Cloning, heterologous expression, and activity analysis of NADPH-cytochrome
P450 reductase from the Chinese white rabbit

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ABSTRACT
Cytochrome P450 reductase (CPR) is an integral component of the P450 oxidoreductase system (P450s). It serves as the electron donor for most cytochromes in P450s, which are involved in the metabolism of foreign compounds and the synthesis of endocrine hormones and have tremendous biotechnological potential for the synthesis of pharmaceuticals and fine chemicals. However, commercially available CPR is very expensive, and heterologous expression in Escherichia coli is a more affordable way to obtain enough CPR. In the present study, a full-length cDNA encoding a CPR was isolated from the liver of the Chinese white rabbit using reverse transcription-polymerase chain reaction (RT-PCR). The cDNA contains a 2,040-bp open reading frame, which is predicted to encode an enzyme of 679 amino acids. The deduced peptide shares 99.5% amino acid similarity with CPR of Oryctolagus cuniculus, showing that the Chinese white rabbit is a close genetic relative of the European rabbit. The cloned CPR has the typical hallmarks, including an N-terminal membrane anchor and flavin adenine dinucleotide (FAD)-, flavin mononucleotide (FMN)- and nicotinamide adenine dinucleotide phosphate (NADPH)-binding domains. An N-terminally truncated protein was heterologously expressed in E. coli BL21 (DE3) cells and purified, and the specific activity of the recombinant enzyme was determined. The enzyme activity analysis indicated that electrons were passed from NADPH to Cyt C at a rate of 2.3174 μmol/(min/mg protein). The present study provides an efficient procedure for preparing large amounts of recombinant CPR, which would facilitate the synthesis of pharmaceuticals and fine chemicals with P450s.

KEYWORDS
Cytochrome P450 reductase; cloning; heterologous expression; activity analysis; Chinese white rabbit

Introduction
Cytochrome P450 oxidoreductase systems (P450s) are found in all eukaryotes and some prokaryotes [1]. P450s can catalyse a variety of chemical reactions using a wide range of hydrophobic substrates [2,3]. This property makes them well suited for the synthesis of fine chemicals, such as drugs [4,5]. However, the typical P450s catalytic cycle requires an electron donor, the nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase (CPR; EC 1.6.2.4), which is an integral part of P450s. CPR contains several conserved domains, such as the flavin mononucleotide (FMN)-, flavin adenine dinucleotide (FAD)- and NADPH-binding domains. An electron is transferred from NADPH to FAD and FMN by CPR and is subsequently donated to cytochrome P450 enzymes [6,7], such as heme oxygenase, cytochrome b5 and cytochrome c.

CPRs have been identified and functionally characterized in several mammalian species, including rat [8], New Zealand white rabbit [9], guinea pig [10], mouse [11], hamster [11], human [12], horse and koala [13]. It was believed that there is only one CPR protein-encoding gene in each species [9], and the reaction catalysed by CPR is the rate-limiting step among the many oxidation reactions catalysed by microsomal enzymes [14].

However, CPR is very expensive. Here, we are interested in characterizing CPR with a goal of reconstituting a cytochrome P450 oxidoreductase system with sufficient activity. In the present study, a cDNA containing the full coding region of CPR from Chinese white rabbit (Oryctolagus cuniculus) was isolated, and the CPR protein was over-expressed in Escherichia coli. The preliminary results provide a solid foundation for future functional studies of rabbit CPRs and a reconstituted cytochrome P450 oxidoreductase system.

Materials and methods

Bacterial strains and plasmids
Competent E. coli DH5α and BL21 (DE3) cells were obtained from Transgen Biotech Co., Ltd. (Beijing, China)
and were used for cloning and protein expression, respectively. Plasmid pMD™19-T, which was used for cloning PCR (polymerase chain reaction) fragments, and pET28b, which was used for protein expression, were purchased from Novagen (Darmstadt, Germany). All restriction endonucleases, the PrimeScript RT reagent Kit, DNA ligation kit, plasmid extraction kits and agarose gel extraction kit were purchased from TaKaRa Bio. Inc. (Dalian, China). The DNA molecular weight marker and protein marker were purchased from Transgen Biotech. Cytochrome c (Cyt C) from equine heart was obtained from Sigma (St. Louis, MO, USA).

Rabbit hepatic tissues
A Chinese white rabbit was obtained from the Chengdu Zaofeng rabbit farm (Chengdu, China), and was used as a source of rabbit liver. The liver was quickly removed, immediately frozen in liquid nitrogen and stored at −80 °C until use.

Extraction of total RNA from fresh rabbit liver
Total RNA was isolated from fresh rabbit liver using the RNAeasy Mini Kit (Qiagen, Hilden, Germany). First, freshly obtained rabbit liver was placed into a porcelain mortar, liquid nitrogen was added and the tissue was ground. This was repeated four times, and then the subsequent steps for total RNA isolation were conducted according to the manufacturer’s protocol. RNA samples were treated with a DNase I from the RNAase-Free DNase Set (QIAGEN) to remove potential contaminating genomic DNA. The quality and concentration of the RNA sample was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and gel electrophoresis.

Reverse transcription-polymerase chain reaction (RT-PCR)
The procedures used for reverse transcription and PCR are summarized in Figure 1, and the sequences of the primers used for reverse transcription and PCR are shown in Table 1. The first-strand cDNA was synthesized in a 20-μL reaction mixture containing 1 μg of total RNA, 1 mmol/L of each deoxyribonucleoside triphosphate (dNTP), 100 pmol of random primer (hexa-deoxyribonucleotide mixture) and 25 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (TaKaRa, Dalian, China). Using the first-strand cDNA as the template, the 5'-segment of the CPR gene was amplified using the Redu-F/redu-R1 PCR primer pair, with the following amplification program: 95 °C for 5 min, 36 cycles of 95 °C for 50 s, 63 °C for 50 s and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. The amplifications were performed in a C1000 TM thermal cycler (Bio-RAD, California, USA). Similarly, the Redu-F1/redu-R primer pair and the first-strand cDNA were used to amplify the 3'-segment of the CPR gene.

Construction of the CPR expression vector
Primers F and R1 were used to amplify the 5'-portion of the gene (1346 bp, the DNA fragment was named FR1). Primers Redu-F1 and Redu-R1 were used to amplify the 3'-portion of the gene (the DNA fragment obtained was named FR1). Then, these two DNA fragments (FR1 and FR1) were ligated separately into the pMDTM19-T vector (TaKaRa) to generate plasmids pMD-FR1 and pMD-FR1, respectively. Plasmid pMD-FR1 was digested with NotI and XhoI, and the obtained fragment was ligated into the pET-28a(+) expression vector that had been linearized with NotI and XhoI to generate the plasmid pET-FR1. Plasmid pMD-FR1 was digested with Ndel and NotI, and the obtained fragment was ligated into pET-FR1 that had been linearized with Ndel and NotI to generate the expression plasmid pET-CPR.

CPR expression
To express the CPR protein, the recombinant cells were activated in Luria–Bertani (LB) medium (10 mL) containing 50 μg/mL kanamycin, at 37 °C for 16 h with shaking at 200 r/min. The culture was then inoculated into 500 mL of LB medium containing kanamycin (50 mg/mL) and grown at 25 °C, 200 r/min. When the optical density at 600 nm (OD600) of the cells reached 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 0.5 mmol/L to induce protein expression. After incubation at 16 °C for 16–20 h, the cells were harvested by centrifugation at 3000 × g for 10 min at 4 °C. The cell pellets were washed with 10 mL of 50 mmol/L Tris-HCl buffer (pH 7.0) and resuspended in the same buffer. The cell pellets and crude cell lysates were used directly to determine protein expression. CPR protein was expressed as a C-terminal His-tagged fusion protein.

| Primer | Sequence (5’–3’) |
|--------|-----------------|
| Redu-F | CTGCATATGCGGAGCTCCAGCGGACAC (Ndel) |
| Redu-R | GTAATCCAGACTGATCCACCCATCCGGGGATAG (XhoI) |
| Redu20-F | CTGCATATGCTCGTCAGTCCGAGTCAGCAG (Ndel) |
| Redu30-F | CTGCATATGCCTCGTCATCGTGGGCGGATG (Ndel) |
| Redu-F1 | CATCTCCGAGACTCCGCCT |
| Redu-R1 | AGCCAGCTCACAGCTGCTGATG |

Table 1. Primers used in this study.
Figure 1. Construction of the CPR expression plasmid pET-CPR. CPR cDNA was obtained from rabbit liver by RT-PCR. Fragments containing the 5' and 3' halves of the CPR gene were obtained by PCR and ligated separately into the pMD19-T vector. Finally, the two cloned segments of the CPR gene were ligated together into pET28a to obtain the expression plasmid pET-CPR.
All purification steps were carried out at 0–4 °C. E. coli cells containing pET-CPR grown in 0.5 L of LB medium were harvested by centrifugation at 7000 x g for 10 min, washed once with Tris-NaCl buffer (25 mmol/L Tris, 100 mmol/L NaCl, pH 7.4) and resuspended in 10 mL of the same buffer. The cell suspension was lysed by sonication on ice, and centrifuged at 12,000 x g for 30 min. The supernatant was applied to a HisTrap column (GE Healthcare) and eluted with a linear gradient of 0–300 mmol/L imidazole in Tris-NaCl buffer (50 mmol/L Tris·HCl, 200 mmol/L NaCl, pH 7.4). The elution fractions were analyzed by 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to detect the protein. The CPR-positive fractions were pooled and desalted with a centrifugal filter device (YM-30, 30,000 MWCO; Millipore, Ireland) using 50 mmol/L Tris·HCl, pH 7.4 as the buffer. The concentration of the obtained protein was measured with an AnalytikJena ScanDrop 100 (Germany).

**Determination of P450 enzyme activity**

The activity of the purified recombinant CPR was assayed by monitoring the increase of the absorbance at 550 nm when Cyt C was reduced [15,16]. In brief, in a 96-well ELISA plate, we added a 0.2-mL reaction mixture containing 16 U of 0.5 mmol/L horse heart Cyt C (10 mmol/L in PBS, pH 7.7) and 1 μg of purified recombinant CPR. The reaction was initiated by adding 2 μL of 10 mmol/L NADPH (final concentration, 10 μmol/L). The time-dependent absorption increase in triplicate samples was monitored on a Bio-Rad Model 680 microplate reader (Eppendorf, Hamburg, Germany). Cyt C_red was calculated using the following formula [16]: nmol Cyt C_red per min = ΔA550/min/0.021.

**Sequence analysis**

The presence of a signal peptide was detected using SignalP-4.1 at the Center of Biological Sequence Analysis (http://www.cbs.dtu.dk/services/SignalP-4.1/), and transmembrane domain prediction was performed using the TMHMM 2.0 server (http://www.cbs.dtu.dk/services/TMHMM/).

**Results and discussion**

**Analysis of the p450 rabbit reductase gene**

The primers used to amplify the p450 reductase-encoding gene (listed in Table 1) were designed according to the *Oryctolagus cuniculus* genome sequence (http://www.genome.jp/kegg-bin/show_organism?org=ocu). The full coding region (2040 bp; GenBank accession number: KT989504) was isolated from Chinese white rabbit liver. It is predicted to encode a peptide of 679 amino acids. Alignment of the amino acid sequence revealed that it shares 99.5% homology with the p450 reductase of *Oryctolagus cuniculus* (Table 2), and it shares high similarity with orthologs from other mammals, which all contain conserved functional binding domains, including the FMN-, FAD-, NADPH- and substrate-binding domains (Figure 2).

**Expression and purification of China white rabbit P450 reductase**

To better understand the physiological roles of CPR and explore the use of this CPR to reconstitute a cytochrome P450 oxidoreductase system with sufficient activity, we heterologously expressed an N-terminally truncated enzyme in *E. coli* Rosetta (DE3) cells. Using plasmid pET-F1R as a template, and primers redu20-F, redu30-F, and redu-R1, we amplified the 5’ portion of the gene (the Table 2. Amino acid similarities of CPR from Chinese white rabbit and other mammals.

|        | ChCPR | EqCPR | SuCPR | CaCPR | CrCPR | MuCPR | OrCPR | RaCPR | PhCPR |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| ChCPR  | 100   | 90.4  | 89.9  | 90.4  | 89.3  | 89.9  | 99.5  | 90.4  | 84.8  |
| EqCPR  | 100   | 100   | 92    | 90.8  | 90.1  | 90.4  | 90.5  | 90.7  | 84    |
| SuCPR  | 100   | 100   | 91    | 90.6  | 90.1  | 90.4  | 90.1  | 90.7  | 83.2  |
| CaCPR  | 100   | 100   | 90.4  | 90.4  | 90.4  | 90.8  | 91.5  | 91.5  | 82.7  |
| CrCPR  | 100   | 100   | 95.5  | 89.3  | 89.3  | 96    | 83.3  | 83.7  | 83.7  |
| MuCPR  | 100   | 98.3  | 89.9  | 89.9  | 89.9  | 98.3  | 89.9  | 84.8  | 84.8  |
| OrCPR  | 100   | 100   | 100   | 100   | 100   | 100   | 100   | 100   | 100   |
| RaCPR  | 100   | 100   | 100   | 100   | 100   | 100   | 100   | 100   | 100   |
| PhCPR  | 100   | 100   | 100   | 100   | 100   | 100   | 100   | 100   | 100   |

Note: ChCPR, China white rabbit (KT989504); EqCPR, *Equus caballus* CPR (horse, EU447660); SuCPR, *Sus scrofa* CPR (Pig, L33893); CrCPR, *Sus scrofa* CPR (Pig, L33893); MuCPR, *Mus musculus* CPR (house mouse, D17571); OrCPR, *Oryctolagus cuniculus* CPR (Rabbit, X04610); RaCPR, *Rattus norvegicus* CPR (Norway rat, M12516); PhCPR, *Phascolarctos cinereus* CPR (koala, EU924793).
DNA fragment obtained was named F1R, and the obtained PCR products, with N-terminal regions shortened by 20 and 30 amino acids compared with the full CPR-encoding region, were named CPRd20 and CPRd30, respectively. After digestion with NdeI and NotI, the N-terminally truncated fragments were ligated to pET-F1R, which was digested with the same restriction endonucleases. The recombinant plasmids were named pET-CPRd20 and pET-CPRd30, respectively. The constructs were confirmed by DNA sequencing. The above expression constructs were then transformed into E. coli BL21 (DE3) cells.

Figure 2. Multiple sequence alignment of CPR from Chinese white rabbit and other mammals.

Figure 3. Transmembrane region analysis of CPR from China white rabbit.
SDS-PAGE analyses of the cell lysates revealed that *E. coli* (Rosetta) effectively expressed the CPR protein without apparent degradation (Figure 4). The protein expressed by the cells containing pET-CPR-30, which is missing the N-terminal 20 amino acids, was only found as inclusion bodies (data not shown), whereas a portion of the protein produced by the cells containing pET-CPR-30 was expressed in the cell lysate in a soluble form (lanes 2 and 3). After purification, the purity of the CPR protein was determined to be >90% (lane 4).

**Analysis of rabbit P450 reductase activity**

The ability of the recombinant CPR to function as an electron donor for Cyt C was examined. When p450 reductase is mixed with Cyt C, an electron is passed from p450 reductase to the latter, and Cyt C is reduced. The reduced Cyt C (CytC_red) has a particular absorption peak at 550 nm; therefore, P450 reductase activity can be calculated based on the change per minute in the absorption at 550 nm. Figure 5 shows that the fusion protein CPR-30 can accept electrons from NADPH and transfer them to Cyt C. The activity of the recombinant P450 reductase was 2.3174 μmol/(min/mg protein).

It is thought that Chinese white rabbit originated in Europe and was introduced in China through the Silk Road. The inhabitants of ancient China spent more than 2,000 years on the domestication of this rabbit, and a fine variety was obtained; because of its white fur, it is known as the Chinese white rabbit. Therefore, the Chinese white rabbit should be closely related to the European rabbit *Oryctolagus cuniculus*. In the present study, primers for amplifying the P450 reductase gene of the Chinese white rabbit were designed based on the *Oryctolagus cuniculus* genome sequence. After reverse transcribing the RNA into first-strand cDNA, two primer pairs were used to amplify the 5' and 3'-portions of the gene. Sequence analysis showed that it shares 99.5% homology with the p450

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**Figure 4.** Analysis of the expression of a recombinant rabbit P450 reductase fusion protein by SDS-PAGE. Lane M, protein marker; lane 1, lysate of *E. coli* cells containing the empty-vector pET28a(+); lane 2, whole *E. coli* BL21(DE3) cells containing pET-CPR-30 after induction with IPTG; lane 3, cell lysate of *E. coli* BL21(DE3) cells containing pET-CPR-30 (DE3); lane 4, purified protein.

**Figure 5.** Analysis of p450 reductase activity as the change in CytC_red A_{550} over time.
reduce the redox balance of the cytochrome P450 system, thereby facilitating the synthesis of pharmaceuticals. The authors describe the cloning of the redox enzyme, CPR, from Chinese white rabbits and its expression in Escherichia coli. They note that the CPR has a high redox potential and can transfer electrons to a variety of P450 substrates. The study also includes a discussion of the use of CPR in biocatalytic processes, such as the production of pharmaceuticals and the detoxification of environmental pollutants.

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