

Study on the protein expression of CYP3A4 and CYP2C18

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Abstract. Background As a drug enzyme, CYP450 has important significance in clinical treatment and the combined use of drugs, but the current research has not yet achieved high efficiency and large amount of expression in vitro. Therefore, the expression of CYP450 in vitro has become a hot spot. Objective Study the in vitro high-efficiency expression and time optimization of CYP3A4 and CYP2C18. Methods (1) CYP3A4 and CYP2C18 yeast recombinants were induced, and 24h, 48h and 72h bacterial liquid were collected respectively, to obtain the crude enzyme solution at different time periods, and then the enzyme solution at different time periods was subjected to SDS-PAGE protein electrophoresis to obtain its target bands. The results of comparative analysis between the protein bands and the Marker were observed by observing the gel running results. Finally, the enzyme activity was determined by liquid chromatography. Results The expressed proteins of CYP3A4 and CYP2C18 yeast recombinants were successfully induced. The target bands were obtained by SDS-PAGE protein electrophoresis and the optimal expression time was analyzed. Finally, the activity of the products was verified by liquid chromatography. Conclusion CYP3A4 and CYP2C18 yeast recombinants were successfully expressed in this experiment, and the most abundant expression of CYP3A4 and CYP2C18 was found at 72h by comparing the position of electrophoresis bands. At the same time, it was also verified that the expressed products were active, and CYP3A4 had the highest activity at 48h, which could be used for the study of CYP450 metabolism in vitro.

Keywords: CYP450 3A4 enzyme; CYP450 2C18 enzyme; Protein expression; enzyme activity determination.

1. Introduction

Cytochrome P450 (CYP450) is generally referred to as pharmacokinase, which is short for drug metabolizing enzyme and plays an important catalytic role in the metabolism of drugs [1] and is widely present in various organisms, including mammals, bacteria, fungi, and humans, as a superfamily of a class of ferrous heme-thiolate proteins with molecular weights in the range of 40-60 KDa, and the complex has a 450 nm maximum characteristic absorption spectrum [2]. In humans, CYP450 is the main metabolizing enzyme of drugs mainly distributed in hepatic microsomes [3-4] and is also expressed in the gastrointestinal tract, kidney, lung and placenta. It is involved in the metabolism of 70%-80% of clinical drugs. Therefore, it is often referred to as a drug enzyme [5]. In addition, CYP450 also has a very important role in plant abiotic stress and detoxification metabolism [6] as well as in the synthesis of natural drugs.

A lot of attention has been paid to the study of CYP450, and while the functional and structural [7] analysis of CYP450 has been carried out, the cloning and expression analysis of CYP450 [8]-[9] and the application of CYP450 in clinical treatment, such as the treatment of cardiovascular diseases [10] and tumor treatment [11], have also been studied in China, a. CYP450 has also been intensively studied abroad, including the role of CYP450 in breast cancer [12] and its importance in drug combination therapy [13]. Current studies have shown that the main metabolic enzymes involved in the metabolism of drugs in humans include CYP3A4, CYP2C9, CYP1A2, CYP2C19, CYP2E1, CYP2A6, and CYP2D6 [14]. Among them, CYP3A4 enzyme system has the strongest effect [15], accounting for more than 50% of CYP450 enzymes, and the substrates metabolized by CYP3A4 are very extensive, including macrolide antibacterial drugs and antifungal drugs [16], CYP3A4 has a large number of plastic active sites that can match different chemical substrates, and changes in the activity or content of CYP3A4 enzymes directly affect the metabolism in vivo CYP2C18 is also involved in drug metabolism [18], but is expressed at low levels in liver tissues, and at the RNA level, it is a major CYP2C in skin and lung tissues [19-20], and expression of

CYP2C18 in yeast metabolizes (S)-methylphenidate tropine, warfarin, etc. In addition, there are studies at home and abroad that have shown that CYP2C18 has significant antitumor effects [21]. However, its antitumor effect was not fully expressed and further studies are needed.

Recently, several scholars have conducted in-depth studies on the gene polymorphism of CYP450 [22] and its metabolism of drugs, especially the metabolism of CYP450 with various drugs and its related applications in disease treatment [23], but for now, most of the CYP450 extraction is limited to the use of microsomes from animal livers [24], which is not only expensive but also has low sample content, so It is necessary to establish a reliable and efficient extraction method for CYP450, which is beneficial for its research in metabolism. In this experiment, we will perform protein expression of CYP3A4 enzyme as well as CYP450 2C18 enzyme by induction of yeast, preparation of cell crushing solution and obtaining enzyme crude solution as well as protein electrophoresis and enzyme activity assay, which will provide the basis for efficient in vitro expression of the above two enzymes. This has important implications for the study of drug metabolism.

2. Methods

2.1 Yeast induction

Preparation of YPD medium, BMMY medium and BMGY medium: weigh the appropriate amount of reagents, place them in a beaker, add distilled water and mix well, then dispense them into triangular flasks and seal the sealing film, put them in an autoclave for sterilization.

Yeast inoculation: a. Take 10 μ L of the original bacterial solution on the ultra-clean bench and inoculate it into 10 m of LYPD liquid medium, shake until uniform and seal the sealing film, and incubate overnight in a water-tight incubator at 30 °C and 250 rpm. b. Take 1 m of LYPD medium and 20 mL of BMGY medium in a triangular flask and incubate at 30 °C and 250 rpm in a water-tight incubator. Incubate until OD600 is 3-6. c. Then transfer BMGY medium to a sterile 50mL centrifuge tube, centrifuge at 5000rpm for 5min and discard the supernatant. d. Resuspend the bacteria with BMMY medium until the suspension OD600 is 1.0. e. Take 10mL of bacterial solution into a sterile 200mL triangular flask and incubate in an incubator with shaking, taking samples every 24h, i.e. 24h, 48h, 72h respectively, while adding 0.5mL of methanol after each sample. The extracted bacterial solution was kept in the refrigerator at -20°C for freezing.

2.2 Preparation of protein electrophoresis samples

Cell crushing: Take out the spare bacterial solution, wait for melting, put it in a high-speed freezing centrifuge and centrifuge, put the supernatant in another centrifuge tube for spare, resuspend the precipitate with 1/2 volume of distilled water of the original bacterial solution, take the cell suspension in a centrifuge tube, put the centrifuge tube with bacterial solution in an ice water bath, and ultrasonic crushing with an ultrasonic crusher to finally obtain the cell crushing solution. The cell crushing solution was placed in a high-speed frozen centrifuge and the supernatant was taken.

Preparation of cell-broken supernatant protein electrophoresis sample: take 45ul of crude enzyme solution, add 15ul of 4×Protein SDS PAGE Loading Buffer, mix well and put it in a constant temperature metal bath at 99°C for denaturation for 10min, leave it to cool to room temperature and put it in -20°C refrigerator for storage.

Preparation of protein precipitation electrophoresis samples: Take 50 μ L of cell crushing solution, put it in a high-speed refrigerated centrifuge for 2 min at 10000rpm, discard the supernatant, and add 50 μ L of distilled water to the precipitate for resuspension. Subsequently, 45 μ L of the resuspension solution was taken and 15 μ L of 4×Protein SDS PAGE Loading Buffer was added. After mixing well, the sample was denatured in a constant temperature metal bath at 99 °C for 10 min, cooled to room temperature, and then stored in a refrigerator at -20 °C.

2.3 Preparation of SDS-PAGE gel and protein electrophoresis

Protein electrophoresis electrode buffer preparation and protein electrophoresis staining solution preparation

Preparation of protein electrophoresis decolorization solution: take 70mL of 95% medical ethanol, 50mL of glacial acetic acid and 380mL of distilled water, mix them well and store them at room temperature.

Configuration of 12% SDS-PAGE separation gel (10mL) and 5% SDS-PAGE concentrated gel (5 mL): take appropriate amount of reagents and mix well (APS and TEMED are added last, shake well immediately after adding, and immediately perfuse into the gap of two glass plates)

Gel preparation: select two pieces of glass plates without defects, wash and dry the two pieces of flat glass, and properly installed, after the preparation of 1mL of separation gel according to the formula, take 300-400 μ L of separation gel from both sides of the glass plate smoothly into the gap between the two glass plates, and then add 100 μ L of isopropyl alcohol to eliminate the air bubbles in the gap between the two glass plates. Wait for about 40 min, the separation gel is basically cohesive and can seal the gap between the bottom of the two glass plates. Slowly pour out the covered isopropyl alcohol and carefully absorb the residual isopropyl alcohol with filter paper, then prepare the concentrated gel according to the recipe, quickly infuse the concentrated gel in the gap between the two glass plates, and then immediately insert a clean small comb to ensure that the concentrated gel fills the gap of the comb and there must be no air bubbles. Again, after 40min at room temperature, the concentrated glue is basically polymerized. Remove the comb carefully. Mount the glass plate with gel on the vertical electrophoresis tank, and finally pour in the electrode buffer.

Sample loading: Firstly, carefully aspirate the electrode buffer with a pipette gun to rinse the comb wells to ensure there is no residual gel for sample loading, then carefully add 2 μ L of sample to the comb wells separately, adding Marker to the first well and Pasteur Piccolo to the second well for control observation. The subsequent wells were added to the supernatant, cytosol and cytosol of the same time period, respectively.

Electrophoresis: Cover the electrophoresis instrument with a lid after sample addition, plug in the power supply, adjust the voltage to 80V, stop electrophoresis when the bromophenol blue indicator moves to the lower end of the gel, during which the condition of the electrophoresis instrument should be paid attention to at all times.

Staining and decolorization: After electrophoresis, recover the electrode buffer, take off the gel, put it into the staining tank with good stain, and shake and stain overnight. When the color of the decolorization solution is dark, replace the decolorization solution in time until the background color becomes white. If the coloring is not satisfactory, you can repeat the coloring and decolorize again.

The finished decolorized gel will be photographed to observe the protein expression effect.

Determination of enzyme activity

Testosterone analysis conditions: column: Welchrom® C18, UF-AA, 250 mm*4.6 mm*5 μ m; mobile phase: acetonitrile:0.1% formic acid in water=6:4; flow rate: 0.8 mL/min; detection wavelength: 230 nm

HPLC enzyme activity assay:

Then add 300 μ L of the above CYP3A4 cell suspension to make up the volume with phosphate buffer solution (PH7.4) to make up the total volume of the reaction system to 1 mL. After pre-warm incubation in a 37C water bath for 5 min, add the final concentration of 1 mmol/L. After pre-warm incubation at 37C for 5 min, the reaction was initiated by adding NADPH at a final concentration of 1 mmol/L. The reaction was incubated for 30 min and then terminated in a low temperature refrigerator at -20°C.

Sample processing: The terminated sample was centrifuged at 15110 r/min (25000 g) for 15 min, the supernatant was extracted from the sample, passed through an activated C18 solid phase extraction column, eluted with 3 mL of methanol, the eluate was collected in a 37°C water bath and blown dry with N₂, the residue was dissolved with 200 µL of 50% methanol and injected into a 40 µL sample for detection and analysis. For the determination, a standard curve and quality control (QC) samples were established along with each batch of samples, which were regularly dispersed in the analytical order of the samples to be tested.

Configuration of standard solution: 200mg of TS control and 1.60mg of 6β-OHT control were weighed precisely and placed in a 10.0mL volumetric flask, dissolved with methanol and diluted to the scale, and shaken well to obtain 200µg/mL of TS stock solution and 160µg/mL of 6β-OHT stock solution, which were stored in a 4°C refrigerator. Dilute with methanol to the desired concentration before use

The peak area values of each group of testosterone were detected by HPLC, so as to obtain the difference between the peak areas of samples and standards, which led to the amount of substrate conversion, and finally the enzyme activity of each group of CYP3A4 was calculated indirectly.

Definition of CYP3A4 activity: Under the above reaction conditions, the amount of enzyme catalyzing 1 µmol of testosterone in 1 min was defined as a unit of enzyme activity (unit U).

Enzyme activity calculation formula: $M = (A_{\text{standard}} - A_{\text{sample}}) / A_{\text{standard}} \times T \times V \times C_{\text{standard}} \times V_{\text{total}}$ style in: M is enzyme activity (unit U/mL).

3. Results

3.1 Protein expression of enzymes

3.1.1 Protein expression of CYP3A4 and CYP2C18 after 24 incubation

(1) Protein expression of CYP3A4:

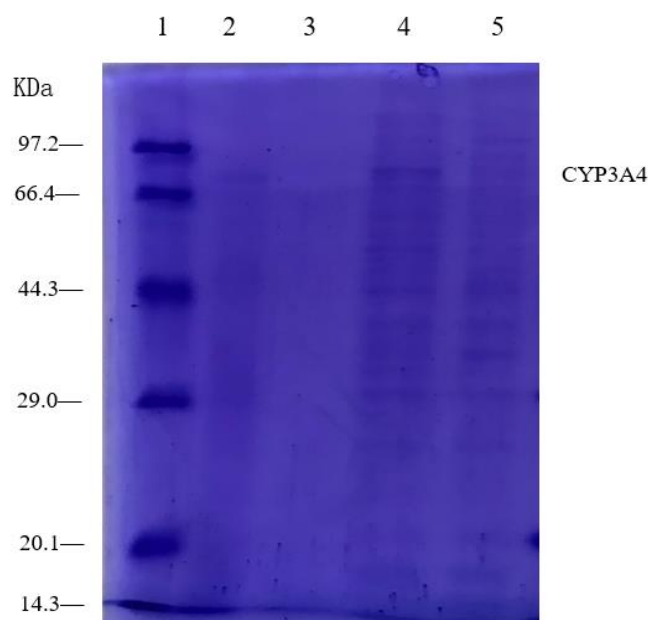


Figure 1 Protein expression of CYP3A4 24h

Note: The horizontal coordinates are the five different lanes and the vertical coordinates are the KDa values of the bands appearing in the Marker

lane 1; Marker; lane 2; Bilibi GS115 24h cytosol;

lane 3; CYP3A4 24h supernatant; lane 4; CYP3A4 24h cytosol;

Lane 5: CYP3A4 24h cytosol.

Analysis of the results: As shown in Figure 1, the control with GS115 at the corresponding time indicates that the results are normal and reliable. The protein expression of supernatant has no bands,

indicating that the supernatant does not contain protein and there is no soluble expression, while the cytosol and cytosol of cell crushing solution have bands, and the cytosol bands are deeper, indicating that the protein expression of CYP3A4 is successful after 24h of culture, and the protein is mostly present in the cytosol, but the bands are still not clear This indicates that 24h is not the best time for expression and further incubation is needed to find the best time optimization scheme. The protein band of CYP3A4 was calculated to be approximately at 75.0 KDa.

(2) Protein expression of CYP2C18

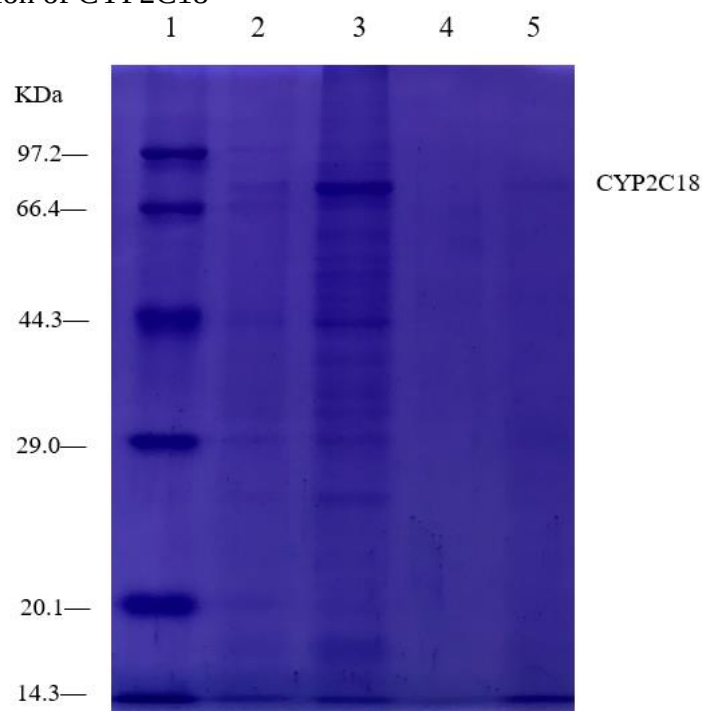


Figure 2 Protein expression of CYP2C18 24h

Note: The horizontal coordinates are the five different lanes and the vertical coordinates are the KDa values of the bands appearing in the Marker

lane 1; Marker;

lane 2; CYP2C18 24h cytosol;

lane 3; CYP2C18 24h cytosol;

lane 4; CYP2C18 24h supernatant;

Lane 5; Bilibi GS115 24h cytosol.

Analysis of the results; as shown in Figure 2, the control with GS115 at the corresponding time shows that the results of running gel are normal and reliable, the protein expression of supernatant has no bands, indicating that the supernatant does not contain protein and has no soluble expression, while the cytosol and cytosol have bands, and the bands of cytosol are deeper, indicating that the protein expression of CYP2C18 after 24h of culture is successful, and the protein is mostly present in the cytosol, but the bands are not clear, which can It can be concluded that 24h of CYP2C18 culture is not the best expression time, and further culture expression is needed to find the best time optimization scheme. The protein band of CYP2C18 was calculated to be approximately at 75.0 KDa.

3.1.2 Protein expression of CYP3A4 and CYP2C18 after 48h incubation

(3) Protein expression of CYP3A4

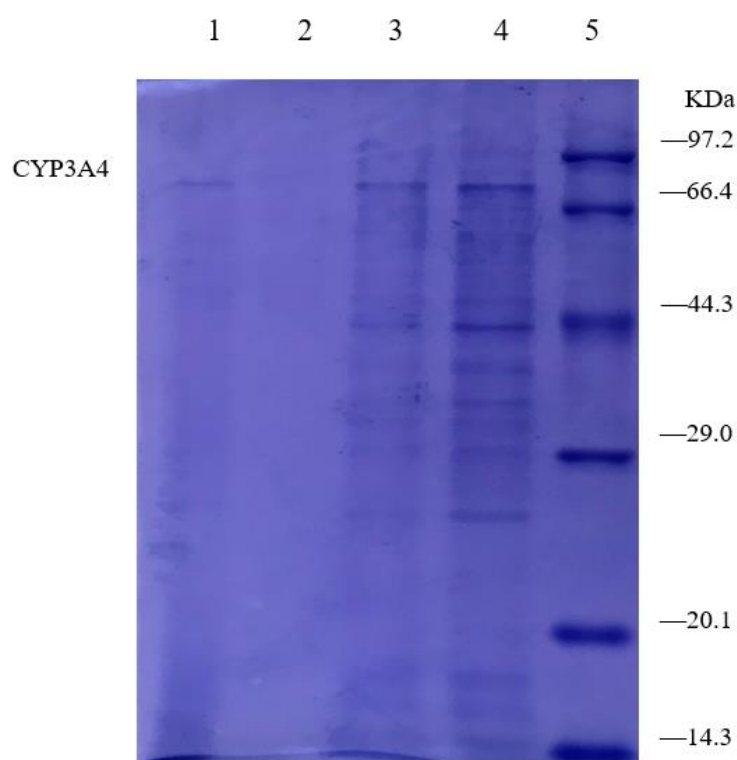


Figure 3 Protein expression of CYP3A4 for 48h

Note: The horizontal coordinates are the five different lanes, and the vertical coordinates are the KDa values of the bands appearing in the Marker

lane 1: Bilibi GS115 48h cytosol;

lane 2: CYP3A4 48h supernatant;

lane 3: CYP3A4 48h cytosol;

lane 4: CYP3A4 48h cytosol;

lane 5; Marker

Analysis of the results; as shown in Figure 3, the control with GS115 at the corresponding time shows that the results of running gel are normal and reliable, the protein expression of supernatant still has no bands, indicating that the supernatant does not contain protein, no soluble expression, while the cytosol and cytosol have bands, and the bands are clearer compared to the culture 24h, and the bands of cytosol are deeper, indicating that the protein expression of CYP3A4 after 48h of culture is successful. And compared to culture 24h protein content is more, the protein is mostly present in the cytosol, but it is possible that it is not the best expression time, so still need to carry out further culture expression, after calculation can be obtained CYP3A4 protein band is located roughly at 75.0.

(4) Protein expression of CYP2C18

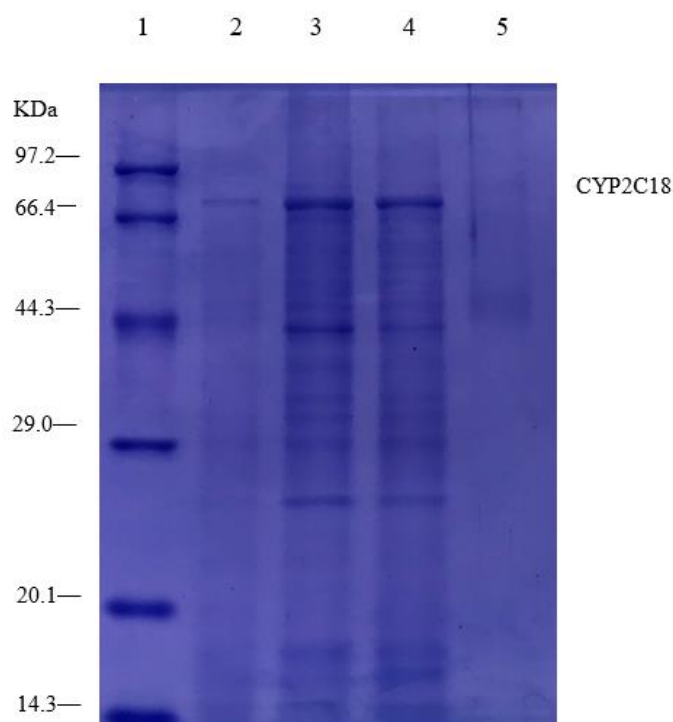


Figure 4 Protein expression of CYP2C18 for 48h

Note: The horizontal coordinates are the five different lanes and the vertical coordinates are the KDa values of the bands appearing in the Marker

lane 1: Marker;

lane 2; Bilobic yeast GS115 48h cytosol

lane 3; CYP2C18 48h cytosol;

lane 4; CYP2C18 48h cytosol

Lane 5: CYP2C18 48h supernatant.

Analysis of the results; as shown in Figure 4, the control with GS115 at the corresponding time shows that the results of running gel are normal and reliable, the protein expression of supernatant still has no bands, indicating that the supernatant does not contain protein, no soluble expression, while the cytosol and cytosol have bands, and the bands are clearer, and the cytosol bands are deeper, indicating that the protein expression of CYP2C18 after 48h of culture is successful. Compared with culture 24h protein content is more, the protein is mostly present in the cytosol, but it is possible that it is not the best expression time, so further culture expression is still needed, and the protein bands of CYP2C18 can be calculated to be roughly located at 75.0KDa.

Protein expression of CYP3A4 and CYP2C18 after 72h incubation

(5) Protein expression of CYP3A4

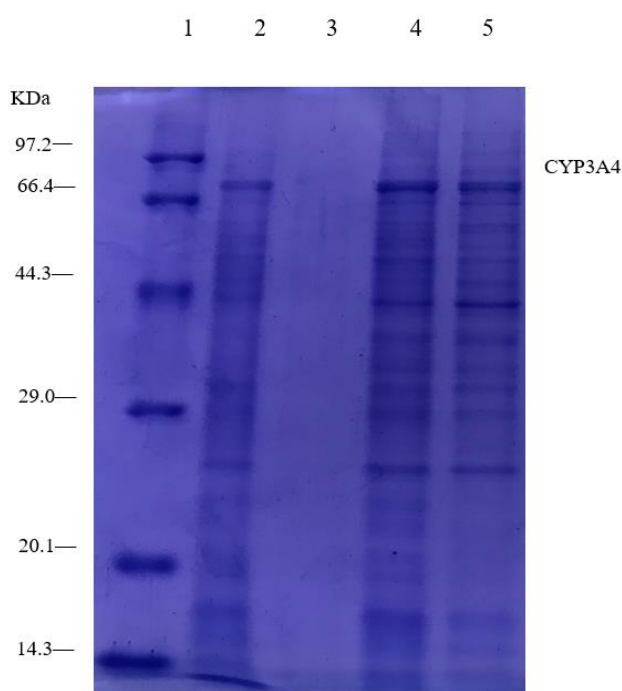


Figure 5 Protein expression of CYP3A4 72h

Note: The horizontal coordinates are the five different lanes, and the vertical coordinates are the KDa values of the bands appearing in the Marker

lane 1: Marker;

lane 2: Bilobic yeast GS115 72h cytosol;

lane 3: CYP3A4 72h supernatant;

lane 4: CYP3A4 72h cytosol;

Lane 5: CYP3A4 72h cytosol.

Analysis of the results; as shown in Figure 5, the control with GS115 at the corresponding time shows that the results of running gel are normal and reliable, and the protein expression of supernatant still has no bands, indicating that the supernatant no longer contains protein. There was no soluble expression, while there were bands in the cytosol and cytosol, and the bands were obviously clear compared with 24h and 48h, and the bands in the cytosol were deeper, indicating that the protein content expressed by 72 hours of incubation was higher, and the protein was mostly present in the cytosol, which could be used as the best expression time, and the protein bands of CYP3A4 were calculated to be roughly located at 75.0 KDa.

CYP2C18 protein expression in culture for 72h

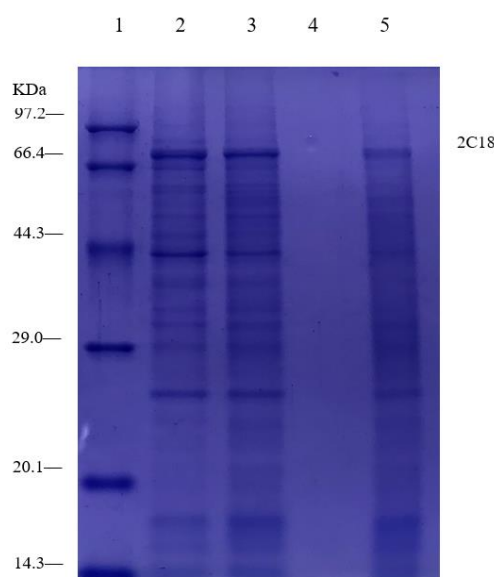


Figure 6 Protein expression of CYP2C18 72h

Note: The horizontal coordinates are the five different lanes, and the vertical coordinates are the KDa values of the bands appearing in the Marker

lane 1; Marker; lane 2: CYP2C18 72h cytosol;

lane 3; CYP2C18 72h cytosol;

lane 4; CYP2C18 72h supernatant

Lane 5: Bilibi GS115 72h cytosol

Analysis of the results; as shown in Figure 6, the control with GS115 at the corresponding time shows that the results of running gel are normal and reliable, and the protein expression of supernatant still has no bands, indicating that the supernatant no longer contains protein. There was no soluble expression, while there were bands in the cytosol and cytosol, and the bands were obviously clear compared with 24h and 48h, and the bands in the cytosol were deeper, indicating that the protein content expressed by 72 hours of incubation was higher, and the protein was mostly present in the cytosol, which could be taken as the best expression time, and the protein bands of CYP2C18 were calculated to be roughly located at 75.0 KDa.

CYP3A4 to CYP2C18 final ratio

Table 1 KDa values of CYP3A4 and CYP2C18 at different time periods

KDa\H	24h	48h	72h
CYP3A4	75.0	75.0	75.0
CYP2C18	75.0	75.0	75.0

Note: The data represent the positions of the bands of CYP3A4 and CYP2C18 in different time periods.

[CYP3A4] Substrate: Testosterone

Chromatographic column: Welchrom® C18, UF-AA, 250 mm*4.6 mm*5 µm

Mobile phase: acetonitrile:0.1% formic acid water=6:4

Flow rate: 0.8 mL/min

Detection wavelength: 230nm

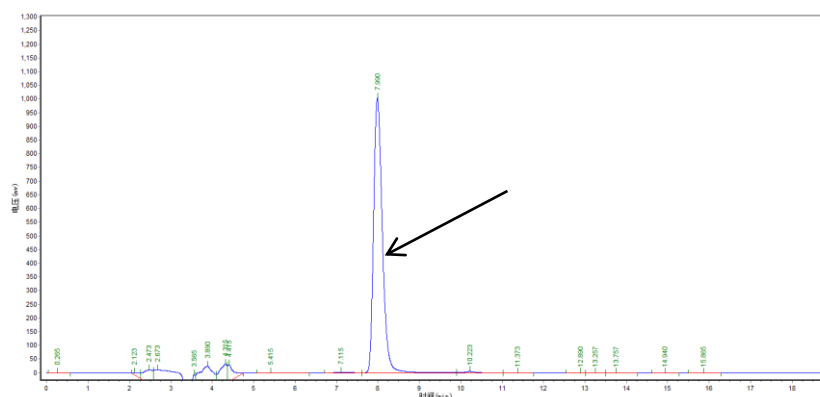


Figure 7 Results of enzyme activity analysis of CYP3A4

Note: Horizontal coordinates are time and vertical coordinates are voltage
24h 75U/L; 48h 136U/L; 72h 96U/L.

Analysis of results: As shown in Figure 7, CYP3A4 enzyme activity was successfully determined and the highest enzyme activity was observed at 48h.

CYP2C18 enzyme activity was not measured for the time being.

4. Discussion

CYP3A4, as the most abundant CYP450 enzyme isoform in liver, and CYP2C18, as the predominant CYP2C in skin and lung tissues, are both involved in the metabolism of various drugs, but for the current study, the extraction of CYP450 is limited to the microsomes of animal liver, and in order to obtain a more reliable and efficient extraction method, in vitro studies on CYP450 should be targeted. In order to obtain a more reliable and efficient extraction method, a more recent study on the in vitro expression of CYP450 should be carried out. In this experiment, we successfully completed the efficient protein expression of CYP3A4 and CYP2C18, and compared the protein expression effects at 24h, 48h and 72h, and concluded that the protein expression of CYP2C18 and CYP3A4 was the highest at 72h, and the enzyme activity of CYP3A4 was the highest at 48h. However, the protein expression of CYP450 was only limited to that in *B. pseudomallei*, and it did not really achieve a large amount of CYP450 expression in vitro, so further in-depth research is needed. In order to realize the effective use of CYP540 in clinical therapy, we should focus on the efficient expression of CYP450 in various cells in the future, not only in animal microsomes, and in this regard, how to achieve the effective and large expression of CYP450 is a major problem in the future research field, and researchers need to propose more conjectures and turn them into reality.

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