

Isolation and Identification of an Esterase from a Mexican Strain of *Boophilus microplus* (Acari: Ixodidae)

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ABSTRACT A strain of Mexican *Boophilus microplus* (Cz) collected near Coatzacoalcos, Veracruz, Mexico, exhibits a moderate, but significant, level of permethrin resistance. Unlike other highly permethrin resistant strains, the Cz strain does not have a mutation within the sodium channel gene that results in target-site insensitivity. However, the Cz strain possesses a substantial increase in general and permethrin esterase activity relative to highly permethrin resistant and control strains suggesting the involvement of a metabolic esterase(s) in the expression of permethrin resistance. We report the isolation of a 62.8 kDa protein from Cz strain larvae that we think is the esterase previously reported as Cz EST9. In addition, internal amino acid sequence data obtained from the 62.8 kDa protein suggest that it is the gene product of a previously reported *B. microplus* carboxylesterase cDNA. We propose that the 62.8 kDa protein (Cz EST9) has permethrin hydrolytic activity and as a result plays an important role in Cz strain resistance to permethrin.

KEY WORDS carboxylesterase, insecticide resistance, permethrin detoxification

THE SOUTHERN CATTLE TICK, *Boophilus microplus* (Canestrini), is an important arthropod vector of bovine babesiosis (Smith and Kilborne 1893). In 1943, the U. S. national eradication program for *B. microplus* was declared complete (Graham and Hourigan 1977). However, the tick remains endemic to Mexico, and poses a constant threat for reintroduction along the south Texas border with Mexico. To date, reintroduction of this important disease and vector has been prevented by vigilant surveillance and quarantine along the Texas-Mexico border (George 1996). Cattle entering the United States must be dipped in vats containing the organophosphate (OP) acaricide coumaphos at border import stations. A limited number of compounds are suitable for dipping vat formulation, and one is the pyrethroid permethrin. Increasing reports of *B. microplus* resistance to pyrethroid and OP compounds (Ortiz et al. 1994, Fragoso et al. 1995) raises concerns about the continued effectiveness of the dipping program to prevent reentry of this important disease and disease vector into the United States.

Knowledge of resistance mechanisms is extremely important to mitigate resistance development and prolong the effective life of the control program and to prevent reentry of the tick into the United States. A Mexican *B. microplus* strain was collected in 1994 near Coatzacoalcos, Mexico from a tick population suspected of pyrethroid resistance. The strain was

given the name of Coatzacoalcos (Cz), and is unique among the pyrethroid resistant strains maintained at the Cattle Fever Tick Research Laboratory (CFTRL) in Mission, TX. Pyrethroid resistance is often based upon reduced target site affinity for the toxicant as a result of mutations within the voltage-gated sodium channel gene. He et al. (1999) identified a mutation within the sodium channel gene of *B. microplus* that is diagnostic for pyrethroid resistance. The Cz strain has a moderate level of resistance to permethrin relative to strains that possess the sodium channel mutation. He et al. (1999) were unable to find the diagnostic sodium channel mutation within the Cz strain. The absence of an insensitive target site mechanism in the Cz strain suggests that metabolic enzymes may be involved in the detoxification or sequestration of permethrin. Jamroz et al. (2000) recently reported that the Cz strain possessed general esterase activity and permethrin hydrolytic activity that was ≈ 4.5 -fold greater than the susceptible Gonzalez strain, and ≈ 3.5 times greater than the highly permethrin-resistant Corrales strain that carries the sodium channel mutation (He et al. 1999). An intense band of general esterase activity, unique to the Cz strain, was noted on native PAGE gels of Cz strain extracts and designated esterase Cz EST9 (Jamroz et al. 2000). In addition, it was reported that triphenyl phosphate, an OP that irreversibly inhibits serine dependent esterases, synergized permethrin toxicity of the Cz strain (Miller et al. 1999). The absence of a sodium channel mutation, observed increased general and permethrin esterase activity, and synergism with OPs suggest that a unique

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metabolic esterase is involved in Cz strain permethrin resistance. Therefore, Cz EST9 defined by Jamroz et al. (2000) became a candidate for protein purification so that it could be further investigated as the putative permethrin esterase associated with the Cz strain.

Materials and Methods

Tick Strain. The Coatzacoalcos strain (Cz) of *B. microplus* used in this study is maintained at the USDA Cattle Fever Tick Research Laboratory (CFTRL) in Mission, TX. The Cz strain was established in 1994 at the CFTRL from larvae (designated as the F₁) obtained from the National Parasitology Laboratory in Cuernavaca, Morelos, Mexico. The founding individuals were collected from a site near Coatzacoalcos, Veracruz, Mexico where control failure with pyrethroid acaricides occurred. At the CFTRL, larvae of the Cz strain were selected with increasing concentrations of permethrin at each generation (F₂, 0.05% active ingredient [AI] increased 0.05% at each generation to F₁₁, then increased 0.5% through F₁₇ at 3.0% [AI]), for the first 17 laboratory generations. Resistance was increased 28-fold between the F₂ (LC₅₀ 0.15%) and the F₁₅ (LC₅₀ 4.2%) generations by this selection process (Miller et al. 1999). Resistance has been maintained by selection of subsequent larval generations at 1% (AI) permethrin, a lethal concentration (LC) that kills all susceptible ticks. In this study we used 11-d-old-larvae from the F₃₁ generation for the preparation of a larval protein extract. The F₃₁ generation was not bioassayed with permethrin, however, the F₃₄ generation had an LC₅₀ of 3.8% permethrin.

Tick Extract. Cz larvae (2.3 g) frozen at -80°C were pestled to a powder with a mortar and pestle that had been chilled with liquid nitrogen. The frozen powder was placed into 3 ml of Tris-Borate buffer, pH 8.06, 50 mM Tris, 70 mM boric acid, 2 mM EDTA-disodium, 1 mM dithiothreitol (TBB). Dithiothreitol was included in the buffer to inhibit proteolysis and stabilize esterase activity as suggested by Willadsen and Williams (1976). Tick proteins were extracted for 2 h at 4°C with gentle agitation. Following extraction of tick proteins at 4°C the mixture was centrifuged at 14,000 × g for 30 min in a Sorvall RC5B centrifuge at 4°C. The supernatant fluid was collected and recentrifuged at 25,000 × g for 30 min at 4°C. The resultant supernatant fluid was collected and stored at 4°C for protein determination, enzyme analysis, and ion-exchange chromatography.

Protein Determination. Two methods of protein determination were used in this study with bovine serum albumin (Sigma, St. Louis, MO) as a standard. The protein concentration of the crude extract and partially purified fractions were determined with a micro-BCA (bicinchoninic acid) technique according to the methods of the manufacturer (Pierce, Rockford, IL). The estimation of the protein concentration of purified proteins was accomplished by SDS-PAGE. Quantification of resultant silver stained bands (Blum et al. 1987) was made with the aid of a laser densi-

tometer (LKB Ultrascan XL densitometer, GelScan XL software, Bromma, Sweden).

Enzyme Assays. General esterase activity of fractionated proteins was measured with a modified microplate assay using α -naphthyl acetate as substrate (Jamroz et al. 2000). Briefly, 50 μ l of diluted tick proteins were added to a microplate well. Substrate, (150 μ l) α -naphthyl acetate (α -NA, 5×10^{-4} M in 0.02 M sodium phosphate buffer, pH 7.0), was added to each well and the reaction allowed to proceed for 30 min at 30°C. The enzyme reaction was stopped by the addition of 50 μ l of 0.02 M sodium phosphate buffer containing 0.15% o-dianisidine (tetrazotized) and 1.75% sodium dodecyl sulfate to each well (Devonshire et al. 1986). The color development was allowed to stabilize for 15 min in the dark at room temperature. Plates were read with a CERES UV900HDi plate reader (Bio-Tek Instruments, Winooski, VT). Initial velocity in terms of μ M of α -naphthol produced per min was determined from an α -naphthol standard curve.

Hydrolysis of permethrin was monitored by the detection of the metabolite, 3-phenoxybenzyl alcohol, by high pressure liquid chromatography (HPLC; Jamroz et al. 2000). Fractionated tick proteins (20 μ l) were mixed with 78 μ l of 0.05 M sodium phosphate buffer, pH 7.5, in a Pyrex glass tube (5 by 60 mm). Permethrin (96.2%, 52:48 *cis:trans*-, Burroughs-Wellcome, Research Triangle Park, NC) in methyl cellulose (40 mg/ml) was added (2 μ l) to the Pyrex tubes for a total volume of 100 μ l. The tubes containing the reactants were vigorously shaken with an orbital shaker for 3 h at 37°C. Following incubation, 40 μ l of the reaction mixture was removed from the Pyrex tube and added to 4 ml of acetonitrile (Fisher, Fair Lawn, NJ):water (50:50) for a 1:101 sample dilution. The diluted sample (100 μ l) was injected onto a Water's C18 Radial Compression Column (Nova-Pak, 4 μ m, 5 × 100 mm, Milford, MA). The column was eluted at 0.7 ml/min with a solvent mixture of acetonitrile:water (70:30). The eluent was monitored at 245 nm and the concentration of 3-phenoxybenzyl alcohol produced determined by comparison to a 3-phenoxybenzyl alcohol (Aldrich, Milwaukee, WI) standard curve.

Ion-Exchange Chromatography. Extracted tick proteins were initially fractionated with DEAE Sephacel (Pharmacia, Uppsala, Sweden) anion-exchange chromatography. Tick proteins (10.5 ml at 16.88 mg/ml) were loaded onto the column (2.6 by 21 cm) and eluted with TBB. A linear NaCl gradient (0–1.0 M) in TBB was initiated after the fall-through peak eluted, beginning at tube 26. Individual tubes (3.7-ml fraction volume) were collected and 15 μ l of the contents analyzed for esterase activity (α -NA). Two peaks of esterase activity were pooled, fraction 1 (F1, tubes 59–66), and fraction 2 (F2, tubes 67–97). The pooled fractions were concentrated and desalted with an Amicon 8050 concentration cell (Lexington, MA) using a PM30 membrane, and stored at 4°C in preparation for further purification.

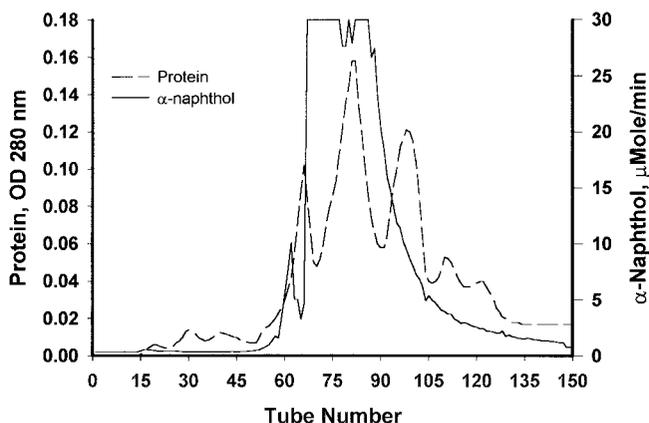


Fig. 1. DEAE-Sephadex anion-exchange fractionation of Cz strain crude proteins. 0–1 M NaCl gradient started at tube 26. Tube volume 3.7 ml. Esterase activity pooled into two fractions: fraction 1 (tubes 59–66) and fraction 2 (tubes 67–97) for further purification. Esterase activity in tubes 67–77, 79, and 82–86, >27.4 μM α -naphthol/min.

Continuous Elution Polyacrylamide Gel Electrophoresis (PAGE). Permethrin esterase activity was purified from pooled anion-exchange fraction, F2, using continuous elution PAGE with the Bio-Rad 491 prep cell according to the manufacturers instructions (Bio-Rad, Hercules, CA). Before electrophoresis, previously concentrated and desalted fraction F2 proteins (≈ 11.5 ml, 4.81 mg/ml) were further concentrated with a Amicon Centriprep-10 concentrator to 1.18 ml (≈ 55.3 mg). A 37-mm-diameter column was used for protein separation with a 6 cm 8% native polyacrylamide gel (30.0:0.8, acrylamide:bis-acrylamide). The concentrated sample (1.18 ml) was combined with 0.571 ml 70% sucrose, 0.05 ml bromphenol blue (0.25%), and 0.199 ml TBB (total volume 2.0 ml, 20% sucrose) and loaded onto the stacking gel (10%) and the run conducted at 40 mAmps constant current. As the tracking dye reached the bottom of the gel, continuous elution fractions (2.0 ml) were collected for 128 tubes. Each fraction was subsequently analyzed for esterase activity (α -NA). Fractions containing significant esterase activity (40 through 94, every third tube) was concentrated for assay of permethrin esterase activity. 500 μl of each fraction was concentrated 10x (Amicon, Microcon-10) and 20 μl of the concentrated fraction was analyzed for permethrin esterase activity as described above. Resultant permethrin esterase activity was pooled into four fractions; fraction 1 (tubes 73–79), fraction 2 (tubes 80–87), fraction 3 (tubes 88–94), and fraction 4 (tubes 95–100). These pooled fractions were concentrated with an Amicon Centriprep-10 concentrator to 2 ml for fraction 1 and 1 ml for fractions 2–4.

Native and Sodium Dodecyl-Polyacrylamide Gel Electrophoresis SDS-PAGE. Fractionated proteins were resolved on PAGE gels (12%, native no SDS or SDS and 2-mercaptoethanol SDS-PAGE) using a modified method of Laemmli (1970). Samples run on SDS-PAGE gels were reduced with 5% 2-mercaptoethanol before electrophoresis. Gels were run at a constant current of 20 mAmps/gel on an LKB 2050 midjet

electrophoresis unit. Resolved proteins were silver stained according to the nonammonical, nonfixing method of Blum et al. (1987). Native gels were stained for general esterase activity using a modification of the method of Hughes and Raftos (1985). Native gels were placed in 100 ml of 0.1 M sodium phosphate buffer, pH 6.6, containing 1.07 mM α -naphthyl acetate as substrate and 1.20 mM Fast Blue BB salt as the dye. The glass dish containing the gel was covered with plastic wrap and placed in a dark incubator at 37°C for 45 min.

Amino Acid Sequencing. The purified fraction 3 (F3) from continuous elution PAGE, containing permethrin esterase activity, was submitted to the Protein Chemistry Laboratory, Department of Biochemistry, Texas A & M University, College Station, TX. Preliminary results indicated that the 62.8 kDa band (putative permethrin esterase) was blocked at the amino-terminus. The 62.8 kDa protein was separated by SDS-PAGE, the band collected and the protein cleaved into representative peptides in situ with endoproteinase Lys-C. Peptides were purified by HPLC and their mass determined by mass spectrometry. Purified peptides were subjected to automated Edman chemistry on a Hewlett-Packard G1000A automated protein sequencer.

Results

Ion-Exchange Chromatography. DEAE-Sephadex anion-exchange fractionation of Cz crude proteins resulted in two peaks of general esterase activity (Fig. 1). Tubes 59–66, containing esterase activity, were pooled into ion-exchange fraction 1, and tubes 67–97, also containing esterase activity, were pooled into ion-exchange fraction 2. Native PAGE revealed that fraction two contained the greater concentration of Cz EST9 (data not shown) as defined by Jamroz et al. (2000).

Continuous Elution Polyacrylamide Gel Electrophoresis (PAGE). Anion-exchange fraction 2 was further fractionated into two peaks of general esterase

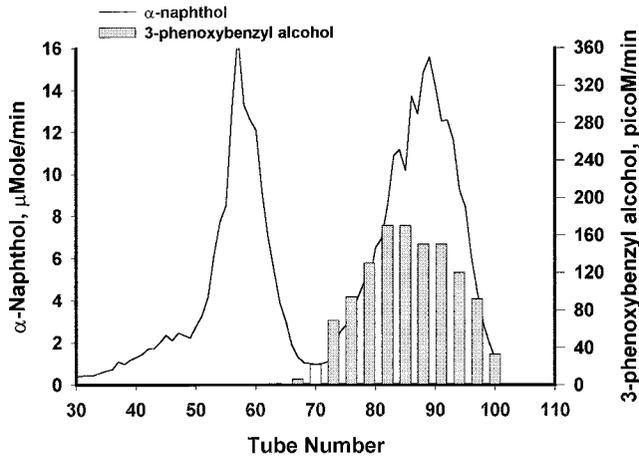


Fig. 2. Continuous elution PAGE fractionation of anion-exchange fraction 2. Analysis of 1 μ l of the contents of each tube (2 ml) for general esterase activity by the formation of α -naphthol. Analysis of 20 μ l of the 10 \times concentrated contents of every third tube from 40 to 100 for permethrin esterase activity by the formation of the permethrin metabolite, 3-phenoxybenzyl alcohol. Pooled permethrin esterase fractions: F1 (tubes 73–79), F2 (tubes 80–87), F3 (tubes 88–94), and F4 (tubes 95–100).

activity by continuous elution PAGE (Fig. 2). Every third tube between tube 40 and 100 was analyzed for permethrin esterase activity. Permethrin esterase activity was limited to the second peak between tube 70 and 100. Peak permethrin esterase activity was found between tubes 80 and 90. Tubes containing permethrin esterase activity were pooled into four fractions, fraction 1 (tubes 73–79), fraction 2 (tubes 80–

87), fraction 3 (tubes 88–94), and fraction 4 (tubes 95–100).

Sodium Dodecyl-Polyacrylamide Gel Electrophoresis SDS-PAGE. Pooled continuous elution fractions were analyzed by SDS-PAGE (Fig. 3). SDS-PAGE revealed two prominent polypeptide bands in fraction 1 through 4. The band at 62.8 kDa was presumptively identified as the putative Cz EST9. The

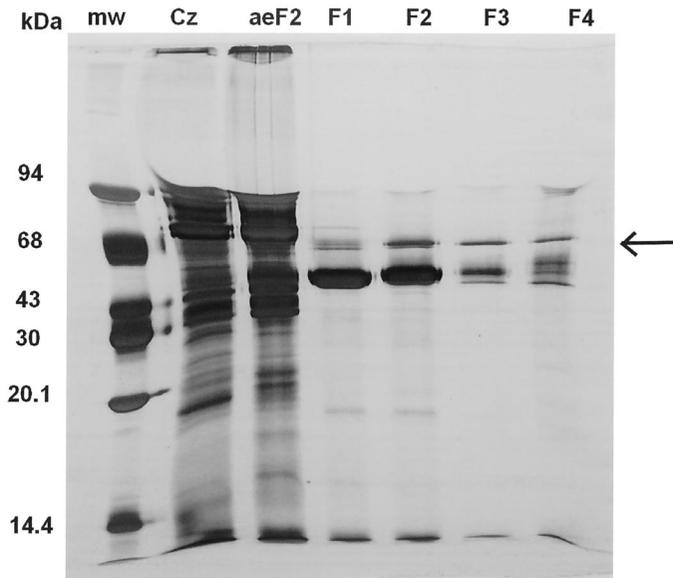


Fig. 3. SDS-PAGE analysis of crude protein extract (Cz), anion-exchange fraction 2 (aeF2), and fractions from continuous elution PAGE (F1–F4). Molecular weight markers (mw) labeled in kDa. F1, 14 ml pooled and concentrated to 1 ml; F2 16–1 ml; F3 14–2 ml; F4, 12–1 ml. Gel loaded with 10 μ g protein for Cz and aeF2, and 4.1 μ l of each concentrated fraction (F1–F4). Total load volumes for Cz, aeF2, and F1–F4 was 10 μ l. Position of the 62.8 kDa protein isolated from F3 for amino acid sequencing is indicated by the arrow. Estimated concentration of 62.8 kDa protein in each loaded fraction, F1 = 0.018 μ g, F2 = 0.076 μ g, F3 = 0.043 μ g, F4 = 0.040 μ g.

Table 1. Comparison of internal peptide sequences of the 62.8 kDa protein and predicted sequences of cDNA clone 13

Peptide	Sequence	Predicted mass	Measured mass
Frac #31	R-A-V-L-M-S-G-T-M-Y-N-I-D-L-W-D-M-V-H-E	2,828	2,853
Clone 13 aa 236-255	R-A-V-L-M-S-G-T-M-Y-N-I-D-L-W-D-M-V-H-E		
Frac #27	V-V-H-V-L-D-N-E-P-V-E-E-Y-V-X-I-P	2,823	2,822
Clone 13 aa 39-55	V-V-H-V-L-D-N-E-P-V-E-E-Y-V-G-I-P		
Frac #15	T-A-L-S-S-W-V-K-F/I-D	nd	nd
Clone 13 aa 378-387	T-A-L-S-S-W-V-K-E-D		

X denotes no amino acid was called in that cycle, amino acid (aa)

51.4 kDa band, so prominent in fractions 1 and 2, declined considerably in fraction 3. The permethrin esterase results for every third tube between tube 40 and 94 are presented in Fig. 2. Permethrin esterase activity was greatest between tube 83 through 91. As the concentration of the 51.4 kDa band decreased considerably in fraction 3 (Fig. 3) and the activity of permethrin esterase did not, the 62.8 kDa protein was presumed responsible for the esterase activity.

Amino Acid Sequencing. Continuous elution pooled fraction 3, containing the enriched 62.8 kDa protein, was concentrated and submitted to the Protein Chemistry Laboratory at Texas A&M University for sequencing of the 62.8 kDa band. Initial sequencing attempts revealed that the amino-terminus of the protein was blocked. Internal peptides were generated by in situ digestion of the 62.8 kDa protein with endo Lys-C. Peptides in sufficient concentration for detection were purified by micro-bore HPLC and their mass determined before sequencing by mass spectrometry. The results of sequencing three internal peptides of the 62.8 kDa protein are presented in Table 1 along with peptide amino acid sequences predicted from endo Lys-C digestion of the clone 13 gene product (Hernandez et al. 2000).

Discussion

In this study we describe the isolation of a 62.8 kDa protein and enrichment of permethrin esterase activity that is thought to be responsible, in part, for the observed pyrethroid resistance associated with the Mexican Cz strain. Because the fraction (F3) that possesses permethrin hydrolytic activity and contains the enriched 62.8 kDa protein is only partially pure, containing a prominent 51.4 kDa contaminant, the permethrin esterase activity of the 62.8 kDa protein is at this time presumptive. However, the 51.4 kDa contaminant that is so prominent in Bio-Rad 491 fractions 1 and 2 (Fig. 3) is reduced in fraction 3, the fraction submitted for sequencing. The apparent decline in concentration of the 51.4 kDa band in fraction 3 (Fig. 3) was not coupled with a comparable decline in permethrin hydrolytic activity (Fig. 2). This result further supports the 62.8 kDa protein as the presumptive permethrin esterase, Cz EST9.

Partially purified pyrethroid hydrolyzing esterases have been previously isolated from Australian *B. microplus* strains (Riddles et al. 1983, De Jersey et al. 1985). A *trans*-permethrin hydrolyzing carboxylester-

ase was identified from the pyrethroid resistant Malchi strain that had an approximate molecular weight of 89 kDa and a pI of 5.5–5.8 (De Jersey et al. 1985). In addition, a carboxylesterase was also partially purified from the Malchi strain that hydrolyzed the α -cyano-substituted *trans*-cypermethrin, but not *trans*-permethrin. This carboxylesterase had an approximate molecular weight of 67 kDa and a pI of 4.6. These carboxylesterases were partially purified by gel filtration chromatography followed by isoelectric focusing.

In the current study, isolation of the 62.8 kDa protein from the Mexican Cz strain allowed us to obtain information regarding the internal amino acid sequence of the protein. Amino acid sequences of peptides generated by in situ digestion of the 62.8 kDa protein with endo-Lys C allowed us to compare those sequences with amino acid sequences of other known *B. microplus* esterases. Hernandez et al. (2000) identified two esterase cDNA sequences designated clone 8 and clone 13 from the Cz strain by polymerase chain reaction (PCR) amplification using degenerate primers. An Internet search of a motif finder, identified clone 8 as a carboxylesterase B2 (soluble) and clone 13 as a carboxylesterase B1 (membrane associated). Two allelic forms of Clone 13 were found to exist in the *B. microplus* population, a wild type and a mutant allele. Clone 13 had an open reading frame of 1632 bases and encoded a protein of 544 amino acids with a predicted molecular weight of 60.6 kDa. They found that clone 13 contained a silent mutation and a base substitution of G→A at nucleotide 1120 that resulted in a change of aspartate (negative charge) for asparagine (polar uncharged). They only found the mutated allele in the Cz strain (Hernandez et al. 2000).

Simulated digestion of the predicted amino acid sequences of clone 8 and clone 13 described by Hernandez et al. (2000) with endo-Lys C predicted sets of peptides and their masses. Actual digestion of the 62.8 kDa protein described in this study with endo-Lys C yielded peptides with similar mass to those predicted for clone 13 (Table 1). Amino acid sequencing of those peptides demonstrated near identity with the corresponding predicted peptide sequence of clone 13. Peptide 31 from the digest of the 62.8 kDa protein had 100% identity (20 amino acids) that represented amino acid 236 through 255. Although we were unable to make a call at amino acid position 53 of peptide 27, there was 100% agreement with the amino acid sequence 39–55. The only sequence difference that was found was at amino acid position 386 of peptide 15, as

we called phenylalanine or isoleucine and the predicted sequence from clone 13 suggested glutamic acid. Unfortunately we were unable to sequence the peptide that contained the mutant site, amino acid 364 asparagine. The similarity in peptide mass and amino acid sequence strongly suggest that the 62.8 kDa protein is the gene product of clone 13 a carboxylesterase B1 membrane associated protein (Hernandez et al. 2000).

Primarily based upon the considerable difference in molecular weight, we think the esterase that we have isolated from the Cz strain is distinct from the esterase with permethrin hydrolytic activity isolated by De Jersey et al. (1985). Although not indicative of molecular identity the concentration of 62.8 kDa protein, as a percentage of total protein, that we isolated in this study was much greater than the 89 kDa esterase isolated by De Jersey et al. (1985). De Jersey et al. (1985) estimated a recovery of 50 μ g of the 89 kDa protein starting with 10 g of tick larvae for a yield of 0.0005% of total larval weight. In the current study we estimate a total yield of 53.7 μ g of 62.8 kDa protein from 2.3 g of tick larvae for a yield of 0.0023% of total larval weight, or a 4.6 times greater yield than from the Malchi strain.

De Jersey et al. (1985) reported the total esterase activities toward *p*-nitrophenyl butyrate and *trans-p*-nitrophenyl-(1*R,S*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (permethrin analog) of extracts of the pyrethroid resistant Malchi strain and the control susceptible were not different. In contrast the Cz strain has been reported by Jamroz et al. (2000) to have 4.5-fold greater general esterase activity and 4.5-fold greater permethrin esterase over the control susceptible Gonzalez strain. Native PAGE analysis of the general esterase activity of the Cz strain yields a characteristic region of heavy staining esterase activity that is not shared by other Mexican strains that have been analyzed (Jamroz et al. 2000). It is from this region of elevated α -naphthyl acetate staining that the 62.8 kDa protein was isolated. At this time, we do not have comparative kinetic data to suggest that this enzyme has acquired increased catalytic activity relative to other and perhaps related esterases (wild type allele). However, we do have evidence that greater amounts of the enzyme are being expressed in the Cz strain as evidenced by our increased yield of purified protein as a percentage of larval weight. In addition, Southern hybridization experiments revealed that clone 13 is more abundant in the Cz strain than the San Felipe or Corrales strains suggesting that gene amplification has occurred in the Cz strain (Hernandez et al. 2000).

Identification and partial sequencing of the 62.8 kDa protein has allowed us to suggest that the 62.8 kDa protein is the gene product of clone 13, a B1 carboxylesterase as described by Hernandez et al. (2000), and representative of Cz EST9 as described by Jamroz et al. (2000). In addition, we propose that the 62.8 kDa protein has significant permethrin esterase activity, and contributes to the observed pyrethroid resistance expressed by the Cz strain.

Acknowledgments

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