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Estimation of Human Flavin-containing Monooxygenases Activity (FMO1) in the Baculovirus Expression Vector System by using S-oxidation of Methimazole

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ABSTRACT – The flavin-containing monooxygenases (FMOs) (EC 1.14.13.8) are NADPH-dependent flavoenzymes that catalyze oxidation of soft nucleophilic heteroatom centers in a range of structurally diverse compounds including foods, drugs, pesticides, and other xenobiotics. In humans, FMO1 appears to be the predominant form expressed in human fetal liver. cDNA-expressed human FMO and human liver microsomal FMO have been observed to *N*- and S-oxygenate nucleophilic nitrogen- and sulfur-containing drugs and chemicals, respectively. In the present study, FMO1 can be expressed in the baculovirus expression vector system at level of 2.68 nmol FMO1/mg of membrane protein. This isoform was examined for its capacity to metabolize methimazole to its S-oxide using thiocholine assay. Kinetic studies of its S-oxide by recombinant human FMO1 result in K_m of 7.66 μM and V_{max} of 17.79 nmol/min/mg protein.

Key words □ Flavin containing monooxygenase, Methimazole, Baculovirus, S-oxidation

Introduction

The microsomal flavin-containing monooxygenase (FMOs; EC 1.14.13.8) catalyze the flavin adenine dinucleotide (FAD)-, NADPH-, and O_2-dependent oxidation of numerous xenobiotics containing nitrogen, sulfur, phosphorous, or selenium heteroatoms[1-3] (Scheme 1.). Although primarily involved in the detoxication process, activation of compounds to the more reactive chemical species may occur and ultimately elicit a toxic response.[4-5] In contrast with the numerous exogenous compouds identified as substrates for the FMOs, relatively few endogenous substrates are known; examples of these include cyteamine[6-7] and the cysteine S-conjugates.[8-9] The dietary compound trimethylamine is converted to its corresponding N-oxide by FMO; however, individuals deficient in FMO(presumably FMO3) may develop trimethylaminuria, a genetic disorder resulting in the excretion of the malodorous free amine.[10-12]

To date, five FMO isoforms(designed FMO1-5) have been identified by amino acid or cDNA sequencing and each represented by a single gene (for reviews, see 25-28). Orthologous genes share at least 80% amino acid identity, whereas homologous FMOs are 52-57% identical. Highly related forms (greater than 98% identity) within a single species are the result of allelic variation.[29]

In addition to the well-documented species and tissue-dependent expression of FMOs, endogenous factors such as developmental status or gender affect expression. Based on mRNA expression, the primary isoform expressed in adult human liver appears to be FMO3; however, FMO1 appears to be the predominant form expressed in human fetal liver.[30] These FMOs are mostly found in major organs of entry such as liver and kidney, and have been purified from pigs, rats, mice, rabbits, and guinea pigs.

The purpose of this study was to express human FMO1 in the baculovirus expression vector system. Recombinant baculoviruses are used to express heterogous genes in cultured insect cells and insect lavae. For large-scale applications, the baculovirus expression vector system is particularly advantageous. Specialized media, transfection reagents, and vectors have been developed in response to recent advances in insect

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cell culture and molecular biology methods.

Materials and Methods

Chemicals Oligonucleotides for PCR were purchased from Integrated DNA Technologes, Inc. All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs(Beverly, MA). Glucose-6-phosphate, NADP+, glucose-6-phosphate dehydrogenase, catalase, and acetylthiocholine hydrochloride were obtained from Sigma Chemical Co. All other reagents were the highest grade available from commercial sources.

Synthesis Thiocholine hydrochloride was prepared by the methanolysis of acetylcholine as follows. 200-mg of acetylcholine dissloved in 2 ml of dry and oxygen-free methanol saturated with HCl was incubated at room temperature for overnight. The reaction was usually completed after only 5 h but prolonged incubation had no detectable effect on yield of thiocholine. After incubation, methanol, methylacetate, and excess HCl were removed under vacuum or by purging with a stream of dry argon. Aqueous 10 mM solutions of thiocholine hydrochloride are stable at 0-4°C for several months.

Construction of recombinant baculovirus To construct FMO1 recombinant baculovirus, pFastBac1(Life Technologies) was incubated with BamHI and XbaI, and the insert was excised by incubation with BamHI and XbaI, then gel-purified and ligated into pFastBac1 to give FMO1/pFastBac1. The recombinant plasmid was transformed into DH10Bac competent cells which contain the bacmid with a mini-attTn7 target site and the helper plasmid. FMO1 recombinant baculovirus was generated by transfection of *Spodoptera frugiperda*(Sf) 9 cells with the corresponding recombinat bacmid DNA obtained *via* site-specific transposition using the Bac-to-Bac system (Life Technologies).

PCR anaysis of recombinant bacmid To confirm the size of an insert, recombinant bacmid DNA was isolated from small-scale culture using an alkaline lysis protocol modified for high-molecular-weight plasmid purification described in the Bac-to-Bac manual, and 1ul was used for PCR with the pUC/M13 amplification primers.

Baculovirus-mediated expression of FMO1 - Sf9 insect cells were grown and passaged in shaker cultures using Sf-900II (Life Technologies) serum free media containing ampotericin B (5 ug/ml), penicillin G(100 units/ml), and streptomycin sulfate(50 ug/ml). For amplification of virus, Sf9 cells were infected at a MOI(multiplicity of infection) of 0.1. For expression, 500 ml of Sf9 cells, grown to a density of 1.5×10^6 cells/ml in a 1 liter Erlenmeyer flask, were infected with virus at a MOI of 7 and incubated by shaking at 130 rpm in an orbital shaker at 27 ± 5°C for 72 hours. Cells were pelleted and resuspended in 50 mlof lysis buffer, 0.154M KCl, 50mM Tris-HCl(pH 7.4) and 0.2mM phenylmethylsulfonylfluoride. And the cells were broken twice in a precooled French Pressure cell (SLM Amincom Urbana, IL) at 10,000psi. The lysed cells were centrifuged at $3,000\times g$ for 15 min at 4°C. The supernatant was saved and microsomal fraction was obtained by centrifugation of the resulting supernatant at $100,000\times g$ for 1 hour at 4°C. Microsomal pellets were resuspended in 10 ml 0.154M KCl, 10-mM HEPES(pH 7.5), 1 mM EDTA and 20%(v/v) glycerol, then stored in aliquots at −80°C until use. Protein concentration was determined by the method of Bradford with Bio-Rad reagents using bovine serum albumin as a standard.

DNA Dot blot analysis For determining the viral titers, DNA dot blot analysis was performed by End-point dilution method. 100ul of each cell lysates were applied to dot blot apparatus, washed, UV cross-linked and hybridized at 60°C with ^{32}P-dCTP labelled human FMO cDNA as probe (*rediprime* DNA labelling system, RPN1633/1634 Amersham LIFE SCIENCE).

Western blot analysis Proteins were separated by SDS-PAGE on 12.5% gels and transferred eletrophoretically to Immobilon-P membrane(Millipore). Blots were probed with a 1:4000 dilution of rabbit anti FMO IgG followed by detection with a 1:4000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase

as described by the supplier (Bio-Rad).

Determination of monooxygenase activity FMO1 activities of microsomes were measured by following substrate-dependent oxidation of thiocholine at 37°C. Reaction medium contained 0.1M potassium phosphate, pH 7.5, 0.25 mM NADP+, 2.5 mM glucose-6-phosphate, 1.5U glucose-6-phosphate dehydrogenase, 130 mM thiocholine, 100U catalase, 2 mM benzylimidazole, and 0.4mM EDTA in a final volume of 2 ml. After a 4- to 6-min temperature equilibrium, the microsomal fractions (1-2 mg protein) were added. About 1 min later, the reaction was initiated by adding the substrates dissolved in water or ethanol. The volume of ethanol added, never more than 15 μL/mL of the reaction mixture, had no detectable effect on the activity measurements. Aliquots(0.4 mL) usually withdrawn at 0, 4, 8 and 12 min were transferred to tubes on ice containing 0.05 mL of 3 M trichloroacetic acid and added 0.5 mL of H_20. After all aliquots were collected, precipitated protein was separated by centrifugation and 0.6 mL clear supernatant liquid was transferred to tubes containing and 0.05 mL of 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB) (10mM). A milimolar absorptivity of 13.6cm-1 for 5-thio-2-nitrobezoate was used to calculate the loss of thiocholine as a function of reaction time.

III. Results and Discussion

FMO is a flavin-containing monooxygenase catalyzes the NADP-dependent oxidative metabolism of many

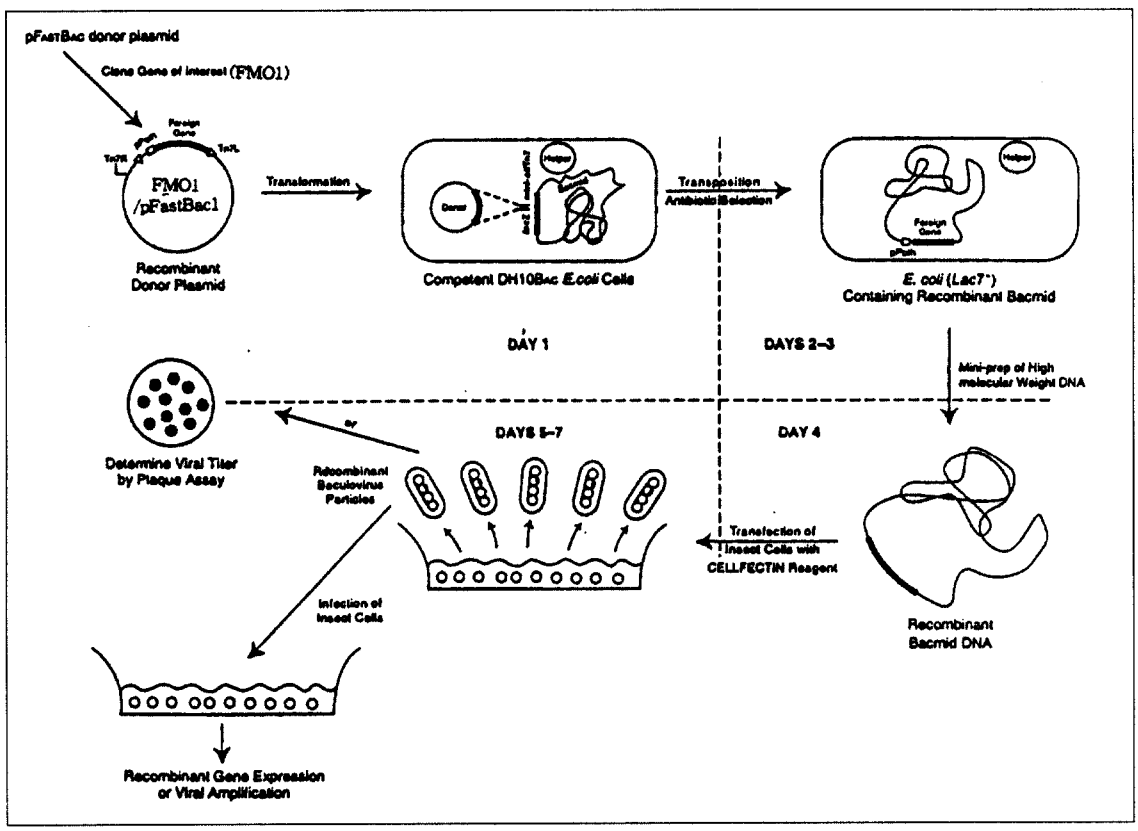
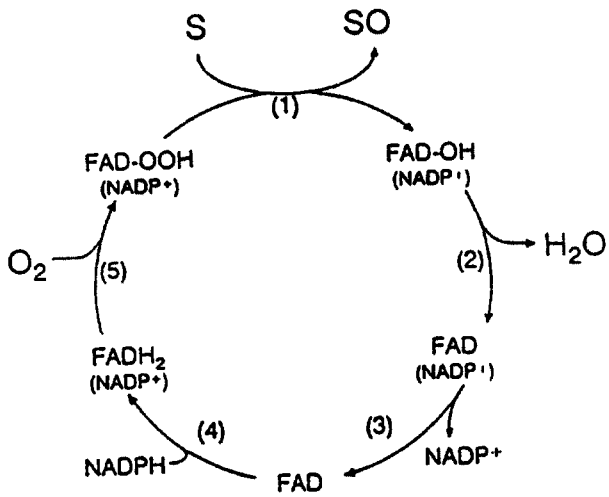


Fig. 1. Generation of recombinant FMOl baculovirues and gene expression. *FMO*1 gene is cloned in to a pFastBacl donor plasmid, and the recombinant GMOl/pFastBacl plasmid is transformed into DH10Bac competent cells which contain the bacmid with a mini-*att*Tn7 target site and the helper plasmid. The mini-Tnt7 element on the pFastBac donor plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of trasposition proteins ptovided by the helper plasmid. Colonies containing FFMO1 recombinant bacmids are identified by disruption of the lacZ α fine. High molecular weight mini-prep DNA is prepared fron selected *E. coli* clones containing the reconbinant bacmid, and this DNA is then used to transfect insect cells.



Scheme 1. Major steps in the catalytic cycle of the porcine liver flavin-containing monooxygenase In step (1), any soft nucleophile S that can contact the enzyme-bound 4a-hydroperoxyflavin is oxygen- ated to SO which is released immediately. After release of H_2O and $NADP^+$ in steps (2) and (3), the oxygenating form of the enzyme is regen- erated by steps (4) and (5). FAD, flavin adenine dinucleotide.

drugs, pesticides, and other foreign compounds(Scheme 1). In general, FMO metabolizes compounds to detoxify them, while there are some known examples of the same enzyme activating pharmacological and toxicological point of view.[1,19] The expression of FMO1 in *E. coli* and Yeast had been attempted in the past, but the result was an insoluble product and enzymatically inactive form. In the present study, the results suggest that FMO1 can be expressed in the baculovirus expression vector system in an enzymatically active form.

Recombinant baculoviruses are widely used to express heterogous genes in cultured insect cells and insect lavae. For large-scale applications, the baculovirus expression vector system is particularly advantageous. Specialized media, transfection reagents, and vectors have been developed in response to recent advances in insecet cell culture and molecular biology methods. The major difference between the naturally occurring in vivo infection and the recombinant in *vitro* infection is that the naturally occurring polyhedrin gene within the wild-type baculovirus genome is replaced with a recombinant gene. These genes are commonly under the control of polyhedrin and p10 promoters. In the late phase of infection, the virions are assembled and budded recombinant virions are released. However, during the very late phase of infection, the inserted heterologous genes are placed under the transcriptional control of the strong AcNPV (*Autographa californica* nuclear polyhedrosis virus) polyhedrin pomoter. Thus, recombinant product is expressed in place of the naturally occurring polyhedrin protein. Usually, the recombinant proteins are processed, modified, and targeted to the appropriate cellular locations.

When the baculovirus expression system technology was introduced in 1983, the baculovirus system has become one of the most versatile and powerful eukaryotic vector systems for recombinant protein expression[20]. More than 600 recombinant genes have been expressed in baculoviruses to date. When the first protein(IL-2) was produced in large scale from a recombinant baculovirus in 1985, use of the baculovirus expression vector system has been increased dramatically.[21] Baculovirus offer the following advantages over other expression vector systems; safety, ease of scale up, high levels of recombinant gene expression, accuracy and use of cell lines ideal for suspension culture.

To subclone the FMO1 cloning region from pET16b to pFastBac1 donor plasmid, insert was both digested with BamHI(5' region) and XbaI(3' region) with gel purified, ligated. FMO1/pFastBac1 was transformed into DH10Bac competent cells which contain the bacmid with a mini-*att*Tn7 target site and the helper plasmid. The mini-*att*Tn7 element on the pFastBac1 donor plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Recombinant bacmid was identified by disruption of the lacZα gene. High molecular weight mini-prep DNA was prepared from selected *E. coli* clones containing the recombinant bacmid (143kb, Fig. 2). It is better to use the polymerase chain reaction (PCR) with the pUC/M13 amplification primers, to confirm the size of an insert(Fig. 3). The primers were directed at sequences on either side of the mini-*att*Tn7 site within the lacZα-compelementaion region of the bacmid. The expected results from the PCR were 2.3kb (bacmid

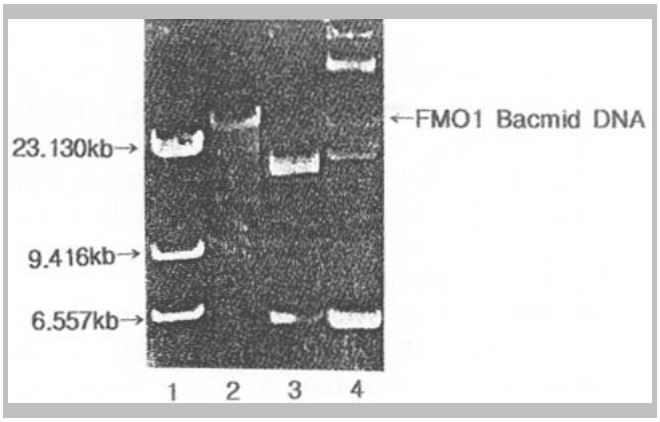


Fig. 2. Agarose gel analysis of mini-prep bacmid DNA. λDNA/Hind III(S, Size marker, Purified bacmid DNA(*Lane1*). Mini-prep of Helper DNA(*Lane2*). Mini-perep of high molecular weight FMO1 bacmid DNA(*Lane3*) DNA electrophoresed at 23V for 12hour in 1% agarose gel containing ethidium bromide.

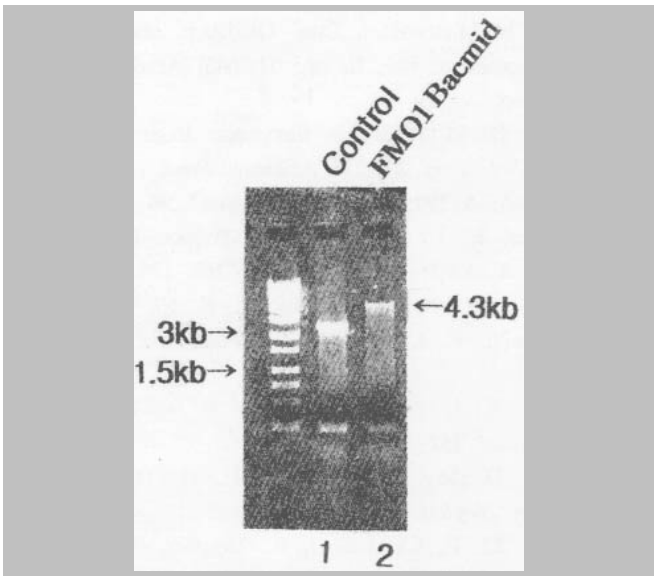


Fig. 3. PCR analysis of FMO Recombinant Bacmid. After transposition of DNA into DH10Bac cells, bacmid DNA was prepared by a modified alkaline lysis method and amplified with pUC/M13 amplification primers. λ/Hind III (*Lane 1*), FMO/pFastBac1 plasmid transposed into DH10Bac cells(*Lane 2*).

transposed with pFastBac1). Insertion of *FMO* gene into pFastBac1 donor plasmids resulted in an increase in the size of the PCR product(4.3kb). This increase in PCR product is corresponded to the size of *FMO* gene(2kb).

For determining the viral titer, DNA dot blot analysis was performed by End-point dilution method with 32P-dCTP labelled FMO1 using cDNA as probe.

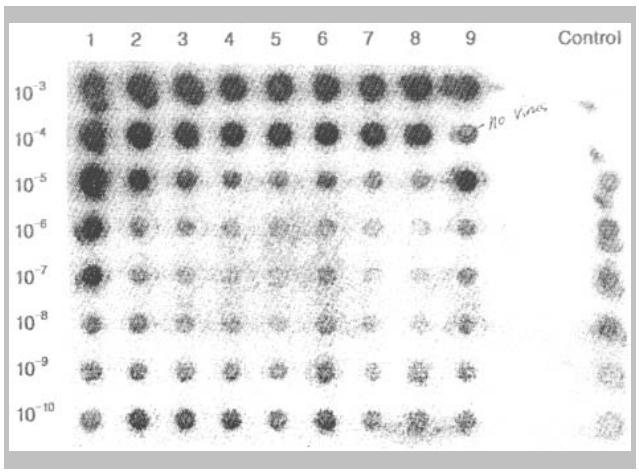


Fig. 4. DNA Dot blot analysis (End-point dilution) of FMO1 in baculovirus. Equal amounts of DNA from each insect cells were hybridized with ^{32}P-labeled human FMO1 cDNA. This viral titer is about 1.3×10^{7} plaque-forming units(pfu)/ml

This step was generally regarded as the most difficult step in the baculovirus expression vector system(Fig. 4). Maximum protein expression was observed 72hour post-infection at 1.5×10^{6}cell/ml with an MOI(multiplicity of infection) of 7. It is important to determine the expression kinetics for product, as many proteins may be degraded by cellular proteases released in cell culture.

Extracts were prepared from the insect cells carrying FMO/Bacmid and analyzed by SDS-PAGE and western blotting with antisera prepared against FMO. The FMO1 could be seen on the stained gel (Fig. 5A), and the bands shown in western blot indicate

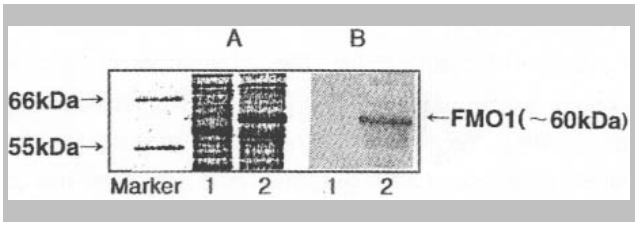


Fig. 5. Expression of Human FMO1 in the Baculovirus system. A, samples(20 μg) of the microsomes from culture of Sf9 cells were electrophoresed on a polyacrylamide gel in the presence of SDS and stained with Coomassie Blue. B, samples(20 μg) of the microsomes from culture of Sf9 cells were electrophoresed on a polyacrylamide gel in the presence of SDS, transferred to Immobilon-P membrane, and visualized by immunostaining, as described under "Materials and Methods." The samples shown are Sf9 cells alone(*lane1*), FMO1(*lane2*).

Table 1. Metabolism of methimazole(0.2 mM) catalyzed by human FMO1 in microsomes from Sf9 cells

Enzyme	K_m(μM)	V_{max}(nmole/min/mg)
Control	[a]NA	[a]NA
FMO1	7.66	17.79

Note. The oxidation of the compounds listed was measured by following substrate-dependent oxidation of thiocholine in 0.1M phosphate, pH 7.4, the NADPH generating system, 1000 units catalase, 300 μM thiocholine, and 0.1 mM EDTA. Kinetic constants were calculated from double reciprocal plots of velocity vs substrate concentration above and below K_m.
[a]NA, no activity detectable at concentrations near the limits of solubility of the compound in the assay medium.

that the human FMO1 was expressed in the baculovirus system (Fig. 5B). Human FMO1 encoded polypeptides of 531 amino acid18,22) and whose molecular mass was calculated to be about 60kD.[23-26] This value was similar to that of the microsomal preparation of human FMO1 as estimated by SDS-PAGE. In fact, the enzyme expressed in the baculovirus expression system showed essentially the same molecular mass on the SDS-PAGE(Fig. 5B). These microsomes were assayed for FMO activity(Table 1). Methimazole is an excellent substrate for most mammalian FMO isoforms. S-oxidase of methimazole by human FMO1 was measured by thiocholine assay. The cells carrying the FMO1/pFastBac1 plasmid appeared to have FMO activity.

In conclusion, according to the data presented, it was clear that cloning and expression of the human FMO1 in the baculovirus expression system was achieved. This method permits the rapid and simultaneous isolation of multiple recombinant viruses and is particularly suited for the expression of protein variants for structure/function studies. We are currently investigating the metabolism of other substrats by the human-expressed FMO isoforms. And the next step in this study will be to purify and characterize it and identify structure of FMO1.

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