

Expression of cytochrome P-450_d by *Saccharomyces cerevisiae*

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Rat liver microsomal cytochrome P-450_d was abundantly expressed in the yeast *Saccharomyces cerevisiae* by using a yeast-*Escherichia coli* shuttle vector consisting of rat liver P-450_d cDNA and yeast acid phosphatase promoter. The expressed cytochrome P-450_d was immunologically crossed with rat liver P-450_d. The hydroxylase activity of estradiol-1,3,5(10)-triene-3,17 β -diol was 11 nmol/min per nmol P-450_d, which is comparable to that reported previously for rat liver P-450_d. The expressed P-450_d content was nearly 1% of total yeast protein as estimated from immunoblotting, hydroxylase activity and optical absorption of the reduced CO form.

Cytochrome P-450 (Yeast) Expression cDNA

1. INTRODUCTION

A family of heme-containing monooxygenases, collectively called cytochrome P-450 (P-450), is widespread throughout nature and plays an important role in a variety of metabolic hydroxylations [1]. Isosafrole induces predominantly a unique P-450, cytochrome P-450_d (P-450_d), in rat liver microsomes [2]. P-450_d has an efficient catalytic activity toward the 2-hydroxylation of 17 β -estradiol. This activity is in contrast with those of other forms of rat liver P-450, P-450_a, P-450_b, which are predominantly induced by phenobarbital, and P-450_c, which is predominantly induced by 3-methylcholanthrene [3]. Kawajiri et al. [4]

determined the coding nucleotide sequence of mRNA for P-450_d by sequence analysis of cloned cDNAs. As the first step toward elucidation of a structure-function relationship in the P-450_d molecule, we constructed a direct expression plasmid of P-450_d in the yeast by using cDNA of P-450_d and a yeast-*E. coli* shuttle vector, pAM82 [5], which carries a yeast acid phosphatase promoter. The constructed expression plasmid of P-450_d allowed high expression of P-450_d in the yeast up to 1% of the total yeast protein. The expressed P-450_d of the yeast was identical to rat liver P-450_d in terms of immunocrossing, hydroxylase activity of the 2-position of 17 β -estradiol and optical absorption spectra.

Abbreviations: P-450, cytochrome P-450; P-450_d, cytochrome P-450_d; 17 β -estradiol, estradiol-1,3,5(10)-triene-3,17 β -diol; isosafrole, 5-(1-propenyl)-1,3-benzodioxole; phenobarbital, 5-ethyl-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione; 3-methylcholanthrene, 1,2-dihydro-3-methylbenz(j)aceanthrylene; ampicillin, 6-[D-(2-amino-2-phenylacetoamido)]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo(3,2,0)heptane-2-carboxylic acid

2. MATERIALS AND METHODS

Yeast strain AH22 (*a leu2 his4 can1 cir⁺*) was kindly supplied by Dr K. Matsubara of Osaka University.

P-450_d cDNA was obtained as described [4]. A yeast-*E. coli* shuttle vector, named pAM82, consisting of yeast acid phosphatase was kindly sup-

plied by Dr K. Matsubara of Osaka University. This vector carries marker *arsI*, *2- μ ori*, *leu2* and an ampicillin-resistant marker of *E. coli* plasmid pBR322 [5].

Restriction endonucleases, other nucleic acid modifying enzymes and phosphorylated *Xho*I linker were purchased from Takara Shuzo (Kyoto). Leupeptin and pepstatin A were purchased from Peptide Institute (Osaka). Other reagents used were of the highest guaranteed grade and were used without further purification.

Transformation was carried out as in [5]. *Leu*⁺ transformants were selected on Burkholder minimal medium containing 2% agar. The cultivation and the induction of yeast cells were performed as described in [5]. Microsomes were prepared according to Aoyama et al. [6]. To minimize decomposition of P-450_d in the yeast,

cells were lysed by a French press and spun down [6]. P-450_d was purified from the microsomes according to Hashimoto and Imai [7]. Hydroxylase activity of P-450_d was measured by the method by Ryan et al. [2,3].

3. RESULTS AND DISCUSSION

3.1. Construction of expression vector

cDNA of P-450_d incorporated into pBR322 was doubly digested by exonucleases *Rsa*I and *Acc*I (fig.1). After the sticky end of the *Acc*I site was converted to the flush end by the Klenow fragment of *E. coli* DNA polymerase in the presence of deoxyribonucleoside 5'-triphosphates, the phosphorylated *Xho*I linker was ligated to both sides of the P-450_d cDNA. The P-450_d cDNA with *Xho*I linkers on both ends was digested with *Xho*I to

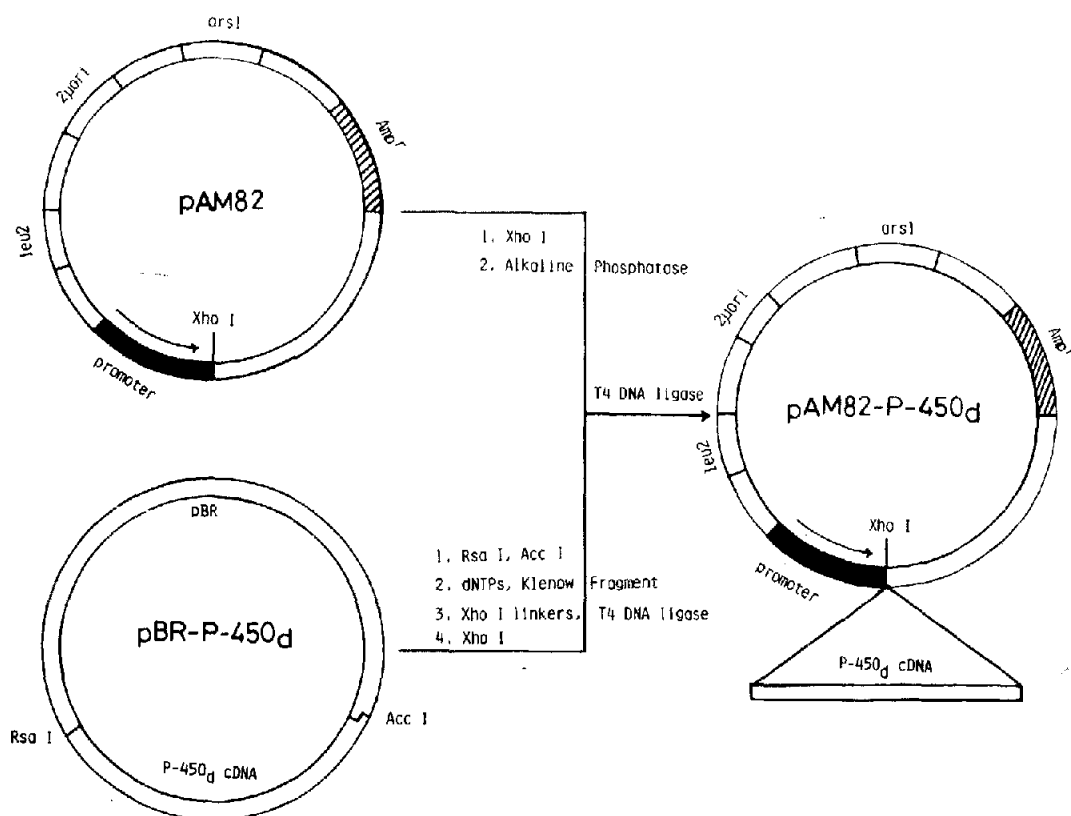


Fig.1. Construction procedure of the expression vector, pAM82-P-450_d. P-450_d cDNA in pBR322 was digested by *Acc*I and *Rsa*I. After the sticky end of the *Acc*I site was filled with dNTPs by the Klenow fragment, phosphorylated *Xho*I linker was ligated to both ends of P-450_d cDNA by T4 DNA ligase. The P-450_d cDNA was cleaved by *Xho*I and then inserted into the *Xho*I-digested pAM82. The expression vector pAM82-P-450_d was thus constructed.

create the *Xho*I sticky ends on both ends of the P-450_d cDNA. The yeast-*E. coli* shuttle vector, pAM82, was digested by *Xho*I, the site of which is located just after the yeast acid phosphatase promoter (fig.1). The P-450_d cDNA having *Xho*I sites was inserted into the *Xho*I-digested pAM82 by T₄ ligase. The P-450_d expression vector constructed as mentioned was transferred into *E. coli*, HB101. Purified P-450_d expression vector was then transferred into the yeast, *S. cerevisiae*.

3.2. Expression of P-450_d in terms of Western blotting

Expression of P-450_d was studied by immunoblotting (Western blotting) using anti-P-450_d IgG and peroxidase-conjugated goat anti-rabbit IgG (fig.2). The SDS-solubilized fraction of the

whole yeast cells containing the P-450_d expression vector, pAM82-P-450_d, showed a clear band (lane 2, fig.2), which reacts with anti-P-450_d IgG, at an *M_r* identical to rat liver P-450_d (lane 1, fig.2). Expression of P-450_d was estimated at nearly 1% of the total yeast protein from immunoprecipitations of authentic rat liver P-450_d. Probably partially decomposed forms of P-450_d were detected in the lower *M_r* regions. The whole yeast cell without the expression vector, pAM82-P-450_d, or with the vector pAM82 did not show any band in Western blotting. To reduce the content of the partially decomposed P-450_d, hemin was added to the induction medium. As can be seen in lanes 2 and 3 of fig.2, the content of the decomposed P-450_d did not decrease considerably. We changed the phosphate concentration in the first culture for cell

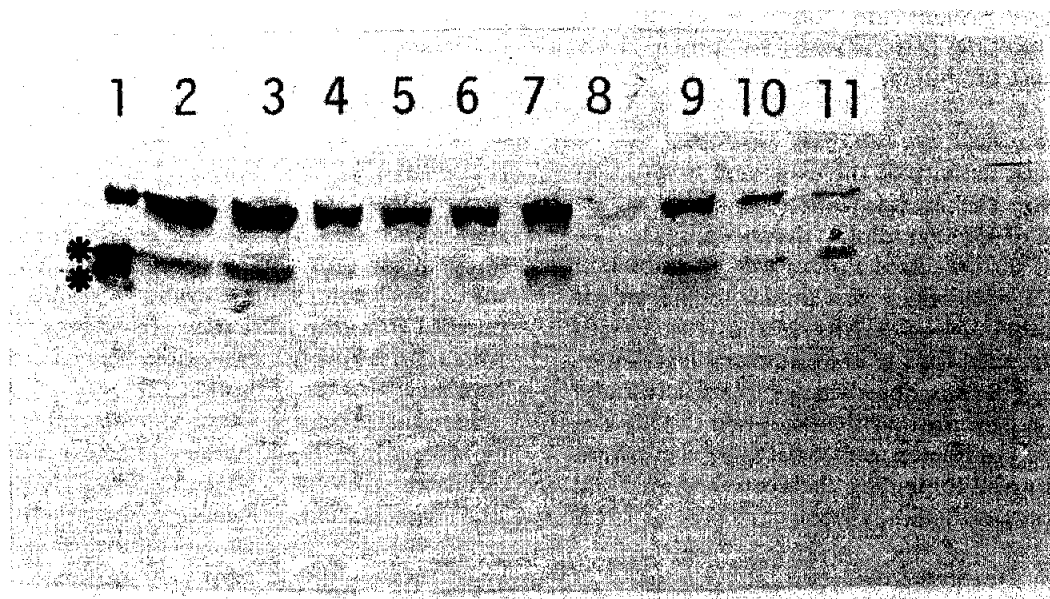


Fig.2. Western blotting of rat liver P-450_d (lane 1) and P-450_d expressed in the yeast AH22 (lanes 2–11). Lanes: 1, rat liver P-450_d, 0.3 µg protein; 2, yeast cultured in 1 l low-phosphate minimal medium after the first cultivation in 1 l high-phosphate (1.5 g KH₂PO₄/l) Burkholder minimal medium in the presence of 50 mg hemin; 3, yeast cultured as for lane 2 in the absence of hemin; 4, yeast cultured in 1 l low-phosphate minimal medium after the first cultivation in a high-phosphate (37.5 g KH₂PO₄/l) minimal medium. 250 ml of the first cultivation was used for the induction in the low-phosphate medium; 5, yeast cultured as in lane 4, but the phosphate content of the first medium was 7.5 g/l; 6, yeast cultured in the same way as lane 4, but the phosphate content of the first medium was 1.5 g/l; 7, yeast cultured first in low-phosphate minimal medium. We did not change the content of phosphate; 8, yeast cultured in 1 l low-phosphate minimal medium after first cultivation in 1 l high-phosphate (1.5 g KH₂PO₄/l) minimal medium; 9, yeast cultured as in lane 8. But 250 ml of the first culture solution was used for the second cultivation; 10, yeast cultured as in lane 8. However, 63 ml of the first culture solution was used for the second cultivation; 11, yeast cultured as for lane 8. But 16 ml of the first culture solution was used for the second cultivation. Protein contents of the yeast applied for electrophoresis were nearly 10 µg. Asterisks for lane 1 indicate degraded forms of the native rat P-450_d.

growth (lanes 4–7, fig.2). Expression of P-450_d did not seem to be strongly dependent on the phosphate concentration in the first culture. The content of the decomposed P-450_d was also not changed by varying the phosphate concentration in the first culture. The ratios of the culture volume for cell growth to that for induction appeared to be important for protection against degradation of the product. Therefore, we changed these ratios by reducing the first culture volume to improve the expression efficiency and to reduce the content of the decomposed P-450_d (lanes 8–11, fig.2). An optimum ratio of the culture volume for cell growth to that for induction was 1/4 so that we could obtain the highest expression of P-450_d with minimal decomposition.

To minimize the decomposition of P-450_d in the yeast, we added the inhibitors, leupeptin and pepstatin A. Significant improvement in the Western blotting patterns was not observed by adding these inhibitors.

3.3. Hydroxylase activity of 17 β -estradiol

Enzymatic activity of the expressed P-450_d was studied for the hydroxylation reaction at the 2-position of 17 β -estradiol as described in [2]. The activity of the expressed P-450_d was 11 nmol/min per nmol P-450_d which was similar to that of rat liver P-450_d [2]. The activity of the yeast P-450_d was not detected for the mitochondrial fraction of the yeast, but was observed for the microsomal fraction, suggesting that the expressed P-450_d was localized in the microsomes. Microsomes prepared from the yeast containing the vector pAM82 did not show any hydroxylation activity of 17 β -estradiol.

3.4. Optical absorption of the expressed P-450_d

When whole yeast cells transfected with the P-450_d expression vector were treated with CO gas and sodium dithionite, an absorption maximum at 448 nm was observed (fig.3), suggesting that P-450_d is produced in the yeast to a considerable extent. On the other hand, the yeast harboring the vector pAM82 did not give rise to the absorption maximum around 450 nm. From the absorption magnitude of the reduced CO form of P-450_d in the yeast, the amount of the expressed P-450_d is estimated to be 1% of the total yeast protein, which is consistent with the estimation of ex-

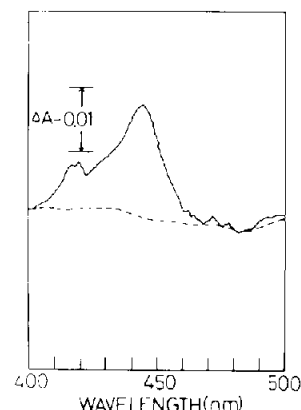


Fig.3. Difference absorption spectrum of the reduced CO form of P-450_d which was expressed in the yeast harboring the expression vector pAM82-P-450_d (—). The spectrum was taken for 1 ml yeast solution which was suspended with distilled water after the yeast cells in the 50 ml cultivated solution were harvested. The yeast cells harboring the vector pAM82 did not show a band around 450 nm (---).

pressed P-450_d from Western blotting. Microsome preparations from the yeast containing the expression vector also gave rise to an absorption maximum at 448 nm, while mitochondria did not show the absorption band around 450 nm. P-450_d purified from the yeast was a ferric high-spin type with a Soret absorption maximum at 392 nm in accordance with that reported [2].

In conclusion, we have demonstrated that a cloned rat liver P-450_d gene can be expressed efficiently in yeast cells under the control of the yeast acid phosphatase promoter. Oeda et al. [8] reported a high expression of rat liver P-450_c in the yeast under the control of a yeast alcohol dehydrogenase promoter. The efficiency of the expression of P-450_d in the yeast of our system is as high as that of P-450_c by using the alcohol dehydrogenase promoter [8]. We attempted to express P-450_d by using the yeast alcohol dehydrogenase promoter, but did not succeed in the high expression of P-450_d for unknown reasons.

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