial fluorescence of the samples was used to assess the efficiency the solubilisation.

Purification

Solubilised receptors were incubated for 1 h at room temperature with chelating Sepharose (1–2 ml) charged with 0.3 M nickel acetate. The resin was then poured in an 8 ml plastic column, and washed with 50 ml of the initial buffer. Proteins bound to the resin were subsequently eluted with a step imidazole gradient (3 × 4 ml). Eluted fractions were analyzed for their fluorescence at 508 nm.

SDS-PAGE and western blot analysis

Proteins were separated by SDS-PAGE using 10% (v/v) acrylamide gels and visualized by silver nitrate staining. For immunoblot analysis, proteins were transferred to an immun-Blot membrane (Bio-Rad) after SDS-PAGE. Antigens were probed with the monoclonal anti c-myc antibody (Sigma, clone 9E10) as a primary antibody, and with a secondary anti-mouse horseradish peroxidase-coupled antibody (Jackson Immunoresearch) as described in detail previously (Sarramegna et al. 2002b). Protein molecular weight markers (116, 66, 45, 35, 25, 18, and 14 kDa) were from Fermentas.

Results

Strain selection

The three β-AR subtypes, β1-AR, β2-AR and β3-AR, were expressed in the methylotrophic yeast *Pichia pastoris* as a fusion protein with EGFP at the *N*-terminus and c-myc and 6-his tags at the *C*-terminus. A c-myc tag was added to make western blot revelations and 6-his tag allows performing affinity chromatography on a nickel column. EGFP is a useful reporter since it allows to screen by fluorescence the positive clones on zeocin-selection plates and to follow the production of the recombinant protein in shacked flask during the methanol induction phase. After electrotransformation, cells were laid down on minimal methanol plates made with white agar. Positive clones for each subtype were selected for their intense green fluorescence intensity through a blue filter.

Kinetic of expression and expression levels

The selected clones were grown in shake-flasks until the late growth phase where cellular densities reach 1–3 × 10^9 cells/ml. At this stage, cells were harvested and the growth medium was exchanged with the induction medium. As revealed by the analysis of cell fluorescence at 508 nm, the maximum of production for all the β-AR subtypes was reached between 20 and 25 h after the beginning of induction (Fig. 1). GFP-β2-AR and GFP-β3-AR expressing cells showed identical induction patterns with an exponential expression phase persisting for 10 h followed by a plateau of expression. This behaviour

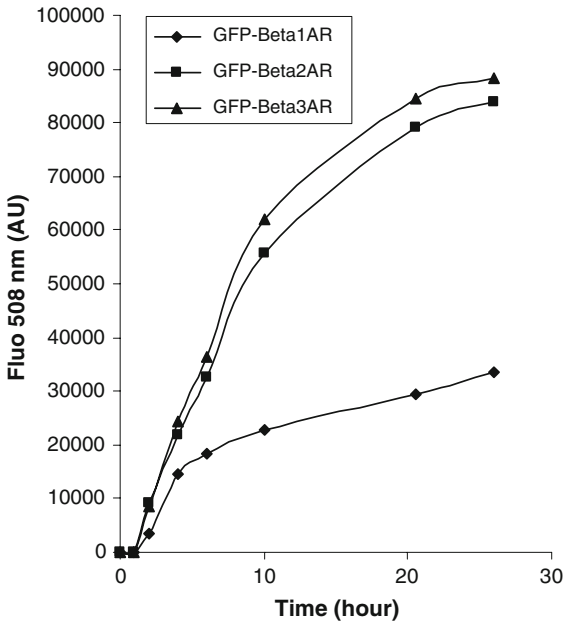

Fig. 1 Kinetic expression of beta adrenergic receptor subtypes. *Pichia* cells fluorescence was followed at 508 nm

was also observed for the human mu-opioid receptor expressed in *Pichia pastoris* (Sarramegna et al. 2002a). On the contrary, the GFP-β1-AR total expression was only 1/3 of the two other subtypes and the exponential expression was less elongated 6 h. Expression levels were determined by fluorescence measurement of the cells and reached up to 4 mg/l for GFP-β1-AR and 10 mg/l for GFP-β2-AR and GFP-β3-AR. Since EGFP (MW = 26.5 kDa) account for ~35% of the molecular weight of the fluorescent receptors, we obtain 2.6 mg/l for-β1-AR and 6.5 mg/l for β2-AR and β3-AR.

Solubilisation of receptors

We previously reported several data on the solubilisation and purification of the human mu-opioid receptor (Sarramegna et al. 2005). We have thus employed optimized conditions for the solubilisation and purification of the three β-AR subtypes. The enriched-receptor containing fraction obtained after cell breaking and differential centrifugation at 10,000*g* was dissolved in a buffer containing 8 M urea and 0.1% SDS to ensure proper solubilisation of the receptors. The efficiency of solubilisation was total since a centrifugation at 10,000*g* did not produce any pellet. The buffer was then mixed for 1 h with the nickel phase and the interaction efficiency was determined by measuring the fluorescence before and after the contact with the nickel phase. Interaction efficiencies were as follow: 35% for GFP-β1-AR, 53% for GFP-β2-AR and 37% for GFP-β3-AR. These values can be explained by a too brief time contact between receptors and the nickel phase or by an over saturation of the phase. Nevertheless, at this step, we were able to solubilise 0.9 mg/l for-β1-AR and 3.4 mg/l for β2-AR and β3-AR.

Purification of receptors

After interaction, the nickel phase was extensively washed with the initial buffer. This cleaning step was followed with an elution step using imidazole in the buffer (25, 50, 100, 300 mM). Each fraction was further characterized by fluorescence measurements (Fig. 2), silver nitrate staining (Fig. 3) and immunoblotting (Fig. 3). Fluorescence intensity was measured at 508 nm and revealed elution efficiencies

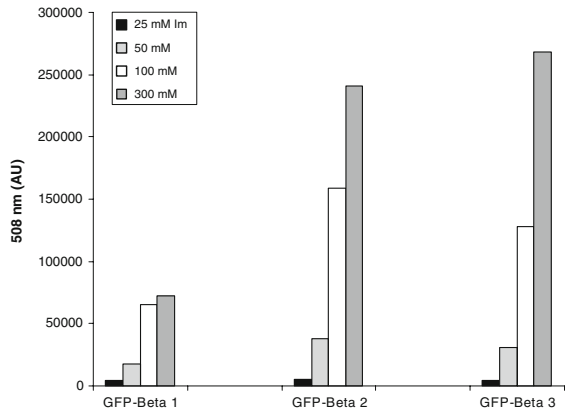

Fig. 2 Purification of beta adrenergic receptor subtypes. Elution efficiencies were followed by fluorescence at 508 nm. Receptors were mainly found in the 100 mM and 300 mM imidazole fractions

of each concentration of imidazole. Elution with the lowest concentration of imidazole (25 mM) resulted in low recovery of fluorescence, between 1 and 2% of total for the 3 β-AR subtypes. Moreover, no specific recognition of receptor was revealed by immunoblotting. On the contrary, an important number of proteins were detected after silver nitrate staining leading to conclude that 25 mM imidazole in the buffer is essential to remove unspecific-bound proteins from the nickel resin. By fluorescence, the 50 mM imidazole fractions represent ~7–11% of total and we can also observe the occurrence of foreign protein contaminations as for the 25 mM fractions. Nevertheless, these proteins seems to be identical to those observed in the 25 mM fraction and should be eliminated by increasing the number of washes at 25 mM imidazole. The 100 mM imidazole fractions account, by fluorescence, for ~30–40% of the total whereas the 300 mM imidazole fractions contained ~45–60% of fluorescence. The GFP-β1-AR, GFP-β2-AR and GFP-β3-AR have predicted molecular weight of 81.2, 76.6, and 73.5 kDa, respectively and the apparent molecular weights observed on the SDS-PAGE gels (Fig. 3) are consistent with these values. In the 100 mM and 300 mM imidazole fractions, the GFP-β1-AR presented mainly, on the SDS-PAGE gel (Fig. 3a), a single band which was also detected by western blotting. An upper band was also detected by western blotting in the two fractions, but was undetectable on the silver nitrate gel. This protein band certainly corresponds to aggregates and is mainly due

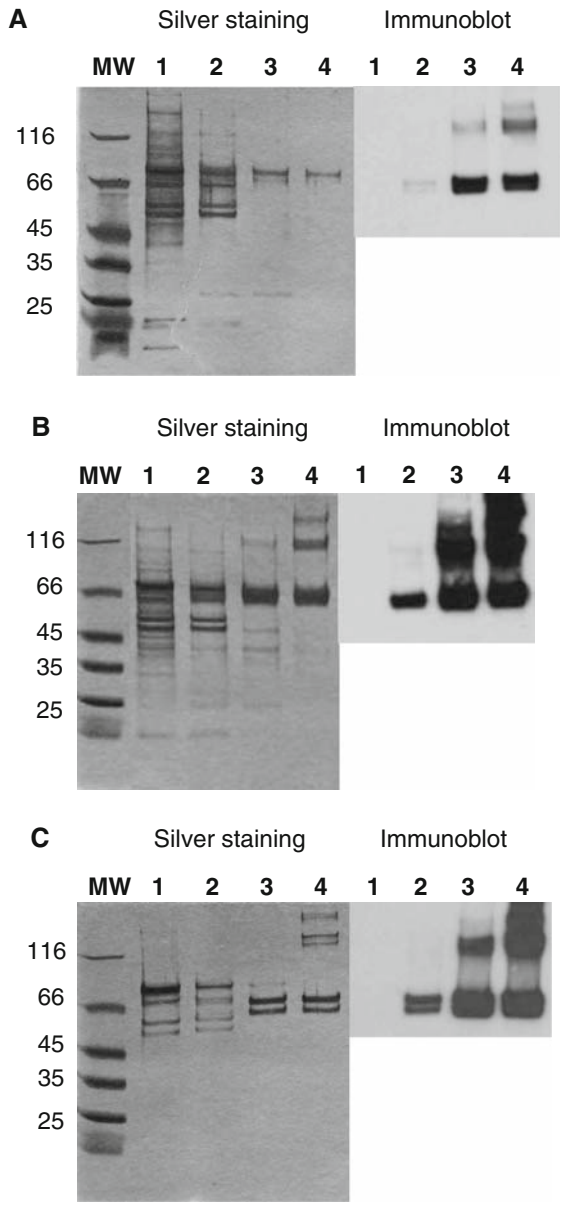

Fig. 3 Silver nitrate staining (left) and immunoblotting (right). (**a**) β1 adrenergic receptor, (**b**) β2 adrenergic receptor, (**c**) β3 adrenergic receptor. MW; Molecular Weight, 1, 25 mM; 2, 50 mM; 3, 100 mM; 4, 300 mM imidazole

to artefactual migration of the receptor within the SDS-PAGE gel, a commonly observed feature with GPCRs (Perret et al. 2003). In the same manner, for GFP-β2-AR, a major single band was detected by silver nitrate staining (Fig. 3b) in the 100 mM fraction whereas multimers were observed for the 300 mM fraction. The GFP-β3-AR also presented an interesting pattern on gel (Fig. 3c) but the protein bands were visualized as doublets. This aspect on gel can represent *N*-terminal truncated forms of the receptor. For the three β-AR subtypes, the major part of the receptor was found in the 100 and 300 mM fractions and represented 90% of the total.

Discussion

Heterologous expression is an unavoidable step to obtain β-ARs in large quantities enough to perform 3D structural biology experiments, The human β2-AR has been expressed in various host strains from *Saccharomyces cerevisiae* where it reached 115 pmol/mg membrane proteins to *E coli* where the expression levels were lower (Sarramegna et al. 2003). The other human subtypes β1-AR and β3-AR were less studied and their expression levels were in the fmol/mg membrane proteins range when expressed in heterologous systems (Chapot et al. 1990; Duport et al. 2003). The β1-AR which structure has been very recently solved by X-ray crystallography was from Turkey and its stability was increased by mutagenesis (Serrano-Vega et al. 2008). The availability of pure receptors from baculovirus-infected insect cells was a key for the determination of the two crystallographic structures (Rosenbaum et al. 2007; Warne et al. 2008). The expression yield of the turkey β1 adrenergic receptor in baculovirus-infected insect cells reached 1.2 mg/l. This receptor was purified to homogeneity giving a yield of 0.5 mg/l of pure receptor (Warne et al. 2003). For the human β2 adrenergic receptor, expression and purification yields were 1 mg/l and 0.25 mg/l, respectively (Kobilka 1995). We demonstrate here that *Pichia pastoris* can be an alternative host to express and purify high amounts of human receptors. Moreover the presence of EGFP in the fusion protein represents an extraordinary tool to follow receptors from selection of clones to purification of the protein. We were, thus, able to purify 0.8 mg/l for β1-AR and 3 mg/l for β2-AR and β3-AR. The β2-AR was already purified in *Pichia pastoris* with a lower yield of 0.09 mg/l but the receptor was not fused to EGFP (Noguchi and Satow 2006). All the cell cultures were realized in shacked flasks. Fermentation of *Pichia pastoris* cells has been optimized over years and it is possible routinely to obtain 100 g/l of dry cell weight

whereas 5 g/l are obtained in flasks. The functionality of a recombinant GPCR is an important issue as recently discussed (Brillet et al. 2008). In *Pichia pastoris*, the three beta-adrenergic receptor subtypes in fusion with EGFP were not able to bind specific ligands (data not shown) as for the human mu-opioid receptor fused to EGFP (Sarramegna et al. 2005). Nevertheless, in the course to obtain structural information on GPCR, two strategies can be followed: the first one preserves the receptor functionality during the solubilisation and purification steps, as for β1 and β2 adrenergic receptors expressed in baculovirus-infected insect cells (Kobilka 1995; Warne et al. 2003; Cherezov et al. 2007; Rasmussen et al. 2007; Rosenbaum et al. 2007; Warne et al. 2008). The second strategy is focused on the refolding of an initially inactive and unfolded form of the receptor and it was realized for receptors such as leukotriene B4, 5HT-4, and OR5 olfactory receptors (Sarramegna et al. 2006). Thus, the avability of pure receptors in large quantities is essential for systematic refolding studies and biophysical characterization such as circular dichroism (Muller et al. 2008).

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