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CYTOCHROME P450 2A1, 2E1, AND 2C9 cDNA-EXPRESSION BY INSECT CELLS AND PARTIAL PURIFICATION USING HYDROPHOBIC CHROMATOGRAPHY

JAMES GROGAN,* MAGANG SHOU, ERICKA A. ANDRUSIAK, SHINJI TAMURA, JEROEN T. M. BUTERS, FRANK J. GONZALEZ and KENNETH R. KORZEKWA

Laboratory of Molecular Carcinogenesis, National Cancer Institute, Bethesda MD 20892, U.S.A.

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Abstract—High-level expression of three cloned cytochrome P450 enzymes was accomplished using the baculovirus-insect cell expression system. The amount of enzyme expression was enhanced by cell infections in the presence of medium-supplements containing hemin and by growth in suspension cultures. Human cytochromes P450 2E1 and 2C9 and rat cytochrome P450 2A1 were partially purified from cell extracts using hydrophobic interaction and hydroxyapatite chromatography. The resulting enzymes were of estimated molecular masses similar to those reported previously and analyzed by PAGE. Reconstitution of enzyme activity resulted when the enzymes were incubated together with NADPH–cytochrome P450 reductase, phospholipid, NADPH, and appropriate substrates. The cytochrome P450 activity of the partially purified enzymes was comparable to that of the corresponding enzymes expressed in the vaccinia virus-Hep G2 system. These results provide evidence for a general means of obtaining cytochrome P450 enzymes for mechanistic, immunochemical, and biophysical investigations.

Key words: cytochrome P450; cDNA expression; protein purification; baculovirus; hydrophobic chromatography; insect cells

The cytochrome P450 enzymes are a superfamily of monooxygenases responsible for the oxidation of a wide variety of endogenous and exogenous compounds. The importance of these enzymes in drug metabolism, toxicology, carcinogenesis, and steroid and fatty acid metabolism has made the P450s one of the most widely investigated classes of enzymes. Initial studies of these enzymes were complicated by the presence of multiple forms in tissue preparations, and their microsomal localization. The eventual purification and reconstitution of individual mammalian forms provided evidence for enzyme multiplicity and allowed for the characterization of substrate and product selectivities.

The cloning and expression of the cytochrome P450 enzymes have resulted in the characterization of a large number (>300) of P450 enzymes, including many of the human forms [1]. Cytochrome P450s have been expressed successfully in bacterial, yeast, insect, and mammalian cells [2]. Each expression system has unique benefits and disadvantages based on expression levels, the utility of the expressed protein *in situ*, the fidelity with which the system produces post-translational modifications similar to the native protein, and the practical considerations of ease of preparation and cost. For example, expression of P450s in Hep G2 cells with vaccinia virus vectors has the advantage that saturating levels of NADPH-cytochrome P450 oxidoreductase and cytochrome b_5 are present in the host cells [3]. They also express epoxide hydrolase activity at high levels [4]. However, in this system, expressed enzyme concentration is too low for routine purification of cytochrome P450 enzymes, and the toxicity of the virus makes investigations of *in situ* cellular toxicity problematic. Human lymphoblastoid cell lines engineered to express P450 enzymes have been described [5], which are more suitable for *in situ* toxicity and drug metabolism studies, but these cells also express levels of enzyme too low for purification.

Bacterial [6], yeast [7, 8], and insect [9–12] expression systems have been demonstrated to produce quantities of cytochrome P450 enzymes suitable for purification. These systems require co-expression of NADPH–cytochrome P450 oxidoreductase [13, 14] to obtain turnover numbers on the order of the same enzymes expressed in mammalian cells. However, purified enzymes from the bacterial, yeast, and insect expression systems may be investigated by reconstitution of activities that are otherwise difficult to observe or differentiate from other P450 enzymes present. Baculovirus expression is particularly attractive for expression leading to purification and reconstitution of P450s because of the relative ease of Sf9 cell culture, the expression of enzyme that includes post-translational modifications of mammalian proteins, and the synthesis of membrane proteins for ready extraction. In this report, we investigate insect cell expression of three cytochrome P450s and their partial protein purification.

MATERIALS AND METHODS

Generation, maintenance, and expression of recombinant proteins

Construction of recombinant viruses containing the CYP2A1, CYP2E1, and CYP2C9 genes was as described previously for vaccinia virus [3] and baculovirus [10, 13, 15]. Expression of P450s in spinner flasks was

* Corresponding author: Dr. James Grogan, Ross University, School of Medicine, Portsmouth, Commonwealth of Dominica. Tel. (809) 445-5355; FAX (809) 445-5383.

by the following method: 0.4 L of *Spodoptera frugiperda* cells (Sf9; Invitrogen Corp., San Diego, CA) at 2×10^6 cells/mL in log phase growth was harvested by centrifugation at 1000 *g* for 10 min. The pelleted cells were resuspended gently into 5 mL medium A. Medium A consisted of 1.0 L IPL-41 insect medium, 100 mL fetal bovine serum (heat-inactivated), 20 mL tryptose phosphate broth, 10 mL pluronic f-68 (Life Technologies, Inc., Gaithersburg, MD). Addition of gentamycin to a final concentration of 0.1 mg/mL had no effect on levels of expression of recombinant proteins. The recombinant virus was added to a final multiplicity of infection of 2. Typically, 10 mL of virus stock at 1.5×10^8 pfu/mL was added into the concentrated cell suspension. The virus-cell infection mixture was left at room temperature for 40–60 min and then added to a sterile spinner flask containing 0.4 L of medium A. Spinner flasks were maintained at 27 ± 1.5° with spinner rotation at 60–80 rpm.

For hemoprotein expression, a media-supplement containing hemin was added to the spinner flask 24–48 hr post-infection. This supplement was prepared as previously described [10] or as a hemalbumin conjugate prepared essentially as described [16]. The latter supplement was routinely prepared as follows: 2 mg of equine hemin (Sigma Chemical Co., St. Louis, MO) was added into 2 mL of 0.1 N NaOH containing 35 mg/mL Na_2HPO_4. The resulting solution was added dropwise into 5 mL of 43 mg/mL bovine serum albumin (Sigma). The pH of the resulting solution was adjusted to 7.2 with 1 N HCl (final concentration: 0.29 mg/mL hemin, 0.44 mM hemin; with a hemin to albumin molar ratio of 1:1). The hemalbumin supplement was sterile-filtered (0.2 μm) and added to spinner flasks (0.4 L; Bellco Glass, Inc., Vineland, NJ) to a final concentration of 1 μg/mL. Although concentrations of hemalbumin as high as 20 μg/mL hemin were tolerated by Sf9 cells, no increase in expression (CO difference spectral P450) was observed at concentrations above 2 μg/mL hemin. At 72–96 hr post-infection, cells were harvested by centrifugation at 1200 *g* for 10 min. Pelleted cells were resuspended in PBS and analyzed for P450 content as described by Omura and Sato [17]. Harvested cells were stored as concentrated pellets at −80° until further use.

Trichoplusia ni cells (Hi-five; Invitrogen Corp.) [18] were also used to express P450s. These cells were grown to confluence (which included nascent cell clusters) in polystyrene roller bottles having a surface area for cell growth of 850 cm^2. Medium A (80 mL) was necessary for growth at a bottle rotation of 1 turn/30 sec and 27°. Cells were infected with 5 mL Sf9-generated viral stock in 10 mL fresh medium. After 1 hr, 80 mL medium A containing hemalbumin at 0–20 μg/mL hemin was added to each roller bottle. At 96 hr post-infection, cells were harvested from the inside walls of the roller bottles with a cell scraper.

Partial purification of recombinant protein

All samples were maintained at 4° or on ice with the exception of the column purification steps, which were at room temperature. Cell pellets were pooled and sonicated gently on ice until >95% of the cells were disrupted, as assessed using a hemocytometer under the microscope. Sonication buffer contained 0.1 M KP_i, pH 7.4, 20% (v/v) glycerol, 0.35 mM dithiothreitol (DTT), and 2 mM EDTA (buffer A). Protein concentrations after sonication of cells were 10–15 mg/mL (BCA method; Pierce Chemical Co., Rockford, IL). The cell suspension was centrifuged at 400,000 *g* for 12 min in a Beckman tabletop ultracentrifuge (model TL-100: Beckman Instrument Co., Palo Alto, CA). The resulting pellets containing the hemoprotein were resuspended by homogenization in buffer A containing 0.2% (w/v) sodium cholate (Calbiochem Co., San Diego, CA) at a cholate/protein ratio of 0.1 (w/w). This suspension was stirred for 2 hr, and the resulting suspension was centrifuged at 400,000 *g* for 12 min. The resulting pellets were resuspended by homogenization in buffer A containing 1.0% (w/v) sodium cholate at a cholate/protein ratio of 1.4 (w/w) and stirred at 4° overnight.

Centrifugation of the 1% cholate suspension at 400,000 *g* for 12 min resulted in a clear supernatant containing hemoprotein. This material was diluted with 4 vol. of buffer A or dialyzed against buffer A containing 0.2% (w/v) sodium cholate overnight. The sample was filtered through a 1.0 μm filter (Millipore Corp., Bedford, MA). Smaller pore size filters resulted in very slow filtration, while unfiltered samples resulted in excessive column back-pressure for fast performance liquid chromatography (FPLC).

An FPLC (Pharmacia LKB Biotechnology, Piscataway, NJ) system equipped with post-effluent monitors for absorption at 405 nm and for pH (at 7.4) was used for all column purification steps. Octyl-sepharose chromatography was conducted in a 1.6 × 60 cm column equilibrated with buffer A containing 0.2% (w/v) sodium cholate. Samples were back-loaded into a 50-mL superloop by a peristaltic pump before column loading using P-500 pumps. After loading, the retained protein was washed with 100 mL buffer A at 1.0 mL/min. P450 was eluted with a linear gradient of buffer A containing 0.2% (w/v) sodium cholate to buffer A containing 0.2% (w/v) cholate and 2% (w/v) Lubrol PX (Sigma) for 300 min at a flow rate of 1 mL/min. The 405 nm absorbing fractions were pooled and dialyzed overnight against 40 vol. of buffer B [10 mM KP_i, pH 7.4, 20% (v/v) glycerol, 0.35 mM DTT containing 0.15% (w/v) sodium cholate]. The resulting sample was loaded at 0.5 mL/min onto an HPLC hydroxyapatite column (Mitsui Toatsu; 7.6 × 100 mm equipped with FPLC-HPLC adaptors) equilibrated with buffer B. The column was washed extensively with buffer B after loading the sample to remove the Lubrol detergent. P450 was eluted with a linear gradient of buffer A to buffer B at a concentration of 0.5 M KP_i, pH 7.4.

Immobilized artificial membrane chromatography (IAM-PC; Regis, Inc., Morton Grove, IL) of cholate extracts was as previously described [19] using a semi-preparative IAM-PC column fitted with FPLC-HPLC adaptors. Extracts containing 1% cholate were diluted with buffer A to a final cholate concentration of 0.6% (w/v). Cytochrome P450 eluted as a broad peak at 1 to 1.2% Lubrol in buffer A when using a flow rate of 1 mL/min and conditions as described [20]. The eluting hemoprotein was dialyzed and chromatographed on hydroxyapatite as described above. Hemoprotein eluting from the hydroxyapatite column was concentrated using Centriprep-30 with 30 kDa cutoff membranes (Amicon Inc., Beverly, MA). The concentrated protein was dialyzed extensively against buffer C [containing 20% (w/v) glycerol, 0.1 M KP_i, pH 7.4] to minimize detergent concentration. The resulting solutions were centrifuged

at 400,000 *g* for 12 min to remove precipitates formed during concentration and detergent removal.

SDS–PAGE was performed as previously described [21] with 7.5% (w/v) acrylamide gels (Bio-Rad, Inc., Hercules, CA) in both running and stacking gels. Apparent molecular weight estimates were made by comparison of relative mobilities of sample protein to standard molecular weight markers (Mid-range standards; Integrated Separation Systems, Inc., Hyde Park, MA). Semi-log plots of standards had r^2 >0.99.

Measurement of P450 enzymatic activity

Assays for the 7α-hydroxylation of testosterone have been described previously for both the reconstituted system [22] and for the vaccinia expression system [23]. CYP2C9 activity was determined using tolbutamide as a substrate. Assays with the vaccinia expressed enzyme have been reported previously [24], and purified CYP2C9 was reconstituted with 10 μg dilauroylphosphatidylcholine: b_5 at a molar ratio of 1:3:1.

A nitroanisole demethylase assay was used to determine CYP2E1 activity. Briefly, 50 pmol of vaccinia expressed or reconstituted enzyme (P450:reductase: cytochrome b_5 at a molar ratio of 1:3:1) was incubated with 150 μM nitroanisole (Aldrich Chemical Co., Milwaukee, WI). Reactions were initiated by the addition of 0.1 mL of 8 mg/mL of NADPH, resulting in a total volume of 1 mL. Reactions were terminated after 30 min by the addition of 2.5 mL dichloromethane. An aliquot of 5 nmol 2,3,5,6-$^2H_4$-nitrophenol (Cambridge Isotope Laboratories Inc., Andover, MA) in methanol was added as an isotopic internal standard to each incubation. Incubations were extracted, and the dichloromethane layer was removed and dried over magnesium sulfate. The sample was concentrated to 100 μL and derivatized with *t*-butyldimethylsilyltrifluoroacetamide (Regis Chemical Co., Morton Grove, IL) containing 1% *t*-butyldimethylsilylchloride. The derivatized phenols were quantitated by GC-MS using an Alltech AT-1 column (30 m × 0.25 mm, 1.0 μm film thickness; Alltech Associates, Inc., Deerfield, IL) and a Hewlett Packard 5971 Mass Selective Detector using selected ion monitoring detection. Samples were injected at 50°, followed by a temperature gradient of 30°/min to 150° followed by 10°/min to 280°. The derivatized nitrophenols eluted at 16.3 min and were quantitated by integrating the peak areas at mass to charge ratios (*m/z*) of 253 and 257.

RESULTS

Conditions for maximum expression of cytochrome P450 enzymes in the baculovirus expression system were determined using spinner flasks containing 0.4 L *S. frugiperda* (Sf9) cells in log phase growth. Optimal expression was found when uninfected cells were harvested for viral inoculation in a concentrated cell suspension. For infection of *Trichoplusia ni* (Hi-five) cells, in roller bottles, the highest cytochrome P450 expression was obtained when confluent cells were inoculated in 15 mL of fresh medium containing viral inoculum just sufficient to immerse a cell layer of the rotating roller bottles (15 mL for 850 cm^2 cell growth area). After 1 hr, fresh medium was added into the inoculation mixtures to support expression of recombinant protein.

Expression of cytochrome P450 enzymes by Sf9 and *T. ni* cultures was enhanced by addition of a medium-supplement containing hemalbumin complex as compared with expression in the presence of hemin-NaOH medium-supplement. Results of cell expression of cytochrome P450 2E1 by *T. ni* cells in the presence of hemin-NaOH or, alternatively, hemalbumin are shown in Fig. 1. A sample of cells from each roller bottle was analyzed for cytochrome P450 content by sonication and CO difference spectroscopy 4 days after viral infection. When insect medium was not supplemented with hemin, a minimal expression of cytochrome P450 2E1 was obtained, resulting from the native heme biosynthetic capacity of the insect cells. The expression of CYP2E1 in the presence of 2 μg/mL hemin-NaOH medium-supplement was increased sharply above the level obtained with no supplement added. However, further addition of hemin-NaOH supplement decreased holoenzyme expression. This result is in accord with the previously reported optimal concentration of hemin-base reported for CYP2A1 expression in Sf9 cells [10]. Previous investigation of hemin-NaOH assisted expression of CYP2A1 suggested that the advantages of this medium-supplement were limited by cellular toxicity above 4 μg/mL hemin [15]. Therefore, we investigated the possibility of using a supplement that may supply cofactor to cells in a more gradual manner.

The expression of CYP2E1, even in the presence of 0.2 μg/mL hemin as hemalbumin, also resulted in a sharp increase in functional holoenzyme synthesis compared with no-supplement controls (Fig. 1). Expression of CYP2E1 with hemalbumin supplement surpassed that of cells with hemin-NaOH supplemented medium. Similar results were obtained with Sf9 cells in spinner cultures, resulting in our routine use of this reagent for hemoprotein expression. The optimal range of expression using hemalbumin supplement is 0.2 to 4 μg/mL hemin as hemalbumin using a heme to albumin molar ratio of 1:1. P450 expression at the highest concentrations of hemalbumin shown in Fig. 1 still produced high levels of enzyme compared with cells in the absence of supplements. However, in these cells the intensity of the 420 nm peak in CO difference spectra was several times that of the 450 nm peak. In CO difference spectra of enzyme produced in the presence of 0.2 μg/mL hemin as hemalbumin, the intensity of the 420 nm peak was low compared with that of the 450 nm peak. Because the 420 nm peak represents uncharacterized hemin conjugates and "free" hemin, we routinely used hemalbumin at a concentration of 1 μg/mL in medium in order to minimize these impurities.

Partial purification of the Sf9-expressed cytochrome P450 enzymes was accomplished rapidly, using a procedure that resulted in a 10- to 20-fold purification over starting material. The specific content of the purification starting material ranged from 0.2 to 0.3 nmol P450/mg protein for the three enzymes described in Table 1. These specific contents represent 20–40 nmol of cytochrome P450 expressed in a 0.4-L spinner flask of log phase cells. Extraction, the initial step in cytochrome P450 purification, resulted in a sizable loss of yield with little improvement in specific content. Analysis by SDS–PAGE of the individual fractions suggested that much of the lost yield may be accounted for by protein remaining in the final membrane fraction after solubilization. Difference spectra of all supernatants and resuspended pellets during the first (0.15% cholate) and second (1%

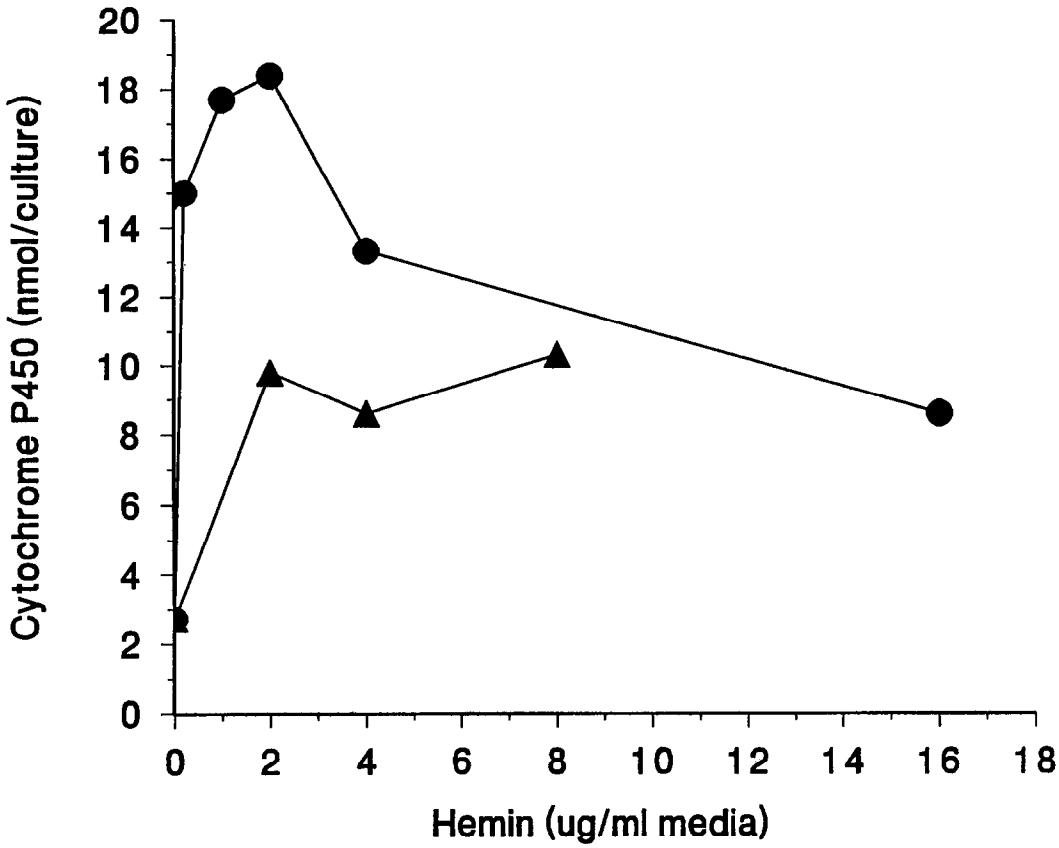

Fig. 1. Cytochrome P450 2E1 expression in *T. ni* cells. Cells were grown in roller bottle culture, as described in Materials and Methods, with hemin as hemalbumin (●) or hemin as hemin-NaOH (▲) supplements added at 24 hr after infection with recombinant baculovirus. The cytochrome P450 2E1 expression is the total P450 content for each roller bottle culture estimated by CO difference spectroscopy of cell sonicates (ordinate, nmol/culture). Difference spectra were recorded, as described in Materials and Methods, for cells harvested 4 days after infection.

Table 1. Purification of P450s expressed in Sf9 cells*

Enzyme	Sample	P450 (nmol)	Specific content (nmol/mg)	Recovery (%)
CYP2A1	Sonicated cells	133	0.23	100
	1% Cholate extract	50	0.24	38
	Concentrated protein	21	4.4	16
CYP2E1	Sonicated cells	232	0.20	100
	1% Cholate extract	66	0.23	28
	Concentrated protein	30	2.4	13
CYP2C9	Sonicated cells	194	ND†	100
	1% Cholate extract	82	ND‡	43
	Concentrated protein	25	4.9	13

* Purifications were as described in Materials and Methods using Sf9 cells as the starting material. Total enzyme (P450) and specific content of cytochrome P450 were determined from CO difference spectra. Protein concentrations were determined by the BCA (Pierce Inc., Rockford, IL) method using suitable corrections for detergent concentrations in samples. Results were obtained using octyl-Sepharose chromatography. Similar purification results were obtained when immobilized artificial membrane chromatography was substituted for octyl-Sepharose chromatography of the extracts.

† ND = not determined; a specific content of 0.29 nmol/mg was determined in a smaller preparation.

‡ A specific content of 0.56 nmol/mg was determined in a smaller preparation.

cholate) extractions revealed that loss of active enzyme could not be accounted for by discarded material, suggesting that cytochrome P450 may be inactivated during the extractions. The initial 0.15% cholate wash was necessary to remove the loosely associated membrane proteins that otherwise copurified with cytochrome P450.

The generation of back-pressure during column purification procedures made necessary the filtration of all samples, and filtration and degassing of buffers. Even after these precautions, pressures in the range of 10–20 bar were typical. Using the conditions described in Materials and Methods, P450s eluted at 0.2 to 0.6% Lubrol on Octyl-Sepharose chromatography and at 1.0 to 1.2% Lubrol on immobilized artificial membrane chromatography. Dialysis in low cholate and phosphate buffer was necessary for binding of these samples to hydroxyapatite. After hydroxyapatite elution and concentration, the yield of active enzyme recovered was in the range of 13–16% of the starting material with specific contents of 2–5 nmol/mg protein. Although the specific content is not comparable to the highest specific contents reported for P450s, we have found these preparations suitable for reconstitution studies or for starting material in enzyme-specific column purification procedures, such as ion-exchange chromatography. As more P450s will be expressed in Sf9 cells, some of them with entirely unknown properties, such as non-specific partial purification may have broad utility.

Individual fractions characterized in Table 1 for each of the P450s were also analyzed by SDS–PAGE. Panel A of Fig. 2 indicates the progress of the purification for cytochrome P450 2A1. A prominent band was observed in SDS–PAGE gels of Sf9 cells expressing CYP2A1 at an M_r of 49.8 kDa in samples of sonicated cells (see Fig. 2, panel A). Comparison of sonicated cell samples with 1% cholate extracts (cf. panel A, lanes labeled "cell sonicate" and "1% extract," respectively) suggests a partial purification of CYP2A1. SDS–PAGE analysis of the column-purified sample (panel A, lane labeled "concentrate") indicated that it contained few impurities, and this was reflected by the higher specific content of the sample in Table 1. The estimated apparent M_r value of CYP2A1 (see Table 2) was in accord with previous reported values allowing for 5% error (48 kDa; [15]). Protein samples of CYP2E1 and CYP2C9 purifications were also analyzed by gel electrophoresis and are shown in panels B and C of Fig. 2, respectively. The corresponding apparent M_r values of these enzymes are also given in Table 2. The putative molecular mass values derived from the cDNA sequences for CYP2A1, CYP2E1, and CYP2C9 are 56.0, 55.6, and 56.8 kDa, respectively (Protein Identification Resource database). Typical difference spectra of the purified proteins had 420 nm peak intensities of 10% relative to 450 nm intensities in CO difference spectra, suggesting that denatured holoenzyme is not a major impurity in these preparations. Absorbance maxima for the partially purified enzymes are also reported in Table 2 from CO-reduced versus reduced difference spectra.

The catalytic activities for the three partially purified preparations are shown in Table 2. Turnover numbers for vaccinia expressed P450s are included for comparison. Enzyme activities analyzed for both the vaccinia virus expression system and for the purified preparations of the baculovirus expression system compared well for each of the three enzymes.

DISCUSSION

The rapid evolution of knowledge regarding P450 cDNA-expression already suggests that an expression system favored for all P450 enzymes is unlikely to emerge. Of the cytochrome P450 expression systems in common use, yeast, *Escherichia coli*, and insect cells produce active enzyme in cellular concentrations sufficient for purification [6, 7, 10]. Special considerations

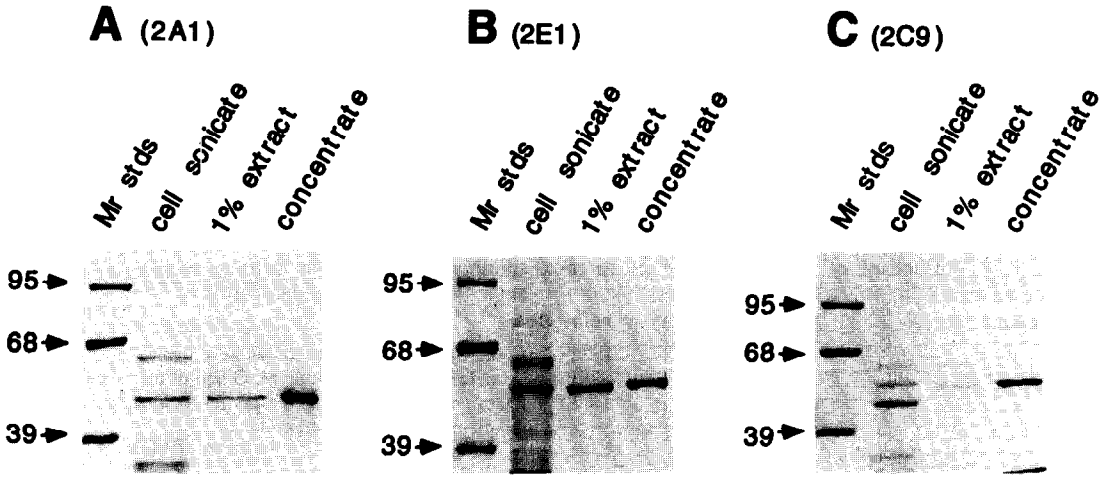

Fig. 2. SDS–PAGE analysis of purification samples. PAGE analysis was by the method of Laemmli [21], using 7.5% acrylamide in running and stacking gels for each of the cytochrome P450 enzymes analyzed: panel A, CYP2A1 (rat); panel B, CYP2E1 (human); and panel C, CYP2C9 (human). Staining was with Coomassie R250 (ICN Biomedicals, Inc., Irvine, CA). Cell samples indicated above each lane correspond to the same samples characterized in Table 1, with the exception of CYP2C9 samples, which are the corresponding samples of a purification resulting in a concentrate fraction with a specific content of 2.7 nmol/mg protein. Cell sonicates and 1% extracts are 10–60 μg protein/lane, and concentrates are 7 μg/protein/lane. M_r standards in kDa are indicated in the left margin of each panel for the markers in the left-most lane of each gel. Apparent M_r estimates for each enzyme are given in Table 2.

Table 2. Physical properties and catalytic activities of expressed P450s*

Enzyme	λ_{max} (nm)	M_r, app (kDa)	Hep G2 catalytic activities (min^{-1})	Sf9 catalytic activities (min^{-1})
CYP2A1	451	49.8	20.5 (0.5)†	16.6 (2.0)
CYP2E1	450	55.7	2.34 (0.01)	1.46 (0.01)
CYP2C9	450	55.0	1.93 (0.08)	1.50 (0.11)

* Maximal absorbance was obtained from difference spectra of purified proteins analyzed for P450 content as described in Materials and Methods. Apparent molecular mass values (M_r, app) were determined by gel electrophoresis. Activities reported are turnover numbers of reconstituted cytochrome P450 activity in standard activity assays. CYP2A1 activity is testosterone 7α-hydroxylase, CYP2E1 activity is nitroanisole demethylase, and CYP2C9 activity is tolbutamide hydroxylase as described in Materials and Methods.

† Values in parentheses represent the standard error.

for expression of high levels of cytochrome P450s include: (1) the endogenous levels of cytochrome P450 enzymes present in the host cells, (2) factors that may limit heme insertion into apoenzyme and membrane association of the holoenzyme, and (3) the chemical similarity of expressed versus native enzymes.

We have not detected P450 in wild-type Sf9 cells. The baculovirus systems reported here produced P450 at levels of 0.2 to 0.3 nmol/mg cellular protein and have been shown elsewhere to exceed these levels [12]. Endogenous cytochrome P450 enzymes in host cells become insignificant contaminants when cDNA-directed expression reaches high relative levels.

Among considerations for P450 expression are those of hemin incorporation into apoenzyme and membrane localization of the expressed protein. Although the capacity of insect cells to produce sufficient heme for high level hemoprotein synthesis is feeble, it has been demonstrated previously that hemin medium-supplements may bolster endogenous heme content [15]. Optimal expression of several cytochrome P450 enzymes [10] and the hemoprotein myeloperoxidase [25] has also been obtained using hemin supplements for Sf9 expression. However, failure of heme incorporation into cytochrome b_{558} expressed in Sf9 cells by media supplementation suggests that this methodology may not be generally successful [26]. We report here that hemalbumin medium-supplement enhanced active hemoprotein expression above that obtained with hemin-NaOH medium-supplement. Increased expression using this protein–heme conjugate may result from a more gradual uptake of the cofactor during the period of protein expression, lessening Sf9 toxicity. Similarly enhanced expression was obtained with hemalbumin for the expression of cytochrome b_5 in Sf9 cells (data not shown).

Cytochrome P450 enzymes are associated with the endoplasmic reticulum membrane in their native condition. High level expression of P450 enzymes in Sf9 cells may exceed the capacity of the cells for membrane insertion into the endoplasmic reticulum. This possibility has been raised by the finding of P450 aggregates within deformed portions of endoplasmic reticulum and nuclear regions of Sf9 cells expressing a rabbit CYP2B [9]. Enzyme that is devoid of an appropriate lipid environment may become an inactive protein contaminant and difficult to separate from active enzyme during purification. This consideration may be relevant to the low yields obtained in the initial cholate extractions (see Table 1). Formation of active P450 may also be restricted by P450-specific membrane environments and/or heme incorporation requirements as has been shown when P450scc apoprotein accumulates in Sf9 mitochondria [27].

The purification of several cytochrome P450 enzymes to apparent homogeneity has been reviewed [28]. A primary consideration in purification of P450 from tissue sources is the removal of copurifying cytochrome P450 enzymes, which behave similarly when subjected to chromatographic procedures. This consideration is minimized when purification starting materials are derived from overexpression in host cells with low endogenous levels of P450s. However, the yields resulting from individual extraction or chromatographic methods are not expected to be altered. We have investigated the use of octyl-Sepharose chromatography or, alternatively, IAM-PC on Sf9 cell extracts as a means of obtaining partially purified P450s. The efficacy of these procedures for three cytochrome P450 enzymes appears to be uniform, resulting in enzymes in the 2–5 nmol/mg range of specific content, which compares favorably with previous reports using IAM-PC [19]. Yields after detergent removal using hydroxyapatite chromatography were 13–16% of the starting material. Further purification may be accomplished by tailoring purification procedures to the characteristics of individual enzymes by inclusion of ion-exchange chromatography, albeit with lower expected yields.

We have shown that the cytochrome P450 enzymes expressed in Sf9 cells have apparent molecular masses in the range of those expected for these enzymes (see Table 1). CYP2A1 was shown previously to behave in an anomalous fashion by PAGE analysis with an apparent M_r of 48 kDa [29]. This result is reproduced here for the cDNA expressed enzyme, while CYP2E1 and CYP2C9 both have significantly greater apparent M_r values. The enzymes also have characteristic CO difference spectra and enzymatic activities similar to the enzymes expressed in Hep G2 cells (see Table 2). The difference in turnover numbers between enzymes expressed in Sf9 cells and those expressed in Hep G2 cells may result from the differing lipid environments and reductase concentrations in the two systems. These results indicate that the Sf9-expressed and reconstituted enzymes are similar in structure and activity to the same enzymes expressed in a mammalian cell line.

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