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Identification, Expression, and Purification of a Pyrethroid-hydrolyzing Carboxylesterase from Mouse Liver Microsomes*[S]

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Carboxylesterases are enzymes that catalyze the hydrolysis of a wide range of ester-containing endogenous and xenobiotic compounds. Although the use of pyrethroids is increasing, the specific enzymes involved in the hydrolysis of these insecticides have yet to be identified. A pyrethroid-hydrolyzing enzyme was partially purified from mouse liver microsomes using a fluorescent reporter similar in structure to cypermethrin (Shan, G., and Hammock, B. D. (2001) *Anal. Biochem.* 299, 54–62 and Wheelock, C. E., Wheelock, A. M., Zhang, R., Stok, J. E., Morisseau, C., Le Valley, S. E., Green, C. E., and Hammock, B. D. (2003) *Anal. Biochem.* 315, 208–222) and subsequently identified as a carboxylesterase (NCBI accession number BAC36707). The expressed sequence tag was then cloned, expressed in baculovirus, and purified to homogeneity. Kinetic constants for a large number of both type I and type II pyrethroid or pyrethroid-like substrates were determined. This esterase possesses similar kinetic constants for cypermethrin and its fluorescent-surrogate (k_{cat} = 0.12 ± 0.03 *versus* 0.11 ± 0.01 s^{-1}). Compared with their *cis*- counterparts, *trans*-permethrin and cypermethrin were hydrolyzed 22- and 4-fold faster, respectively. Of the four fenvalerate isomers the (2*R*)(α*R*)-isomer was hydrolyzed at least 1 order of magnitude faster than any other isomer. However, it is unlikely that this enzyme accounts for the total pyrethroid hydrolysis in the microsomes because both isoelectrofocusing and native PAGE indicate the presence of a second region of cypermethrin-metabolizing enzymes. A second carboxylesterase gene (NCBI accession number NM_133960), isolated during a cDNA mouse liver library screening, was also found to hydrolyze pyrethroids. Both these enzymes could be used as preliminary tools in establishing the relative toxicity of new pyrethroids.

Because of the removal of organophosphates from the market, pyrethroids are now the major class of insecticides used in the United States with more than 1200 metric tons used in American agriculture in 1997 (USGS Pesticide National Synthesis Project, available at ca.water.usgs.gov/pnsp). Both agricultural and residential usage is continuing to grow (4, 5), leading to increased human exposure to these compounds. Pyrethroids are ester-containing insecticides that generally have low acute oral mammalian toxicity due to their rapid metabolism (6, 7). The major routes of pyrethroid metabolism in humans include ester hydrolysis by carboxylesterases (8) and oxidation by cytochrome P450s (8–10) and alcohol dehydrogenases (11). Carboxylesterases are also responsible for the hydrolysis of a large number of other endogenous and xenobiotic ester-containing compounds such as long chain, acyl-CoA esters, and many drugs (12). Their importance in the hydrolysis of pyrethroids in mammals was first demonstrated via the use of carboxylesterase inhibitors (8). These observations were further confirmed when Suzuki and Miyamoto (13) isolated a pyrethroid-hydrolyzing carboxylesterase from rat liver.

Although pyrethroids have relatively low mammalian toxicity, a natural variation in esterase activity or the presence of other esterase substrates and/or inhibitors may compromise the ability of an organism to detoxify these pesticides (12). Furthermore, both the levels of cytochrome P450s and carboxylesterases have been shown to be significantly lower in prenatal/newborn rats than in adults (14, 15). These data suggest an increased susceptibility of fetuses and young infants to pyrethroids.

Although it has been established that carboxylesterases are involved in the detoxification of pyrethroids (16), most of the work on pyrethroid toxicity in mammals has been based solely on investigations in whole animals (16) or liver microsomes (8, 17). Therefore, the specific carboxylesterases that hydrolyze pyrethroids have not yet been characterized (18). Several carboxylesterase isozymes with various substrate specificities have been isolated from a variety of organisms (12). However, none have displayed any specificity for pyrethroids. Therefore, we decided to identify a pyrethroid-hydrolyzing esterase(s) and to clone, express, and purify it for further characterization. This esterase was isolated from a common laboratory mouse strain (Swiss-Webster) due to the use of mice as animal models for studying the effects of both endogenous and xenobiotic compounds (19). By isolating specific carboxylesterases we may begin to understand their relative importance in the hydrolysis of a range of different esters.

MATERIALS AND METHODS

Chemicals—All substrates used in this study are numbered according to the system outlined in Fig. 1. The compounds (*R*/*S*)-α-cyano(6-methoxy-2-naphthyl)-methyl acetate (**A1**), (*R*/*S*)-α-cyano(6-methoxy-2-naphthyl)-methyl-(*R*/*S*)-*trans*/*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (**A2**), (*R*/*S*)-α-cyano(6-methoxy-2-naphthyl)-methyl-(*R*)-(−)-2-(4-chlorophenyl)-3-methyl butanoate ((α*R*/*S*)(2*R*)-**A3**), (*R*/*S*)-α-cyano(6-methoxy-2-naphthyl)-methyl-(*S*)-(+)-2-(4-chlorophenyl)-3-methyl butano-

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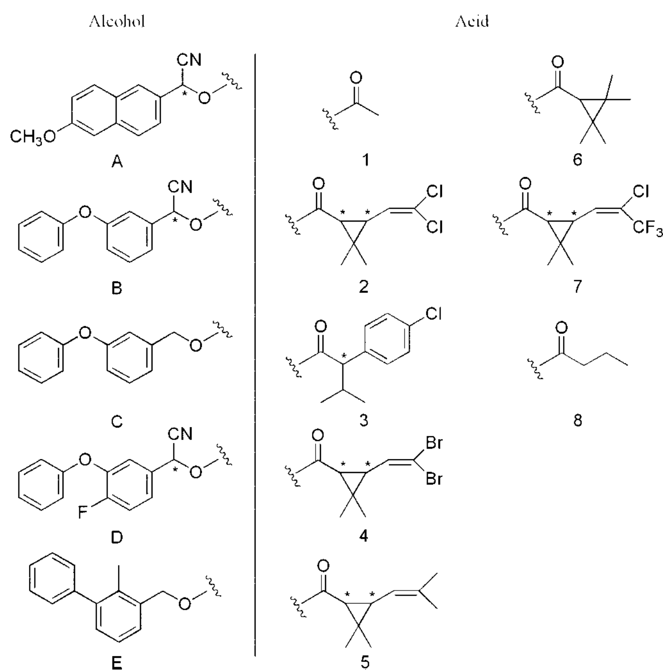

FIG. 1. **Nomenclature scheme for the various pyrethroids and pyrethroid mimics used in this study.** The alcohol moiety is indicated by a *letter*, and the acid moieties are indicated by a *number*. Stereogenic centers are indicated by an *asterisk*.

ate ((αR/S)(2S)-**A3**), and fenvalerate isomers ((αR)(2R)-**B3**), ((αR)(2S)-**B3**), ((αS)(2R)-**B3**), ((αR)(2R/S)-**B3**), and ((αS)(2R/S)-**B3**) were all previously synthesized in this laboratory (1, 2, 20). All fenvalerate isomers were ~96% enantiomeric excess. Malathion, cypermethrin (**B2**), esfenvalerate ((αS)(2S)-**B3**), (*trans*/*cis*)-permethrin (**C2**), and (*cis*)(αS)(1R,3R)-deltamethrin ((*cis*)(αS)(1R,3R)-**B4**) were obtained from Chem Services Inc. (West Chester, PA). Both *cis*- and *trans*-permethrin (**C2**) were gifts from ICI Agrochemicals. Both *cis*- and *trans*-cypermethrin (**B2**), α-cypermethrin ((αR)(1S,3S)- and (αS)(1R,3R)-**B2**) and ζ-cypermethrin ((αS)(1R/S,3R/S)-**B2**) (S/R ratio, 89.7/10.3) were gifts from FMC Corp. (Princeton, NJ). All isomers unless otherwise stated were ~99% of the isomer mixture indicated. All other chemicals used in this study were either purchased from Sigma-Aldrich or Fisher. Synthesis of compounds **A4–8** is outlined in the supplemental material.

Isolation of a Cypermethrin-hydrolyzing Carboxylesterase—The following procedures were performed at 4 °C unless otherwise stated. Livers from male Swiss-Webster mice (Charles River Laboratories Inc., Wilmington, MA) were perfused with 1.15% KCl (~10 °C) and homogenized (2 × 30 s, 10,000 rpm, Polytron homogenizer, Brinkmann) in Buffer A (20 mM sodium phosphate buffer, pH 7.4, 5 mM EDTA, and 1 mM 1-phenyl-2-thiourea). The supernatant was collected by centrifugation of the homogenized sample (10,000 *g*, 20 min). The pellet was resuspended in buffer A (ratio 1:10), and the supernatant was collected by centrifugation (10,000 × *g*, 20 min). After centrifugation of the combined supernatants (100,000 × *g*, 1 h), the microsomes were washed in fresh buffer A, re-pelleted (100,000 × *g*, 1 h), resuspended in 10 mM sodium phosphate, pH 7.0, 20% glycerol, 0.5 M EDTA, and 1 mM 1-phenyl-2-thiourea, and stored at −80 °C.

Microsomes were solubilized using the previously described protocol (21). Briefly, octyl pyranoglucoside (final concentration, 1% w/v) was added to the microsomes, which were then placed on a rotating wheel for 1 h. The resulting protein mixture was concentrated, mixed 1:1 with Tris-glycine native sample buffer (Invitrogen), loaded onto a 7% polyacrylamide gel tube, and run overnight on a preparative electrophoresis unit (Mini Prep Cell, Bio-Rad). The pyrethroid activity was screened with **A2**. Active fractions were pooled and concentrated by filtration (30-kDa cutoff; Millipore, Billerica, MA).

Identification of a "cypermethrin-hydrolyzing carboxylesterase" was achieved via separation of the semi-purified protein on two-dimensional PAGE. A pH 4–7 immobilized pH gradient strip (13 cm, Amersham Biosciences) was rehydrated overnight in a solution (250 μl) containing semi-purified protein (10–15 μg), 5% glycerol, and 2% immobilized pH gradient (4–7) buffer and then focused on a 2117 Multiphore II IEF[1] unit (LKB, Bromma, Sweden). A second SDS-PAGE dimension was run by first equilibrating the strip in SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, and bromphenol blue) with 1% (w/v) dithiothreitol for 15 min. This solution was then replaced with SDS equilibration buffer that included 0.25% (w/v) iodoacetamide, and the strip was equilibrated for 15 min. This strip was then loaded onto a 10% SDS-PAGE gel and sealed with 1% IsoGel-agarose. The gel was run at a constant current of 30 mA in a vertical slab gel unit (Hoeffer) at 10 °C. After the isoelectrofocusing step, a second immobilized pH gradient strip was cut into ~1–2-mm slices and screened for **A2** activity. The strip was then aligned with the SDS-PAGE gel, and the appropriate spots were sequenced. In-gel trypsin digestion followed by peptide mass-mapping and mass spectrometry protein sequencing were performed by the Molecular Structure Facility (University of California at Davis).

cDNA Library Screening—The mRNA from Swiss-Webster mouse liver (~4 g, pooled from 4 mice) was isolated via a two-step procedure in which total RNA was initially extracted (RNeasy and Oligotex Kits, Qiagen, Valencia, CA). First strand cDNA was synthesized using Superscript reverse transcriptase II (Invitrogen) and the BD SMART mRNA amplification Kit (BD Biosciences) according to the manufacturer's procedure. The cDNA generated was used as the template in the subsequent PCR reaction. PCR primers were designed to one of the sequenced peptides, NLFGSEDLK (5′-AATTTGTTYGGTTCAGAGGATCTGAAG-3′) and, in addition, to either the conserved catalytic serine region IFGESAGGT (5′-TGTGCCACCTGCTGACTCTCCAAAAAT-3′) or the C-terminal region of the putative mouse carboxylesterase (NCBI accession number BAC36707) (5′-CTACAACTCTTTGTGCCTCTCCTGAGA-3′). A DNA fragment (399 bp or 1.4 kilobases) was amplified with *Taq* DNA polymerase (Invitrogen) using the above primers and employing the following conditions: 95 °C for 3 min, 30 cycles of 95 °C for 45 s, 45 °C for 5 min, and 72 °C for 2 min, and completed with 72 °C for 10 min. The DNA fragment was ligated into a pCR2.1-TOPO treated vector (TA cloning kit, Invitrogen), and the sequence was verified (DNA Sequencing Facility, University of California at Davis).

Approximately 1% of a mouse (C57Bl/6J) liver cDNA library (1 × 10^7 independent clones) (Invitrogen) was screened using the horseradish peroxidase-labeled PCR fragment generated above (ECL, Amersham Biosciences). Potentially positive clones were transformed into *Escherichia coli* DH5α and verified by DNA sequencing.

Cloning, Expression, and Purification of the Pyrethroid-hydrolyzing Carboxylesterase—An Expressed Sequence Tag (Clone ID 1450336, Invitrogen) was purchased that contained the gene (NCBI accession number BC055062) corresponding to the protein identified by mass spectrometry. The gene was excised from the original vector (pME18S-FL3) using StuI/XbaI and ligated into a similarly cut vector pBacPAK8 (BD Biosciences). The recombinant baculovirus containing the esterase gene was generated (22) and expressed in High five *Trichoplusia ni* cells (2 liters, 1 × 10^6/ml) with a multiplicity of infection of ~0.1. At 72 h post-infection the cells were pelleted (2,000 × *g*, 20 min), resuspended in 100 mM Tris-HCl, pH 8.0 (containing 1 mM dithiothreitol, 1 mM EDTA, and 1 mM 1-phenyl-2-thiourea), and homogenized (2 × 30 s; Polytron homogenizer, Brinkmann). The supernatant that was generated after centrifugation (10,000 × *g*, 20 min) underwent further centrifugation (100,000 × *g*, 1 h) to pellet the microsomal fraction. The latter supernatant was diluted 5-fold with buffer B (20 mM Tris HCl, pH 8.0, 1 mM dithiothreitol), loaded onto a DEAE ion exchange column (3 × 15 cm; Amersham Biosciences), and washed in the same buffer. After a second wash with 75 mM NaCl in Buffer B, the esterase was eluted with 125 mM NaCl in Buffer B and detected by measuring *p*-nitrophenyl acetate (*p*NPA) hydrolysis. After concentration, the targeted protein was further purified using a preparative isoelectrofocusing unit (Bio-Rad; pH 3–8). The esterase-containing fractions were then combined, mixed with an equal volume of Buffer B containing 4 M KCl, and loaded onto a phenyl-Sepharose column (1.6 × 9 cm; Amersham Biosciences) that had been equilibrated in advance with buffer B containing 2 M KCl. The esterase was eluted at ~50 mM KCl by running a linear salt gradient (2 to 0 M KCl) and was then stored at −80 °C. The enzyme could be purified in a similar fashion from the microsomal fraction generated after the second centrifugation step (100,000 × *g*). In this case, solubilization (1% (w/v) octyl glucopyranoside) was necessary before application on the DEAE column.

Characterization of the Carboxylesterases—Protein concentration

[1] The abbreviations used are: IEF, isoelectrofocusing; *p*NPA, *p*-nitrophenyl acetate; GCMS, gas chromatography-mass spectroscopy.

was determined with the Pierce BCA assay (Pierce) using bovine serum albumin as the standard. SDS-PAGE and native PAGE were performed using 12% Tris-glycine gels (Invitrogen), whereas IEF PAGE was performed using IEF 3–7 gels (Invitrogen). The pI was estimated by incubating 2.5-mm IEF gel strips at room temperature for 30 min in water (500 μl) and measuring the pH with a pH meter.

De-glycosylated protein was obtained by employing N-glycosidase F (New England Biolabs, Beverly, MA) using the procedure provided by the manufacturer. Briefly, the esterase (100 μg) was denatured with the addition of 0.5% SDS and 1% β-mercaptoethanol (final concentration) for 10 min at 100 °C. After cooling on ice, 50 mM sodium phosphate, pH 7.5, 1% Nonidet P-40 (final concentration), and *N*-glycosidase F (5000 units) were added, and the consequent solution (final volume 100 μl) was incubated for 1 h at 37 °C.

Enzyme Activity Assays—Esterase activity was detected using a number of different substrates: *p*NPA, malathion, individual pyrethroid isomers or racemic mixtures, and fluorescent-pyrethroid surrogates (**A1–8**). Because all isomers or isomer mixtures were at least 98% of the stereochemistry indicated, quantities were sufficient to satisfy Michaelis-Menten conditions. Briefly, *p*NPA hydrolysis was monitored for 2 min at 405 nm according to the methods of Ljungquist and Augustinsson (23), as modified in Wheelock *et al.* (24). The malathion assay was conducted according to the method previously described (25, 26). GCMS assays (2) were modified by adding 1 μl of substrate (**B2–4**, **7**, **C2**, **D2**, and **E7**; 25 mM in ethanol) to 0.5 ml of the enzyme (1–30 μg/ml). The enzyme mixture was incubated for 20 s to 10 min depending on the substrate. Ethyl acetate (250 μl) and brine (250 μl) were added to each sample, after which the mixture was vortexed. An internal standard (3-(4-methoxy)-phenoxybenzaldehyde; 80 μM final concentration) was added to 100 μl of the ethyl acetate solution, and this solution was analyzed by GCMS. The method detection limits for 3-phenoxybenzaldehyde and 4-fluoro-3-phenoxybenzaldehyde were calculated by heat-killing the carboxylesterase (3 μg) and running a mock assay without the addition of substrate. The assay solutions were spiked with either 3-phenoxybenzaldehyde or 4-fluoro-3-phenoxybenzaldehyde (0.5 μM final concentration; 5× lowest detectable standard), brine (250 μl) and ethyl acetate (250 μl) were added, and the mixture was vortexed. This procedure was repeated seven times, and the ethyl acetate extract of each replicate was analyzed by GCMS. Fluorescent assays were conducted by measuring the production of 6-methoxynaphthaldehyde for 9 min after the addition of the substrates (**A1–8**) using an excitation wavelength of 330 nm (bandwidth, 35 nm) and an emission wavelength of 465 nm (bandwidth, 35 nm) (2). Protein concentration varied from 1 ng to 50 μg for these fluorescent assays. All assays were performed in buffer B at 30 °C. No more than 10% of the substrate was hydrolyzed during the assay, and solvent content never exceeded 1% of the total assay volume.

RESULTS

Isolation of a Pyrethroid-hydrolyzing Carboxylesterase from Mouse Liver—To isolate a "pyrethroid-hydrolyzing esterase" microsomes were prepared from murine livers. Cypermethrin and **A2** were employed to characterize the behavior of esterases on both native and IEF PAGE. At least 90% of the **A2** activity was associated with esterases located in the lower section of both native and IEF gels (Fig. 2). Further analysis revealed a second region of pyrethroid-hydrolyzing esterases accounting for 50–60% of the total cypermethrin activity (Fig. 2). Because the esterases that migrate faster on native PAGE (Fig. 2*A*) had higher apparent hydrolase activity specific for cypermethrin and **A2** when compared with *p*NPA (5–10% of the total *p*NPA activity), we concentrated on purifying a pyrethroid hydrolase from this area using **A2** as a reporter. Effective separation of the pyrethroid-hydrolyzing carboxylesterase(s) from the majority of the other esterases was achieved using a preparative electrophoresis unit (Table I). To separate the remaining proteins, IEF PAGE was employed together with SDS-PAGE to run a two-dimensional gel. Three protein spots on this two-dimensional gel were found to have **A2** hydrolysis activity.

Identification of a Pyrethroid-hydrolyzing Carboxylesterase—Mass spectral sequence analysis of the protein spot with the highest pyrethroid activity produced two peptide sequences (PYTEEEE; LQFWTK) that were identical to sequences in a putative carboxylesterase (NCBI accession number BAC-36707). A third peptide (QNDNLFGSEDLK) was also found to correspond to the same putative carboxylesterase with a one-amino acid difference from the reported sequence (reported sequence, QNDNLMGSEDLK). The remaining two protein spots that possessed pyrethroid activity were also analyzed. Although they contained some of the same peptides that corresponded with the first carboxylesterase, they were both contaminated with other proteins, and therefore, no conclusions were drawn (peptides observed were GFFSTGDQHAK and QQNLVHFGG; residues that are different in NCBI accession number BAC36707 are underlined).

Screening cDNA Library—Using the information gathered from protein sequencing we attempted to isolate the corresponding gene (NCBI accession number BC055062) from a mouse liver cDNA library using a probe generated by PCR. Although the gene of interest was not isolated from these attempts, two other putative carboxylesterase genes were found (NCBI accession numbers NM_133960 and NM_144930).

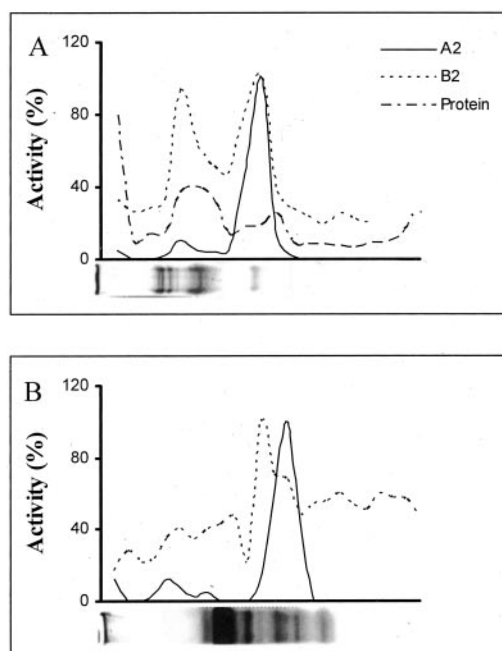

FIG. 2. **To determine the properties of the protein(s) responsible for the hydrolysis of pyrethroids, solubilized mouse liver microsomes were loaded onto native PAGE (*A*) or IEF PAGE (*B*) and stained with α and β-naphthyl acetate.** Pyrethroid-hydrolyzing activity using **A2** and cypermethrin (**B2**) was found by cutting a second unstained lane from the same gel into 2.5-mm slices. These slices were soaked in buffer B and assayed according to the method outlined under "Materials and Methods." Activities were normalized to a percentage for easy comparison (by setting the highest value of each assay at 100%). Approximately 54% of the original **A2** hydrolysis activity was recovered.

TABLE I
Partial purification of a pyrethroid-hydrolyzing carboxylesterase from mouse liver microsomes

	Total protein	Total activity[a]	Specific activity	Purification fold	Activity
	mg	*nmol/min*	*nmol/min/mg*		*%*
Microsomes	22	ND[b]	ND		
Solubilized microsomes[c]	13	2.3	0.18	1.0	100
Preparative electrophoresis	0.054	0.79	15	81	34

[a] Enzyme activity was measured using **A2** as described under "Materials and Methods."

[b] Not determined.

[c] Microsomes were solubilized according to the methods of Huang *et al.* (21).

TABLE II
Purification of a recombinant pyrethroid-hydrolyzing carboxylesterase BAC36707 expressed in baculovirus

Step	Total activity[a]	Total protein	Activity	Specific activity	Purification fold
	nmol/min	*mg*	*%*	*nmol/min/mg*	
Crude	1900 ± 230	3600 ± 130	100	0.54 ± 0.06	1.00
10,000 × *g*	1200 ± 57	2100 ± 45	63	0.56 ± 0.03	1.04
Supernatant 100,000 × *g*	980 ± 80	1700 ± 31	51	0.58 ± 0.05	1.08
DEAE	200 ± 11	31 ± 3	11	6.5 ± 0.9	12
Preparative IEF (3–8)	280 ± 12	3.5 ± 0.1	14	78 ± 10	146
Phenyl-Sepharose	159 ± 1	1.4 ± 0.1	8	110 ± 6	209

[a] Enzyme activity was measured using **A2** as described under "Materials and Methods."

Expression and Purification—The pyrethroid-hydrolyzing carboxylesterase (NCBI accession number BAC36707) was expressed by infecting *T. ni* High five cells with the recombinant baculovirus. Esterase activity was subsequently found in both the microsomal pellet and the supernatant of centrifugation (100,000 × *g*), and thus, the esterase was purified separately from both sources. The most effective step in the purification of this esterase was performed by the preparative IEF unit, which increased the specific activity of the enzyme 10-fold (Table II). The two additional carboxylesterase genes were both expressed and purified in a similar fashion to the first (data not shown).

Characterization of the Pyrethroid-hydrolyzing Carboxylesterase—The N-terminal sequence (DSASPIRNTH) confirmed that the expressed protein gave an exact match to the mature protein with the removal of the signal peptide (27 amino acids). IEF PAGE analysis revealed 3 separate bands that had an average pI of 5.8 ± 0.2, similar to that determined from the mouse liver microsomes. Initial experiments using mass spectrometry indicated that the esterase was heavily glycosylated, and hence, it was difficult to determine the mass of the protein. SDS-PAGE indicated that the purified enzyme had two equally intense bands. Only the lower of the two bands remained after removal of the glycosylation, suggesting that approximately half of the protein was not glycosylated.

Kinetic Analysis of the Pyrethroid-hydrolyzing Carboxylesterase—To determine the specificity of the esterase BAC36707 toward pyrethroids, the kinetic constants of a number of general esterase substrates were compared with both pyrethroid mimics (**A2**, **A3**, and **A5**) and cypermethrin (Table III). For this particular enzyme **A2** seems to be an excellent mimic of cypermethrin because there is little difference between these two substrates (K_m and k_{cat}). In addition, the esterase was found to have similar K_m values to other pyrethroids, such as ($\alpha R/S$)(2R)-**A3** and **A5** but with much lower k_{cat} values. Comparison to acetate substrates such as **A1** and *p*NPA suggests that the enzyme predominantly recognizes the acid component of the pyrethroid rather than the alcohol. Although *p*NPA has an extremely high k_{cat} value (94 ± 2 s^{-1}), the K_m value for this substrate was ~200–600-fold higher than that of the pyrethroids.

Specific activity was determined for both type I and II pyrethroids and their mimics (Table IV). In general, the highest specific activity in all the purified enzymes and solubilized microsomes was associated with esters with simple acids such as acetate and butyrate (*p*NPA, **A1** and **A8**). Of the pyrethroids, those that contained either a chrysanthemic acid (**A5**) or a close derivative (**A2**, **B2**, **D2**) were found to be hydrolyzed fastest by the carboxylesterases BAC36707 and NM_133960. Also, a distinct difference was observed in the hydrolysis of **A5**, which contains a chrysanthemic acid, and **A6**, which contains a tetramethylcyclopropane carboxylate.

To examine the importance of the chlorine atoms in cypermethrin hydrolysis, they were replaced with both methyl groups (**A5**) and other halogens (deltamethrin, **A4**, λ-cyhalothrin, and **A7**). Apart from **A5**, the hydrolysis of these compounds by esterase BAC36707 was significantly reduced, suggesting that the esterase has a specific preference for the size and properties of the chlorine atoms. Although commercial deltamethrin is only one optical isomer, **A4** is racemic and, hence, a better comparison of the substitution effects of bromine for chlorine.

Two other mouse liver carboxylesterases (NM_133960 and NM_659179) were compared with the pyrethroid esterase BAC36707. In general, carboxylesterase NM_133960 was found to have a similar pyrethroid hydrolysis profile as esterase BAC36707 (Table IV). The only striking difference was the lack of stereoselectivity of carboxylesterase NM_133960 for λ-cyhalothrin, whereas the esterase BAC36707 had a slight preference for the *cis* isomer. The second of the two carboxylesterases, NM_659179, did not hydrolyze any of the pyrethroid substrates or their mimics, although it did show high activity with the other substrates (*p*NPA, **A1** and **A8**). This observation confirmed that not all carboxylesterases hydrolyze pyrethroids.

It has been previously determined in mammals that there is a preference for *trans*-pyrethroids containing the chrysanthemic acid (or its halogenated derivatives) over *cis* (8, 17). For esterase BAC36707, *trans*-cypermethrin and permethrin were hydrolyzed ~4- and 22-fold faster, respectively, than their *cis* counterparts (Table V). Interestingly, α-cypermethrin, which is a mixture of two of the *cis* isomers (Fig. 3), was hydrolyzed slower than all four isomers of the *cis*-cypermethrin. The specific activities of racemic cypermethrin and ζ-cypermethrin (four isomers with the *S* configuration at the α-cyano center) were found to be relatively similar, suggesting that the stereochemistry at the α-cyano center does not have a major influence on the hydrolysis of cypermethrin by this esterase.

In a previous paper we showed that there was a preference in both human and mice microsomes for ($\alpha R/S$)(2R)-**A3** over ($\alpha R/S$)(2S)-**A3** (2). Esterase BAC36707 also favored the 2R isomer, which was metabolized 100-fold faster than the corresponding 2S (Table IV). Such a large difference was not observed when the fenvalerate (**B3**) isomers were analyzed (Table V). In addition, αR-fenvalerate was more easily hydrolyzed than the αS-isomer.

DISCUSSION

Because it has been determined that the majority of carboxylesterase activity in mammals is associated with the microsomal fraction of the liver (12), a pyrethroid-hydrolyzing carboxylesterase was partially purified from liver microsomes. Compound **A2** (1, 2), a fluorescent substrate similar in structure to cypermethrin, was employed to purify the esterase. **A2** was used because this assay is faster than the end point GCMS assay employing the actual pyrethroids (2). This rapid fluorescent assay reduced the overall length of the purification process and consequently limited enzyme degradation. However, one of the shortcomings of this mimic was that it failed to identify esterases that specifically recognized the alcohol component of cypermethrin. This was evident on both native and IEF PAGE

TABLE III
Kinetic constants determined for the purified recombinant pyrethroid-hydrolyzing carboxylesterase (NCBI accession number BAC36707)

Substrate	K_m	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	$\mu M^{-1}\ s^{-1}$
p-Nitrophenyl acetate	286 ± 16	94 ± 2	0.33 ± 0.01
Cypermethrin (**B2**)	0.50 ± 0.15	0.12 ± 0.03	0.24 ± 0.06
A1	NM[a]	NM	1.2 ± 0.1
A2	2.2 ± 0.2	0.11 ± 0.01	0.040 ± 0.004
(α*R*/*S*) (2*R*)-**A3**	0.89 ± 0.08	0.067 ± 0.012	0.077 ± 0.021
A5	0.96 ± 0.13	0.099 ± 0.005	0.11 ± 0.01

[a] NM, not measurable. K_m and k_{cat} values could not be calculated due to substrate insolubility.

TABLE IV
Specific activity for the hydrolysis of various substrates by the purified, recombinant carboxylesterases (NCBI accession numbers BAC36707, NP_659179, and NM_133960) and solubilized mouse microsomes

Substrate	BAC36707	NP_659179	NM_133960	Microsomes[a]
	nmol/min/mg	*nmol/min/mg*	*nmol/min/mg*	*nmol/min/mg*
p-Nitrophenol acetate	140,000 ± 9400	34,000 ± 3200	17,000 ± 730	1820 ± 94
Malathion	ND[b]			
Cypermethrin (**B2**)	134 ± 14	ND	72 ± 15	0.7 ± 0.2
λ-Cyhalothrin (**B7**)	10.5 ± 1.4		6.4 ± 1.4	
Cyfluthrin (**D2**)	179 ± 12	0.38 ± 0.08	56 ± 3	0.11 ± 0.04
(*cis*)(α*S*)(1*R*,3*R*)-Deltamethrin (**B4**)	0.98 ± 0.21			
Bifenthrin (**E7**)	2.5 ± 0.9		2.6 ± 0.1	
A1	63,000 ± 3500	200 ± 2	6600 ± 330	1310 ± 130
A2	113 ± 5	0.81 ± 0.11	61 ± 1	0.50 ± 0.01
(α*R*/*S*)(2*R*)-**A3**	65 ± 11	0.16 ± 0.10	20 ± 1	0.26 ± 0.02
(α*R*/*S*)(2*S*)-**A3**	0.29 ± 0.02	ND	0.63 ± 0.03	ND
A4	3.4 ± 0.1	ND	2.7 ± 0.2	ND
A5	96 ± 5	1.0 ± 0.2	29 ± 1	0.33 ± 0.01
A6	3.8 ± 0.1	1.3 ± 0.3	2.9 ± 0.2	ND
cis-**A7**	6.3 ± 0.4	ND	13 ± 1	0.10 ± 0.02
trans-**A7**	2.4 ± 0.1	ND	14 ± 1	0.10 ± 0.01
A8	240,000 ± 10,000	640 ± 100	21,000 ± 3000	2960 ± 110

[a] Microsomes were solubilized according to the methods of Huang (21).
[b] ND, not detected. The method detection limit for 3-phenoxybenzaldehyde is 0.15 μM; for 4-fluoro-3-phenoxybenzaldehyde, it is 0.12 μM.

TABLE V
Specific activities for pyrethroid isomer-specific hydrolysis

Substrate	BAC36707[a]
	nmol/min/mg
Cypermethrin (**B2**)	134 ± 14
cis-Cypermethrin	60 ± 6
trans-Cypermethrin	246 ± 16
α-Cypermethrin	45 ± 3
ζ-Cypermethrin	95 ± 14
(α*R*/*S*)(2*R*)-Fenvalerate (**B3**)	29 ± 4
(α*R*/*S*)(2*S*)-Fenvalerate	0.94 ± 0.31
(α*S*)(2*S*)-Fenvalerate	ND[b]
(α*R*)(2*S*)-Fenvalerate	1.4 ± 0.3
(α*R*)(2*R*)-Fenvalerate	75 ± 13
(α*S*)(2*R*)-Fenvalerate	7.2 ± 1.1
Permethrin (**C2**)	239 ± 27
cis-Permethrin	27 ± 5
trans-Permethrin	597 ± 22

[a] Purified carboxylesterase, NCBI accession number BAC36707.
[b] ND, not detected.

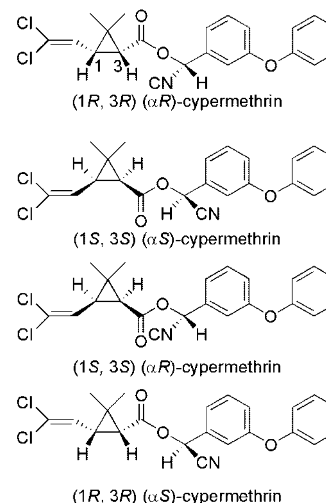

FIG. 3. **Enantiomers of *cis*-cypermethrin.** (1*S*, 3*S*)(α*R*)-cypermethrin and (1*R*, 3*R*)(α*S*)-cypermethrin are the two isomers of α-cypermethrin.

(Fig. 2) in which the hydrolysis of **A2** indicated one predominant pyrethroid-hydrolyzing peak, whereas a second peak in both gels showed cypermethrin-hydrolysis activity. In addition, high protein concentration has been observed to quench the fluorescence of the reporter group (2) and may contribute to our inability to observe the second pyrethroid-hydrolysis peak in native PAGE (Fig. 2*A*).

After partial purification, protein sequencing identified this protein as a carboxylesterase (NCBI accession number BAC36707). Employing the carboxylesterase classification system proposed by Satoh and Hosokawa (12) this carboxylesterase belongs to the CES 2 family. It is strongly homologous with carboxylesterases AT41 (NCBI accession number BAA23605; 83% identity, 89% similarity) found in golden hamster (*Mesocricetus auratus*) (27) and human carboxylesterase 2 (71% identity, 82% similarity) (28).

The translated genetic sequence indicated that the esterase contained the following characteristics of carboxylesterases. First, the esterase has the catalytic triad common to all α/β-hydrolase fold proteins; that is, a serine nucleophile (residue 201), a histidine base (430), and a glutamic acid (318). Second, it contains a C-terminal retention sequence (HKEL) characteristic of proteins retained on the luminal side of the endoplasmic reticulum (29, 30). Third, the esterase was predicted to have a cleavable signal peptide of 27 amino acids at the N terminus.

The signal peptide cleavage site was observed to be identical to that found in the hamster AT41 carboxylesterase gene. Carboxylesterase signal peptides are usually hydrophobic (31) and are cleaved from the protein as it passes through the endoplasmic reticulum membrane (32). Finally, carboxylesterases are usually glycosylated. Unlike those in the CES 1 family, CES 2 carboxylesterases usually have only one glycosylation site (27). In the case of the pyrethroid-hydrolyzing carboxylesterase the glycosylation site was found at residue 249 of the mature protein. This corresponds to the same position in both hCE 2 and hamster AT41.

After the cloning, expression, and purification of esterase BAC36707, we wanted to test if the protein had the same relative characteristics of the originally isolated, pyrethroid-metabolizing esterase. The recombinant enzyme was found to behave similarly to the isolated esterase on both IEF and native PAGE (data not shown). Together with the observed **A2** hydrolysis activity, the recombinant enzyme was judged to be the same esterase as that isolated from mouse liver microsomes.

During the expression and purification of the esterase it was found that the protein was not as closely associated with the microsomes as expected. After homogenization and centrifugation at 100,000 × *g*, 63% of the enzyme was found in the cytosol, whereas only 11% was found in the microsomes. During the course of the purification, SDS-PAGE revealed that although the protein had similar specific activities when purified from either the microsomes or the cytosol, the two proteins appeared to be slightly different sizes (data not shown). Additionally, in both the microsome- and cytosol-purified proteins there were two bands at ~60 kDa. The difference in apparent molecular mass is thought to be due to a number of factors, one of which is inefficient glycosylation of the protein. Similar results have been observed with other carboxylesterases expressed in insect cells (33). A second reason for the mass discrepancy may be inefficient processing of the N-terminal sequence, which was primarily a problem in the microsome-purified sample. Homogeneity could be attained by expressing the protein in a controlled fashion, in which both the signal sequence and the C-terminal retention sequence are removed.

Two other putative carboxylesterases were isolated during the screening of a cDNA library with fragments of the isolated esterase (NCBI accession number BAC36707). One of these carboxylesterases (NCBI accession number NP_598721) is also in the CES 2 family and has relatively high sequence similarity to the original esterase (BAC36707) (74% identity, 83% similarity), whereas the second of these esterases was closer to mouse esterase 22 (70% identity, 79% similarity) (34), which is in the CES 1 family. Both these carboxylesterases were expressed, purified, and then used for comparison with the original carboxylesterase.

All the recombinant carboxylesterases were found to hydrolyze esters with simple acids, such as acetate and butyrate (*p*NPA, **A1** and **A8**), much faster than any of the pyrethroid substrates (Table IV). Although these substrates are chemically easier to hydrolyze, the acid component of the substrate is also important. This is evident with carboxylesterase NP_659179, which hydrolyzes *p*NPA 170-fold faster than **A1**, although both contain acetate moieties. In contrast, there is only an approximate 2-fold difference between the hydrolysis rates of these two substrates with carboxylesterases BAC36707 and NM_133960.

The recombinant pyrethroid-hydrolyzing carboxylesterases BAC36707 and NM_133960 reflected some of the trends observed in previous pyrethroid studies involving either whole animals/cells or liver microsomes. Type I pyrethroids, which do not contain an α-cyano group (*e.g.* permethrin), are known to be less toxic than type II pyrethroids (*e.g.* cypermethrin) to both whole animals (35) and to cells (36). This was supported by the observation that both BAC36707 and NM_133960 hydrolyzed permethrin faster than cypermethrin (Table IV). In addition, it was observed that both these enzymes hydrolyzed cypermethrin at least 10–100-fold faster than bifenthrin, λ-cyhalothrin, or deltamethrin. The acute oral toxicity (in corn oil) of these pyrethroids in rats is ~2–3 times higher than that of cypermethrin (35). This suggests that there is a direct link between an increase in carboxylesterase-catalyzed hydrolysis rates of pyrethroids and a decrease in toxicity. That stated, it has previously been shown that the relative toxicological effects of pyrethroids are not solely based on their detoxification (3).

It has previously been suggested that the hydrolysis of *trans*- and *cis*-pyrethroids are likely to be catalyzed by different carboxylesterases (17). Comparison of the hydrolysis rates of *cis*-and *trans*-permethrin (Table V) revealed that *trans* was hydrolyzed ~22-fold faster; which is similar to the hydrolysis ratios found in mouse liver microsomes (42.5-fold) (17). In addition, this supports data indicating that in general the *cis*-pyrethroids are more toxic than *trans* (8) and that this is due in part to their relative rates of detoxification. Interestingly, the difference in hydrolysis rates between *cis*- and *trans*-cypermethrin was not as great as for *cis* and *trans*-permethrin (permethrin 22-fold *versus* cypermethrin 4-fold). This observation may be due to the additional stereocenter introduced by the α-cyano group.

Analysis with each of the four fenvalerate isomers also supports previous studies (37, 38). In those studies both the $(\alpha S)(2R)$- and $(\alpha R)(2R)$-fenvalerate were metabolized by mouse liver microsomes at similar rates, whereas with our esterase BAC36707 there is at least a 10-fold preference for the $(\alpha R)(2R)$ enantiomer (Table V). In addition, significant hydrolysis of the $(\alpha S)(2S)$-enantiomer was observed in the previous study (~5-fold less than the $(\alpha S)(2R)$-isomer), which in contrast could not be hydrolyzed at all by esterase BAC36707. These slight differences are undoubtedly due to the presence of other enzymes that can hydrolyze different isomers of fenvalerate. $(\alpha S)(2S)$-Fenvalerate is commercially sold as esfenvalerate and has been shown to be more toxic than other pyrethroids, such as permethrin and cypermethrin (35). Although $(\alpha S)(2S)$-fenvalerate is a more effective insecticide than other pyrethroids, our work would predict it to have higher murine toxicity due to its resistance to degradation by carboxylesterases.

The likelihood of other carboxylesterases being involved in the hydrolysis of pyrethroid or pyrethroid-like compounds is supported by a comparison of tetramethylcyclopropane carboxylate (**6**) with chrysanthemate (**5**). In a previous study (8) microsomes were found to hydrolyze a tetramethylcyclopropane carboxylate (**6**) considerably faster (relative to that of chrysanthemate (**5**)) than either of the two recombinant enzymes, BAC36707 and NM_133960. In addition, one would expect some stabilization of the ester carbonyl over the tetrahedral intermediate or transition state due to the aromatic nature of the cyclopropane. Such stabilization should be greater with the isobutylene substitution of chrysanthemic esters (**5**) over tetramethyl ester (**6**).

No malathion hydrolysis activity was observed for the esterase BAC36707. This confirmed earlier work in which microsomes from a number of different mammals were separated by chromatofocusing and the fractions were analyzed using a variety of substrates (39). It was discovered that in rat and rabbit liver microsomes both malathion and fenvalerate were hydrolyzed by the same peaks, in contrast to mouse.

In isolating, cloning, and expressing a specific carboxylesterase involved in pyrethroid-hydrolysis, we have demonstrated

that this enzyme is likely to be involved in the metabolism of these pesticides. There is at least a 25–300-fold increase in the specific activity from the mouse microsomes to the purified pyrethroid-hydrolyzing esterases, suggesting that these enzymes comprise only a small portion of the total esterases (less that 0.5%). The esterase BAC36707 accounts for ~30–40% of the total cypermethrin hydrolysis activity in mouse liver and, hence, may be a useful tool in determining the toxicity of new pyrethroids. In addition, the fluorescent assays employing **A2–7** were found to be excellent tools to mimic the hydrolysis of pyrethroids by these enzymes.

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