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High-Level Expression of Human Cytochrome P450 3A4 by Co-Expression with Human Molecular Chaperone HDJ-1 (Hsp40)

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Cytochrome P450 (CYP) 3A4 is of great interest because of its important roles in the oxidation of numerous drugs and xenobiotics. HDJ-1, a molecular chaperone in human, is known to assist the correct folding of unfolded proteins. To achieve a high yield of recombinant human CYP3A4 in *Escherichia coli*, the CYP3A4 encoding gene was co-expressed with the chaperone HDJ-1, under the control of an inducible *tac* promoter in a bicistronic format. The levels of expression of the CYP3A4 in the bicistronic construct reached up to 715 nmol (liter culture)$^{-1}$ within 16 h at 37°C, which was about a 3.3-fold increase compared to that of the CYP3A4 alone without the HDJ-1. By co-expression with HDJ-1, the catalytic activity of CYP3A4 was also increased by ~15-fold. The amount of activity increase was similar to that of the CYP production at the whole cell level. The present over-expression system may be useful for the rapid production of large amounts of active CYP3A4 in *E. coli*.

Key words: CYP3A4, HDJ-1, Co-expresion, Chaperone

INTRODUCTION

Cytochromes P450 (CYP or P450) are the major enzymes involved in the oxidation of xenobiotic chemicals and endogenous substrates (Wrighton and Stevens, 1992), and multiple forms of CYPs are present in mammals (Guengerich, 1995). Human CYP3A4 is generally agreed to be the most abundant CYP enzyme present both in the liver and small intestine, two of the major sites for oxidation of xenobiotic chemicals (Guengerich, 1995). The enzyme has very broad substrate specificity, and catalyzes the metabolism of approximately 50% of therapeutic agents (Tang and Stearns, 2001).

Recombinant human CYP enzymes have proved to be useful for drug metabolism research; and thereby, many heterologous expression systems have been developed (Guengerich and Parikh, 1997). Bacterial expression systems, especially *Escherichia coli* cells, have also been widely used for the production of various CYP enzymes to obtain high yields of the proteins. Moreover, to achieve maximal catalytic activity, as well as protein expression, various strategies are employed, such as co-expression of CYP with NADPH-P450 reductase (Iwata *et al.*, 1998), *N*-terminal modification (Pritchard *et al.*, 1997) and the use of fusion proteins (Parikh and Guengerich, 1997). Similarly, it was reported that the expression of CYP3A7 was elevated by the presence of molecular chaperone GroEL, which is known to assist the correct folding of proteins in *E. coli* (Inoue *et al.*, 2000).

In this study, an over-expression method has been provided for human CYP3A4 in *E. coli* achieving a high production of the CYP3A4 enzyme by co-expression with HDJ-1, a molecular chaperone in humans. HDJ-1 (also called Hsp40) is a human homologue of bacterial heat shock protein DnaJ (Nagata *et al.*, 1998), and is also known to regulate the formation of protein aggregation, protein folding and translocation (Bao *et al.*, 2002; Freeman and Morimoto, 1996).

MATERIALS AND METHODS

Materials

The $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, testosterone, δ-aminolevulinic acid (δ-

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ALA) and 6β-hydroxytestosterone were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant rat NADPH-P450 reductase was expressed in *E. coli,* and purified as previously described (Hanna *et al.*, 1998).

Construction of CYP expression plasmids

The cDNA for human CYP3A4 with *N*-terminal truncation (Gillam *et al.*, 1993) was subcloned into the expression vector, pCW, to generate pCYP3A4. The HDJ-1 gene in the pET21d HDJ-1, an expression vector for HDJ-1 in *E. coli*, was amplified by PCR. To construct a co-expression vector for CYP3A4 and HDJ-1, the PCR amplification was designed to include a *Sal*I restriction enzyme site followed by a ribosomal binding site and 6 nucleotide spacer (5' AGGAGGGCTAAC 3'), as shown in Fig. 1. The resulting PCR product was purified, digested with *Sal*I, and then ligated into the pCW3A4 vector treated with *Sal*I. The nucleotide sequence of the entire region, including CYP and HDJ-1, was analyzed by dideoxy sequencing (Sanger *et al.*, 1980).

Expression of recombinant CYP3A4 and preparation of membranes

E. coli DH5aF'IQ cells were transformed with the expression plasmid. The transformed cells were grown in Terrific Broth (TB) containing 0.2% bactopeptone (w/v), supplemented with 100 μg ampicilline/mL, 1.0 mM thiamine and trace elements (Sandhu *et al.*, 1993); 50 μM $FeCl_3$, 1 mM $MgCl_2$ and 2.5 mM $(NH_4)_2SO_4$. When the optical density of the culture at 600nm was approximately 0.4~0.5, protein expression was induced by the addition of 1 mM IPTG and 1.5 mM δ-ALA. Every 3 h, a portion of the culture was sampled, and expression level of CYP quantitated by Fe^{2+}-CO *versus* Fe^{2+} difference spectra (Omura and Sato, 1964). Expression was allowed to proceed for 24 h at 37°C. A bacterial membrane fraction was prepared, as described previously (Gillam *et al.*, 1993).

Enzymatic assay

Whole cells and membrane fractions were analyzed for the expressed CYP concentrations by Fe^{2+}-CO *versus* Fe^{2+} difference spectroscopy, as previously described (Omura and Sato, 1964), in 100 mM Tris-Cl at pH 7.4, containing 20% glycerol (v/v), 10 mM CHAPS and 1 mM Na-EDTA, using a Shimadzu UV-1650 PC spectrophotometer. The testosterone 6β-hydroxylation activity was determined for CYP3A4 with membrane fractions, as described elsewhere (Arlotto *et al.*, 1991). The activity assay was performed in 100 mM potassium phosphate buffer (pH 7.4) in a reaction volume of 500 μL. The membrane fraction (0.4 μM CYP3A4) and NADPH-cytochrome P450 reductase (2 μM) were mixed in the presence of the testosterone substrate. The reaction was initiated by the addition of an NADPH-generating system, the sample incubated at 30°C for 10 min and the reaction stopped by the addition of 50 μL 1.0 M HCl containing 2.0 M NaCl. The resulting product was extracted and analyzed by HPLC, with UV detection at 240 nm.

Other methods

Protein concentrations were estimated using a bicinchoninic acid procedure, according to the manufacturers directions (Pierce, Rockford, IL). Rabbit anti-human CYP3A4 was used in the presented immunoblotting experiments. The specificity and properties of similar preparations have been reported elsewhere (Distlerath *et al.*, 1985). The antiserum was adsorbed with *E. coli* proteins before use to remove background staining.

RESULTS AND DISCUSSION

Several strategies for the expression of recombinant human CYPs in bacteria have been developed, and are widely used (Guengerich and Parikh, 1997). It was provided that GroEL, a molecular chaperone, increases the expression level of CYP3A7 in *E. coli* (Inoue *et al.*, 2000). As a result of widening the search of other chaperones

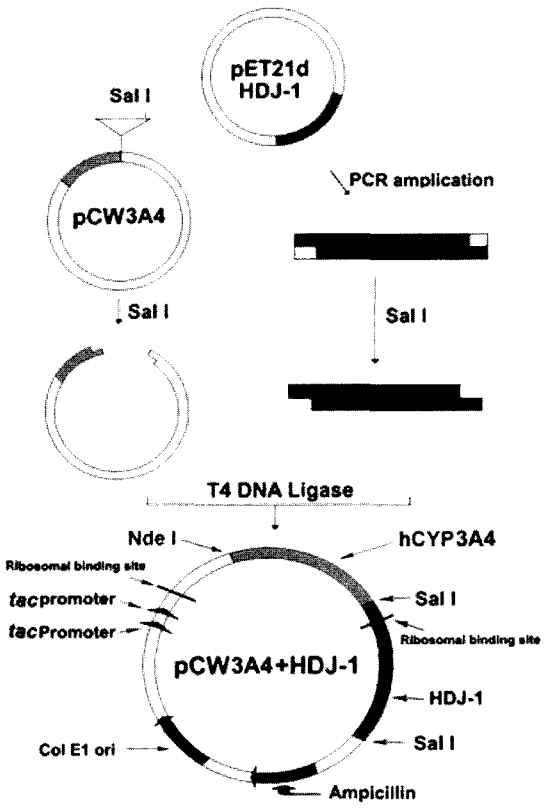

Fig. 1. Construction of the co-expression plasmid for human CYP3A4 and HDJ-1 in a bicistronic format

that affect the level of CYP production, a bicistronic construct expressing both CYP3A4 and HDJ-1 was prepared, as outlined in Fig. 1.

Fig. 2 shows the induction time-dependent expression level of recombinant CYP3A4 in *E. coli* cells at 37°C. Regardless of the presence or absence of chaperone HDJ-1, the CYP production was elevated with increasing induction time, with the maximal yield obtained at around 12~16 h when assayed by the Fe^{2+}-CO *versus* Fe^{2+} difference spectra of whole *E. coli* cells. However, further incubation decreases the level of CYP production, but the reason for this is not clear.

By the co-expression with HDJ-1, the production level of CYP3A4 in whole cells was increased ~3.3-fold compared to that of CYP3A4 without HDJ-1. Concomitantly, the incorporation of CYP3A4 into membranes was also stimulated. Table I summarizes the maximal production level of CYP3A4. More interestingly, when HDJ-1 was co-expressed, the induction time-dependent decrease of the production level was less significant than in the case of CYP3A4 alone. At present, however, it is not clear how HDJ-1 exerts its function to induce resistance against the time-dependent decrease of CYP expression. The expression of CYP3A4 was also confirmed by Western blot analysis (Fig. 3). These results suggest the possibility that the expression of holoenzyme of CYP3A4 is dependant on the status of protein folding, with HDJ-1 assisting the correct folding of the CYP3A4 in *E. coli*. As a control experiment, the production of HDJ-1 was also checked using antiserum against the protein (results not shown).

The catalytic activity of CYP3A4 was assayed with membrane fractions, and the result is summarized in Table II. As expected, the activity was stimulated ~15-fold

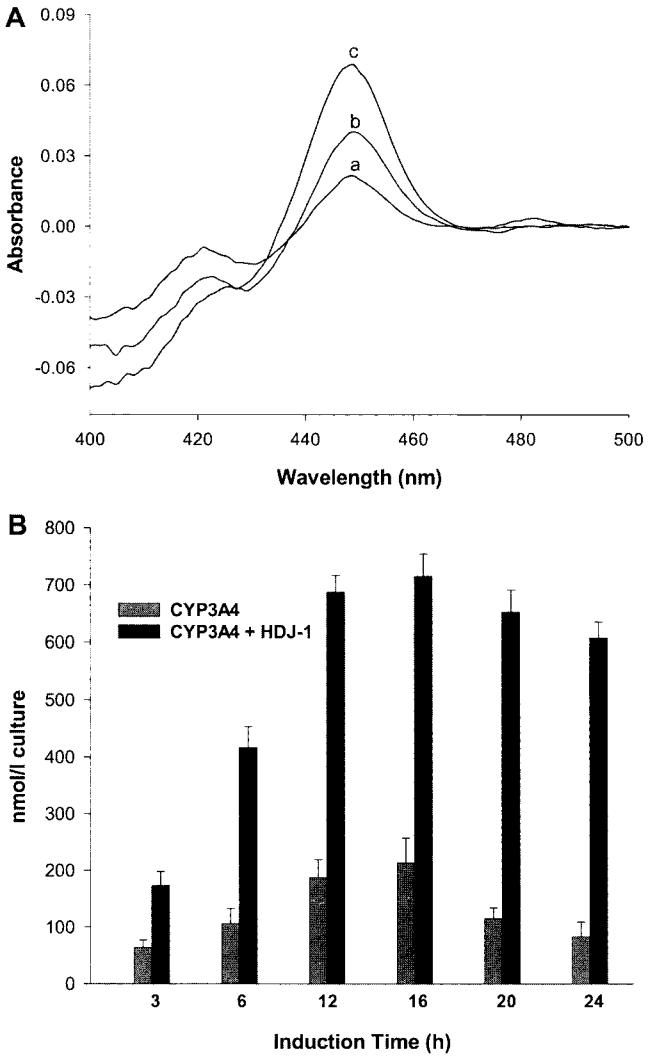

Fig. 2. Induction time-dependent expression level of recombinant CYP3A4. Figure (A) represents the Fe^{2+}-CO *versus* Fe^{2+} difference spectroscopy, with increasing induction time, and shows the expression of CYP3A4 from the construct of CYP3A4-HDJ-1 co-expression in whole *E. coli* cells. Line a, 3 h; line b, 6 h and line c, 16 h. The absorption intensity was maximized at 449nm. In figure (B), error bars represent S.D from three independent determinations.

Table I. Maximal expression yields of CYP proteins in *E. coli*[a]

Construct	Expression level		
	Whole cells (nmol/liter culture)	Membranes (nmol/liter culture)	Membrane CYP content (nmol/mg protein)
CYP3A4 alone	214 ± 32[b]	79 ± 8	0.23 ± 0.05
CYP3A4 + HDJ-1	715 ± 52	306 ± 31	0.86 ± 0.07

[a] Expression was carried out at 37°C for 16 h in *E. coli* DH5aF'IQ in the presence of 1.0 mM IPTG and 1.5 mM δ-ALA.
[b] Results are expressed as means ± SD, from three independent experiments.

Table II. Activities of recombinant CYP3A4 expressed in *E. coli*

Construct	Testosterone 6β-hydroxylation activity[a]
CYP3A4 alone	2.0 ± 0.5[b]
CYP3A4 + HDJ-1	30 ± 3

[a] Activities were determined with bacterial membranes, and are represented in nmol/min/nmol P450.
[b] Results are expressed as means ± SD, from three independent experiments.

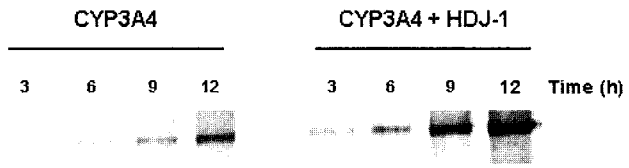

Fig. 3. Immunoblot analysis of CYP3A4 expression in whole *E. coli* cells, in an induction time-dependent manner. The blot was probes with rabbit anti-human CYP3A4 primary antibody and HRP-linked rat anti-rabbit IgG secondary antibody.

by the co-expression of HDJ-1, compared with the value for CYP alone. The amount of activity increase was similar to that of the CYP production at the whole cell level. This result suggests that HDJ-1 is involved in the correct folding of CYP3A4, as well as its incorporation into membranes, as has been suggested.

CONCLUSION

The successful construction of an expression system for the production of recombinant human CYP3A4 in *E. coli* is reported. The expression level and catalytic activity were enhanced by co-expression with human molecular chaperone HDJ-1 in a bicistronic format. The present system is very rapid compared with other reported bacterial expression methods, which usually adopt long culture times (48~72 h) at lower temperatures to avoid the formation of inclusion bodies of the expressed CYP proteins (usually under 30°C). The production level of CYP3A4 and its catalytic activity were also compatible. Our expression system was not accompanied by any insoluble protein aggregates at the elevated temperature (37°C), suggesting that molecular chaperone HDJ-1 assists the proper folding of expressed CYP proteins. Further works should be undertaken to elucidate the roles of HDJ-1 in combating the problem of inclusion body formation.

ABBREVIATIONS

CYP or P450, cytochrome P450; *E. coli*, *Escherichia coli*; IPTG, isopropyl-β-D-thiogalactopyranoside; δ-ALA, δ-aminolevulinic acid; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; HPLC, high-performance liquid chromatography.

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