



Cytochrome P450 Purification and Immunological Detection in an Insecticide Resistant Strain of German Cockroach (*Blattella germanica*, L.)

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A German cockroach strain, *Munsyana* (MA) had 80-fold resistance to the pyrethroid insecticide *cypermethrin*, 4.5-fold greater total cytochrome P450 content and 2.5-fold greater cytochrome P450-mediated *N*-demethylation of 4-chloro-*N*-methylaniline compared to the susceptible Johnson Wax (JWax) strain. Immobilized artificial membrane high performance liquid chromatography (IAM-HPLC) of microsomal proteins from the MA strain enriched cytochrome P450 greater than 70-fold. Following purification, a single protein band of $M_r = 49,000$ (P450 MA), was detected by silver-staining SDS PAGE gels. Antiserum to the purified protein from the MA strain (anti-P450 MA) was produced in mice. Anti-P450 MA inhibited cytochrome P450-mediated *N*-demethylation by 4-fold in both MA and JWax strains. In Western blots of microsomal proteins, anti-P450 MA differentiated single MA and JWax individuals by recognizing an M_r 49,000 protein band in only the MA strain. In JWax cockroaches, the M_r 49,000 band was only detectable in Western analysis following induction with pentamethylbenzene (PMB). PMB induction also increases *N*-demethylation 2.6 and 8.0-fold in the MA and JWax strains, respectively. These results are consistent with the hypothesis that insecticide resistance in the MA strain is due to over-expression of a cytochrome P450. © 1998 Elsevier Science Ltd. All rights reserved

Blattella germanica Antiserum Cytochrome P450 Pyrethroid resistance Immobilized artificial membrane chromatography

INTRODUCTION

Insecticide resistance is a significant obstacle to effective control of the German cockroach (*Blattella germanica*, L.) (Cochran, 1995). Resistance to pyrethroid insecticides, poses a particular threat because of the few alternatives available. The implication of German cockroach allergens as inducers of human asthma (Chapman *et al.*,

1997), adds emphasis to the need for effective control measures and an understanding of resistance mechanisms in this species.

In 1994, the *Munsyana* strain of German cockroach was collected from a public housing site in Indiana. This strain has 80 and 5-fold resistance at LD₅₀ to the pyrethroid insecticide *cypermethrin* and the organophosphate insecticide *chlorpyrifos*, respectively (Scharf *et al.*, 1997). Synergism of pyrethroid toxicity by the cytochrome P450 inhibitor piperonyl butoxide, suggests that resistance is mediated by cytochrome P450 in this strain. The ability to characterize and detect the cytochromes P450 responsible for insecticide resistance in the *Munsyana* strain of German cockroach would be an important tool for resistance management.

Cytochromes P450 are a superfamily of hemoproteins that occur in all cellular organisms (Nelson *et al.*, 1996). Many cytochromes P450 metabolize apolar xenobiotics (Gotoh, 1993) and cytochromes P450 are the most com-

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mon mechanism of insecticide detoxication in insects (Hodgson, 1985). Advances in Immobilized Artificial Membrane chromatography (IAM-HPLC; Pidgeon *et al.*, 1991a, b) have overcome many obstacles to rapidly and efficiently isolating cytochromes P450. The objectives of this study were to purify cytochromes P450 from the German cockroach, use the purified protein to produce antiserum and to determine if the antiserum could be used to distinguish resistant and susceptible cockroaches.

MATERIALS AND METHODS

Insect strains and rearing

The *Munsyana* strain (MA) was derived from German cockroaches collected from a public housing site in Muncie, Indiana in 1994. Prior to collection, this strain had been exposed to formulated cypermethrin (Demon WP®, Zeneca, Wilmington, DE) for 8 years and had become very difficult to control. The eighty-fold resistance to cypermethrin (measured at LD₅₀) in the MA strain was reduced by 90% by pre-treatment with piperonyl butoxide (Scharf *et al.*, 1997).

The Johnson Wax susceptible strain (JWax) was used for all comparisons to the MA strain in this study. The JWax strain was isolated in the late 1930s, before the introduction of synthetic organic insecticides (Koehler and Patterson, 1986). All test insects were maintained at 20–25°C and 65–70% RH with harborage, unlimited water and HT-8604 laboratory diet (Harlan Teklad, Milwaukee, WI).

Reagents

All organic reagents were purchased from Sigma Chemical (Saint Louis, MO) or US Biochemical (Cleveland, OH) unless otherwise specified. All inorganic reagents were analytical grade. Water used for all experiments was deionized by a MilliQ deionizer (Millipore, Marlborough, MA). Ultra-pure glycerol for HPLC and microsome preparations was purchased from Fisher Scientific (Pittsburgh, PA); Lubrol WX was purchased from Serva; and 4-chloro-*N*-methylaniline, 4-chloroaniline, pentamethylbenzene (PMB) and piperonyl butoxide (PBO) were purchased from Aldrich (Milwaukee, WI). Sodium cholate was twice recrystallized prior to its use (Guengerich, 1978).

Buffers and solutions

Homogenization buffer: 0.1 M sodium phosphate, pH 7.5. *Resuspension buffer*: Homogenization buffer containing 30% v/v glycerol. *Buffer A*: 0.1 M potassium phosphate, pH 7.25, 20% v/v glycerol, 1 mM EDTA, 0.6% w/v sodium cholate. *Buffer B*: Buffer A + 2.0% w/v Lubrol WX. *SDS PAGE sample buffer*: 0.5 M tris, 13% v/v β-mercaptoethanol, 10% w/v SDS, 53% v/v glycerol, and 0.1% w/v bromophenol blue. *Sodium hydrosulfite solution*: 220 mM sodium hydrosulfite in water. *PBO stock solution*: 295 μM piperonyl butoxide (PBO)

in ethanol. *NADPH generating system*: Resuspension buffer containing 2.5 mM glucose-6-phosphate, 0.05 units glucose-6-phosphate dehydrogenase, 0.5 mM NADP⁺ and 7.5 mM magnesium chloride. *Demethylation assay reaction mixture*: NADPH generating system containing 10 μM 4-chloro-*N*-methylaniline. *Stop solution*: 134 mM *p*-dimethylaminobenzaldehyde (PDAB) in 1.0 N H₂SO₄.

Preparation of microsomes

All tissues and homogenates were kept at 4°C throughout all procedures, except during IAM-HPLC separations (ca. 2 h at room temperature). Frozen, adult male German cockroaches with heads removed were homogenized in 9.0 ml homogenization buffer in a Duall 24 homogenizer (Kontes, Vineland, NJ). The homogenate was centrifuged at 11,000 *g* for 10 min. The supernatant was filtered through glass wool then centrifuged at 106,000 *g* for 60 min. The resulting microsomal pellets were re-suspended in 2.5 ml resuspension buffer in a Duall 22 homogenizer and stored at –70°C. For cytochrome P450 protein purification by IAM-HPLC, six microsomal preparations of 50 adult male MA cockroaches each were pooled. For microsomal preparations of single insects, individual adult male cockroaches were homogenized in 2.0 ml homogenization buffer with microsomes obtained as described above and resuspended in 20 μl of homogenization buffer. For all other procedures, microsomes were prepared from 25–30 adult males of each strain, resuspended in 1.2 ml resuspension buffer, and divided into 100 μl aliquots for storage at –70°C.

Solubilization of microsomes

Microsomal proteins were solubilized and prepared for chromatography as described by Pidgeon *et al.* (1991a, b) and Otto *et al.* (1991). Microsomal suspensions (determined to be free of cytochrome P420 by CO difference spectra) were solubilized at a concentration of 1.5–2.5 mg/ml microsomal protein (final concentration) by adding microsomes dropwise to Buffer A (containing 3 mg sodium cholate per mg microsomal protein) while stirring at 4°C. The mixture was stirred for 30 min, centrifuged at 105,000 *g* for 60 min to remove particulate matter, filtered through a 0.2 micron membrane, then used immediately for IAM-HPLC.

Purification of cytochrome P450 proteins

A 12 micron, 15 cm Immobilized Artificial Membrane (IAM.PC) analytical HPLC column, with an IAM guard column and 0.2 micron frit (Regis, Morton Grove, IL) was equilibrated with Buffer A. The solubilized microsomes were divided into 4 samples and chromatographed separately as described in Marcus *et al.* (1990a,b) and Otto *et al.* (1991). Solubilized microsomes were loaded onto the column with a 10 ml super loop (Pharmacia). Flow rate was 0.5 ml/min and back pressures were maintained at less than 800 lb/in² (143 kg/cm²). One milliliter fractions were collected throughout the chromatography.

Optical density of column effluent was monitored at 280 and 405 nm to detect total protein and hemoprotein elution, respectively. Weakly bound proteins were washed from the column by Buffer A. A linear gradient of Buffer B (0–65%) in Buffer A was used to specifically elute cytochrome P450 proteins. A single peak of heme-containing protein (measured by OD 405) was eluted from the column by the gradient at Lubrol WX concentrations between 0.5 and 1.4%. Thirty microlitres of each 1.0 ml hemoprotein-containing fraction was diluted with 15 μ l of SDS PAGE sample buffer and left overnight at 4°C. These samples were electrophoresed on 7.5% SDS-PAGE gels (15 cm \times 1.5 mm) and visualized by silver staining. Fractions from the HPLC separations containing a band with an M_r of 49,000 were pooled and concentrated to 1 ml using a pressurized, magnetically stirred ultrafiltration cell (10 ml capacity) fitted with a 30,000 molecular size cutoff disc membrane (PM30; Amicon, Beverly, MA). The concentrated sample was diluted 1:2 with sample buffer and stored overnight at 4°C. The Lubrol WX detergent was removed from purified cytochrome P450 proteins by preparative SDS-PAGE gels (7.5% acrylamide; 15 cm \times 1.5 mm). Briefly, the purified cytochrome P450 protein was electrophoresed until a pre-stained M_r 50,000 molecular weight marker (protein kinase) traveled approximately 3/4 the length of the gel. Gels were removed from electrophoresis units, rinsed briefly in water, and reversibly stained with zinc (Fernandez-Patron *et al.*, 1992). For zinc staining, gels were incubated for 5 min in 0.2 M imidazole solution and transferred to a 0.3 M zinc sulfate solution until the background became an opaque white (protein bands stained negatively and remained transparent). The protein band at M_r 49,000 was cut from the gel and destained with 3 rinses of citric acid (2% w/v). The purified cytochrome P450 protein was electroeluted (Bio-Rad, Model#422 electroelutor) from the gel strips for 12 h at 5 mA/cell in tris-glycine reservoir buffer. Electroeluted protein was dialyzed against 10 mM Tris, pH 7.4 for 24 h at 4°C to remove SDS. Dialyzed protein from all purifications was pooled and concentrated in a speedvac concentrator (Savant), yielding 260 μ g of purified cytochrome P450 protein. All protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical Company, Rockford, IL) according to the directions of the manufacturer.

Antiserum production

Polyclonal antiserum to the purified cytochrome P450 protein was prepared by the Antibody Production Core Facility of Purdue University. Prior to immunization, blood was drawn by a retro-orbital procedure into a capillary tube (containing 10 μ l citrate buffer), from a methoxyfluorane anesthetized BALB/c mouse. The blood was centrifuged to remove large particulate matter, and the remaining serum was used for pre-immune controls. Immunizations were performed by making four interscapular-subcutaneous injections of the dialyzed and con-

centrated cytochrome P450 antigen. The first immunization occurred the week of the pre-immunization bleed, and the second, third and fourth injections occurred at 3, 4 and 5 wk (respectively). The first injection contained 20 μ g antigen in 200 μ l phosphate buffered saline (PBS) and an equal volume of Freund's complete adjuvant. The 3 booster injections contained 10 μ g antigen in 200 μ l PBS and an equal volume of Freund's incomplete adjuvant. At 4 wk, a 10 μ l sample of serum was obtained and tested to confirm antibody production. At 5 wk the mouse was sacrificed and its blood serum, which served as the antibody source, was isolated as described above.

Mini-gel SDS-page

Prior to loading, protein samples were diluted 1:1 in sample buffer, and placed in boiling water for 5 min. Ten percent polyacrylamide gels, 5 cm \times 0.75 mm, and 2 cm stacking gels (1 cm wells) were poured using the BioRad mini-gel system. Proteins were electrophoresed at 200 V until the dye front completely eluted from the gel (approximately 35 to 40 min).

Induction of cytochrome P450

A 0.2% (w/v) solution of the cytochrome P450 inducer, pentamethylbenzene (PMB; Brattsten and Wilkinson, 1973; Neal and Reuveni, 1992), was prepared by dissolving 20 mg PMB in 100 μ l acetone, and adding to 9.9 ml water. Adult male MA and JWax roaches (30 per strain) were provided with 5 ml of PMB solution as their only source of water, and untreated laboratory chow as their only source of food. After 96 hours, insects were frozen and microsomes prepared as described above.

Western blot analysis

Microsomal proteins were separated by SDS-PAGE using mini-gels as described above. Proteins were transferred from gels to nitrocellulose membranes (Hybond ECL, Amersham, Buckinghamshire, England) for 1 h at 100 V and 4°C using a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad, Hercules, CA). The transfer buffer consisted of 25 mM tris and 192 mM glycine in a solution of 20% v/v methanol in water (pH 8.3). Protein transfer was monitored by staining a strip of the blot with Ponceau S (Salinovich and Montelano, 1986). Membranes were blocked for 1 hr in PBST (PBS + 0.05% Tween-20) containing 1% casein, washed 2 times for 5 min in PBST, incubated for 1 hr with 10 ml anti-cytochrome P450 serum (described above) in PBST (1:5000), washed once for 15 min and 2 times for 5 min in PBST, incubated for 1 hr in 5 ml peroxidase-conjugated secondary antibody (Sigma A-5278 goat anti-mouse, St. Louis, MO) in PBST (1:1500) and washed once for 15 min and 4 times for 5 min in PBST. Antibody conjugate was detected with an enhanced chemiluminescence (ECL) kit (Amersham, Buckinghamshire, UK) following the manufacturer's instructions, by exposing film to the light emitting-ECL reaction for 30 s.

Carbon monoxide difference spectra

Total cytochrome P450 content was quantified by the dithionite-reduced carbon monoxide difference spectra, modified from Omura and Sato (1964). For each replicate, 100 μ l of microsomal suspension (containing approximately 1.0 mg of total protein) was placed in a glass tube, followed by 1.2 ml of resuspension buffer and 400 μ l of fresh sodium hydrosulfite solution. The contents of the tube were then equally divided into two matched, 1.0 ml quartz spectrophotometer cuvettes, placed in a Perkin Elmer Lambda 3 dual beam UV spectrophotometer (Uberlingen, Germany) and background corrected. Carbon monoxide was bubbled into one cuvette for 1 min, and both cuvettes were then scanned from 500–380 nm. The difference in absorbance between 490 and 450 nm was converted to pmol cytochrome P450 per mg microsomal protein using an extinction coefficient of 91 $\text{mM}^{-1} \text{cm}^{-1}$ and by correcting for microsomal protein content. Average cytochrome P450 content between strains was statistically compared using the Ryan-Q test ($\alpha = 0.05$, $n = 3$; SAS Institute, 1990).

In vitro demethylation and inhibition

Demethylation of the model substrate 4-chloro-*N*-methylaniline was quantified following the method of Kupfer and Bruggerman (1966), using 50 μ l of microsomal resuspension per assay (containing approximately 500 μ g total protein). For inhibition studies, microsomal suspensions were incubated with either 1 μ l of serum (either pre- or post-immunized) or 10 μ l of PBO stock solution for 1 h at 4°C. For controls lacking an NADPH generation system, an equivalent volume of resuspension buffer was substituted. Metabolism was initiated by adding 400 μ l of reaction mixture to microsomes and terminated after incubation for 10 min at 37°C by adding 750 μ l of stop solution. Tubes were centrifuged for 15 min at 11,000 *g* at 4°C. The product, 4-chloroaniline, was quantified by comparing absorbance of supernatants at 445 nm to a simultaneously determined standard curve. Average demethylation activities were statistically compared using the Ryan-Q test ($\alpha = 0.05$, $n = 3$; SAS Institute 1990).

RESULTS

Cytochrome P450 quantitation

Total cytochrome P450 content, as determined by carbon monoxide difference spectra, was 4.6-fold higher in the resistant MA strain than the susceptible JWax strain (Fig. 1). Feeding cockroaches the cytochrome P450 inducer, PMB, greatly increased the variability of cytochrome P450 content in microsomal preparations, but did not significantly induce total cytochrome P450 content. Based on the highest cytochrome P450 content (264 pmol/mg microsomal protein) and an approximate molecular weight of 50,000 Da, the cytochrome P450 content is at most 1.3% of any microsomal preparation.

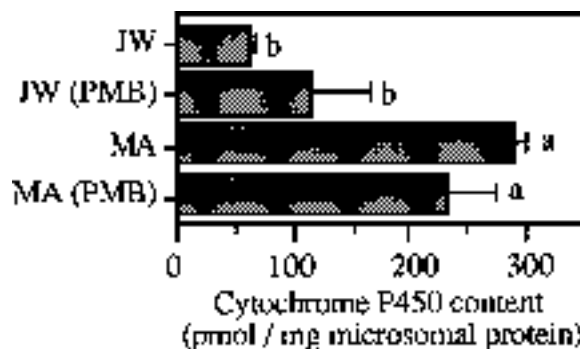


FIGURE 1. Average cytochrome P450 content of microsomes from insecticide resistant and susceptible cockroach strains. Abbreviations: JWax, Johnson Wax strain (susceptible); MA, Munsyana strain (resistant); PMB, pentamethylbenzene induced. Means followed by the same letter are not significantly different by the Ryan-Q test ($\alpha = 0.05$, $n = 3$).

This percentage would be difficult to detect by SDS-PAGE and no differences in banding patterns of microsomal proteins were seen between JWax and MA on a silver stained gel (not shown).

IAM chromatography

Most of the microsomal protein loaded onto the IAM column eluted when washed with Buffer A (Fig. 2). A hemoprotein peak, as indicated by absorbance at 405 nm, eluted as the Lubrol WX detergent concentration increased. The absorbance increase at 280 nm after hemoprotein elution (Fig. 2) is due to the detergent Lubrol WX at higher concentrations, and is not indicative of protein elution. Elution profiles of four HPLC separations of 89, 89, 50 and 47 mg microsomal protein were similar to that shown in Fig. 2.

Examination of purified proteins by SDS-PAGE

Hemoprotein-containing fractions from IAM chromatography were examined by silver stained SDS-PAGE gels (Fig. 3A). By conservative estimates based on the staining profile, greater than 95% of the protein in the hemoprotein peak fractions was concentrated in a single band of approximately M_r 49,000. This represents greater than 70-fold enrichment of cytochrome P450 in a single step.

Traces of contaminating protein and Lubrol WX detergent were removed by preparative SDS-PAGE. Following electroelution, dialysis and concentration as described above, 260 μ g of purified cytochrome P450 was obtained. Only a single protein band (detection limit 2 to 5 ng) was visible following SDS-PAGE and silver staining of 160 ng of the final sample (Fig. 3B).

Antiserum production

ELISA to antigen coated wells, using serum (10 μ l) obtained after the third immunization of purified MA strain protein, confirmed an immune response in the mouse. Following one subsequent immunization (i.e. at 5 wk), antiserum (anti-P450 MA) was collected in a ter-

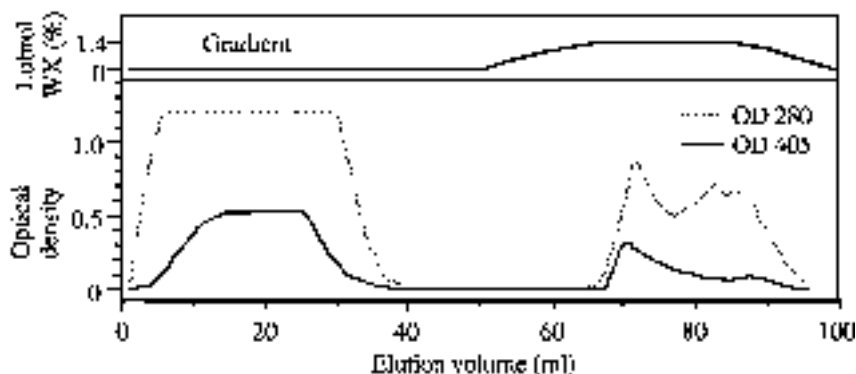


FIGURE 2. Purification of cytochrome P450 from cockroach microsomes by IAM-HPLC preparative chromatography. A typical chromatograph, showing elution of total protein (OD_{280}) and hemoproteins (OD_{405}) along a Lubrol WX gradient (0–1.4%). See text for details.

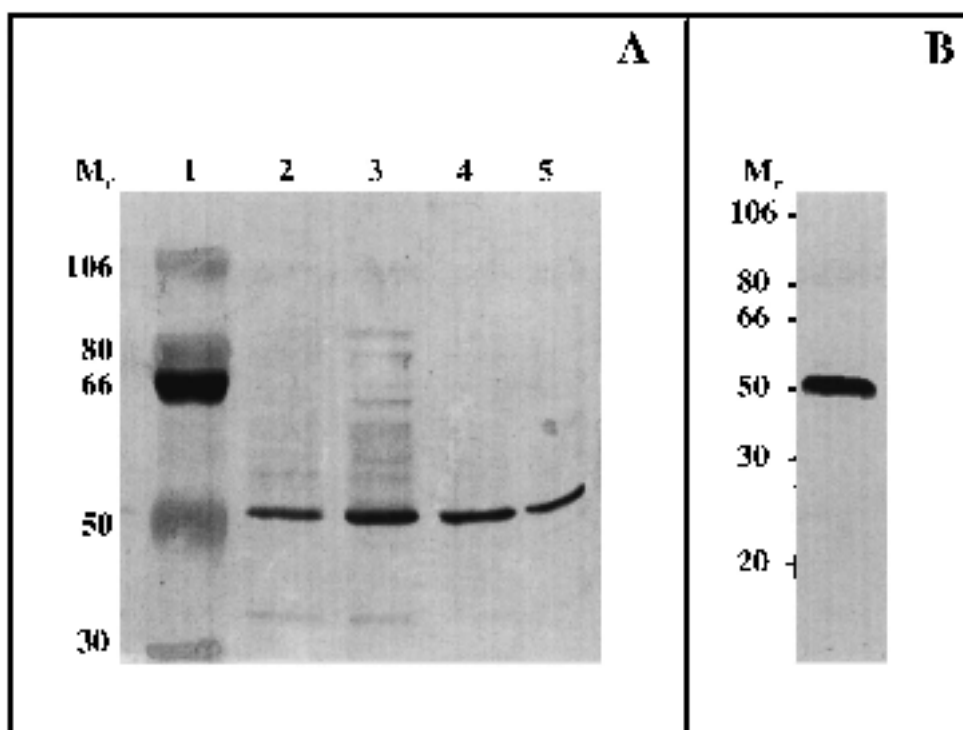


FIGURE 3. Silver stained SDS polycrylamide gels containing hemoprotein (i.e. cytochrome P450) enriched fractions of the resistant MA strain. Panel A: lane 1 contains protein standards (M_r 30,000, 50,000, 66,000, 80,000 and 120,000); lanes 2–5 depict a typical series of hemoprotein fractions as eluted by high Lubrol WX concentrations during IAM-HPLC. Panel B: final protein band after SDS PAGE purification and dialysis.

minimal bleed of the immunized mouse. Optimal dilution of anti-P450 MA for Western analysis of microsomal protein (50 μ g per lane) was determined to be 1:5000.

Demethylation activity, inhibition and induction

Microsomal *N*-demethylase activity was 2.5-fold higher in the resistant MA than susceptible JWax cockroaches (Fig. 4). The lack of *N*-demethylase activity in the absence of an NADPH generating system and the 4- to 5-fold inhibition by PBO demonstrate that this activity is predominantly mediated by cytochrome P450. Anti-P450 MA (1 μ L/132 pmol cytochrome P450) inhibited activity in both JWax and MA cockroaches by 4-fold. Activity in controls containing pre-immunized mouse

serum was 90% and 106% of a no-serum control in MA and JWax, respectively. Inhibition of *N*-demethylase activity by the anti-P450 MA serum strongly suggests the presence of antibodies specific to the cockroach cytochrome P450 form(s) responsible for catalyzing *N*-demethylation.

The cytochrome P450 inducer, PMB, increased *N*-demethylation activity 3-fold in MA and 8-fold in JWax cockroaches. Following induction, maximal levels of *N*-demethylation activity are similar between the JWax and MA strains. This provides further evidence that *N*-demethylation is cytochrome P450-mediated in this species, and that these cytochromes P450 are responsive to induction.

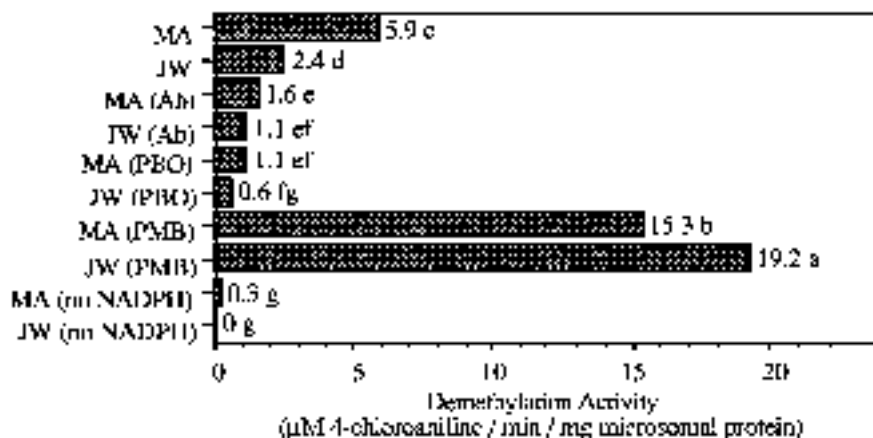


FIGURE 4. Average 4-chloro-*N*-methylaniline demethylation activity by susceptible and resistant cockroach microsomes. Abbreviations: JWax, Johnson Wax strain (susceptible); MA, Munsyana strain (resistant); PMB, pentamethylbenzene induced; PBO, piperonyl butoxide inhibited; Ab, microsomes incubated with anti-P450 MA; no NADPH, reaction contained no NADPH generating system. Means followed by the same letter are not significantly different by the Ran-Q test ($\alpha = 0.05$, $n = 3$).

Western blotting

In Western blots of uninduced microsomal proteins, anti-P450 MA recognized a single protein band of M_r 49,000 from only MA strain cockroaches (Fig. 5). Microsomal protein quantities loaded per lane ranged from 10 to 50 μg (containing an estimated 16–80 and 4–18 ng cytochrome P450 for MA and JWax cockroaches, respectively). No bands were detected in JWax microsomes at the 30 s exposure, however, a faint band was visible at M_r 49,000 for the highest concentration (50 μg) at a 1 min exposure. Following PMB induction, the M_r 49,000 band from JWax appears equally as intense as in MA microsomes, at both concentrations examined (10 and 50 μg).

Fig. 6 demonstrates the ability to differentiate resistant (MA) and susceptible (JWax) individuals by Western blotting, using 15 μg of microsomal protein per lane. All MA individuals examined had prominent bands at M_r 49,000. Bands from MA individuals were similar in intensity, however, there appears to be heterogeneity in the population as two bands were detectable in one individual (M_{12}). All bands from the JWax individuals (except J_{12}) were less intense than the least intense band of the MA individuals. The intensity of protein bands from the majority of JWax individuals varied from faint to absent. No second band was detected in any of the JWax individuals investigated.

DISCUSSION

Utility of IAM chromatography

This is the first use of IAM-HPLC (Pidgeon *et al.*, 1991a,b) to purify cytochromes P450 from an insect. IAM-HPLC has previously been successful for purifying cytochromes P450 from rat liver, and labile isoforms from rat kidney and adrenal microsomes (Pidgeon *et al.*, 1991a; Otto *et al.*, 1991). Cytochrome P450 is thought to adhere to the IAM column via an interaction between a hydrophobic site on the enzyme and the IAM matrix. The similarities among membrane-bound cytochromes P450 from insects and other organisms apparently extend to interactions with IAM. The successful purification of German cockroach cytochrome P450 suggests that IAM chromatography may be useful for purifying cytochrome P450 from other insect taxa.

Cytochromes P450 have been purified from insects by other methods. Ronis *et al.* (1988) partially purified 4 house fly cytochromes P450 using 5 types of conventional column chromatography; Wheelock and Scott (1989) purified one cytochrome P450, one cytochrome b_5 , and one cytochrome P450 reductase from house flies using polyethylene glycol fractionation and 2 types of HPLC; Sundseth *et al.* (1989) partially purified 2 fruit fly cytochromes P450 using 3 types of column chromatography; and Feyereisen *et al.* (1989) partially purified

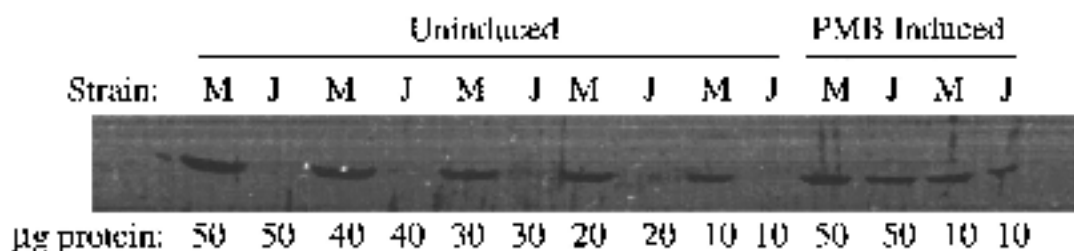


FIGURE 5. Western analysis of cytochrome P450 from insecticide resistant (M) and susceptible (J) cockroach strains from mass homogenates of uninduced and pentamethylbenzene (PMB) induced, adult male cockroaches. Microsomal protein quantities loaded per lane were 50, 40, 30, 20 and 10 μg . All bands are at approximately M_r 49,000.

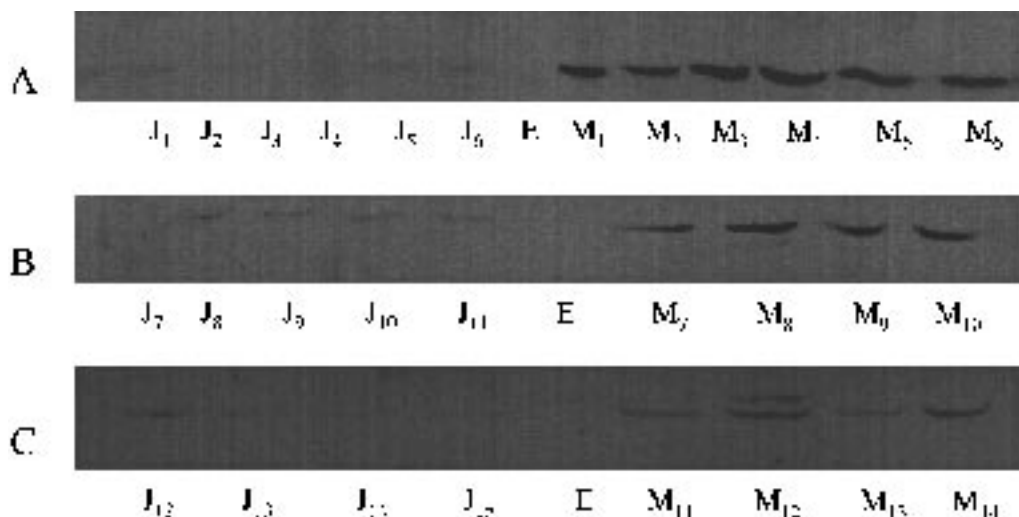


FIGURE 6. Western analysis of cytochrome P450 from individuals of insecticide susceptible and resistant cockroach strains (15 μ g microsomal protein per lane). A, B and C represent blots of different SDS polyacrylamide gels. Abbreviations: J_{1-15} , individuals of the susceptible JWax strain; M_{1-14} , individuals of the resistant MA strain; E, empty lanes (no protein loaded). All bands are at approximately M_r 49,000.

1 house fly cytochrome P450 using 2 types of column chromatography. In comparison to these methodologies, IAM-HPLC rapidly provides almost complete purification from a single column.

The method of Wheelock and Scott (1989) has an advantage over IAM chromatography in that it also provides purified cytochrome b_5 and cytochrome P450 reductase. However, it may be possible to recover these proteins after IAM chromatography, with the removal of other microsomal proteins (including cytochrome P450) making subsequent purification of cytochrome b_5 and cytochrome P450 reductase less difficult. Difficulties associated with IAM-HPLC include the requirement that the column be properly conditioned, proper filtration and back-pressure ahead of the IAM column need to be maintained, and detergent removal. The Lubrol WX detergent used to elute cytochrome P450 from the IAM column is not dialyzable and is difficult to remove from purified protein samples. Substantial loss of yield occurred in procedures subsequent to IAM-HPLC, and improvements in these procedures could significantly improve overall recovery. Advantages of IAM chromatography include recovery of functional proteins (after detergent removal), speed of purification (approximately 2 hr), and an ability to concentrate cytochromes P450 from dilute samples.

Cohen *et al.* (1992) purified a cytochrome P450 from *Papilio polyxenes* by electroeluting xanthotoxin inducible bands from polyacrylamide gels. This technique was possible because of the high level of cytochrome P450 expression after induction. Unfortunately, the cytochromes P450 of many insects are expressed at levels too low to be distinguishable on polyacrylamide gels. However, IAM-HPLC is a powerful tool for rapid purification of cytochromes P450 that could likely be extended to analysis of cytochrome P450 of other insect species.

Composition of IAM protein peak

Analysis of insects by molecular techniques (Scott *et al.*, 1994) suggests that insects have multiple cytochromes P450, perhaps 20 or more per species. However, a single protein band was detected by SDS-PAGE following elution from the IAM-HPLC. The single band could result from multiple cytochromes P450 of similar molecular weight, the preferential retention and elution of a single cytochrome P450, or a single cytochrome P450 that is present in much greater amounts than other cytochromes P450. All cytochromes P450 investigated to date behave similarly on IAM-HPLC (Pidgeon *et al.*, 1991a; Otto *et al.*, 1991), making the separation of closely related cytochrome P450 isoforms by IAM-HPLC unlikely. While IAM-HPLC is an excellent approach for rapidly purifying cytochromes P450 from other contaminating proteins, resolution of individual isoforms generally requires an additional chromatographic or electrophoretic step. Other isolations of cytochrome P450 from phenobarbital-induced (Feyereisen *et al.*, 1989) or LPR resistant house flies (Wheelock and Scott, 1989) have recovered single major cytochrome P450 proteins. Total cytochrome P450 is 2.5-fold higher in the MA strain than in the JWax strain. If the entire increase is due to a single cytochrome P450, then it would be at least 60% of the total cytochrome P450. If the other 40% were divided among 20 or more cytochromes P450, those forms might not be detected. It is possible that IAM-HPLC of microsomal protein from uninduced JWax cockroaches would allow detection of other cytochromes P450.

Anti-p450 MA

Antiserum produced from the purified protein band (anti-P450 MA) recognizes the cytochrome P450 responsible for *N*-demethylation of 4-chloro-*N*-methylaniline

(in both the JWax and MA strains), inducible cytochrome P450 in the JWax strain, and a constitutively expressed protein in all individuals of the MA strain. Two MA individuals had an additional band that was recognized by the polyclonal anti-P450 MA. These bands could contain a similar epitope to the cytochrome P450 or be recognized by separate antibodies within the polyclonal anti-P450 MA. Although anti-P450 MA clearly distinguishes resistant MA from susceptible JWax individuals, it has yet to be determined if this distinction is based on recognition of the cytochrome P450 responsible for insecticide metabolism. Further characterization of anti-P450 MA is in progress, including studies of immunoinhibition of pyrethroid and model substrate metabolism and screening of pyrethroid selected JWax X MA hybrids. Additional information on the numbers of cytochromes P450 that are detected by anti-P450 MA will come from screening other cockroaches that have cytochrome P450-mediated resistance and by screening a German cockroach cDNA expression library.

There are a number of parallels between anti-P450 MA and antisera to cytochromes P450 isolated from other resistant insect strains. Wheelock and Scott (1990) and Sundseth *et al.* (1989) have used LPR strain house fly polyclonal, and Hikone-R fruit fly strain monoclonal anti-cytochrome P450 (respectively) to identify differences in band intensity between mass preparations of resistant and susceptible strains. Anti-LPR and anti-Hikone-R inhibit several cytochrome P450 metabolic activities (Wheelock and Scott, 1992b; Scott and Lee, 1993; Sundseth *et al.*, 1989). Both anti-LPR (Scott and Lee, 1993) and anti-P450 MA recognize an inducible cytochrome P450 in susceptible strains. Anti-LPR inhibits pyrethroid insecticide metabolism (Wheelock and Scott, 1992a; Zhang and Scott, 1996), clearly establishing the involvement of that cytochrome P450 (CYP6D1) in insecticide resistance.

Most cytochrome P450 mediated resistance may be due to over-expression of cytochrome P450 resulting from a mutation in a trans-acting factor which regulates expression, rather than a mutation to the cytochrome P450 gene itself (Waters and Nix, 1988; Carino *et al.*, 1994). This is true for CYP6D1, which is both constitutively over-expressed in the resistant LPR house fly strain and inducible in susceptible strains (Liu and Scott, 1996). Over-expression of CYP6D1 is linked with *cis*- and *trans*-acting factors on autosomes 1 and 2 which are not cytochrome P450 protein-encoding sequences (Liu and Scott, 1996). PBO synergism of pyrethroid toxicity, elevated cytochrome P450 content, elevated cytochrome P450-mediated *N*-demethylation activity in resistant cockroaches, and discrimination between, resistant, susceptible and induced susceptible cockroaches by anti-P450 MA are all consistent with the hypothesis that resistance in the MA strain is mediated by cytochrome P450 over-expression. Determining whether or not the over-expression of cytochrome P450 MA is caused by a

mutation to a *trans*-acting regulatory sequence will require characterization at the molecular level.

Resistance detection

A method is needed to monitor the frequency of over-expressed cytochrome P450 in field- and laboratory-selected populations using 20 or fewer insects. Presently, resistance assessment methods for the German cockroach involve either insecticide bioassays (Scharf *et al.*, 1995) or assays of model substrate metabolism (Valles and Yu, 1996; Anspaugh *et al.*, 1994), both of which require relatively large numbers of insects and an acquired degree of expertise. Detection of cytochrome P450 mediated resistance is possible using anti-P450 MA in Western blot analysis of single insects. However, this particular Western blot analysis requires preparing microsomes from individual insects, SDS PAGE, and immunoblotting. A less time consuming immunoassay such as ELISA or dot blot of crude homogenates from whole insects could be developed to routinely assess cockroach populations.

REFERENCES

- Anspaugh D.D., Rose R.L., Koehler P.G., Hodgson E. and Roe R.M. (1994) Multiple mechanisms of pyrethroid resistance in the German cockroach. *Pestic. Biochem. Physiol.* **50**, 138–148.
- Brattsten L.B. and Wilkinson C.F. (1973) Induction of microsomal enzymes in the southern armyworm. *Pestic. Biochem. Physiol.* **3**, 393–407.
- Carino F.A., Koener F.A., Plapp F.W. and Feyereisen R. (1994) Constitutive over-expression of the cytochrome P450 gene CYP6A1 in a house fly strain with metabolic resistance to insecticides. *Insect. Biochem. Molec. Biol.* **24**, 411–418.
- Chapman, M. D., Vailes, L. D., Hayden, M. L., Platts-Mills, T. A.E. and Arruda, L. K. (1997) Cockroach allergens and their role in Asthma. In *Allergy and Allergic Diseases* (Edited by Kay A. B.), pp. 940–949. Blackwell Science Press, Oxford, UK.
- Cochran, D. G. (1995) In *Understanding and Controlling the German Cockroach* (Edited by Owens J. M. *et al.*), pp. 171–196. Oxford Press, Oxford, England.
- Cohen M.B., Schuler M.A. and Berenbaum M.R. (1992) A host-inducible cytochrome P450 from a host-specific caterpillar: molecular cloning and evolution. *Proc. Nat. Acad. Sci. USA* **89**, 10920–10924.
- Fernandez-Patron C., Castellanos-Serra L. and Rodriguez P. (1992) Reverse staining of sodium dodecyl sulfate polyacrylamide gels by imidazole-zinc salts: sensitive detection of unmodified proteins. *Biotechniques* **12**, 564–573.
- Feyereisen R., Koener J.F., Farnsworth D.E. and Nebert D.W. (1989) Isolation and sequence of cDNA encoding a cytochrome P450 from an insecticide-resistance strain of the house fly. *Proc. Nat. Acad. Sci. USA* **86**, 1465–1469.
- Gotoh, O. (1993) Evolution and differentiation of cytochrome P450 genes. In *Cytochrome P450 (2nd Edition)* (Edited by Omura T. *et al.*), Kodansha, pp. 255–272. Tokyo, Japan.
- Guengerich F.P. (1978) Separation and purification of multiple forms of microsomal cytochrome P450. *J. Biol. Chem.* **253**, 7931–7939.
- Hodgson, E. (1985) In *Comprehensive Insect Physiology, Biochemistry, and Pharmacology (Vol. II Pharmacology)* (Edited by Kerkut G. A. and Gilbert L. I.), pp. 225–322. Pergamon, Elmsford, NY.
- Koehler P.G. and Patterson R.S. (1986) A comparison of insecticide susceptibility in seven non-resistant strains of the German cockroach. *J. Med. Entomol.* **23**, 298–299.
- Kupfer D. and Bruggerman L.L. (1966) Determination of enzymatic

- demethylation of *p*-chloro-*N*-methylaniline - assay of aniline and *p*-chloroaniline. *Anal. Biochem.* **17**, 502–512.
- Liu N. and Scott J.G. (1996) Genetic analysis of factors controlling high-level expression of cytochrome P450, CYP6D1, cytochrome b₅, cytochrome P450 reductase, and monooxygenase activities in LPR house flies. *Biochem. Genet.* **34**, 133–148.
- Marcus C., Wilson N., Jefcoate C., Wilkinson C. and Omiecinski C. (1990a) Selective induction of cytochrome P450 isozymes in rat liver by 4-*N*-alkylmethylenedioxybenzenes. *Arch. Biochem. Biophys.* **277**, 8–16.
- Marcus C., Wilson N., Keith I., Jefcoate C. and Omiecinski C. (1990b) Selective expression of cytochrome P450 isozymes by 4-*N*-alkylmethylenedioxybenzenes in rat lung cells. *Arch. Biochem. Biophys.* **277**, 17–25.
- Neal J.J. and Reuveni M. (1992) Separation of cytochrome P450 containing vesicles from the midgut microsomal fraction of *Manduca sexta*. *Comp. Biochem. Physiol.* **102C**, 77–82.
- Nelson D.R., Koymans L., Kamataki T., Stegeman J.J., Feyereisen R., Waxman D.J., Waterman M.R., Gotoh O., Coon M.J., Estabrook R.W., Gunsalus I.C. and Nebert D.W. (1996) P450 superfamily — update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**, 1–42.
- Omura T. and Sato R. (1964) The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **239**, 2370–2378.
- Otto S., Marcus C., Pidgeon C. and Jefcoate C. (1991) A novel adrenocorticotropin-inducible cytochrome P450 from rat adrenal microsomes catalyzes polycyclic aromatic hydrocarbon metabolism. *Endocrinology* **129**, 97–982.
- Pidgeon C., Stevens J., Otto S., Jefcoate C. and Marcus C.B. (1991a) Immobilized artificial membrane chromatography: rapid purification of functional membrane proteins. *Anal. Biochem.* **194**, 163–173.
- Pidgeon, C., Marcus, C., Alvarez, F. (1991b) In *Applications of Enzyme Biotechnology* (Edited by Kelly J. W. and Baldwin, T. O.), Plenum, p. 201. New York, NY.
- Ronis M.J., Hodgson E. and Dauterman W.C. (1988) Characterization of multiple forms of cytochrome P450 from an insecticide resistant strain of house fly. *Pestic. Biochem. Physiol.* **32**, 74–90.
- Salinovich O. and Montelano R.C. (1986) Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **156**, 341–348.
- SAS Institute (1990) *SAS/STAT User's Guide (Vols. 1 and 2)*. SAS Institute, Cary, NC.
- Scharf M.E., Bennett G.W., Reid B.L. and Qui C. (1995) Comparisons of three insecticide resistance detection methods for the German cockroach. *J. Econ. Entomol.* **88**, 536–542.
- Scharf M.E., Kaakeh W. and Bennett G.W. (1997) Changes in an insecticide resistant field-population of German cockroach following exposure to an insecticide mixture. *J. Econ. Entomol.* **90**, 38–48.
- Scott J.A., Collins F.H. and Feyereisen R. (1994) Diversity of cytochrome P450 genes in the mosquito *Anopheles albimanus*. *Biochem. Biophys. Res. Comm.* **205**, 1452–1459.
- Scott J.G. and Lee S.S.T. (1993) Purification and characterization of a cytochrome P450 from insecticide susceptible and resistant strains of housefly, before and after phenobarbital exposure. *Arch. Insect Biochem. Phys.* **24**, 1–19.
- Sundseth S.S., Kennell S.L. and Waters L.C. (1989) Monoclonal antibodies to resistance-related forms of cytochrome P450 in *Drosophila melanogaster*. *Pestic. Biochem. Physiol.* **33**, 176–188.
- Valles S.M. and Yu S.J. (1996) Detection and biochemical characterization of insecticide resistance in the German cockroach. *J. Econ. Entomol.* **89**, 21–26.
- Waters L.C. and Nix C.E. (1988) Regulation of resistance-related cytochrome P450 expression in *Drosophila melanogaster*. *Pestic. Biochem. Physiol.* **30**, 214–227.
- Wheelock G.D. and Scott J.G. (1989) Simultaneous purification of a cytochrome P450 and cytochrome b₅ from the house fly. *Insect Biochem.* **19**, 481–488.
- Wheelock G.D. and Scott J.G. (1990) Immunological detection of cytochrome P450 from insecticide resistant and susceptible house flies. *Pestic. Biochem. Physiol.* **38**, 130–139.
- Wheelock G.D. and Scott J.G. (1992a) The role of cytochrome P450 LPR in deltamethrin metabolism by pyrethroid resistant and susceptible strains of house flies. *Pestic. Biochem. Physiol.* **43**, 67–77.
- Wheelock G.D. and Scott J.G. (1992b) Anti-cytochrome P450 antiserum inhibits specific monooxygenase activities in LPR house fly microsomes. *J. Exp. Zool.* **264**, 153–158.
- Zhang M. and Scott J.G. (1996) Cytochrome b₅ is essential for cytochrome P450 6D1-mediated cypermethrin resistance in LPR house flies. *Pestic. Biochem. Physiol.* **55**, 150–156.

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