Protein Expression and Purification xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Affinity purification of recombinant human cytochrome P450s 3A4 and 1A2 using mixed micelle systems

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ARTICLE INFO

Article history:
Received 16 April 2014
and in revised form 20 May 2014
Available online xxxx

Keywords:
Affinity purification
Cytochrome P450
Catalytic activity
Mixed micelle

ABSTRACT

Recombinant cytochrome P450 (CYP or P450) enzymes are useful for drug metabolism research and thereby many expression and purification systems have been developed. Here, we provide a method for the purification of human P450s 3A4 and 1A2 expressed in *Escherichia coli* using mixed micelles containing anionic phospholipids. This method does not require any protein-tagging system for protein isolation and has a further advantage that the purification is concomitantly conducted with reconstitution of the enzymes into a phospholipid environment, which is crucial for the catalytic activity assay of P450 enzyme. This method may also be applied to high-throughput catalytic assays of the enzymes because the purification procedures can be undertaken in a 96-well plate.

Introduction

Cytochrome P450 (CYP or P450)[1] enzymes are mainly involved in the oxidation of xenobiotic chemicals and endogenous substrates [1], and multiple forms of P450s are present in mammals [2]. Human CYP3A4, the most abundant P450 enzyme present in both the liver and small intestine, is of great interest because the enzyme has been known to catalyze the metabolism of approximately 50% of therapeutic agents [3]. Human CYP1A2, located predominantly in the liver, also plays an important role in the metabolism of a variety of compounds including the activation of carcinogenic aryl and heterocyclic amines [4].

The production of recombinant proteins in a highly purified form is crucial for many protein research fields. For this purpose, various strategies using affinity-tags such as FLAG, HA, GST, and poly-His have been developed [for review, [5]]. These affinity-tag systems enable rapid isolation (usually one-step) of diverse proteins by chromatographic capture and elution procedures and thereby are widely adapted as a reliable protein purification strategy. However, each affinity-tag purification method is carried out under its specific buffer conditions, which could affect the protein of interest. Additionally, the tertiary structure and/or biological activities of the fusion proteins may be changed by the tags depending on their chemical properties such as amino acid composition [6]. Furthermore, the tag should be removed by an appropriate cleavage method for other applications, e.g., crystallization of protein, which is cumbersome process.

Recombinant human P450 enzymes have proved to be useful for drug metabolism research, and thereby many reports have been suggested for the heterologous expression and purification methods of these enzymes [7]. In addition, several assay systems have been developed to reconstitute P450s into membranous millennium using artificial or natural lipids. These strategies mimic the cellular environments of P450 enzymes and are important for efficiently measuring their catalytic activities. In the present study, we provide a purification method accompanying simultaneous reconstitution of P450s 3A4 and 1A2 using mixed micelles without an affinity-tag and column chromatography.

Materials and methods

Materials

NADP$^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, testosterone, 7-ethoxyresorufin, 6β-hydroxytestosterone, and octyl-β-D-glycopyranoside (*n*-octylglucoside) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Immobilized avidin

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[1] *Abbreviations used:* CYP or P450, cytochrome P450; *E. coli*, *Escherichia coli*; GST, glutathione-*S*-transferase; HA, human influenza hemagglutinin; HPLC, high-performance liquid chromatography; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; 16:0 biotinyl-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl); 16:0–18:1 PA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate; 16:0–18:1 PS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; 16:0–18:1 PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

http://dx.doi.org/10.1016/j.pep.2014.05.010
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on 6% agarose beads and streptavidin-coated 96-well plates were purchased from Pierce Biotechnology (Rockford, IL, USA). All phospholipids including biotinyl-PE were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Recombinant rat NADPH-P450 reductase was expressed in *Escherichia coli* and purified as described [8].

Preparation of mixed micelles and lipid bilayers

Based on previous results regarding micelle formation using lysophospholipids and their critical micelle concentrations [9,10], 49 mol% lyso-PC (8:0, 10:0, 11:0, or 12:0 lyso-PC), 1 mol% 16:0 biotinyl-PE, and 50 mol% 16:0–18:1 phospholipid (16:0–18:1 PA, 16:0–18:1 PS, or 16:0–18:1 PC) were used as the standard mixed micelle components. At the expense of 16:0–18:1 phospholipids, lipid compositions consisting of 99 mol% 10:0 lyso-PC and 1 mol% 16:0 biotinyl-PE were also used. To investigate the effect of artificial membranes with a lipid bilayer structure on P450s 3A4 and 1A2 purification, lipid mixtures containing 99 mol% 16:0–18:1 phospholipid and 1 mol% 16:0 biotinyl-PE were used. After the appropriate amounts of lipids were dissolved in chloroform, the solvent was evaporated under a stream of argon gas. To prepare mixed micelles, the dry lipids were hydrated in a buffer solution (25 mM Tris–HCl, pH 7.4, 100 mM NaCl) by vortex mixing and subsequent brief sonication in a probe-type sonicator (30 s). The LUVs composed of 99 mol% 16:0–18:1 phospholipids and 1 mol% 16:0 biotinyl-PE were prepared by an extrusion method as described [11].

Expression and purification of P450 enzymes

Human liver P450s 3A4 and 1A2 were expressed in *E. coli* with the pCW plasmid containing each corresponding cDNA as described [12,13]. After expression of proteins, bacterial membrane factions were prepared, as described [12]. To solubilize the membrane fractions, the samples were mixed with 1% (w/v, final concentration) *n*-octylglucoside, a nonionic dialyzable detergent [14], and further incubated in a bath sonicator at 30 °C for 10 min. To isolate recombinant P450 enzymes, mixed micelles or liposomes (1.5 mM as a final phosphate concentration) were added to the solubilized fractions (total protein concentration, ~12 mg/mL) and the samples were incubated at 30 °C for 20 min with gentle shaking. The *n*-octylglucoside concentration was diluted to 0.15% (w/v) by mixing the micelles (or liposomes) with the protein solutions. Immobilized avidin on beads (50 μl as a bed volume) was then added to 1 mL of the samples, and they were further incubated at 30 °C for 20 min. After centrifugation of the samples (10,000×g, 30 °C, 5 min), the pellets were resuspended in a reaction buffer (100 mM potassium phosphate, pH 7.4, 100 mM NaCl). To remove any protein nonspecifically bound to the micelles (or liposomes) and/or the beads, washing steps (the centrifugation, the removal of supernatant fraction, and the resuspension of samples with a reaction buffer) were undertaken. The resuspended samples were further analyzed by determining protein concentration and SDS–PAGE using a 10% polyacrylamide gel to verify protein purification. The samples were also used to measure P450 catalytic activities without the removal of beads. To calculate a fold-purification by catalytic activity with statistical significance, streptavidin-coated 96-well plates were used: the samples (membrane fractions and lipids) were incubated at 30 °C for 20 min in the plate with gentle shaking. The supernatant fractions were discarded after centrifugation of the samples (10,000×g, 30 °C, 5 min). The washing steps described above were then repeated twice. The remaining steps were the same as those using the avidin-beads.

Catalytic activity assay

Testosterone 6β-hydroxylation activity was determined for CYP3A4 with the membrane fractions and the purified proteins, as described [15]. The activity assay was conducted in the reaction buffer with total sample volume of 200 μl in both cases for the slurry beads and 96-well plate. The micelle-bead complex containing CYP3A4 (0.4 μM as a P450 concentration) and NADPH-cytochrome P450 reductase (2 μM) were mixed in the presence of testosterone as the substrate. The reaction was started by adding an NADPH-generating system, and after incubating the sample at 30 °C for 10 min with gentle agitation, the reaction was stopped by adding 20 μl of 1.0 N HCl containing 2.0 M NaCl. The resulting product was extracted and analyzed by HPLC with UV detection at 240 nm. The catalytic activity of CYP1A2 was measured with ethoxyresorufin *o*-deethylation activity as described [16]. The activity assay was undertaken using the same buffer and the same assay conditions as those used to measure CYP3A4 activity. The reaction was started by adding an NADPH-generating system in the presence of 7-ethoxyresorufin as the substrate, and after incubating the sample at 30 °C for 10 min, the reaction was stopped by mixing the reaction sample with 200 μl of cold methanol. The formation of resorufin was monitored spectrofluorometrically [16].

Other methods

P450 concentrations in whole cells and membrane fractions were quantitated by Fe^{2+}–CO versus Fe^{2+} difference spectra as described [17]. Protein concentrations were determined by the Bradford method [18]. Phosphoglyceride concentrations were determined by a phosphorus assay [19]. Statistical data are expressed as a mean ± standard error (S.E.) of five independent trials, and analyses were performed using Student's *t*-test. A *p*-value <0.05 was considered significant.

Results and discussion

Purification of CYP3A4 using mixed micelles containing anionic phospholipids

We previously found that P450s 3A4 and 1A2 specifically bind to liposomes containing anionic phospholipids such as PA and PS [11,20]. These results suggest that affinity purification of the P450 enzymes can be accomplished by using a membranous system containing specific lipid(s), which has already been reported for partial purification of diacylglycerol kinase [21]. To test this possibility, *E. coli* membrane fractions containing over-expressed CYP3A4 or CYP1A2, which did not have any affinity-tag such as poly-His, HA, or FLAG, were incubated with mixed micelles containing lyso-PC and anionic phospholipid. Biotinyl-PE was also incorporated to precipitate and recover the micelles.

After pulling down the micelles with an avidin-containing matrix, the amount of bound CYP3A4 and the enzyme activity were measured. First, the fold-purification for CYP3A4 was approximately 854 when a mixed micelle consisting of 49 mol% 10:0 lyso-PC, 1 mol% biotinyl-PE, and 50 mol% 16:0–18:1 PA was used (Table 1). For the fold calculation, the statistic results could be obtained using streptavidin-coated 96-well plate. When purification was repeated with the avidin-beads in a slurry state instead of the well plate using the same ratio of avidin/total protein amount in both cases, the fold was further increased to 1048 on average, although the enhanced value was statistically within the error range of that of the 96-well plate. These increases in fold-purification have important meaning in that the enzyme could be isolated from membrane fractions by a one-step procedure without using a combination of affinity-tags and subsequent column chromatography, which are typically performed for recombinant protein purification. A further advantage of using mixed micelles is that the purification and reconstitution processes of the enzyme