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Research Communication

OPTIMIZATION OF SOLUBLE EXPRESSION, PURIFICATION AND PRELIMINARY CHARACTERIZATION OF CYTOCHROME P450 OF THE LIVER FLUKE *OPISTHORCHIS FELINEUS*

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Abstract: Cytochrome P450 (CYP450) forms an enzyme superfamily that is ubiquitously distributed in nature and represents one of the largest and oldest gene superfamilies. CYP450 is important for the biotransformation of sterols and xenobiotics including drugs and is useful in the synthesis and degradation of signaling molecules. *cyp*450 gene encodes heme monooxygenases with specific substrate range and have numerous paralogues in most eukaryotes including free-living flatworms. This research work focuses on the human liver fluke *Opisthorchis felineus,* which is an emerging cause of hepatobiliary diseases such as cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis. The *cyp*450 gene was cloned in pET28a and was expressed in *Escherichia coli*. The insoluble of CYP450 was purified using a nickel affinity column with chelating Ni-NTA Sepharose matrix after solubilizing with sodium lauroyl sarcosinate (Sarkosyl). The affinity purified recombinant of CYP450 was further purified using size exclusion chromatography (SEC) to apparent homogeneity (~5 mg/L). Following SEC, of CYP450 was stable and observed to be a ~50kDa monomeric protein. Large quantities of the protein will guide in its biochemical and biophysical characterization, which may lead to the identification of new inhibitors of of CYP450.

Keywords: Solubility; Cytochrome P450; Detergent; Ethanol; Insoluble protein.

Introduction

Opisthorchiasis is a helminth infection affecting mainly the hepatobiliary system and pancreas of fish-eating mammals including humans. The causative agents of opisthorchiasis are two species of liver flukes, the trematodes belonging to the family Opisthorchiidae – *Opisthorchis felineus* (Rivolta, 1884) and *O. viverrini* (Poirier, 1886). Infection occurs when eating raw freshwater fish infested with helminth larvae (Pakharukova and Mordvinov, 2016). According to the Food and Agriculture Organization and the World Health Organization (FAO/WHO, 2014), Opisthorchiidae liver flukes rank 8th overall in global health importance among 24 food-borne parasites. *O. viverrini* is endemic in Southeast Asia while *O. felineus* is spread in Russia, Kazakhstan and several European countries. The International Agency for Research on Cancer classified the *O. viverrini* as group 1 agents that are carcinogenic to humans (IARC/WHO, 2012) and a major factor that induce cholangiocarcinoma in endemic regions (Sithithaworn *et al*., 2014). Of the total of 17 million people infested with opisthorchid flukes, at least 1.6

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million people have been reported to be infected with *O. felineus* (Keiser and Utzinger, 2009). Adult flukes feed on epithelial cells of the intrahepatic bile ducts and live long. Complications of this infection include hepatobiliary diseases such as hepatomegaly, cholangitis, cholecystitis, periductal fibrosis, and gallstones (Maksimova *et al*., 2017; Pakharukova and Mordvinov, 2016). Chronic infection is a strong risk factor for cholangiocarcinoma (Honjo *et al*., 2005; Zheng *et al*., 2017).

Cytochrome P450 (CYP450) enzymes are heme containing monooxygenases and are abundant in nature, from bacteria to plants and mammals (Ortiz de Montellano, 2005; Verma *et al*., 2011). They represent one of the largest and oldest gene superfamilies (Degtyarenko and Archakov, 1993; Klingenberg, 1958). CYP450s were first found to be abundant in mammalian liver, where they play an essential role in drug metabolism (Klingenberg, 1958; Omura and Sato, 1964). Human genome contains around 55 CYP450 enzymes, whereas the rice genome has 400 CYP450 enzymes and the *Arabidopsis* genome has over 286 enzymes (Xu *et al*., 2001). CYP450s display a variety of biological functions ranging from degradation and synthesis of endogenous molecules as well as detoxification of environmental carcinogens (Nebert and Russell, 2002). These enzymes are one of the most versatile redox proteins (Zhou, 2008). More than 80% of drugs undergo the steps of biotransformation mediated by CYP450. They are also functionally important for invading pathogens for their survival in the host. In mammals, CYP450s plays multiple roles such as in drug metabolism, xenobiotics, synthesis of steroid hormones, metabolism of fat soluble vitamins, and the conversion of polyunsaturated fatty acids to biologically active molecules (Denisov *et al*., 2005). In plants, CYP450s have roles in hormone biosynthesis and degradation of herbicides while they control the larval development via hormone biosynthesis in insects and also provide resistance to insecticide. These enzymes are also linked to the synthesis of unique sterol like metabolites, e.g., oxysterols and catechol estrogen, known specifically from *O. felineus* and *Schistosoma haematobium* (Gouveia *et al*., 2013; Vale *et al*., 2013).

Parasitic flatworms, including liver flukes (Opisthorchiidae, Fasciolidae), blood flukes (Schistosomatidae), and cestodes (Taeniidae), encode only one *cyp*450 gene and genes for flavin monooxygenases are absent (Pakharukova *et al*., 2012). Apparently, in these parasites, CYP450 is the main enzyme that has a monooxygenase activity towards xenobiotic substances (Pakharukova *et al*., 2015). This reduced number of paralogous*cyp*450 genes in parasitic flatworms indicates simple parasitic lifestyle enabled by high stability of their host environment and extensive exploitation of host metabolism. Recently, CYP450 was found to be essential for parasite survival and egg development and is a novel drug target against parasitic trematodes (Pakharukova *et al*., 2015; Ziniel *et al*., 2015). In *O. felineus*, CYP450 (OfCYP450) has been identified (Pakharukova *et al*., 2012) and was found to have the ability to metabolize xenobiotics. Localization studies have revealed the OfCYP450 activity in excretory channels, and suppression of *cyp*450 mRNA by RNAi was found to induce morphological changes in the excretory system and increase mortality rates of the flukes. The results suggested that the CYP450 function is linked to metabolism and detoxification. Certain azole inhibitors like ketoconazole, miconazole, and clotrimazole were found to inhibit the activity of this enzyme (Mordvinov *et al*., 2017).

Protein expression in *Escherichia coli* provides the ability to obtain large amounts of recombinant proteins rapidly. Prokaryotic expression vectors are extensively used for the expression and purification of recombinant proteins. However, the expression and production of full-length recombinant protein in soluble form are associated with certain difficulties. In the production of recombinant protein, the limiting factors include no expression, insoluble protein production, and improper purification. Some of the strategies used for enhancing the expression level and for large-scale recombinant protein production are the use of osmotic stress and the use of mild detergent for solubility. Most of the work that has been done on CYP450 enzyme is in culture or intact parasite. The enzyme has not been purified in soluble form using recombinant DNA technology due to its high tendency to form insoluble aggregates. In the present work, we report cloning, successful expression, and purification of OfCYP450. We mainly focus on the optimization of expression and solubility of OfCYP450 so that we can further characterize the functional role of this enzyme in *O. felineus*.

Materials and Methods

Enzymes, buffers and deoxynucleotide triphosphate (dNTPs) were purchased from New England Biolabs, MA, USA. The molecular biology

kits and nickel-nitrilotriacetic acid (Ni– NTA) agarose matrix were purchased from Qiagen, CA, USA. All others chemicals and reagents were purchased either from the Sigma- Aldrich chemical company, St. Louis, MO, USA or Sisco Research Laboratories, Mumbai India and were of the highest purity available. Media for bacterial culture were purchased from Himedia Laboratories, Mumbai, India.

Cloning of Ofcyp450 - Primers were designed based on *O. felinus* CYP450 sequence (GenBank ID: JF920147). To improve the bacterial expression of protein, the 5'-terminal sequence of OfCYP450 was modified according to Porubsky *et al* (Porubsky *et al*., 2008). The region encoding the transmembrane domain at the N-terminus of the protein (MWLQSLCEYLPEAAIVVLVCYGIFLFIRHLFELRC) was deleted and a sequence coding for AKKTSSKGK was placed. The modified clone into pUC57 sequence was supplied from SintezGene (Saint Petersburg, Russia). The sequence was cloned at the EcoRI/XhoI restriction sites into pET28 vector to generate the recombinant construct pET28a-cyp450. Cloning was performed using primers (F:CGATCGAATTCATGGCGAAAAAGACCTCCT; R:AGATACTCGAGTTAATGATGATGATGGAAT) and standard cloning techniques. The cloned sequence was tested for the absence of mutations by Sanger assay. The recombinant plasmid was transformed into chemically competent *E. coli* Codon+ expression cells by the heat shock method and then spread onto agar plate containing kanamycin to allow selection of colonies that successfully incorporated the plasmids. Plasmid DNA was extracted using QIAprep Midiprep plasmid purification kit (Qiagen, USA) and was confirmed by sequencing.

Expression of OfCYP450-A single colony was picked from the transformed lysogeny broth (LB) agar plate and was inoculated into 5 mL LB broth containing 50 µg/mL kanamycin in an autoclaved culture vial. It was incubated at 37 °C for overnight with continuous shaking at 180 rpm. Next day, 50 µL of the primary culture was inoculated in six 5 mL LB broth containing 50 µg/mL kanamycin and 1% ethanol. One vial was taken as un-induced control. All cultures were incubated at 37 °C with shaking until the OD_{600} reached 0.6-0.8. The culture was then induced by using 0.5 mM and 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) with 0.5 mM $FeCl_3$. The culture vials were incubated at 37 °C and 16 °C with continuous shaking at 180 rpm for over night. The cells were harvested by centrifugation at 8000 rpm for 10 min and resuspended in lysis buffer (150 mM phosphate, pH 7.6, 300 mM NaCl containing protease inhibitor cocktail) sonicated and then analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Solubility optimization of OfCYP450 using different temperature and different concentrations of Sarkosyl - The expressed recombinant OfCYP450 protein was observed only in the insoluble fraction of the cell lysate. We used 0.1 to 1 M of arginine and glutamine and different concentration of urea. Detergent like Triton X-100 (0.1-1%), Tween-20 (0.1-1%), and NP-40 (0.1-1%) were used in order to solubilise the protein. None of the approaches work. Later we optimized the temperature and added the detergent sarkosyl to make the protein soluble for purification. The culture were grown at different temperatures (37 °C, 20 °C and 16 °C) for overnight and were divided into different aliquots and centrifuged. The pellet were resuspended into in 300 µl of lysis buffer (150 mM phosphate, pH 7.6, 10 mM â-mercaptoethanol, 300 mM NaCl containing protease inhibitor cocktail and different concentration of sarkosyl (0%, 0.05%, 0.1%. 0.2% and 0.3%) were added and sonicated. The samples were centrifuged at 13000 rpm for 10 min. The supernatants were taken out, and the pellets were resuspended in the same lysis buffer. All the samples were tested by SDS–PAGE for solubility. The lowest sarkosyl concentration (0.2%) that was able to solubilize the protein was selected and used for mass culture purification with 1% ethanol at 16 °C.

Purification of recombinant OfCYP450 - The 500 mL culture which was grown at 16 °C for overnight at 180 rpm was pelleted by centrifugation and dissolved in 30 mL of lysis buffer. The cells were lysed by sonication with pulse-rest cycle (30 cycle; 30 s pulse at 50% amplitude with 30 s interval after each pulse). The crude lysate was then centrifuged at 12000 rpm for 30 min at 4 °C. The supernatant was filtered through a 0.45 µm polyvinylidene difluoride (PVDF) membrane before loading into the column. Ni-NTA resin was prepared in a column containing 2 mL of volume to purify the protein. The supernatant was loaded into the column and passed through the Ni-NTA agarose matrix. The column was washed with 30 mL of equilibration buffer (150 mM phosphate pH 7.6, 300 mM NaCl containing 0.2% sarkosyl). The column was washed with increasing concentration of

imidazole containing 0.2% of sarcosyl and the protein was eluted with elution buffer containing 500 mM of imidazole containing 0.2% of sarkosyl. The purified recombinant protein was run into the SDS-PAGE using 12% polyacrylamide gel to check the purity of recombinant protein. The protein was dialysed against the dialysis buffer (150 mM phosphate, pH 7.6, 300 mM NaCl and 10% glycerol) for overnight at 4 °C. The recombinant protein was quantified using Bradford method.

Western Blotting - Purified recombinant OfCYP450 protein containing His-tag was subjected to SDS-PAGE on 12% polyacrylamide gel and was transferred to 0.45 µm PVDF membrane for 3 h at 50 mV. Membrane was then blocked in 5% skimmed milk for 16 h at 4 °C. After blocking, the membrane was incubated with mouse anti-His antibody (1:3000 dilution). Membrane was washed thrice with PBS, pH 7.4 and then incubated with anti-mouse IgG alkaline phosphatase antibody (1:1000). Both primary and secondary antibodies were diluted into PBS containing 3% BSA. Membrane was again washed with PBS pH 7.4 and the protein band was detected with the BCIP/NBT-Blue liquid substrate and analysed using gel documentation system.

Determination of molecular weight - The molecular weight of native OfCYP450 was determined by gel exclusion chromatography on Superdex™ 200 10/300 GL column (manufacturer's exclusion limit 600 kDa for proteins) on an ΔKTA-FPLC (GE HealthCare Biosciences, USA). The column was first equilibrated with equilibration buffer (150 mM phosphate, pH 7.6, 300 mM NaCl and 10% glycerol) at room temperature and loaded with 500 µL of purified OfCYP450 and was eluted at a flow rate of 0.3 mL/min. OfCYP450 was detected by its absorbance at 280 nm. thyroglobin (MW, 669 kDa), ferritin (MW, 440 kDa), aldolase (MW, 158 kDa), conalbumin (MW, 75 kDa), ovalbumin (MW, 44 kDa), carbonic anhydrase (MW, 29 kDa), ribonuclease (MW, 13.7 kDa) and aprotinin (MW, 6.5 kDa) were used as molecular weight standards and were obtained from GE HealthCare Biosciences, USA.

CD measurement - Far-UV CD spectra were recorded on a JASCO J-810 spectropolarimeter calibrated with ammonium(+)-10-camphor sulfonate. Spectra were collected using a 1 mm path length cell at a scan speed of 20 nm/min, a response time of 1 s and a bandwidth of 2 nm. 2.0 µM protein was used for the studies in the above-mentioned buffer. The spectra were averaged over five scans to eliminate signal noise.

Results

Molecular cloning of Ofcyp450 gene

The *Ofcyp*450 sequence was amplified using PCR and was later sub-cloned into an expression vector, i.e., pET28a(+) vector. Restriction digestion and sequencing of the gene confirmed the positive clone (Figure 1).

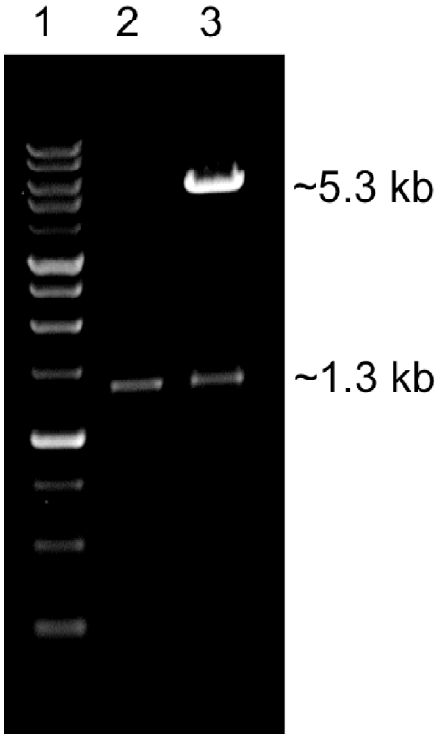

Figure 1: **PCR amplification and restriction digestion of *Ofcyp450*. Lane 1, 100 bp DNA Ladder. Lane 2, amplified PCR product (1329 bp). Lane 3, pET28a-*cyp450* digested with EcoRI and XhoI; upper band is pET28a vector backbone (~5.3 kb) while lower band is *Ofcyp450* gene (~1.3kb). The bands were visualised with ethidium bromide using 1% agarose gel electrophoresis**

Expression of OfCYP450

Inducer concentration (IPTG), expression time, and expression temperature were optimized from time course expression experiment at low (0.1 mM) and high (1mM) inducer concentration and with time from 4 h to 16 h. Several reports suggest that the expression of recombinant protein is enhanced in the presence of ethanol (1% v/v) (Chhetri *et al.*, 2015a; Chhetri *et al.*, 2015b; Chhetri *et al.*, 2015c). Our

results revealed that in the presence of 1 mM IPTG concentration, 0.5 mM $FeCl_3$, and 1% ethanol, recombinant CYP450 was expressed well (Figure 2). Recombinant CYP450 expressed more at 37 °C as compared to 16 °C (Figure 2) but was present in the insoluble fraction.

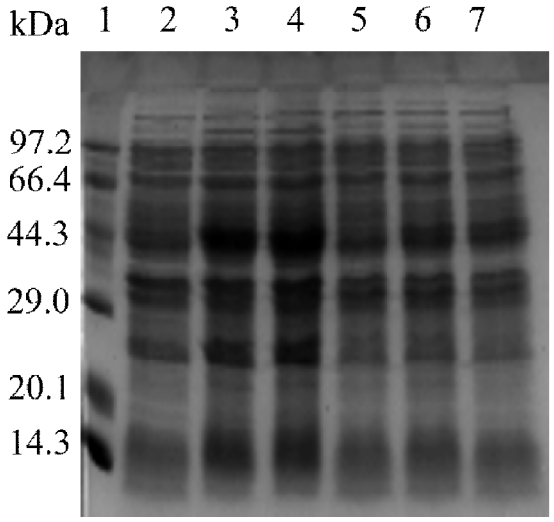

Figure 2: **Expression of OfCYP450. Protein samples were separated by SDS-PAGE (12%) and stained with coomassie brilliant blue. Lane 1, molecular weight marker. Lane 2, OfCYP450 uninduced control at 37 °C. Lane 3, OfCYP450 induced at 37 °C with 0.5 mM IPTG. Lane 4, CYP450 induced at 37 °C with 1 mM IPTG. Lane 5, CYP450 uninduced control at 16 °C. Lane 6, CYP450 induced at 16 °C with 0.5 mM IPTG. Lane 7, CYP450 induced at 16 °C with 1 mM IPTG. The culture were grown in the presence of 1% ethanol and 0.5 mM $FeCl_3$ for overnight**

Solubility optimization of OfCYP450

From the above experiment, it was shown that recombinant OfCYP450 protein was overexpressed at 37 °C but present predominantly in cell pellet, indicating that the protein was mainly insoluble in the form of inclusion bodies (Figure 3). In order to solubilize the protein, the culture was grown at 16 °C and solubilized using an ionic detergent (sarkosyl). Different concentrations of sarkosyl (0.05%, 0.1%, 0.2%, and 0.3%) were used. 0.2% sarkosyl was used to solubilize the protein; this is lowest sarkosyl concentration that can solubilize the protein.. After sonication, both the supernatant and pellet fraction were analyzed by SDS-PAGE, and the bands are shown in Figures 4.

Purification of OfCYP450

His-tag recombinant OfCYP450 protein was purified using an immobilized metal affinity

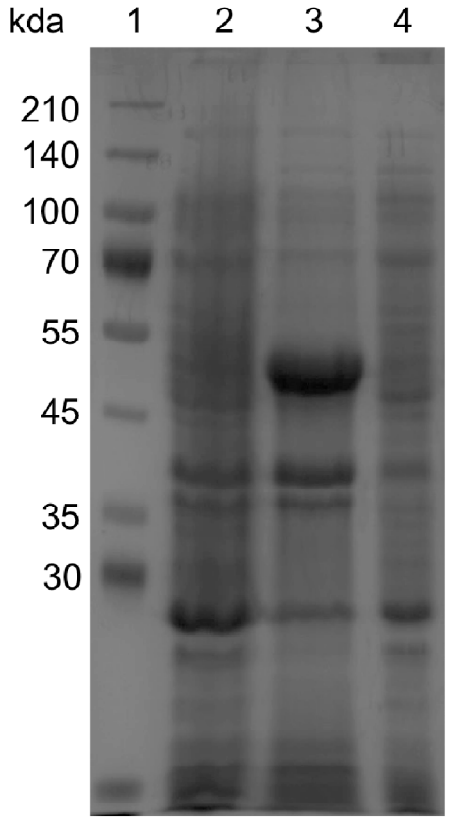

Figure 3: **Solubilization of the protein using 0.2% sarkosyl at 37 °C. Lane 1, molecular weight marker. Lane 2, OfCYP450 uninduced control. Lane 3, OfCYP450 induced pellet fraction. Lane 4, OfCYP450 induced supernatant fraction. The culture was grown at 37 °C. The bands were analysed by 12% SDS-PAGE**

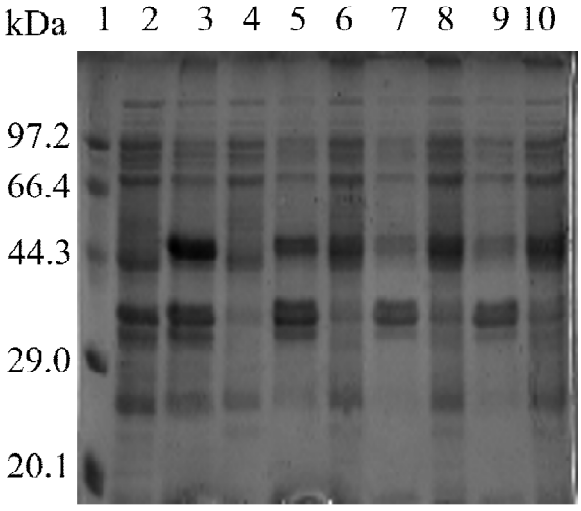

Figure 4: **Soluble and insoluble fractions of OfCYP450 cell lysate after treatment by sarkosyl. Lane 1, Molecular weight marker. Lane 2, OfCYP450 uninduced control. Lane 3-10, insoluble and soluble fractions after treatment of 0.05%, 0.1%, 0.2%, 0.3% sarkosyl respectively. The culture were grown at 16 °C for overnight**

chromatography (IMAC) on Ni-NTA resin column. The recombinant protein was successfully purified with some minor impurities as observed in SDS-PAGE (Figure 5). The purified protein was further subjected to second step of purification using SEC on a Superdex™200 10/300 GL column. It was observed that protein was purified to apparent homogeneity (Figure 6). The His-tag purified recombinant OfCYP450 protein was confirmed with immunoblotting using anti-His antibody (Figure 7).

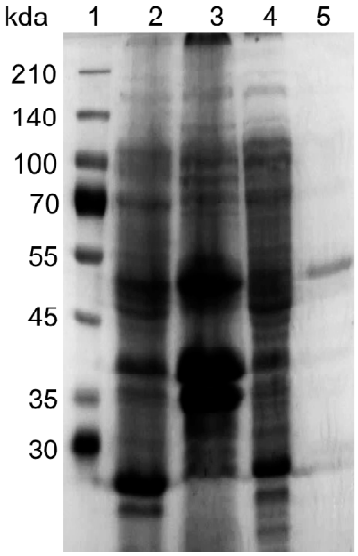

Figure 5: **SDS-PAGE analysis of OfCYP450 uninduced, induced and IMAC purified protein. Lane 1, Molecular weight marker. Lane 2, OfCYP450 uninduced control. Lane 3, OfCYP450 induced pellet fraction. Lane 4, OfCYP450 induced supernatant fraction. Lane 5, OfCYP450 IMAC purified protein**

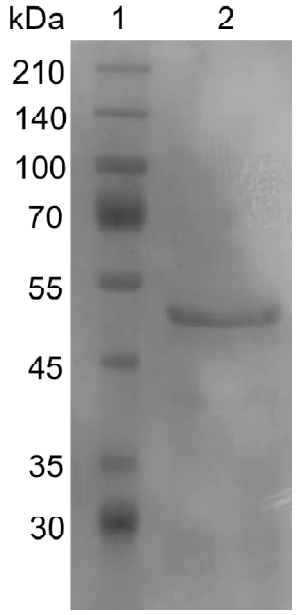

Figure 6: **SEC Purified OfCYP450. Lane 1, Molecular weight marker. Lane 2, SEC purified OfCYP450 protein**

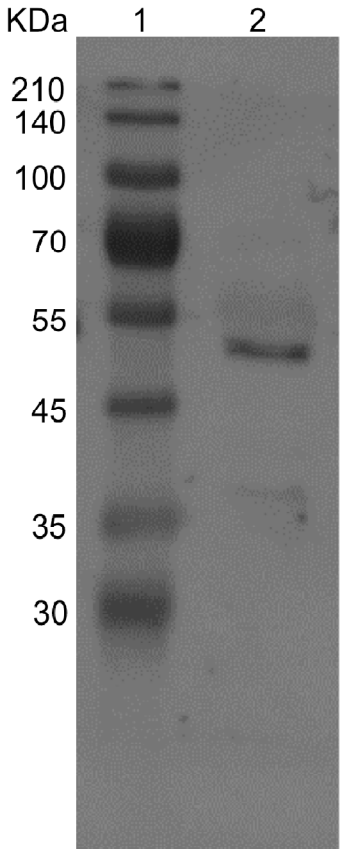

Figure 7: **Western blot analysis of OfCYP450. Lane 1, Molecular weight marker. Lane 2, Western blotting detection of OfCYP450 protein with a mouse anti-his antibody**

Molecular weight and subunit structure of recombinant OfCYP450

SEC was used to determine the molecular mass and oligomeric status of the purified recombinant OfCYP450. Gel filtration of the recombinant protein on Superdex S-200 column calibrated with various molecular weight standards showed a single peak with an elution volume of 15.4 mL (Figure 8), corresponding to a molecular mass of ~50kDa, which is similar to that obtained from SDS-PAGE. These observations suggest that CYP450 of *O. felineus* exists as a monomer under experimental conditions.

Structural features of OfCYP450

Far-UV CD is an excellent tool for rapid determination of the secondary structure and folding properties of proteins. For OfCYP450, a far-UV CD spectrum characteristic of a protein having both α-helices and β-sheets secondary structures, with dominance of β-sheet structures was observed (Figure 9).

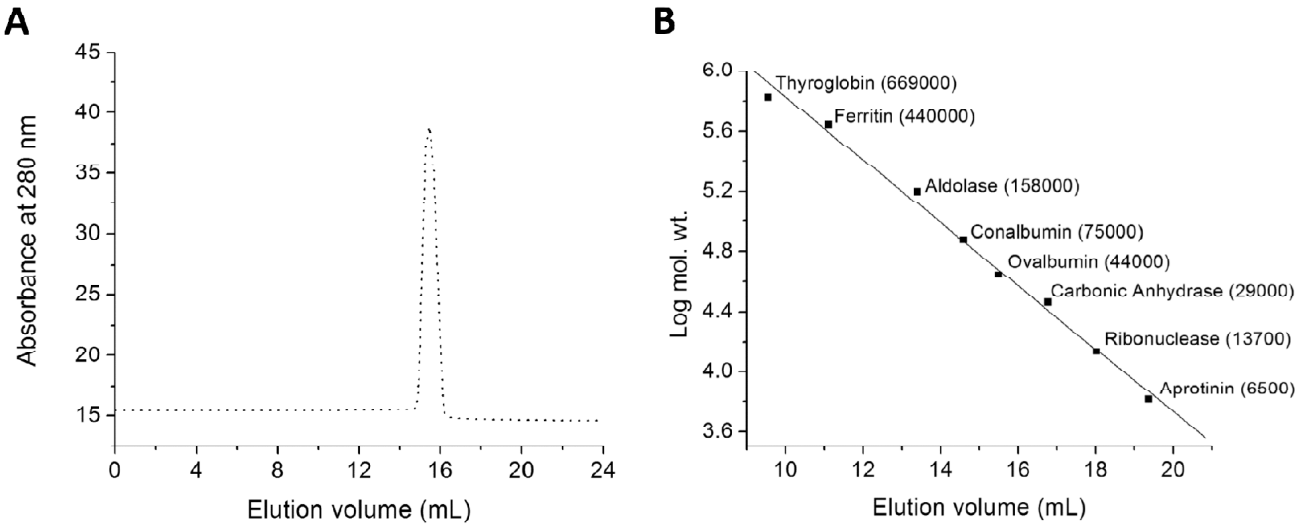

Figure 8: Determination **of native molecular weight of OfCYP450. (A). OfCYP450 was loaded onto a Superdex™ 200 10/300 GL column after purification and was eluted at a rate of 0.3 mL/min. Peak indicate OfCYP450 absorbance at 280 nm. (B). Graph shows the column calibration curve**

Table 1
Protein yield with or without sarkosyl at 16 °C

Sonication and purification condition	*Crude cell pellet/L culture (g)*	*Total protein/L culture (mg)*	*Soluble Protein yield after purification/L (mg)*
Without sarkosyl	2.16	~148	Nil
With 0.2% sarkosyl	2.16	~160	4.5

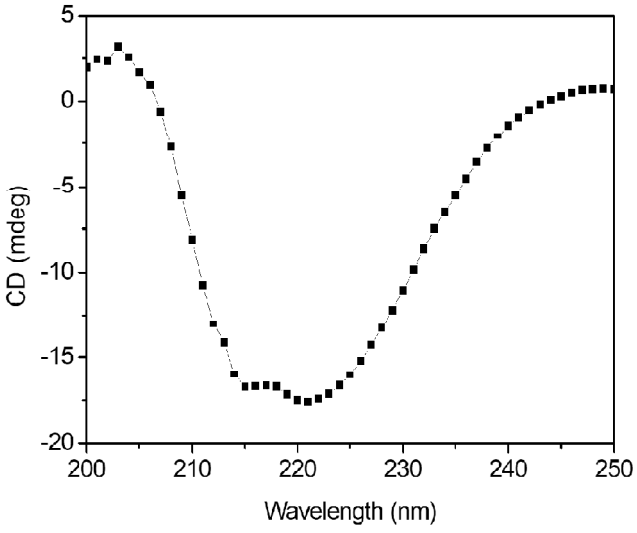

Figure 9: **Far UV-CD spectrum of native OfCYP450**

Discussion

The experimental conditions for expression should be optimized to obtain a high production of recombinant proteins. Different parameters such as cell density at the time of induction, post induction interval, growth temperature, concentration of inducer and the type of medium mainly contribute to the optimization of recombinant protein expression and production (Gopal and Kumar, 2013; Sorensen and Mortensen, 2005). Induction conditions should be optimized in such a way that induction can be commenced at the beginning of early log phase when the OD reaches between 0.6-0.8 at 600 nm wavelength for the complete expression of target protein (Berrow *et al*., 2006).

In the present study, we successfully cloned our gene of interest (CYP450 from *O. felineus*) into pET28a(+) vector and transformed into *E. coli* codon^{-+} cells. pET28a(+) vector contains bacteriophage T7 promoter and a His-tag sequence at the C-terminal region. Generally, the His-tag sequence do not affect protein structure but only facilitate the binding of recombinant protein to the Ni-NTA matrix (Carson *et al*., 2007). The best expression of recombinant OfCYP450 was obtained in the LB broth medium with initiation of induction at 0.6 OD_{600} with 1 mM of IPTG, 0.5 mM $FeCl_3$ and 1% ethanol at 37 °C for over night post induction.

After we obtained the overexpression of OfCYP450, the next obstacle was solubility problem as OfCYP450 is a membrane protein present in the insoluble fraction. In order to solubilize the protein and to avoid the formation of inclusion bodies, several approaches including the use of additives such as arginine, glutamine, urea, etc. have been utilized (Oganesyan *et al*., 2007). However, none of these approaches affected OfCYP450 solubility, and whole OfCYP450 protein was found in the pellet or inclusion body fraction. Thus, we tried the detergent treatment to the pellet to get soluble OfCYP450 protein. It has been well documented that the use ionic or non-ionic detergent can solubilize the protein from inclusion bodies. Initially, we tried several detergents like Triton X-100 (0.1-1%), Tween-20 (0.1-1%) and NP-40 (0.1-1%), but no solubility was observed. After all these, we used sarkosyl in the range of 0.1-1% that helped in solubilization. Sarkosyl has many desirable properties that contribute to the wide use for the solubility and purification of protein (Frankel *et al*., 1991; Tao *et al*., 2010). It is an anionic surfactant, and a small amount is helpful in solubilizing proteins. It forms micelles of small size and also does not interfere with the spectroscopic concentration measurement (Chisnall *et al*., 2014). Sarkosyl can solubilize the protein in low concentration without compromising its native characteristics such as secondary structure and enzymatic properties (Zhuo *et al*., 2005). In order to solubilize and prevent protein from denaturation, we optimized the minimum sarkosyl concentration that gives the maximum protein in the soluble fraction (0.2%). Sarkosyl soluble fraction was bound to Ni-NTA agarose, and the OfCYP450 protein was easily purified following this protocol. The recombinant protein using this protocol maintained a monomeric state in solution as revealed by gel filtration chromatography.

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Abbreviations

SEC, size exclusion chromatography; Ni–NTA, nickel–nitrilotriacetic acid; IMAC, immobilized metal affinity chromatography, CYP450, Cytochrome P450.

Conflict of Interest

The authors declare that there are no competing interests.

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