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# Detection of mutations in Colorado potato beetle : acetylcholinesterase gene responsible for resistance to carbofuran.

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# DETECTION OF MUTATIONS IN COLORADO POTATO BEETLE ACETYLCHOLINESTERASE GENE RESPONSIBLE FOR RESISTANCE TO CARBOFURAN

A Thesis Presented

by

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ABSTRACT

DETECTION OF MUTATIONS IN COLORADO POTATO BEETLE ACETYLCHOLINESTERASE GENE RESPONSIBLE FOR RESISTANCE TO CARBOFURAN

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This study describes N-methyl carbofuran resistance in a CPB field strain (BERTS) collected from Little Creek, DE. The BERTS strain was found to be highly resistant to N-methyl carbofuran and susceptible to the methoxy-organophosphate, azinphosmethyl. Previous studies conducted on an azinphosmethyl-resistant CPB strain (AZ-R) characterized the resistance mechanism to be acetylcholinesterase (AChE) site-insensitivity. This site-insensitivity was correlated to the coexpression of two point mutations in the AChE gene.

BERTS CPB were sorted via AChE enzyme profiles into BERTS-R (resistant to carbofuran) and BERTS-S (susceptible to carbofuran) substrains. Comparison of AChE complimentary DNAs of the two substrains revealed the presence of the S291G mutation, previously found in AZ-R CPB, and a novel mutation, I392T. The S291G mutation was found in both BERTS-S and BERTS-R CPB, however the I392T mutation was present

only in BERTS-S CPB. It is hypothesized the I392T mutation serves to negate the effect of the S291G mutation responsible for AChE insensitivity to N-methyl carbofuran.

A number of studies have indicated that insecticide insensitive forms of AChE, which result in N-methyl carbamate and methoxy-organophosphate resistance, have increased susceptibilty to insecticide analogs with larger alkyl groups. This study shows the BERTS strain to be 90% more sensitive to N-propyl carbofuran than a susceptible strain (SS). N-propyl carbofuran was found to be approximately 4.5 times more toxic than N-methyl carbofuran in BERTS CPB in topical application bioassays. Given such data, it is proposed that insecticide analogs with larger alkyl groups are practical means of control of CPB resistant to N-methyl carbamates via AChE site insensitivity. Detection of resistance in the field can be accomplished with molecular techniques such as SSCP and minisequencing.

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# CHAPTER 1

# INTRODUCTION

Colorado potato beetle (*Leptinotarsa decemlineata* Say) (CPB), is one of the most significant agricultural pests in the world, responsible for extensive damage on potato, tomato, and eggplant crops in North America and elsewhere. It is notorious for quickly developing resistance to any insecticide used for its control. CPB are resistant to all major groups of insecticides including cyclodienes, organochlorines, organophosphates (OPs), carbamates, and pyrethroids (Forgash, 1984). In addition, there are reports of CPB resistance to more modern pesticides including the chloronicontinyl insecticide, imidacloprid, as well as avermectins, and *Bacillus thuriengenesis* (Grafius and Bishop, 1996; Clark *et al.*, 1992). Thus, CPB has consistently been one of the first insects to develop resistance to new insecticides, and exhibits all major resistance mechanisms, including phamacokinetic, metabolic, physiological/behavior and site-insensitivity factors (Argentine *et al.*, 1994). In 1991, Ioanndis *et al.* reported highly diverse resistance patterns to OPs, carbamates, and pyrethroids in field strains of CPB located in a relatively narrow geographic area indicating the availability of a variety resistance mechanisms. Because of these features, CPB is a choice pest for study and ideal model for insecticide resistance, as well as economically important. This study is part of a larger effort focused on CPB as a model for the elucidation of insecticide resistance mechanisms and management.

One mechanism of CPB resistance to OP and carbamate insecticides is site-insensitivity at the target enzyme, acetylcholinesterase (AChE) (Oppenorth, 1985;

Soderlund and Bloomquist, 1990). AChE is responsible for the rapid hydrolysis of the excitatory neurotransmitter acetylcholine into acetic acid and choline once it is released into the synaptic gap. The catalytic center of AChE contains two subsites: the esteratic and anionic subsites. Within the catalytic center, 3 amino acids, serine, glutamine, and histidine, have been found crucial for substrate/enzyme interaction. In addition, there is a peripheral anionic binding site approximately 50 Å from the catalytic center which can bind substrate in high concentrations and down regulate the AChE activity (high substrate inhibition). Both OP and carbamate insecticides function as competitive inhibitors of AChE. Inhibition of AChE leads to a build-up of acetylcholine in the synaptic gap and ultimately results in saturation of acetylcholine receptors (AChR) in the post-synaptic membrane. Persistent stimulation of this receptor in insects leads to involuntary movements, paralysis, and death (Buchel, 1983).

Although, OPs and carbamates share a similar site of action, they differ in their mechanism or mode of action at the $K_3$ rate constant governing reactivation of the acylated enzyme. OPs phosphorylate the enzyme at the catalytically significant serine in the esteratic subsite. Dephosphorylation of the enzyme can take hours or days, resulting in extremely small $K_3$ values. Carbamates carbamoylate AChE. Carbamoylated AChE is hydrolyzed back to its active form in minutes, resulting in intermediate $K_3$ values, but significantly slower then the $K_3$ value that is apparent with deacylation following ACh binding. Hence, OPs are thought of as irreversible inhibitors, while carbamates are reversible inhibitors (Buchel, 1983).

The research presented here is based on initial studies of CPB resistance to the methoxyorganophosphorus insecticide, azinphosmethyl. Because of the similarity in

mode of action, it was decided to study carbamate resistance, specifically to the methyl carbamate, N-methyl carbofuran.

The first carbamate insecticide, carbaryl, was introduced in 1958. Carbamates, in general, have low toxicity to non-target species. Their main area of use is in agriculture, as carbamates are systemically active, meaning they are translocated in the xylem of plants. Thus, carbamates can control pests of roots and shoots that may be difficult to reach using broadcasted insecticides (Buchel, 1983).

Ioannidis *et al.* (1992) first characterized a field population of carbofuran resistant CPB. The resistance was found to be autosomal and monofactorial, resulting in a decrease in AChE sensitivity. A similar resistance profile (a target site-insensitivity mechanism) was determined in an azinphosmethyl-resistant laboratory strain of CPB (Argentine *et al.*, 1994).

Resistance to azinphosmethyl (136-fold) in a nearly isogenic CPB strain (AZ-R) was determined to be autosomal and essentially monofactorial with at least two contributing factors. Biochemical mechanisms of azinphosmethyl resistance included a small enhancement in oxidative metabolism, slightly reduced cuticle penetration, and major target site-insensitivity due to an altered AChE (Argentine *et al.*, 1994). Enzyme kinetics of AChE are easily assessed by the Ellman reaction, in which the hydrolyzed product, thiocholine, undergoes a subsequent reaction that produces color measurable by a UV spectrophotometer (Ellman, 1961). The level of absorbance directly reflects the activity of the enzyme.

The altered form of AChE from the AZ-R CPB strain was shown to hydrolyze acetylthiocholine poorly (Zhu *et al.*, 1994). It has been hypothesized that this may

contribute to the reduced fitness associated with the AZ-R strain (Argentine *et al.*, 1989). The AZ-R strain produced 0.56 times as many larvae as a susceptible laboratory strain (SS), 0.51 times as many adult females as the SS strain, and the mean development time was increased by 1.5 d in the AZ-R strain. From this, the fitness of the AZ-R strain was found to be 0.83 relative to the SS strain.

Steroidal glycoalkaloids, such as α-chaconine, α-solanine, and tomatine are toxic secondary plant chemicals produced by potatoes and act as endogenous noncompetitive cholinesterase inhibitors. Inhibitor studies conducted with these glycoalkaloids on "altered" AZ-R and "normal" SS AChE activities revealed variations in sensitivity. The AZ-R AChE was more sensitive to inhibition by α-solanine and tomatine, but less sensitive to α-chaconine (1.3 fold) (Argentine *et al.*, 1994).

Because α-chaconine is the major steroidal glycoalkaloid in native potato, one may speculate that the altered AChE in AZ-R strain of CPB may give these individuals a fitness advantage over the individuals of the SS strain if they are raised on potato cultivars that contains high levels of α-chaconine. A fitness comparison study of AZ-R and SS strains of CPB reared on various potato cultivars revealed an increased intrinsic rate of growth in the AZ-R strain when raised on the NDA 1725-1 cultivar, which has high levels of α-chaconine. The AZ-R strain had a reduced intrinsic rate of growth compared to the SS strain when reared on Russet Burbank potatoes, which have low levels of α-chaconine (Zhu *et al.*, 1995a).

In addition to the observation of decreased hydrolytic activity to the substrate acetylthiocholine, AZ-R AChE exhibited enhanced hydrolytic activity (3-fold) towards butyrylthiocholine and lacked high substrate inhibition. The altered AChE kinetics

showed substrate activation that is characteristic of butyrylcholinesterase. These observations indicated possible changes to both the esteratic subsite of the catalytic center and to the peripheral anionic binding site of AChE in AZ-R (Zhu *et al.* 1995a).

Biochemical and kinetic properties of AChE has been studied in numerous insects including: housefly, *Musca domestica* (Devonshire, 1975, 1984); spring grain aphid, *Schizaphis gramina* (Brestkin *et al.*, 1985); tobacco horn worm, *Manduca sexta* (Lester and Gilbert, 1987); *Drosophila melanogaster* (Gnagey *et al.*, 1987); *Tenebrio molitor* (Lenoir-Rousseaux and Gauton, 1987; Lenoir-Rousseaux *et al.*, 1988); tobacco budworm, *Heliothis virescens* (Brown and Bryson 1992); and *Lygus hesperus* (Zhu and Brindley, 1992). A complete kinetic study on CPB AChE was conducted with purified enzyme. Two forms of the enzyme were found. The major form (92%) was hydrophilic dimer, while 8% was amphiphilic dimer. Subunit molecular weight was 65,000 as determined by SDS-PAGE. The CPB AChE substrate specificity constant ($k_{cat}/K_m$) for acetylthiocholine (ATC ), was 21-fold higher than that for butyrylcholine (BTC). AChE activity was significantly inhibited by high concentrations of ATC, illustrating substrate inhibition (Zhu and Clark, 1994).

A comparative kinetic study of SS versus AZ-R CPB AChE from enzyme preparations, revealed the AZ-R strain had a significantly reduced biomolecular rate constant for azinphosmethyl. The AZ-R strain AChE $K_i$ was approximately 2-fold lower than that seen in the SS strain (8.63 vs. 15.70, respectively) using a crude AChE preparation method (Argentine *et al.* 1994). A subsequent study using a highly purified AChE preparation found AZ-R strain AChE $K_i$ was approximately 16-fold lower than that seen in the SS strain (0.35 vs. 5.59, respectively) (Zhu and Clark, 1995).

Molecular studies on AChE in insects are limited to 3 dipterans, including housefly (Williamson *et al.*, 1996), mosquito, *Anopheles stephensi* (Hall and Malcolm, 1991), and *Drosophilia melanogaster* (Hall and Spierer, 1986; Fournier *et al.*, 1989, 1992; Mutero *et al.*, 1994) and CPB (Zhu and Clark, 1995b). The cDNA encoding CPB AChE consisted of 2900 nucleotides with an open reading frame of 1887 nucleotides, encoding a protein of 629 amino acid residues. Molecular weight was deduced at 67,994, which agreed with previous study data (Zhu and Clark, 1994). The deduced amino acid sequence consisted of a putative signal peptide (= 29 residues) and the mature protein (= 600 residues). The amino acid sequence showed 57% homology to *D. melanogaster* (Hall and Spierer,1986) and 61% homology to *Anopheles stephensi* (Hall and Malcolm, 1991). The sequence contained the catalytic triad amino acid residues (Ser, Glu, and His) as well as the six cysteines, which form the three intra-subunit disulfide bonds. These residues are conserved in other AChEs sequenced from various species.

Genetic variation of the cDNAs for the AChE gene from the near-isogenic AZ-R and SS strains appear as point mutations responsible for the altered AChE structure, which confers the azinphosmethyl resistance (Zhu *et al.*, 1996, Zhu and Clark, 1997). Of the 4 nonsilent mutations discovered, only 2 occurred in multiple subclones. The Arg/Lys mutation (location nt 198, based on Zhu and Clark's published sequence in 1995) occurred in four of six AZ-R beetles sequenced (R30K). The Ser/Gly (location nt 980) was found all six AZ-R beetles sequenced (S291G). The location of the S291G mutation based on *Torpedo* AChE crystal structure (Sussman *et al.*, 1991) and the pharmacokinetic properties of AZ-R AChE (increased butyrylcholinesterase activity and enhanced interaction with bulkier OPs and carbamates) are indicative of an enlarged

esteratic subsite of AChE. The Ser/Gly mutation corresponds to the first amino acid residue of the α-helix, αE'1, located between the residues that make up the catalytic triad and the peripheral anionic binding site. The predicted secondary structure suggests the transition from the turn to the α-helix occurs sooner when glycine is present in the protein. This change is predicted to enlarge the esteratic subsite, and also to simultaneously effect the peripheral anionic binding site (Zhu *et al.*, 1996).

Different resistance patterns can originate from a combination of several point mutations as seen in the AChE *Ace* gene of *Drosophila melanogaster* (Mutero, 1994). Also, different point mutations, which confer the same resistance as seen in the GABA receptor *Rdl* gene leading to cyclodiene resistance, have been reported in sweet potato whitefly (*Bemisia tabaci*), red flour beetle (*Tribolium castaneum*), *Drosophila melanogaster*, *Aedes aegypti*, and the german cockroach (*Blatella germanica*) ( Anthony *et al.*, 1995; Miyazaki *et al.*, 1996; fFrench-Constant *et al.*, 1993; Thompson *et al.*, 1993; and Kaku and Matsumura, 1994).

Multiple point mutations have been reported in the *para*-type sodium channel α-subunit gene. Originally, the L1014F mutation was thought to be the only mutation to cause *kdr*-like resistance to DDT and pyrethroids. This same mutation was detected in diverse insect species including CPB, housefly (*Musca domestica*), german cockroach, tobacco budworm (*Heliothis virescens*), diamondback moth (*Plutella xylostella*), malaria mosquito (*Anopheles gambiae*), and horn fly (*Haematobia irritans*) (Lee *et al*, 1999; Williamson *et al.*, 1996; Dong *et al.*, 1998; Park *et al.*, 1997; Schuler *et al.*, 1998; Martinez-Torres *et al.*, 1998; Guerero *et al.*, 1997). Further studies of this gene led to the discovery of novel mutation points for *kdr*-like resistance, including I253N (*Drosophila*

*melanogaster*), V410M (tobacco budworm), M918T (horn fly and housefly), and T929I (diamondback moth) (Pittendrigh *et al.*, 1997; Park *et al.*, 1997; Williamson *et al.*, 1996; Guerrero *et al.*, 1997; Schuler *et al.*, 1998). Specific molecular biology techniques have proved useful for the efficient detection of such mutations and are useful for the diagnosis of specific types of resistance.

Single-Stranded Conformation Polymorphisms (SSCP) (Anthony *et al.*, 1995; Coustau *et al.*, 1995; Zhang *et al.* 1999) is an indirect detection method of point mutations, as well as for additions and deletions. Regions of DNA, 150-200 base pairs in length, containing a centered mutation site are PCR amplified and then heat denatured. The single-stranded DNA is resolved on a polyacrylamide gel by electrophoresis. Banding patterns in the gel differ based on the presence or absence of the point mutation. A SSCP method was developed to discriminate between SS and AZ-R CPB, which was based on the presence/absence of the S291G point mutation in AChE cDNA and genomic DNA (Zhang *et al.*, 1999).

The advantage of using the SSCP technique is its ability to detect novel alleles, indicated by characteristic banding patterns (Anthony *et al.*, 1995). SSCP is a very rugged, efficient and cheap means to detect mutations. It is not a direct sequencing method, however, and silent mutations and nonsilent mutations not associated with resistance can result in false positive and negative results. Because of this, SSCP results need to be validated by direct sequencing information.

PCR Amplification of Specific Alleles (PASA) is an unequal template amplification method based on the polymerase chain reaction, which can be interpreted for the presence/absence of specific point mutations by adjusting the base composition of

the 3' end of the PCR primer. The desired allele will amplify readily only with a matched primer/template set. This technique has been used for detection of cyclodiene resistance mutations in a variety of insects (Thompson *et al.*, 1993; Steichen and fFrench-Constant, 1994; Anthony *et al.*, 1995; Aronstein *et al.* 1995; Miyazaki *et al.*, 1995). PASA also is an indirect detection of the mutation, is more laborious, is highly sensitive to PCR conditions, and is difficult to accomplish if introns are present.

Certain mismatched primer sets, however, can result in amplification (Kwok *et al.* 1990). To avoid mismatched primer/template amplification, a modified method called competitive PASA (cPASA) can be used. The cPASA reaction conditions are modified such that amplification will only occur if the primer and template set are matched. One modification is to locate the mutation point as the 3'terminal base of the allele specific primer. This location has been found to be crucial for amplification to occur. Another modification is to set up a competitive PCR in the same reaction. Amplification of a non-allele specific product was found to improve Taq polymerase discrimination of mis-matched primer/template, possibly due to the competition between the non-allele specific primer and allele specific primer for Taq. Other PCR parameters, including annealing temperature and magnesium concentration, are optimized. The cPASA method was used for the detection of the S291G mutation in CPB for azinphosmethyl resistance (Zhu and Clark, 1996).

The result was amplification of a 163 base pair (bp) non-allele specific product from all CPB. The presence of a second allele specific 80 bp product was dependent on the sequence of the template matching the primer exactly. Templates from SS CPB showed amplification only when the susceptible primer was used (indicative of an A at nt

198), and no amplification when the resistant primer was used. Conversely, the AZ-R CPB showed amplification only when the resistant primer was used (indicative of a G at nt 198).

The final technique, solid-phase minisequencing, was originally employed to detect a point mutation related to sickle cell anemia (Kallio, 1997). In this assay, a single digoxigenin-labeled nucleotide is incorporated at the mutation point, based on the sequence of the template. The minisequencing reaction is coupled with an enzyme-linked immunoadsorbant assay (ELISA). The result is a detection of the nucleotide incorporated (or not incorporated) by UV detection. The reactions are run in a 96-well format using a microtitre plate for high-throughput.

Minisequencing identifies the sequence directly, is rugged, efficient, and less expensive than automatic sequencing. The technique has been applied to detect point mutations in CPB AChE gene fragments for azinphosmethyl resistance and in sodium channel α-subunit gene fragments for knockdown-type resistance to DDT and permethrin (Zhang *et al.* 1999).

In the thesis research that follows, I have examined CPB resistance to the carbamate insecticide, N-methyl carbofuran, due to a site-insensitivity resistance mechanism. Resistance is associated with a single point mutations within the AChE gene. The study also examines the toxicity of various OP and carbamate analogs on susceptible and resistant CPB strains (SS, AZ-R, and BERTS). Possible resistance management strategies are discussed including the use of resistance diagnostic techniques and alternative resistance-breaking insecticides specifically for control of resistant insects.

# CHAPTER 2

# MATERIALS AND METHODS

## Insect Strains and Rearing Conditions

A carbofuran-resistant strain of CPB (BERTS) was collected from Little Creek, DE by Drs. Judd Nelson and Gaylon Dively, University of Maryland, College Park. Additional strains were collected from Ellwood, MD (Ellwood), Hurlock, MD (Washington Cemetery), Leipsie, DE (Zimmerman), Pocomoke, MD (Hillman), Horntown, VA (Maddox), and La Cereirede, France (France). An insecticide-susceptible strain (SS) was supplied by G. G. Kennedy, North Carolina State University, Raleigh, and was used to assess the magnitude of resistance (Argentine *et al.*, 1989). A near isogenic azinphosmethyl-resistant (AZ-R) strain was selected by backcrossing the multiply-resistant MA strain, collected from Hadley, MA in 1983, with the SS strain and used for comparative purposes (Argentine *et al.*, 1989). The strains were reared in aluminum and fiberglass screen cages (66 by 91 by 50 cm) and fed potato plants (*Solanum tuberosum* L.). Beetle rearing conditions were 27 ±2°C, 50-85% RH, and a photoperiod of 16:8 (L:D).

## Chemicals

All compounds used were technical grade. N-propyl carbofuran (2,3-dihydro-2,2-dimethyl-benzofuran-7-yl propylcarbamate, 95% pure) was a gift from FMC Corp. (Princeton, NJ). Azinphosmethyl-oxon (O, O-dimethyl S-[4-oxo-1,2,3-benzotriazin-3-(4H)-yl methyl] phosphorothioate, 95% pure) was a gift from Bayer Corporation (Kansas

City, MO). Propaphos (4-(methylthio)phenyl dipropyl phosphate, 93.4% pure) was a gift from Nippon-Kayaku Co. Ltd. (Tokyo, Japan). Azinphosmethyl (O, O-dimethyl S-[4-oxo-1,2,3-benzotriazin-3-(4H)-yl methyl] phosphorodithioate, 98% pure), azinphosethyl (O, O-diethyl S-[4-oxo-1,2,3-benzotriazin-3-(4H)-yl methyl] phosphorodithioate, 99.5% pure), N-methyl carbofuran (2,3-dihydro-2,2-dimethyl-benzofuran-7-yl methylcarbamate. 98% pure), methyl paraoxon (O,O-dimethyl O-4-nitrophenyl phosphate, 99.4% pure), ethyl paraoxon (O,O-diethyl O-4-nitrophenyl phosphate, 99.5% pure), methyl parathion (O,O-dimethyl O-4-nitrophenyl phosphorothioate, 98% pure), and ethyl parathion (O,O-diethyl O-4-nitrophenyl phosphorothioate, 98% pure) were purchased from Chem Service (West Chester, PA). All other chemicals were purchased commercially at the highest purity available. Figure 1 illustrates some of these insecticide structures.

Bioassay Procedures

Fourth instars (65-85mg) were used in all bioassays (Argentine *et al.*, 1989). Insecticides were dissolved in acetone and applied to the third abdominal segment of the larvae using a microapplicator with a 100 µl glass microsyringe (Hamilton Co., Reno, NV). Insecticides were applied in 1 µl aliquots. Control larvae were treated with 1 µl of acetone. Up to 25 larvae were placed into a 1-quart glass jar and supplied with cut potato plant stalks secured in a water pik post-treatment. Mortality was assessed 24 h after treatment. An insect was considered dead if it failed to upright itself in 1 min after being turned on its back. Log dose vs. logit mortality data (POLO-PC, LeOra Software 1987) was used to analyze to determine the $LD_{95}$ values of various insecticides for the SS strain.

The SS, AZ-R, and BERTS strains were dosed at the SS LD 95 concentration of insecticide for comparison of mortality.

AChE Preparation and Determination of AChE Activity from Individual CPB (Ellman Reaction)

*AChE preparation.* Fourth instars (65-85 mg) were collected from each strain and starved for 48 h to remove all gut contents. The larva was kept on ice or refrigerated during the remainder of the experiment. Each larva was cut into two pieces between the prothorax and mesothorax using a razor blade. The mesothorax, metathorax, and abdomen were saved at -80°C or used immediately for nucleic acid studies. The head and prothorax from individual larva was homogenized in 200 µl of 0.1 M sodium phosphate buffer (pH 7.5) containing 0.3% (v/v) Triton X-100. The homogenate was centrifuged 10,050 x g for 20 min at 4°C. The supernatant served as a crude extract preparation for AChE activity.

*Three-way reaction for assessment of azinphosmethyl-oxon and carbofuran inhibition of AChE.* A 150 µl aliquot of the supernatant was transferred to a well on the top row of wells of a clear 96-well microplate. After mixing, 50 µl of the supernatant was transferred to each of two wells located directly below the top well using a multi-channel pipettor. Each well in the top row received 100 µl of a solution containing 0.1M sodium phosphate buffer (pH 7.5) with 0.5 mM acetylthiocholine (substrate), 0.4 mM 5,5'-dithio-*bis* (2-nitrobenzoic acid) (DTNB, coloring reagent), and 1% (v/v) acetone. Each well of the second row received 100 µl of a solution identical to that used in the top row but included 50 µM azinphosmethyl-oxon. Each well of the third row received 100 µl of a

solution identical to that used in the top row but included 50 µM carbofuran. AChE activity was assayed for a total of 45 min using a microplate reader (Molecular Devices, Sunnyvale, CA). The AChE activity was determined from changes in the absorbance (OD) at 405 nm over 15 min intervals at room temperature (OD limit = 0.1; read interval = 45 sec). The effect of inhibitors was determined for individual CPB by calculating the percent AChE activity remaining over time.

*Five-way reaction for assessment of N-methyl and N-propyl carbofuran and methyl and ethyl paraoxon inhibition of AChE.* The same basic procedure that was described for the three-way AChE assay was used with the following changes: The head and prothorax were homogenized in 300 µl of buffer, so that five wells could each receive a 50 µl aliquot of the crude AChE preparation. Each well of the top row received the same mixing solution. The wells of the next four rows received identical solutions, with the addition of either 10 µM N-methyl or N-propyl carbofuran or 150 µM methyl or ethyl paraoxon. Concentrations for the latter insecticides were determined in preliminary assays. A range of concentrations for the methyl-substituted insecticides (N-methyl carbofuran and methyl paraoxon) was examined using AChE preparations from SS CPB to predict the $IC_{50}$ values. These $IC_{50}$ values were used for the methyl-substituted insecticides and their respective analogs with larger alkyl groups for the purpose of comparing insecticide size (alkyl substitutions) in relation to their potency on AChE inhibition.

*Three-way reaction for assessment of propaphos and N-methyl carbofuran inhibition of AChE.* The same basic procedure that was described for the three-way AChE assay was used with the following changes: Each well of the top row received the

same mixing solution. Each well of the second row received 100 µl of a solution identical to that used in the top row but amended with 5000 µM propaphos. Concentration of propaphos was determined by a preliminary assay conducted to predict $IC_{50}$ value in SS CPB. Each well of the third row received 100 µl of a solution identical to that used in the top row but amended with 10 µM N-methyl carbofuran.

Total RNA Extraction from Individual CPB

Total RNA was extracted from the mesothorax, metathorax, and abdomen of individual CPB used in the AChE assay. If the body segments were frozen at -80°C, they were first defrosted on ice. Body segments from individual beetles were homogenized with 1 ml of ice cold TRI Reagent (Molecular Research Center, Cincinnati, OH), which contains guanidine thiocyanate and phenol. Homogenates were centrifuged at 12,000 x g for 10 min at 4°C. The supernatants were incubated at room temperature for 5 min to permit complete disassociation of the nucleoprotein complexes, then extracted with chloroform. The lower phase and interphase were set aside for genomic DNA extraction. The remaining top aqueous phase was mixed gently via inversion of the microtube with 0.5 ml of isopropanol to precipitate the RNA. An RNA pellet was obtained by centrifugation at 12,000 x g for 8 min at 4°C. The pellet was washed with 75% ethanol and air-dried for 5-10 min. The pellet was resuspended into diethyl pyrocarbonate (DEPC) -treated water via gentle pipetting and incubated in a 60°C water bath for 12 min. Concentration of RNA was determined by the RNA/DNA Calculator (Pharmacia Biotech San Francisco, CA). RNA was stored as a precipitate at -80°C for up to three months.

cDNA Synthesis

The procedure followed the manufacturer's instructions that were provided in the *SuperScript Preamplification System for First Strand cDNA Synthesis System* (GIBCO BRL, Life Technologies, Grand Island, NY). The RNA/Primer mixture, including 5 µg of RNA, 0.5 µg of primer and DEPC-treated water in a final volume of 12 µl, was incubated at 70°C for 10 min, centrifuged at room temperature at 10,000 x g for 5 sec, and placed on ice for 1 min in a sterile 0.5 ml microtube. Two cDNA reactions were performed for each RNA extract. The first cDNA reaction used an oligo$(dT)_{12\text{-}18}$ primer for the first strand cDNA synthesis. This cDNA reaction resulted in PCR amplification of the SII and SIII fragments (Zhu *et al.*, 1996a). The second cDNA reaction used a specific primer (28-mer, 5'TCTTGAGTTGAACTGCAGAGACATGTTC), which was located internally in the CPB AChE sequence. This more specific priming method was necessary to obtain sufficient amounts of the 5' end of the AChE cDNA for subsequent PCR amplification of the SI fragment.

Each microtube received 7 µl of the reaction mixture, which was composed of 20 mM Tris HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.125 mM of each deoxynucleoside triphosphates (dNTPs) and 0.01 M DTT (dithiothreitol). Microtubes were incubated at 42°C for 5 min prior to addition of 1µl (200 units) of SuperScript II Reverse Transcriptase giving a final volume of 20 µl. The reactions were incubated at 42°C for 50 min. The reverse transcription reaction was terminated by incubation at 70°C for 15 min. RNA, which may interfere in later PCR amplifications, was degraded by addition of 2 units of RNase H, followed by incubation at 37°C for 20 min. The single stranded cDNA was

placed on ice for use as a RT-PCR template or stored at -20°C for up to 2 weeks, or at -80°C for several months. Figure 2 illustrates the cDNA synthesis strategy.

Genomic DNA Extraction

*From RNA extraction with TRI reagent.* The lower and interphase layers from the chloroform extraction step of RNA isolation were used for genomic DNA (gDNA) precipitation with absolute ethanol. gDNA was pelleted by centrifugation at 2,000 x g for 5 min at 4°C. The pellet was washed twice with 0.1 M sodium citrate in 10% ethanol followed by a single wash with 75% ethanol. Pellet was air dried and then resuspended in 8 mM NaOH. Quantity of gDNA was determined by the DNA/RNA calculator.

*From CPB tissue.* CPB tissues from the mesothorax, metathorax and abdomen were homogenized with 1 ml of DNAzol (Molecular Research Center, Inc. Cincinnati, OH). Each homogenate was incubated at room temperature for 10 min to resuspend gDNA into lysate. Homogenate was centrifuged at 12,000 x g for 10 min at 4°C for sedimentation of insoluble tissue fragments, partially hydrolyzed RNA and excess polysaccharides. The supernatant was transferred to a fresh microtube and gDNA precipitated in absolute ethanol. gDNA was pelleted by centrifugation at 1,000 x g for 2 min at 4°C. The pellet was washed twice with 95% ethanol and air-dried. gDNA was dissolved in 8 mM NaOH. The concentration of gDNA was determined by the DNA/RNA calculator. The pH of the gDNA solution was then adjusted to 7.5 by the addition of 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid).

PCR Amplification of the Open Reading Frame of AChE cDNA

The coding region of AChE cDNA was amplified using three sets of specific primers that were designed to amplify the gene in three fragments, SI (1068 bp), SII (622 bp), and SIII (575 bp), with overlapping sequence at their adjacent ends (Zhu *et al.*, 1996). To ensure that enough PCR product was obtained for subcloning, each fragment was amplified in two rounds of PCR using a semi-nested reverse primer technique. For the first round, the forward primer and outer reverse primer were used in a 50 µl PCR mixture containing 5-10 µl of cDNA reaction mixture, 25 pmol of each primer, 0.2 mM dNTPs (each), 50 mM KCl, 10 mM Tris-HCl (pH 10 for SI; pH 9.5 for SII and SIII), $MgCl_2$ (1.5 mM for SI; 2.0 mM for SII and SIII) and 1.25 U *Taq* polymerase.

PCR parameters included initial denaturing at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. First round PCR reaction mixtures were diluted 20X with distilled deionized water and used directly in the second round of PCR. For the second round, the 50 µl PCR reaction mixture contained 5 µl of the diluted first round mixture with 25 pmol of the same forward primer and a semi-nested reverse primer, 0.2 mM dNTPs (each), 50 mM KCl, 10 mM Tris-HCl (pH 10 for SI; pH 9.5 for SII and SIII), $MgCl_2$ (1.5 mM for SI; 2.0 mM for SII and SIII) and 1.25 U *Taq* polymerase. PCR parameters were identical to those used in the first round except for the addition of a final incubation at 72°C for ten min for addition of the 3' T-overhang, necessary for subsequent TA cloning. Amplification took place in the DNA Thermal Cycler 480 (Perkin-Elmer, Foster City, CA). A portion of the PCR mixture was analyzed for product size by agarose gel electrophoresis using pGEM DNA markers (Promega, Madison, WI). The electrophoresis gel employed contained 1.2% agarose in 1X TBE

buffer. Separation was conducted at 70 V for 1.5 h. Amplification products were used directly for subcloning without further purification. Table 1 presents a list of all PCR primers and their sequences.

Subcloning of PCR Products

The procedure followed the manufacturer's instructions provided with the *TA Cloning Kit* (Invitrogen, Carlsbad, CA). Approximately 50 ng of PCR product (with 3' T-overhang) was ligated with 50 ng of pCR 2.1 vector, containing ampicillin resistance and lacZ genes, in a reaction mixture containing 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 50 mM NaCl, 1 μg BSA (bovine serum albumin), 7 mM β-mercaptoethanol, 0.1 mM ATP (adenosine triphosphate), 2 mM DTT, 1 mM spermadine, and 4.0 U of T4 DNA ligase. Ligation reaction was incubated at 14°C overnight. Fifty μl aliquots of thawed TOP10F' competent cells (*E. coli*), in individual vials, were placed on ice. Each vial received 2 μl of 0.5 M β-mercaptoethanol and 2 μl of the ligation reaction. Vials were incubated on ice for 30 min then heat shocked for exactly 30 sec in a 42°C water bath. The vials were placed on ice for 2 min before the addition of 250 μl SOC media ((2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). Vials were shaken horizontally at 225 rpm for 1 h at 37°C. Transformed cells were plated in 50-100 μl aliquots onto LB-Ampicillin plates coated with IPTG (isopropylthio-• -galactoside) and X-gal (5-bromo-4-chloro-3-indoyl-• -D-galactopyranoside) for blue:white screening. Overnight cultures of positive colonies were screened via initial lysis at 96°C for 15 min, followed by PCR amplification with the pCR 2.1 vector primers (M13 Forward (-20) Primer: 20-mer,

5'TTGTAAAACGACGGCCAGTG 3' and the M13 Reverse Primer: 21-mer, 5'CAGGAAACAGCTATGACCATG 3'). PCR products were run on a 1% agarose gel with 1X TBE buffer at 100 V for 1 h with pGEM DNA markers for size determination of insert. The size-verified plasmid in the remaining culture was purified using Wizard Mini-Prep Kit (Promega, Madison, WI) for automated DNA sequencing.

Sequencing and Analysis of AChE cDNA Fragments

Sequencing of subcloned fragments was accomplished using an ABI Prism 377 DNA Sequencer (Applied Biosystems Institute, Perkin-Elmer, Foster City, CA) at New York State Agricultural Experiment Station, Cornell University (Geneva, NY). Each sequencing reaction required 500 ng of plasmid preparation and 4 pmol of primer. Three primers were used to obtain complete sequences of both strands of each DNA fragment. Each sequence is approximately 700 bp. For SII and SIII fragments, the vector pCR 2.1 primers were used, M13 Forward (-20) Primer ( 20-mer, 5'TTGTAAAACGACGGCCAGTG 3') and the M13 Reverse Primer (21-mer, 5'CAGGAAACAGCTATGACCATG 3'). For the SI fragment, both vector primers were used in addition to one internal primer (17-mer, 5'TGAGTGGTACCGCAACC 3'). Sequences were analyzed with SeqEd software (Perkin-Elmer, Foster City, CA), along with a modified version for of ABIView for the PC (courtesy of David Klatte, http://users.cloud9.net/~dhk), and Gene Runner 3.00 (Hastings Software).

Table 1
Location and sequence of oligonucleotide primers used for semi-nested polymerase chain reaction (PCR) following reverse transcription of RNA.

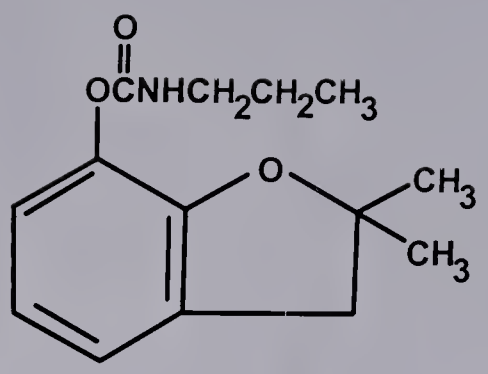
| Fragment | Primer[a] | Location[b] | Oligonucleotide sequence (5' to 3')[c] |
|---|---|---|---|
| SI | Forward | 44 - 61 | TCACTCGGTGAATCGCCC |
| | Reverse A | 1129 - 1102 | GGAATAGGAGTTCCATTGTTGTAGCGAG |
| | Reverse B | 1093 - 1072 | ACGAATTCAGCATCCACTGCTCTCATACAG |
| SII | Forward | 1011 - 1032 | ATGAGCTCTCATACAGGACTGTGGCTGCAA |
| | Reverse A | 1660 - 1633 | TCTTGAGTTGAACTGCAGAGACATGTTC |
| | Reverse B | 1616 - 1595 | ACGAATTCCGTATTCCACTTCATCTCCGTG |
| SIII | Forward | 1551 - 1572 | ATGAGCTCATAGGACGAGCACGTCGTTATG |
| | Reverse A | 2189 - 2162 | GGGTAGAATTACGATTTATAAGTGACCC |
| | Reverse B | 2109 - 2088 | ACGAATTCGAGCATGCGACATTCATACAAG |

[a] Forward and Reverse A primers were used for the first round of PCR. The same Forward and Reverse B primer were used as semi-nested primer set for the second round of PCR (Zhu *et al.*, 1996).

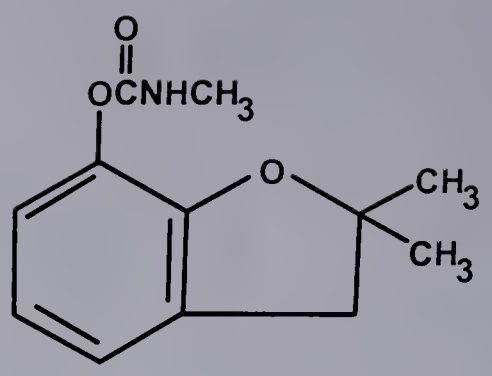
[b] The nucleotide locations were based on the previously published AChE cDNA sequence from the CPB (Zhu and Clark, 1995b).

[c] The underlined nucleotide sequences are inserted restriction sites for subcloning.

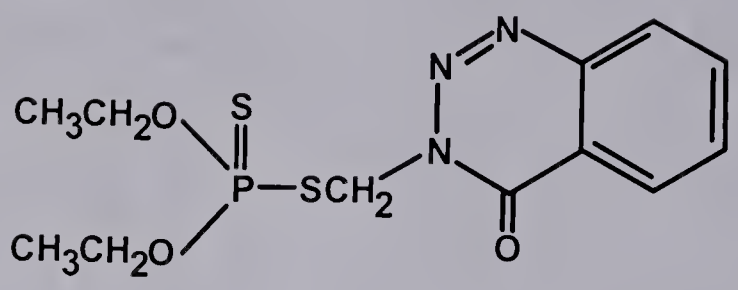
Figure 1: Insecticide structures.



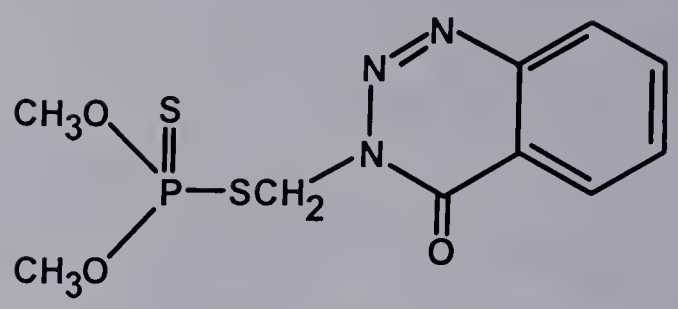

N-propyl carbofuran



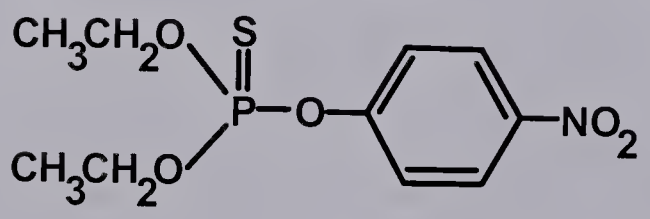

N-methyl carbofuran



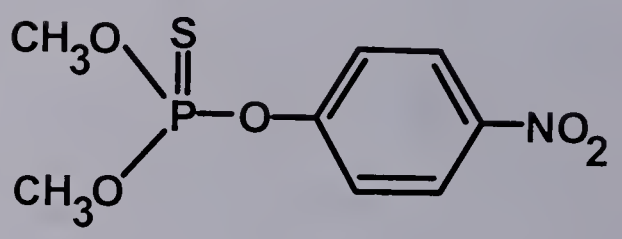

azinphosethyl



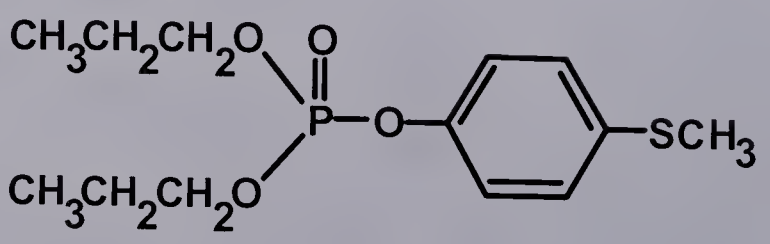

azinphosmethyl




ethyl parathion




methyl parathion




propaphos

Figure 2: Schematic of first-strand cDNA syntheses from CPB total RNA using two different primers. The oligo$(dT)_{12\text{-}18}$ primed cDNA was used as template to obtain SII and SIII fragments of the AChE gene in subsequent PCR amplification. An AChE specific primer was used for an additional first-strand cDNA synthesis. The AChE specific primed cDNA was necessary to obtain sufficient amounts of the AChE SI fragment.

Total RNA Extraction from Individual CPB

First-Strand cDNA Synthesis

With Oligo(dT)$_{12-18}$ Primer

**AChE mRNA** 2100+bp **AAAA**

**Synthesized cDNA** **TTTT**

Up to 2100+ bp

With AChE Specific Primer

**AChE mRNA** 2100+bp **AAAA**

**Synthesized cDNA** **TTCT**

1660 bp

Resultant PCR Amplifications

**TTTT**

SIII

575 bp

+

**TTTT**

SII

622 bp

**TTCT**

SI

1068 bp

# CHAPTER 3

# RESULTS

## Acquisition and Characterization of the BERTS Strain

In the summer and fall of 1996, a collaborative effort with Dr. Galen Dively at the University of Maryland/College Park began, which involved the assessment of insecticide resistance on field populations of CPB. The Maryland group has extensively assessed the types and levels of insecticide resistance in CPB by contact bioassay throughout Maryland, Delaware, and Virginia, as well as in a CPB population from France (Olson *et al.*, 2000). Once insecticide susceptibility/resistance was assessed, egg masses of selected populations were sent to our laboratory and maintained. Fourth instars were assessed for AChE sensitivity to azinphosmethyl-oxon and N-methyl carbofuran via the Ellman reaction.

Overall, 7 field strains were assessed (Table 2 and Fig. 3). Individual CPB sensitivity to insecticides was variable within a strain, making statistically significant differences difficult to determine. However, this type of genetic variability was not unexpected, as it was previously seen in the AChE analysis of individual CPB from the SS strain (Zhu *et al.*, 1996). Although the vast majority of SS CPB are susceptible to azinphosmethyl, a few individuals tested exhibited AChEs insensitive to inhibition by azinphosmethyl-oxon.

Here, the comparison of strains is based on the average susceptibility of the individual CPBs analyzed for each strain. The France and BERTS strains were most sensitive to azinphosmethyl (96.5 and 60.0% mortality, respectively, Table 2). These strains also elicited the lowest AChE activity remaining in the presence of azinphosmethyl-

oxon (7 and 11%, respectively, Fig. 3). The France strain also was highly sensitive to N-methyl carbofuran (90.5% mortality and 10% AChE activity remaining, Table 2 and Fig. 3, respectively).

The correlation between the mortality and AChE inhibition data is less obvious when bioassay results in less than 50% death. Although the Maddox strain showed high resistance to azinphosmethyl in the bioassay (5.75% mortality, Table 2), only 16% of the AChE activity remained in the presence of azinphosmethyl-oxon (Fig. 3). These results indicate that the azinphosmethyl resistance of the Maddox strain is not due to AChE insensitivity, and likely due to other resistance mechanisms.

The bioassay data for N-methyl carbofuran indicate that three of the strains, Maddox, BERTS, and Zimmerman, had high resistance (less than 4% mortality, Table 2). The AChEs from these strains also were relatively insensitive to N-methyl carbofuran (43, 33, and 26% AChE activity remaining, respectively, Fig. 3).

The BERTS strain was selected for further study for the following reasons. The BERTS strain was highly resistant to N-methyl carbofuran but relatively susceptible to azinphosmethyl. It possessed individuals with an AChE that is relatively insensitive to N-methyl carbofuran inhibition but is highly sensitive to azinphosmethyl-oxon inhibition. This type of individual profile was named BERTS-R denoting resistance to N-methyl carbofuran and susceptibility to azinphosmethyl, with respect to AChE insensitivity. Also present in the BERTS population, was a profile type named BERTS-S. These individuals possessed an AChE that was highly sensitive to both insecticides.

Inhibition of AChE Activity from Carbofuran-Resistant and –Susceptible Strains of CPB with N-Methyl Carbofuran and Azinphosmethyl-Oxon

Evaluation of the role of AChE insensitivity in N-methyl carbofuran and azinphosmethyl resistance was determined using individual fourth instars from the unsorted BERTS and sorted BERTS-S and -R strains. Because only the head and prothorax of individual larvae were used in the Ellman assay, the remaining body segments were labeled and stored for future nucleic acid studies. Hence, the AChE cDNA of an individual larva could be correlated to the relative sensitivity of its AChE to specific cholinesterase inhibitors. In this manner, the nucleic acid sequences of the most insensitive AChE, could be compared to the sequences of the most sensitive AChEs in order to detect nonsilent mutations most likely associated with resistance.

Evaluation of the relative insecticide sensitivity of AChE from individual CPB using the Ellman reaction involves a series of absorbance readings taken at 405 nm every 45 seconds between the 15 to 30 min period of time after the reaction has begun in the presence or absence of insecticide inhibitors. The absorbance correlates to the amount of thiocholine that is produced by the AChE-dependent hydrolytic cleavage of the substrate ATC. When the AChE is functioning normally, thiocholine will accumulate with time and result in a positive linear curve (Fig. 4, Control). When the AChE is exposed to insecticides that act as competitive cholinesterase inhibitors, the enzyme can no longer hydrolyze ATC, so the absorbance does not increase or increases at a significantly lower rate. Using this criteria, the BERTS-S and BERTS-R substrains are susceptible to the inhibiting action of azinphosmethyl-oxon (Fig. 4, Azin.). In cases where insecticide resistance is due to a target site-insensitivity mechanism associated with AChE, the

cholinesterase-inhibiting insecticide does not bind efficiently to the target site. Thus, the unbound AChE is free to function relatively normally. A significant amount of AChE activity remains in the presence of N-methyl carbofuran in BERTS-R individuals, but very little activity remains in BERTS-S individuals, indicating the insensitivity and sensitivity of the respective AChEs to carbofuran (Fig. 4, Carbo.).

Identification of point mutations in the AChE cDNA associated with site-insensitivity resistance to N-methyl carbofuran requires a comparison of cDNA from CPBs that expressed carbofuran-sensitive AChEs to CPBs that expressed AChEs that are insensitive to carbofuran inhibition. The heterozygosity of the BERTS strain allowed us to examine both types of individuals within the same strain (BERTS-S and BERTS-R substrains). Using the same strain to evaluate both types of individuals was important, since if more than one strain was used, one may question whether or not a nonsilent mutation was merely a polymorphism in one strain rather than actually associated with carbofuran resistance due to AChE site-insensitivity.

AChE activity was quantified by dividing rate of the accumulation of thiocholine, measured in milli-optical density units per minute (mO.D./min), in a reaction containing insecticide by the rate of the control reaction. This value is multiplied by 100 to determine the percent AChE activity remaining. A total of 299 individual BERTS CPB were analyzed (Table 3). On average, the remaining AChE activity in the presence of 50 μM N-methyl carbofuran was 32.8% of the uninhibited control activity. The remaining AChE activity in the presence 50 μM azinphosmethyl-oxon was only 15.9%. The findings indicate that the BERTS strain was approximately 2-fold more sensitive to the inhibitory action of azinphosmethyl-oxon than to N-methyl carbofuran.

25 individuals were selected from the assayed population to form each of two sorted substrains (Table 3). The BERTS-S substrain was composed of beetles that possessed AChEs that were highly sensitive to the inhibitory action of both N-methyl carbofuran and azinphosmethyl-oxon. Treatments of the AChE of the BERTS-S substrain with 50 μM N-methyl carbofuran resulted in only 0.3% of the activity remaining and only 7.5% remaining in the presence of 50 μM azinphosmethyl-oxon. The BERTS-R substrain was composed of beetles that possessed AChEs that were relatively insensitive to the inhibitory action of N-methyl carbofuran but sensitive to the inhibitory action of azinphosmethyl-oxon. Treatments of the AChE of the BERTS-R substrain with 50 μM N-methyl carbofuran resulted in 61.1% of the activity remaining and only 25.3% remaining in the presence of 50 μM azinphosmethyl-oxon.

Individual beetles of both substrains were further examined as candidates for nucleic acid sequencing of their respective AChE cDNA. The inhibition data of the BERTS population indicated that the pooled AChE was significantly more sensitive to azinphosmethyl-oxon than to N-methyl carbofuran. The AChE of the BERTS-R substrain, however, was significantly more insensitive to azinphosmethyl-oxon than was the AChE from the BERTS-S substrain to azinphosmethyl-oxon (Table 3). The ideal candidates for use in a nucleic acid sequencing analysis to determine mutations associated with N-methyl carbofuran resistance would be BERTS-S and BERTS-R beetles that had little or no resistance to azinphosmethyl-oxon. The high degree of heterogeneity in the BERTS-S and -R substrains, and the ability to determine individual CPB AChE activity profiles in the presence of competitive inhibitors allowed for such ideal candidates to be selected. Selected BERTS-S and BERTS-R CPB had AChE profiles that preserved the

susceptibility to azinphosmethyl-oxon but were substantially different in their susceptibility to N-methyl carbofuran. A total of 6 sorted BERTS-S and 9 sorted BERTS-R beetles were used to obtain sufficient sequence for the mutational analysis. Results of this analysis will be subsequently discussed.

In both sorted BERTS-S and BERTS-R individuals, there is a low amount of AChE activity remaining when treated with azinphosmethyl-oxon (<10%) (Fig. 5). There is, however, a significant difference between the BERTS-S and BERTS-R CPB selected for mutational analysis as is determined by treatment with N-methyl carbofuran. A two-tailed z-test comparing BERTS-S and BERTS-R individuals for AChE activity remaining calculated Z to be equal to –15.02 ($\alpha = 0.05$, z-critical = $\pm$ 1.96), signifying the sorted substrains are statistically different for N-methyl carbofuran sensitivity. The selected BERTS-S individuals show approximately 0-6.5% AChE activity remaining, while the BERTS-R CPB retain up to 65% of normal AChE activity. It is hypothesized that if a common mutation is identified within the AChE gene of all the individuals of either the selected BERTS-S or BERTS-R substrain, it is very likely to be associated with the AChE insensitivity and resistance to N-methyl carbofuran.

Effect of Alkyl Group Substitution on the Susceptibility of CPB to Various OP and Carbamate Insecticides

*Bioassays:* A number of studies on altered AChE, which result in carbamate and organophosphate insecticide resistance due to enzyme insensitivity, suggest that resistant/insensitive forms of AChE have increased susceptibility to insecticide analogs with larger alkyl groups associated with the acid moieties (Yamamoto *et al.*, 1977ab,

1993; Brown and Bryson, 1992; Zhu and Clark, 1995a). The SS, AZ-R, and BERTS strains of CPB were assayed via topical application for resistance/susceptibility to organophosphorous insecticides (azinphosmethyl, azinphosethyl, methyl parathion, ethyl parathion and propaphos) and carbamate insecticides (N-methyl carbofuran and N-propyl carbofuran). Structures of the insecticides are illustrated in Figure 1.

As expected, low mortality (12%) was exhibited by the AZ-R strain with azinphosmethyl and by the BERTS strain with N-methyl carbofuran (8%) and mortality by the SS strain for these insecticides was high (>90%) (Table 4). Azinphosmethyl was approximately 8-times more toxic to the SS strain than to the AZ-R strain, but only approximately 35% more toxic to the SS strain than to the BERTS strain. Carbofuran was approximately 12-times more toxic to the SS strain than to the BERTS strain, but only approximately 2.6-times more toxic to the SS strain than to the AZ-R strain.

Topical application of N-propyl carbofuran on the BERTS strain resulted in 36% mortality, indicating that N-propyl carbofuran is approximately 4.5-times more toxic than N-methyl carbofuran (Table 4). N-propyl carbofuran also produced similar levels of mortality in the SS strain (32% mortality). These data indicate that in a field situation, N-methyl carbofuran would likely eradicate almost all SS CPB, but not the BERTS CPB, thereby increasing the ratio of resistant to susceptible individuals to propagate. Spraying with N-propyl carbofuran, although less potent against the SS CPB, would result in equal selection and not cause such a selection of resistant individuals to occur. The SS strain showed high susceptibility to all OPs tested (80-95%), regardless of the size of the alkyl group (Table 4).

The AZ-R strain elicited higher mortality in the presence of OPs with ethoxy and propoxy alkyl groups compared to their methoxy counterparts (Table 4). AZ-R strain mortality with azinphosethyl was approximately 2.7-times higher than with azinphosmethyl (32 versus 12%, respectively). Ethyl parathion was approximately 12% more toxic than methyl parathion (56 versus 48%, respectively). Propaphos was nearly equal in its mortality response to the SS (96% mortality) and AZ-R (92% mortality) strains. This finding is similar to that already shown in the case of N-propyl carbofuran with the SS and BERTS strains. The usefulness of such negative cross-resistance relationships shown above for these resistance-breaking propyl OPs and carbamates as resistance management tools will be elaborated on in the discussion section.

*Inhibition of AChE from SS, AZ-R, and BERTS strains by various OP and carbamate insecticides:* AChE of the SS strain (n = 64) is nearly 2-times more sensitive to inhibition by methyl paraoxon than ethyl paraoxon and 4.5-times more sensitive to inhibition by methyl paraoxon than propaphos (68 vs. 36 or 15% AChE activity inhibited, respectively, Fig. 6). The AChE of the SS strain is approximately 10-times more sensitive to N-methyl carbofuran than to N-propyl carbofuran (79 vs. 8% AChE activity inhibited, respectively). The AChE of the AZ-R strain (n = 48) is 30% more sensitive to inhibition by ethyl paraoxon (51 versus 36%), 2.2-fold more sensitive to propaphos (33 versus 15%) and 3.6-fold more sensitive to N-propyl carbofuran (29 versus 8%) than the SS strain. The AChE of the BERTS strain is approximately 30% more sensitive to inhibition by propaphos (19 versus 15%) and approximately 90% more sensitive to N-propyl carbofuran (15 versus 8%) than the SS strain. These findings support the hypothesis that

AChEs that are insensitive to methoxy substituted OPs and to N-methyl substituted carbamates show increased inhibition by insecticides with larger alkyl groups and establishes a negative-cross insensitivity relationship to these insecticides and correlates well to their negative-cross resistance relationships established previously (Table 4).

Although AChE inhibition in the AZ-R strain is similar for N-propyl carbofuran and propaphos (29 and 33%, respectively, Fig. 6), bioassay data shows a large difference in susceptibility (Table 4). No mortality occurred in AZ-R CPB dosed with N-propyl carbofuran, yet 92% mortality was seen for propaphos. This phenomenon may be due to the approximately 10-fold difference in discrimating dose, as determined by $LD_{95}$ in the SS strain. N-propyl carbofuran was administered at 15 μg per CPB, while propaphos was administered at 140.7 μg per CPB.

The AChE of BERTS strain (n = 42) is more sensitive to inhibition by N-propyl carbofuran and propaphos than the SS strain. It is approximately 2-fold less sensitive to inhibition by N-methyl carbofuran (39 versus 79%), 40% less sensitive to inhibition by methyl paraoxon (41 versus 68%) and approximately 33% less sensitive to ethyl paraoxon (24 versus 36%) compared with the SS strain (Fig. 6). These findings are consistent with those obtained in topical bioassays. BERTS CPB had only 8% mortality with N-methyl carbofuran, 4% mortality with methyl parathion and 32% mortality with ethyl parathion. However, azinphosmethyl elicited 71% mortality (Table 4). These results indicate that the alcohol moiety of azinphosmethyl also is important in resistance and may be due to the difference in the size and charge of acid moieties of azinphosmethyl versus methyl parathion and N-methyl carbofuran (Fig. 1). The 3-methyl-1,2,3-benzotriazin-4-one

moiety of azinphosmethyl is considerably larger than the nitrophenyl moiety of parathion and the furan moiety of N-methyl carbofuran.

A more compelling comparison is that of the ratios of the AChE activity between insecticides with different alkyl groups. In using a ratio, one can discern the effectiveness of the insecticide with the larger alkyl group relative to that of its smaller counterpart for a given strain. The ratio of the strains' average percent AChE activity remaining in the presence of the insecticide with the larger alkyl group to that in the presence of an insecticide with the smaller alkyl group [i.e. N-propyl carbofuran / N-methyl carbofuran(P/M ratio), ethyl paraoxon / methyl paraoxon (E/M ratio)], reveals a trend. A ratio >1 means that the insecticide with the larger alkyl group is less effective than that with the smaller alkyl group. For both the carbofuran P/M and paraoxon E/M ratios, the SS strain has the highest value (4.38 and 2.00, respectively) indicating the relative ineffectiveness of the insecticides with larger alkyl groups (Table 5). These ratios become smaller for the resistant strains that elicit an acetylcholinesterase insensitivity and increased butyrylcholinesterase activity. Ethyl and methyl paraoxon are nearly equally effective on AZ-R AChE, giving a E/M ratio of 1.02, whereas methyl paraoxon was apparently twice as effective as ethyl paraoxon in the inhibition of SS AChE. The P/M ratio of N-propyl carbofuran to N-methyl carbofuran in the BERTS strain is remarkably lower than that of the SS strain (1.39 compared to 4.38, respectively) (Table 5). The decrease in the ratios indicate an increased effectiveness of insecticides with larger alkyl groups on the resistant AZ-R and BERTS strains.

As a population, the BERTS strain exhibits high resistance to N-methyl carbofuran, but it is not homogeneous for this trait. When individual beetles of the

BERTS strain are sorted into the BERTS-S and BERTS-R substrains, there is a significant difference seen in AChE susceptibilities to N-methyl carbofuran. As previously determined, the AChE of the BERTS-S strain exhibits sensitivity to inhibition by N-methyl carbofuran, whereas the BERTS-R strain is relatively insensitive. Differences in the substrains susceptibility to N-propyl carbofuran also were found (Fig. 7). The AChE of sorted BERTS-S beetles was 2.1-times more sensitive to N-methyl carbofuran inhibition than the sorted BERTS-R beetles. In contrast, the sorted BERTS-R beetles were 1.5-times more sensitive to N-propyl carbofuran inhibition than the sorted BERTS-S beetles. These results support the hypothesis that an altered AChE in the BERTS-R strain shows statistically significant higher levels of inhibition by N-propyl carbofuran when compared to the BERTS-S AChE.

Nucleic Acid Sequence Analysis of Selected BERTS-S and BERTS-R AChE CDNA for the Determination of Mutations Associated with N-Methyl Carbofuran Insensitivity and Resistance

AChE cDNA was synthesized by reverse transcription from RNA of sorted beetles of the BERTS-S and BERTS-R substrains. A semi-nested PCR strategy was used for amplification of the cDNA encoding the AChE gene as three overlapping fragments. The three fragments, "S1" (1068 base pairs, located at the 5' end of the cDNA), "S2" (622 base pairs, located in the middle of the cDNA), and "S3" (575 base pairs, located towards the 3' end of the cDNA) that were PCR amplified are illustrated in Figure 8. Transformed PCR products were screened via agarose gel electrophoresis for presence of an AChE cDNA fragment insert (e.g. SII), prior to growth of adequate amounts of DNA for sequencing (Fig. 9). A total of 4 to 5 complete sequences from each fragment for each of

the sorted BERTS-S and BERTS-R CPB was obtained. The deduced amino acid sequences of the AChE cDNA from BERTS-S and BERTS-R CPB are given in Figure 10 and aligned with the sequence of SS and AZ-R strains.

The nucleic acid sequence analysis occurred in three stages. First the sequences of each fragment were lined up for each of the selected BERTS-S and BERTS-R CPB. Any nucleic acid difference between the sequence obtained from the BERTS cDNAs compared to the previously sequenced AChE cDNA from SS and AZ-R strains of CPB was considered to be a mutation. Each mutation was then determined to be either a silent or nonsilent mutation. A nonsilent mutation is significant since it results in an amino acid substitution that can effect the structure and function of the translated protein. A summary of the mutational analysis of the AChE cDNAs from the sorted BERTS-S and BERTS-R substrains is given in Table 6.

Four nonsilent mutations were identified in the mutational analysis of the BERTS substrains (Table 7). Numbering of the locations of mutations is based on the previously published CPB AChE cDNA sequence by Zhu and Clark (1995b). Both BERTS-S and BERTS-R CPB have a G at nucleotide 198, and matches the SS strain in this respect. The AZ-R strain has an A in the 198 nt location. This nucleotide change confers an amino acid change at residue 30 of an arginine (Arg) for a lysine (Lys) (i.e., R30K). The BERTS and SS strains have an arginine (A<u>G</u>A), a guanidinium-containing basic amino acid ($pK_a$ = 12.5), whereas the AZ-R strain has a lysine (A<u>A</u>A), a α-amino-containing basic amino acid ($pK_a$ = 10.5) (Fig. 10, Table 7). At nucleotide 980, both BERTS-S and BERTS-R CPB have a guanine, which is identical to AZ-R CPB strain, at this location. This substitution differs from the SS strain, which has an A in the 980 location. This nucleotide substitution

alters the 291 codon from AGT (serine, Ser) in the SS strain, to GGT (glycine, Gly) in the BERTS and AZ-R strains (i.e., S291G) (Fig. 10, Table 7). Detailed biochemical and pharmacological studies have coupled the S291G mutation to azinphosmethyl resistance (Zhu *et al.*, 1996; Zhu and Clark, 1997). It is hypothesized that the S291G mutation results in an alteration in the secondary structure of the AChE, which alters both the catalytic esteratic subsite and peripheral anionic site of AChE by modification of the position of the $\alpha$-E'$_1$ helix within the three-dimensional structure of AChE. This modification appears to be due to the difference in size between the serine and smaller glycine residue and the interaction of the free hydroxyl group of serine with other amino acids by hydrogen binding. Obviously, the S291G mutation is a major mutation site associated with both azinphosmethyl and carbofuran resistance. Neverthless, this hypothesis must be expanded to explain why the AChE cDNA from the sorted BERTS strains also had the S291G mutation in spite of the fact that the sorted BERTS AChE was highly susceptible to inhibition by azinphosmethyl-oxon.

There were two additional nonsilent mutations that were not identical in the sorted BERTS-S and BERTS-R CPB substrains. One mutation (C to G) occurred in a single BERTS-R individual (of 5 beetles sequenced) at nucleotide location 1033 and resulted in an aspartic acid (Asp) to lysine (Lys) substitution (D308K) (Table 7). Since this mutation was not consistent with other BERTS-R beetles, it is not likely associated with N-methyl carbofuran resistance. The other nonsilent mutation, however, was consistent for all BERTS-S beetles sequenced. At nucleotide location 1284, a point mutation (T to C) resulted in an amino acid change from an isoleucine to a threonine (I392T). BERTS-R CPB as well as both the AZ-R and SS strains have the T at nucleotide 1284, and share a

common isoleucine residue at this allele (Figs. 10 and 11, Table 7). Threonine is neutral, polar amino acid with a free hydroxyl group while isoleucine is a bulkier structure, neutral, and hydrophobic. The differences in both structure and polarity of threonine versus isoleucine, as well as the predicted location of the I392T mutation within the aromatic gorge associated with butyrylcholinesterase activity (Sussman, 1991), make this mutation a likely candidate for an alteration in the AChE structure which could decrease the binding of hydrophobic insecticides such as N-methyl carbofuran and possibly azinphosmethyl-oxon and alternative substrates such as butyrylcholine. Relationship of the three mutation sites to insecticide resistance will be further developed in the discussion section.

Table 2
Bioassay data for various field strains of CPB using a filter paper exposure test with first instars[a].

| CPB strains | n | Percent mortality (± S.D.) | |
|---|---|---|---|
| | | Azinphosmethyl | N-methyl carbofuran |
| BERTS | 200 | 60.0 (± 5.1) | 2.3 (± 1.0) |
| Ellwood | 200 | 48.5 (± 6.3) | 13.5 (± 3.9) |
| Hillman | 200 | 16.5 (± 4.7) | 43.6 (± 7.8) |
| Maddox | 200 | 5.75 (± 1.5) | 2 (± 1.1) |
| Washington Cemetary | 200 | 25.5 (± 2.6) | 37.0 (± 5.2) |
| Zimmerman | 200 | 37.5 (± 2.0) | 3.5 (± 1.1) |
| France | 200 | 96.5 (± 1.7) | 90.5 (± 13.5) |

[a]Data taken from Olson *et al.*, 2000.

Table 3
Inhibition of AChE activity from unsorted and sorted BERTS strains of CPB by 50 μM N-methyl carbofuran and 50 μM azinphosmethyl-oxon.

| | | % AChE activity remaining[a] | |
|---|---|---|---|
| CPB strains[b] | n | N-methyl carbofuran | Azinphosmethyl-oxon |
| BERTS (unsorted) | 299 | 32.8 (± 17.1) | 15.9 (± 16.4) |
| BERTS-S | 25 | 0.3 (± 0.8)[c] | 7.5 (± 13.5)[d] |
| BERTS-R | 25 | 61.1 (± 4.7) | 25.3 (± 15.7) |

[a] Values are means ± standard deviations of the percent AChE activity remaining determined over the 15-30 min interval of the Ellman reaction assay.

[b] Strains include the unsorted BERTS population, a portion of the population sorted for having the highest inhibition by N-methyl carbofuran (BERTS-S) and a portion of the population sorted for having the least inhibition by N-methyl carbofuran (BERTS-R).

[c] Two-tailed z-test comparing BERTS-S to BERTS-R substrains for % AChE activity remaining was $z = -64.2$ ($\alpha = 0.05$, z-critical = ± 1.96), signifying the sorted substrains are statistically different for N-methyl carbofuran sensitivity.

[d] Two-tailed z-test comparing BERTS-S to BERTS-R substrains for % AChE activity remaining was $z = -4.3$ ($\alpha = 0.05$, z-critical = ± 1.96), signifying the sorted substrains are statistically different for azinphosmethyl-oxon sensitivity.

Table 4
Topical application bioassay[a] of the SS, AZ-R, and BERTS CPB strains with OP and carbamate insecticides with different alkyl groups.

| Insecticide | CPB strains | | |
|---|---|---|---|
| | SS | AZ-R | BERTS |
| | Percent mortality | | |
| N-methyl carbofuran | 92 | 36 | 8 |
| N-propyl carbofuran | 32 | 0 | 36 |
| methyl parathion | 88 | 46 | 4 |
| ethyl parathion | 80 | 55 | 32 |
| propaphos | 96 | 92 | 40 |
| azinphosmethyl | 96 | 12 | 71 |
| azinphosethyl | 88 | 32 | 47 |

[a] Data shows percent mortality at a discriminating dose: 15.0 µg for N-methyl carbofuran and N-propyl carbofuran, 5.2 µg for methyl paraoxon and ethyl paraoxon, 4.5 µg for azinphosmethyl and azinphosethyl, and 140.7 µg for propaphos.

Table 5
Comparison of the ratios of percent AChE activity remaining associated with the SS, AZ-R, and BERTS strains of CPB in the presence of insecticides with propyl, ethyl, and methyl alkyl substitutions.

| | | Percent AChE activity remaining (± S.D.) | | | | Ratios of percent AChE activity remaining | |
|---|---|---|---|---|---|---|---|
| CPB strains | n | propyl carbofuran | methyl carbofuran | ethyl paraoxon | methyl paraoxon | P / M carbofuran[a] | E / M paraoxon[b] |
| SS | 64 | 92 (± 9) | 21 (± 17) | 64 (± 19) | 32 (± 2) | 4.38 | 2.00 |
| AZ-R | 47 | 71 (± 21) | 30 (± 15) | 49 (± 18) | 48 (± 18) | 2.37 | 1.02 |
| BERTS | 42 | 85 (± 22) | 61 (± 21) | 76 (± 20) | 59 (± 19) | 1.39 | 1.29 |

[a] Ratios of average % activity remaining in presence of N-propyl carbofuran divided by that remaining in presence of N-methyl carbofuran.

[b] Ratios of average % activity remaining in presence of ethyl paraoxon divided by that remaining in presence of methyl paraoxon.

Table 6
Summary of mutational analysis of AChE cDNA from sorted BERTS-S and BERTS-R CPB.

| Parameter | CPB strains | |
|---|---|---|
| | BERTS-S | BERTS-R |
| No. of SI fragments sequenced | 4 | 4 |
| No. of SII fragments sequenced | 4 | 5 |
| No. of SIII fragments sequenced | 4 | 5 |
| Total no. of mutations | 25 | 26 |
| No. of silent mutations | 22 | 23 |
| No. of nonsilent mutations | 3 | 3 |

Table 7
Summary of nonsilent mutations found in the SI, SII, and SIII fragments of the AChE cDNA from sorted BERTS-S and BERTS-R CPB.

| cDNA fragment | Location[a] | CPB strains | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BERTS-S | | | | BERTS-R | | | | | SS | AZ-R |
| SI | CPB No. | 1[b] | 4 | 5 | 6 | 3 | 4 | 6 | 7 | | | |
| | nt 198 | AGA | AGA | AGA | AGA | AGA | AGA | AGA | AGA | | AGA | AAA |
| | aa 30 | Arg | Arg | Arg | Arg | Arg | Arg | Arg | Arg | | Arg | Lys |
| SII | CPB No. | 2 | 3 | 4 | 5 | 1 | 2 | 7 | 8 | 9 | | |
| | nt 980 | GGT | GGT | GGT | GGT | GGT | GGT | GGT | GGT | GGT | AGT | GGT |
| | aa 291 | Gly | Gly | Gly | Gly | Gly | Gly | Gly | Gly | Gly | Ser | Gly |
| | nt 1033 | AAC | AAC | AAC | AAC | AAC | AAC | AAG | AAC | AAC | AAC | AAC |
| | aa 308 | Asp | Asp | Asp | Asp | Asp | Asp | Lys | Asp | Asp | Asp | Asp |
| | nt 1284 | ACC | ACC | ACC | ACC | ATC | ATC | ATC | ATC | ATC | ATC | ATC |
| | aa 392 | Thr | Thr | Thr | Thr | Ile | Ile | Ile | Ile | Ile | Ile | Ile |
| SIII | CPB No. | 1 | 3 | 4 | 5 | 2 | 3 | 4 | 5 | 7 | | |
| | | –[c] | – | – | – | – | – | – | – | – | | |

[a] The nucleotide locations were based on the previously published AChE cDNA sequence from the CPB (Zhu and Clark, 1995b).

[b] The insect numbers under each strain correspond to those in Fig. 5.

[c] No nonsilent mutations were found in the analyzed SIII fragments.

Figure 3: Percent AChE activity remaining in the presence of azinphosmethyl-oxon (50 μM, AZIN) and N-methyl carbofuran (50 μM, CARBO) to determine the relative sensitivity of AChE activity of various field strains of CPB. Field strains included BERTS, Ellwood (Ellw), Hillman (Hill), Maddox (Madd), Washington Cemetary (Wash), Zimmerman (Zimm), and France.

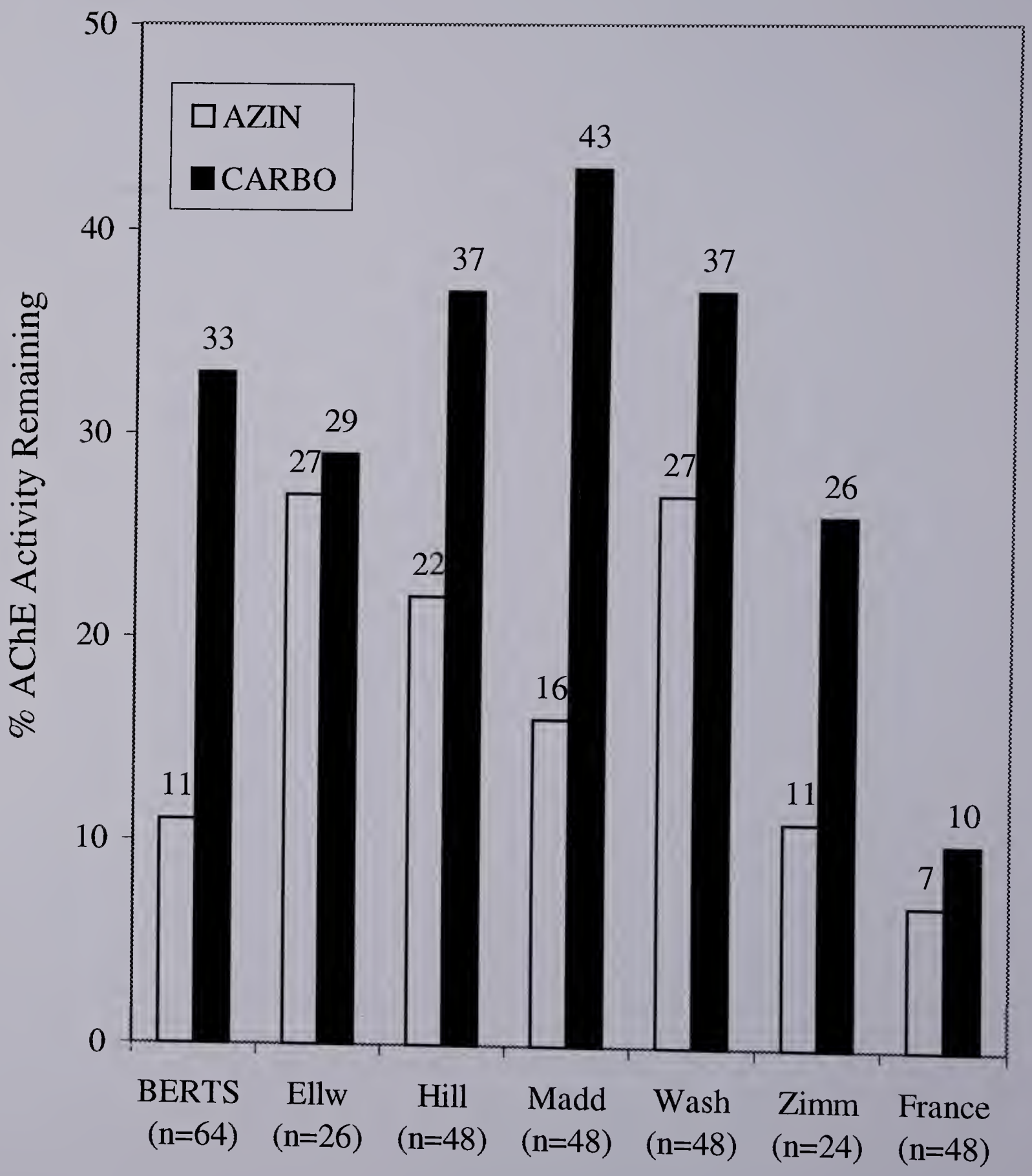

% AChE Activity Remaining
50
40
30
20
10
0
AZIN
CARBO
33
29
27
37
43
37
27
26
22
16
11
10
7
BERTS (n=64)
Ellw (n=26)
Hill (n=48)
Madd (n=48)
Wash (n=48)
Zimm (n=24)
France (n=48)
Field CPB strains

Figure 4: UV spectrophotometric readout (O.D.) of the Ellman reaction using BERTS-S and BERTS-R CPB. The AChE of an individual BERTS CPB is split into three reactions, each containing the substrate ATC and the coloring reagent DTNB. Control reaction has no inhibitors, while the other two reactions contain either of the insecticides azinphosmethyl-oxon (50 μM) or N-methyl carbofuran (50 μM). Each plot contains individual absorbance reading (O.D.) taken every 45 seconds for the 15-30 minute reaction interval.

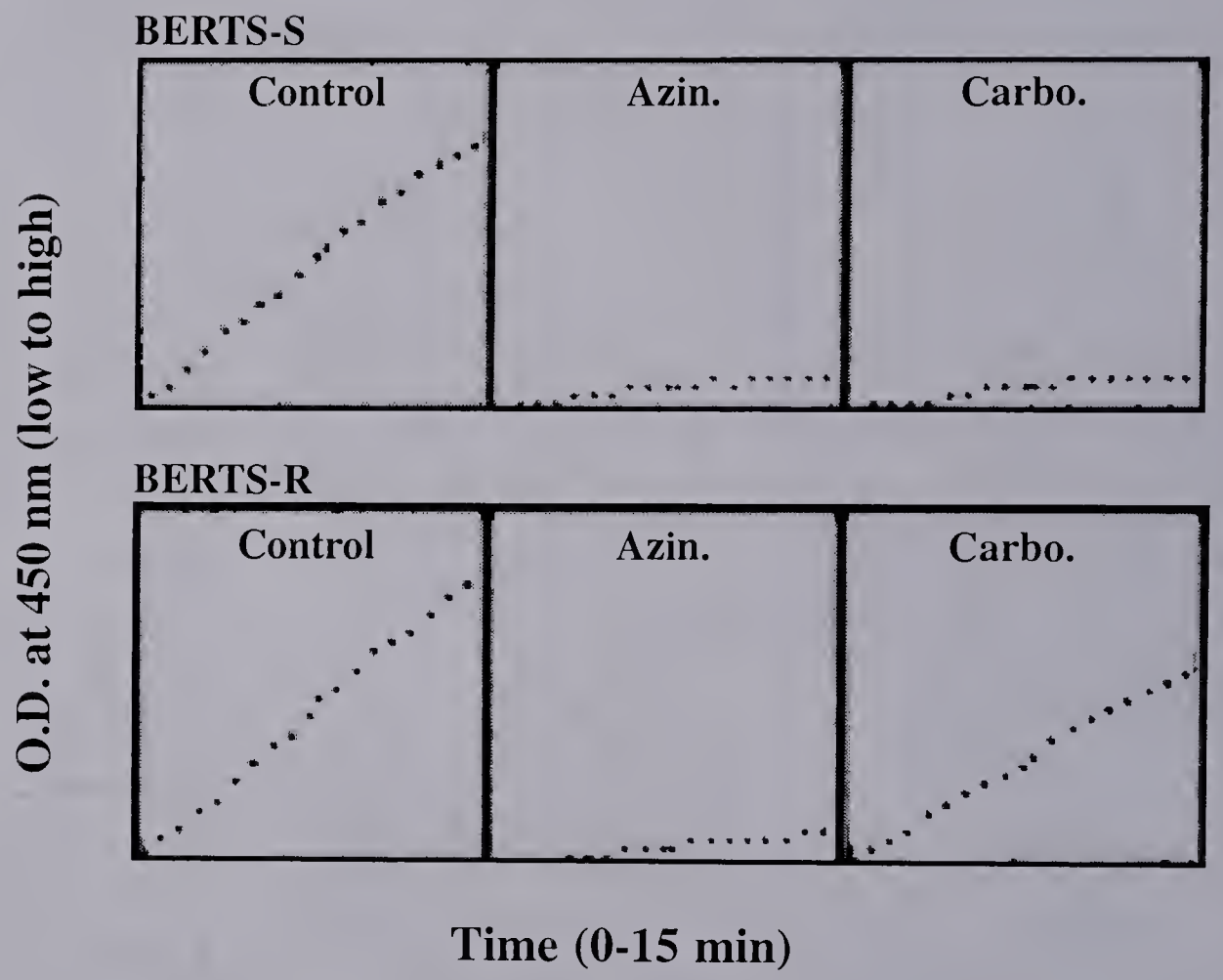
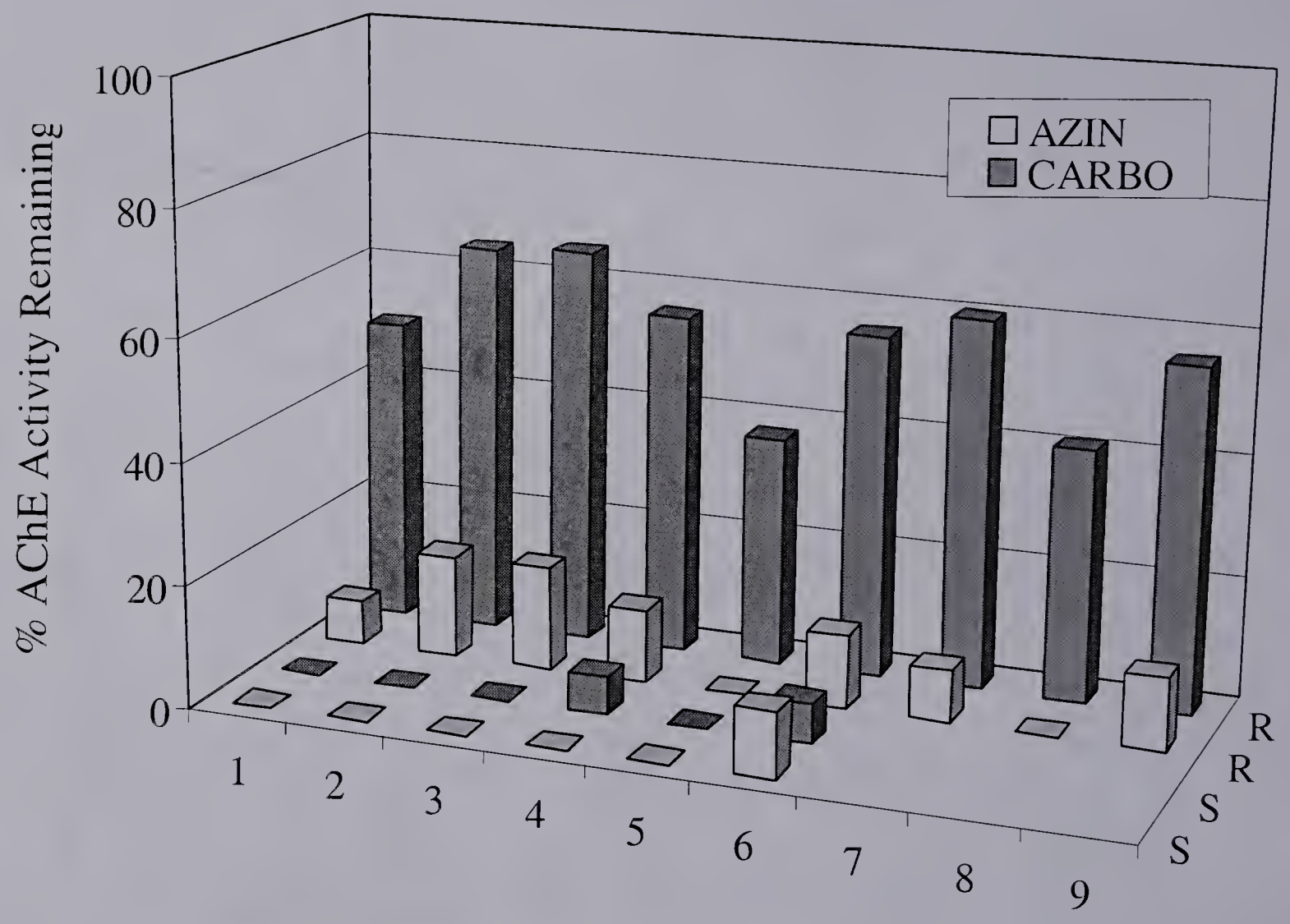

BERTS-S
Control
Azin.
Carbo.
BERTS-R
Control
Azin.
Carbo.
O.D. at 450 nm (low to high)
Time (0-15 min)

Figure 5: AChE activity profiles from selected BERTS-S (S) and BERTS-R (R) CPB incubated with and without N-methyl carbofuran (50 μM) and azinphosmethyl-oxon (50 μM). BERTS-S 1,4,5, and 6 and BERTS-R 3, 4, 6, and 7 individuals were used for molecular analysis of the SI fragment of AChE cDNA. BERTS-S 2, 3, 4, and 5 and BERTS-R 1, 2, 7, 8, and 9 individuals were used for molecular analysis of the SII fragment of AChE cDNA. BERTS-S 1, 3, 4, and 5 and BERTS-R 2, 3, 4, 5 and 7 individuals were used for molecular analysis of the SIII fragment of AChE cDNA.




Selected BERTS-S and BERTS-R CPB

Figure 6: Percent AChE activity inhibited in the presence of 10 μM N-methyl carbofuran, 10 μM N-propyl carbofuran, 150 μM methyl paraoxon, 150 μM ethyl paraoxon, and 5000 μM propaphos using the SS, AZ-R, and BERTS CPB strains.

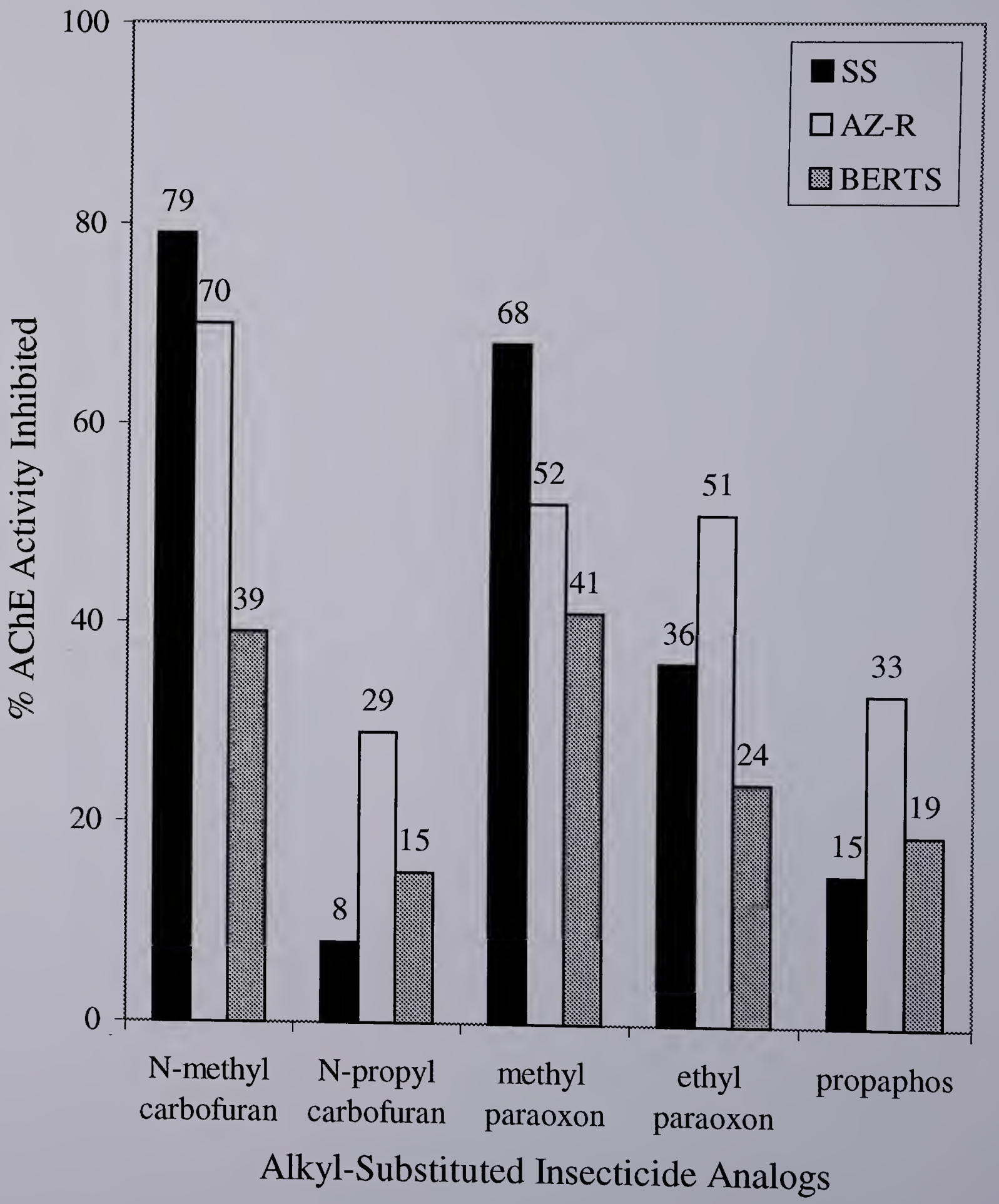

100
80
60
40
20
0
% AChE Activity Inhibited
SS
AZ-R
BERTS
79
70
39
8
29
15
68
52
41
36
51
24
15
33
19
N-methyl carbofuran
N-propyl carbofuran
methyl paraoxon
ethyl paraoxon
propaphos
Alkyl-Substituted Insecticide Analogs

Figure 7: Percent AChE activity remaining in the presence of N-methyl carbofuran (10 μM) or N-propyl carbofuran (10 μM) from a susceptible (BERTS-S) and N-methyl carbofuran-resistant (BERTS-R) strains of CPB (n = 10).

[a] Two-tailed z-test comparing BERTS-S to BERTS-R substrains for % AChE activity remaining was z = -12.9 ($\alpha = 0.05$, z-critical = ± 1.96), signifying the sorted substrains are statistically different for N-methyl carbofuran sensitivity.

[b] Two-tailed z-test comparing BERTS-S to BERTS-R substrains for % AChE activity remaining was z = 4.3 ($\alpha = 0.05$, z-critical = ± 1.96), signifying the sorted substrains are statistically different for N-propyl carbofuran sensitivity.

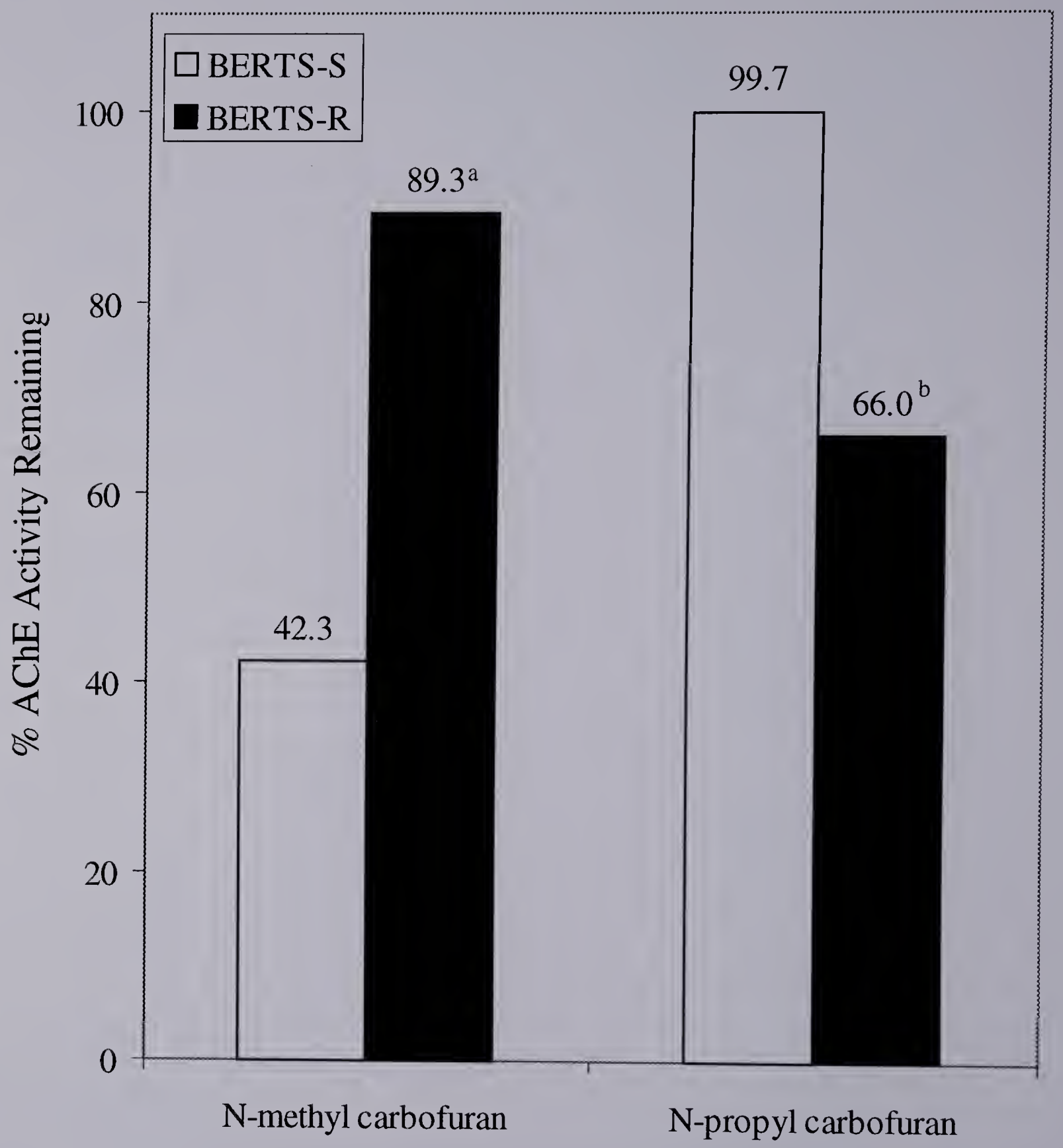

BERTS-S
BERTS-R
100
80
60
40
20
0
% AChE Activity Remaining
42.3
89.3a
99.7
66.0b
N-methyl carbofuran
N-propyl carbofuran
N-Alkyl-Substituted Carbamate Insecticides

Figure 8: Agarose gel electrophoresis of PCR amplification of the SI (1068 bp), SII (622 bp), and SIII (575bp) fragments from the coding region of the AChE cDNA of sorted BERTS-S and BERTS-R CPB. Lanes M, pGEM DNA Marker (Promega). Fragment size in base pairs from top to bottom: 2645, 1605, 1198, 676, 517, 460 and 396. Lane 1, BERTS-S SI. Lane 2, BERTS-R SI. Lane 3, BERTS-S SII. Lane 4, BERTS-R SII. Lane 5, BERTS-S SIII. Lane 6, BERTS-R SIII.

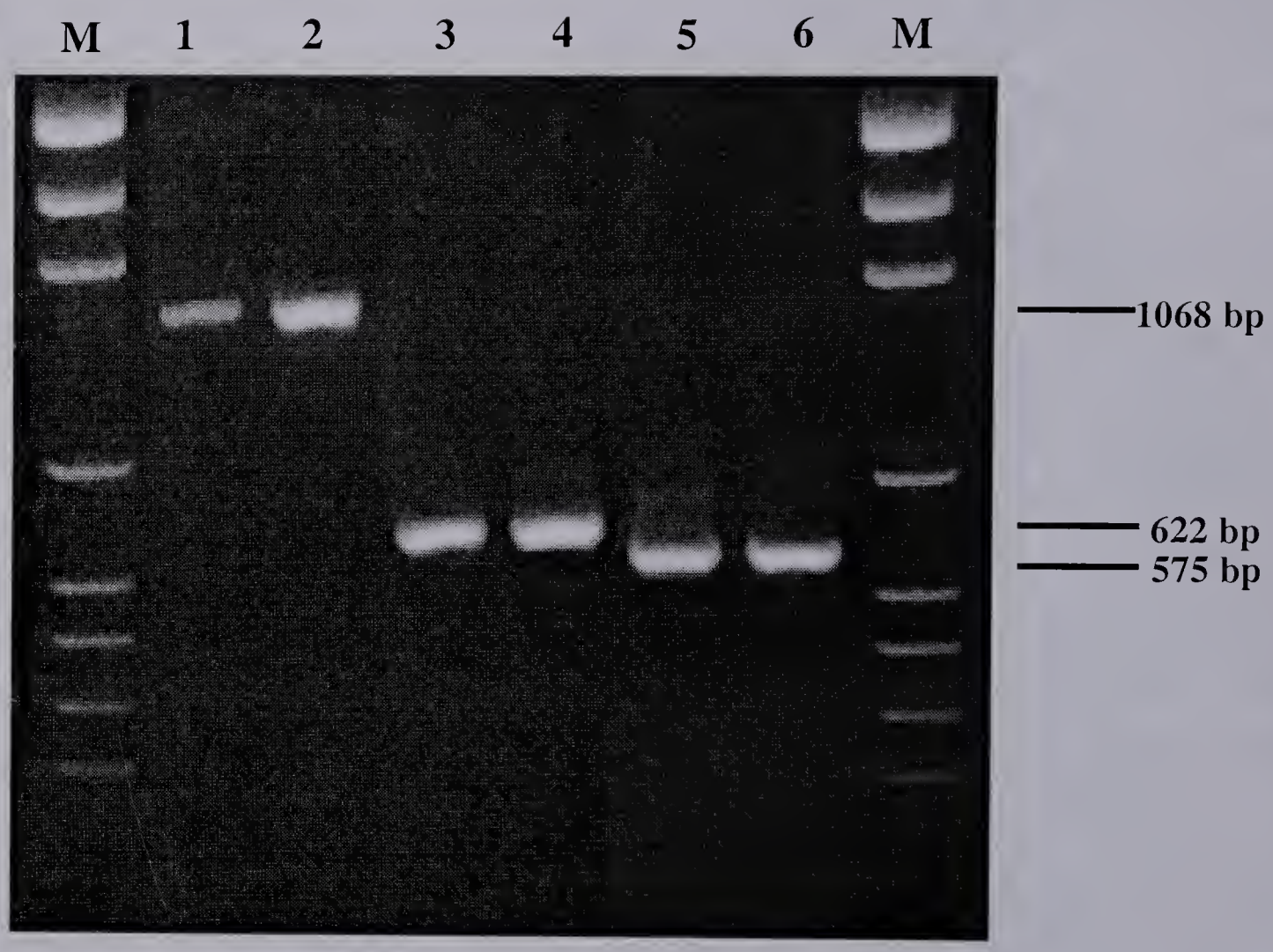

M
1
2
3
4
5
6
M
1068 bp
622 bp
575 bp

Figure 9: Agarose gel electrophoresis of amplification products from a screen for positive transformants of the TA Cloning of the SII (622 bp) fragment of AChE cDNA of selected BERTS-S CPB. Amplification used 5 µl of an overnight culture innoculated with a single white colony with plasmid primers located approximately 100 bp on either side of the insertion site. Lane M, pGEM DNA Marker (Promega). Fragment size in base pairs from top to bottom: 2645, 1605, 1198, 676, 517, 460, 396 and 350. Lanes 1, 2, 3, 4, 8,10 and 11 show amplification of plasmid DNA only, indicative of a false positive. Lanes 5,6,7, and 12 show amplification of plasmid DNA (199 bp) containing the SII insert (875 bp).

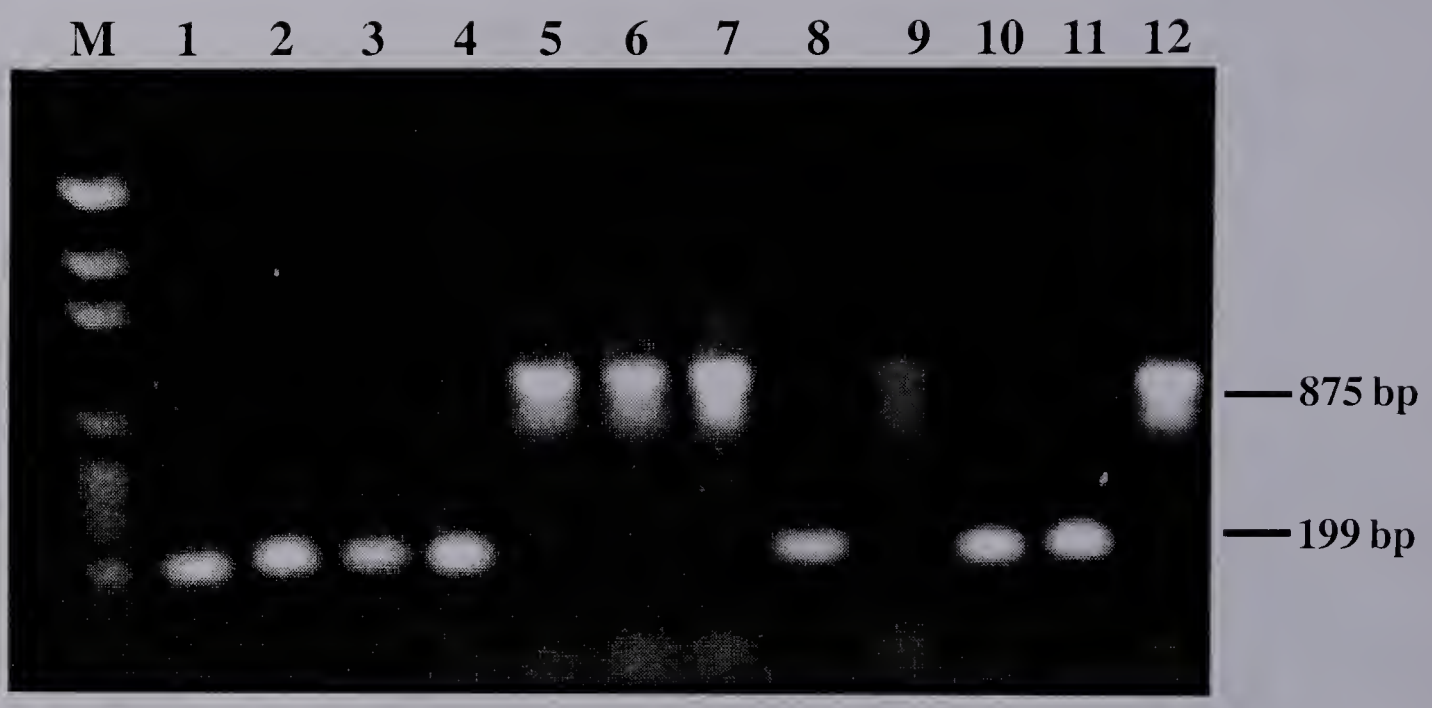
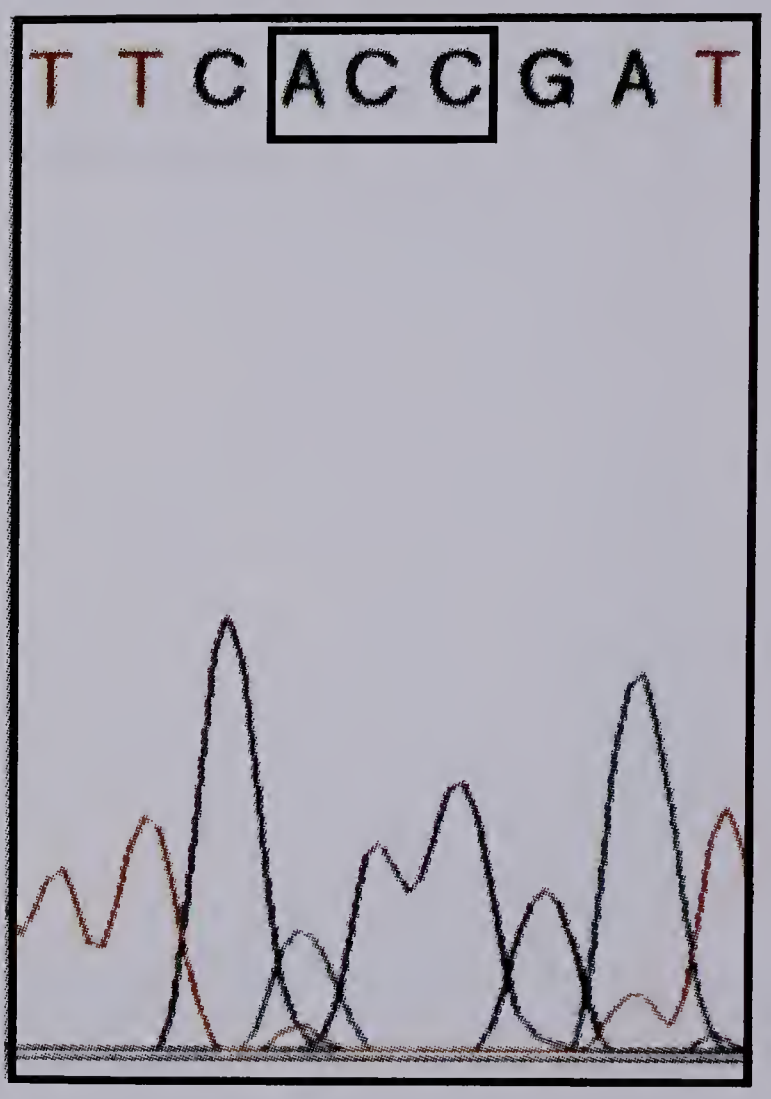

M 1 2 3 4 5 6 7 8 9 10 11 12
875 bp
199 bp

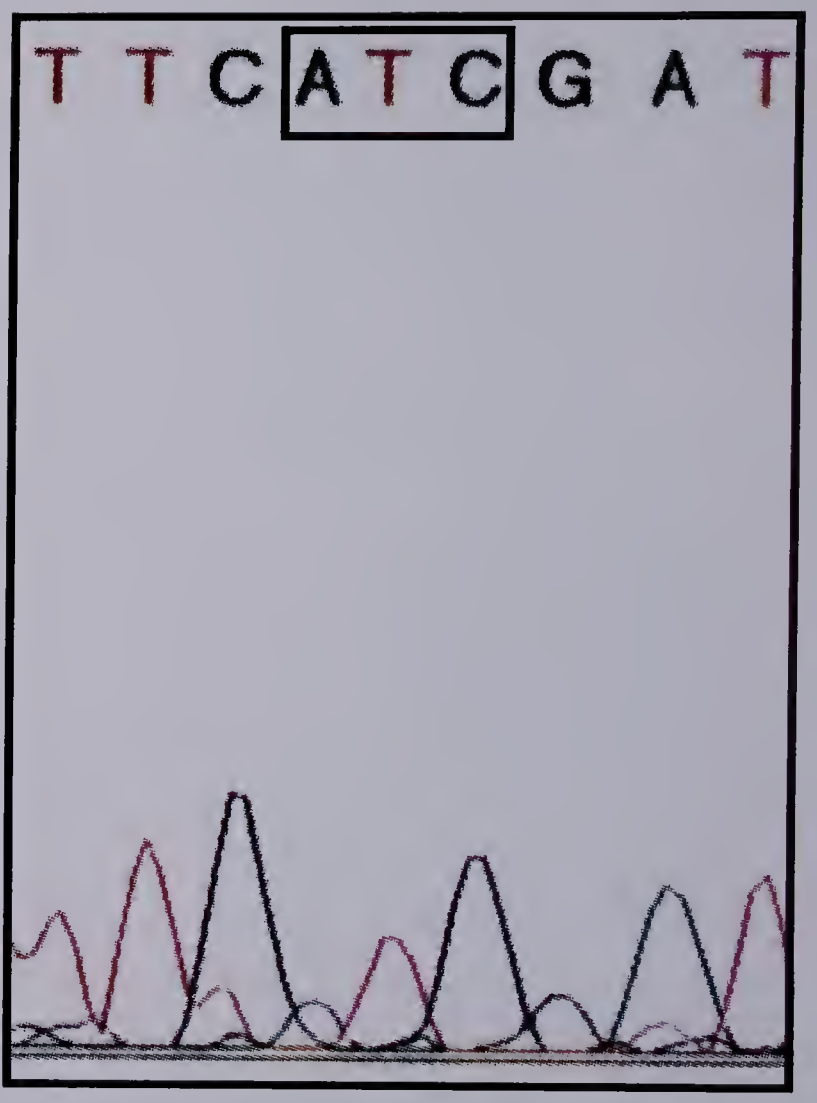
Figure 10: Deduced amino acid sequence of the AChE cDNAs from BERTS-S and BERTS-R CPB aligned with the sequences of SS and AZ-R CPB strains (Zhu and Clark 1995b). The numbering starts with the start codon (M) of the putative signal peptide. Amino acid substitutions due to nonsilent mutations are indicated in bold and are boxed. Both BERTS-S and BERTS-R sequences are identical to the SS sequence at the mutation site located at amino acid residue 30 (Arg to Lys in AZ-R). Both BERTS-S and BERTS-R sequence are identical to the AZ-R sequence at the mutation site located at amino acid residue 291 (Ser to Gly in AZ-R). BERTS-R sequence is identical to both the SS and AZ-R sequence at the mutation site located at amino acid residue 392 (Ile to Thr in BERTS-S). The asterisk symbols indicate the mutation sites. The open and closed arrows indicate the residues probably forming the catalytic triad and the intra-subunit disulfide bonds, respectively.

**CPB**

```
                                                *
SS          MGQLSILCLFVTVCASVCGYSWPSDETTTRPSQFKDFHTDPLVVETTSGLVRGYSKTVL   60
AZ-R        MGQLSILCLFVTVCASVCGYSWPSDETTTKPSQFKDFHTDPLVVETTSGLVRGYSKTVL   60
BERTS-S     MGQLSILCLFVTVCASVCGYSWPSDETTTRPSQFKDFHTDPLVVETTSGLVRGYSKTVL   60
BERTS-R     MGQLSILCLFVTVCASVCGYSWPSDETTTRPSQFKDFHTDPLVVETTSGLVRGYSKTVL   60

                                                          ↓
SS          REVHVFTGIPFAKPPIEQLRFKKPVPIDPWHGILDATKQPNSCFQERYEYFPGFEGEEMW  120
AZ-R        REVHVFTGIPFAKPPIEQLRFKKPVPIDPWHGILDATKQPNSCFQERYEYFPGFEGEEMW  120
BERTS-S     REVHVFTGIPFAKPPIEQLRFKKPVPIDPWHGILDATKQPNSCFQERYEYFPGFEGEEMW  120
BERTS-R     REVHVFTGIPFAKPPIEQLRFKKPVPIDPWHGILDATKQPNSCFQERYEYFPGFEGEEMW  120

                ↓
SS          NPNTNISEDCLYLNIWVPQRLRIRHHADKPTIDRPKVPVLIWIYGGGYMSGTATLDVYDA  180
AZ-R        NPNTNISEDCLYLNIWVPQRLRIRHHADKPTIDRPKVPVLIWIYGGGYMSGTATLDVYDA  180
BERTS-S     NPNTNISEDCLYLNIWVPQRLRIRHHADKPTIDRPKVPVLIWIYGGGYMSGTATLDVYDA  180
BERTS-R     NPNTNISEDCLYLNIWVPQRLRIRHHADKPTIDRPKVPVLIWIYGGGYMSGTATLDVYDA  180

SS          DIIAATSDVIVASMQYRLGSFGFLYLNRYFPRGSDETPGNMGLWDQILAIRWIKDNAAAF  240
AZ-R        DIIAATSDVIVASMQYRLGSFGFLYLNRYFPRGSDETPGNMGLWDQILAIRWIKDNAAAF  240
BERTS-S     DIIAATSDVIVASMQYRLGSFGFLYLNRYFPRGSDETPGNMGLWDQILAIRWIKDNAAAF  240
BERTS-R     DIIAATSDVIVASMQYRLGSFGFLYLNRYFPRGSDETPGNMGLWDQILAIRWIKDNAAAF  240

                    ⇓                                           *
SS          GGDPDLITLFGESAGGGSISIHLISPVTKGLVRRGIMQSGTMNAPWSYMSSERAEQIGKI  300
AZ-R        GGDPDLITLFGESAGGGSISIHLISPVTKGLVRRGIMQSGTMNAPWSYMSGERAEQIGKI  300
BERTS-S     GGDPDLITLFGESAGGGSISIHLISPVTKGLVRRGIMQSGTMNAPWSYMSGERAEQIGKI  300
BERTS-R     GGDPDLITLFGESAGGGSISIHLISPVTKGLVRRGIMQSGTMNAPWSYMSGERAEQIGKI  300

            ↓               ↓
SS          LIQDCGCNVSLLENSPRKVMDCMRAVDAKTISLQQWNSYSGILGFPSTPTIEGVLLPKHP  360
AZ-R        LIQDCGCNVSLLENSPRKVMDCMRAVDAKTISLQQWNSYSGILGFPSTPTIEGVLLPKHP  360
BERTS-S     LIQDCGCNVSLLENSPRKVMDCMRAVDAKTISLQQWNSYSGILGFPSTPTIEGVLLPKHP  360
BERTS-R     LIQDCGCNVSLLENSPRKVMDCMRAVDAKTISLQQWNSYSGILGFPSTPTIEGVLLPKHP  360

                        ⇓                   *
SS          MDMLAEGDYEDMEILLGSNHDEGTYFLLYDFIDFFEKDGPSFLQREKYHDIIDTIFKNMS  420
AZ-R        MDMLAEGDYEDMEILLGSNHDEGTYFLLYDFIDFFEKDGPSFLQREKYHDIIDTIFKNMS  420
BERTS-S     MDMLAEGDYEDMEILLGSNHDEGTYFLLYDFTDFFEKDGPSFLQREKYHDIIDTIFKNMS  420
BERTS-R     MDMLAEGDYEDMEILLGSNHDEGTYFLLYDFIDFFEKDGPSFLQREKYHDIIDTIFKNMS  420

                                                    ↓
SS          RLERDAIVFQYTNWEHVHDGYLNQKMIGDVVGDYFFVCPTNNFAEVAADRGMKVFYYYFT  480
AZ-R        RLERDAIVFQYTNWEHVHDGYLNQKMIGDVVGDYFFVCPTNNFAEVAADRGMKVFYYYFT  480
BERTS-S     RLERDAIVFQYTNWEHVHDGYLNQKMIGDVVGDYFFVCPTNNFAEVAADRGMKVFYYYFT  480
BERTS-R     RLERDAIVFQYTNWEHVHDGYLNQKMIGDVVGDYFFVCPTNNFAEVAADRGMKVFYYYFT  480

                    ⇓
SS          HRTSTSLWGEWMGVIHGDEVEYVFGHPLNMSLQFNSRERELSLKIMQAFARFATTGKPVT  540
AZ-R        HRTSTSLWGEWMGVIHGDEVEYVFGHPLNMSLQFNSRERELSLKIMQAFARFATTGKPVT  540
BERTS-S     HRTSTSLWGEWMGVIHGDEVEYVFGHPLNMSLQFNSRERELSLKIMQAFARFATTGKPVT  540
BERTS-R     HRTSTSLWGEWMGVIHGDEVEYVFGHPLNMSLQFNSRERELSLKIMQAFARFATTGKPVT  540

                                                    ↓
SS          DDVNWPLYTKDQPQYFIFNADKNGIGKGPRATACAFWNDFLPKLRDNSGSEEAPCVNTYL  600
AZ-R        DDVNWPLYTKDQPQYFIFNADKNGIGKGPRATACAFWNDFLPKLRDNSGSEEAPCVNTYL  600
BERTS-S     DDVNWPLYTKDQPQYFIFNADKNGIGKGPRATACAFWNDFLPKLRDNSGSEEAPCVNTYL  600
BERTS-R     DDVNWPLYTKDQPQYFIFNADKNGIGKGPRATACAFWNDFLPKLRDNSGSEEAPCVNTYL  600

SS          SKIRSSSNELLPPSTSLVLIWIMTLLNAL                                 629
AZ-R        SKIRSSSNELLPPSTSLVLIWIMTLLNAL                                 629
BERTS-S     SKIRSSSNELLPPSTSLVLIWIMTLLNAL                                 629
BERTS-R     SKIRSSSNELLPPSTSLVLIWIMTLLNAL                                 629
```

Figure 11: Automated DNA sequencing traces of BERTS-S (left) and BERTS-R (right) AChE cDNA (SII fragment). The thymine to cytosine (T to C) mutation (nt 1284, Zhu and Clark 1995b) results in an amino acid change at residue 392, from an isoleucine (ATC) to a threonine (ACC).


T T C A C C G A T


T T C A T C G A T

# CHAPTER 4

# DISCUSSION

## Type of AChE Alteration Leading to Target Site Insensitivity Based on Alkyl Group Information

Studies of altered AChE as a means of N-methyl carbamate and methoxy-substituted organophosphate insecticide resistance due to enzyme insensitivity suggest that resistant forms of AChE have increased susceptibility to insecticide analogs with larger alkyl groups. In general, only the N-methyl or N,N-dimethylcarbamates are efficacious insecticides due to their inhibition of AChE. Carbamates with larger N-alkyl moieties were found to be inefficient insecticides mainly due to the decrease in the carbamylation rate. The present study establishes an N-propylcarbamate, N-propyl carbofuran, as a potent inhibitor of AChE in CPB resistant to N-methyl carbofuran (BERTS-R). The BERTS strain exhibited the highest mortality in bioassays and the AChE activity from the BERTS-R substrain was most inhibited by N-propyl carbofuran (Table 4, Figs. 6 and 7). The AChE of the BERTS-S substrain was highly susceptible to N-methyl carbofuran, but poorly inhibited by N-propyl carbofuran (Fig. 7).

A number of studies have indicated that insecticide insensitive forms of AChE, which result in N-methyl carbamate and methoxy-OP resistance, have increased susceptibility to insecticide analogs with larger alkyl groups. This illustrates what is known as negative cross-resistance. In AZ-R CPB, the azinphosmethyl insensitive AChE was more susceptible to inhibition by ethyl paraoxon and diisopropyl fluorophosphate (DFP) than the SS AChE (Zhu and Clark, 1995a).

Aryl N-propyl carbamates were found to be potent inhibitors of AChE in Green Rice Leafhopper (GRLH), *Nephotettix cinciticeps*, which were resistant to N-methyl carbamates (Yamamoto *et al.*, 1977a). GRLH susceptible to N-methyl carbamates were poorly inhibited by N-propyl carbamates. Yamamoto also found a synergistic insecticidal action of N-methyl and N-propyl carbamates in GRLH (Yamamoto *et al.*, 1977b). Selection of resistant GRLH with the combination resulted in no resistance to the combination, as well as no increase in resistance to N-methyl carbamates.

Similar findings were reported in a methyl parathion-resistant tobacco budworm strain (Brown and Bryson, 1992). This strain was found to be less sensitive to methoxy OPs and N-methyl carbamates but more sensitive to N-propyl 1-naphthyl carbamate. As with the AZ-R AChE of CPB, the methyl parathion insensitive form of AChE from resistant tobacco budworm lacked high substrate inhibition. There was no evidence that resistance was due to reduced rates of phosphorylation and the authors attributed the resistance to be related to the overall size and shape of the inhibitors: "Perhaps the shapes of the antiresistant inhibitors did not permit their access to the active site of the normal enzyme, while the resistant enzyme had a requirement for this larger size or different shape."

Likewise, negative cross resistance patterns have been reported for sheep blowfly, *Lucilia cuprina* resistant to OPs (Campbell *et al.*, 1998). Campbell evaluated diazinon (ethoxy-OP) resistant and malathion (methoxy-OP) resistant strains of sheep blowfly. Diazinon resistant individuals were found to have 2-times greater resistance factors towards diethoxy OPs than their dimethoxy analogs and malathion resistant individuals showed 2-times greater resistance factors towards dimethoxy OPs than their diethoxy

analogs. Campbell attributes these observations to mutated ali-esterases. Ali-esterases are enzymes responsible for the metabolism of insecticides, however they have some similarity to acetylcholinesterase with regard to the catalytic centers of activity.

The present study further examined the toxicity of OP analogs on the AZ-R strain using methyl paraoxon, ethyl paraoxon, azinphosmethyl, azinphosethyl, and propaphos. Our present findings agree with those previously determined. The AChE of the AZ-R strain is more inhibited by those insecticides with larger alkyl groups including ethyl paraoxon, azinphosethyl, and propaphos and correlate with increased mortality in bioassays (Table 4).

AChE of the SS strain is poorly inhibited by N-propyl carbofuran and yet significant mortality (congruent to that seen in the BERTS strain) occurred in bioassays with this insecticide (Table 4, Fig. 6). Still, a substantial difference is seen in the mortality of the BERTS (8%) and SS strains (92%) in bioassays with N-methyl carbofuran. In a field situation where individuals of both strains reside, the choice of N-methyl carbofuran treatment alone would result in mortality of the majority of SS and a minority of BERTS beetles, thus selecting for the N-methyl carbofuran resistant genes. No such selection would occur if N-propyl carbofuran is sprayed.

The negative cross-insensitivity of AChE to propyl- versus methyl-substituted OP and carbamate insecticides may be due to an alteration in the catalytic center of the enzyme, which increases its ability to bind to insecticides with a larger alkyl substitutions (ethyl or propyl). Kinetic studies on the AChEs purified from the AZ-R and SS CPB strains suggested changes in ligand binding to both the catalytic center and peripheral anionic site (Zhu and Clark, 1995a). Subsequent identification of a point mutation,

S291G, in the AChE gene of the AZ-R CPB strain and its predicted location in the translated protein supported this theory (Zhu *et al.*, 1996a).

Significance of Point Mutations Found in AChE

The mutational analysis of four strains of CPB (BERTS-S, BERTS-R, SS, and AZ-R) has allowed us to speculate on the significance of identified nonsilent point mutations and insensitivity resistance mechanisms at the molecular level. It has been suggested that the susceptible form of AChE binds those insecticides with smaller alkyl groups (methyl) more efficiently than the same insecticide with a larger alkyl group (ethyl or propyl) substitution due to the original point mutation found by Zhu *et al.* (S291G). There is evidence that the A→G point mutation, which causes a residue change of serine (AGT) in susceptible AChE to glycine (GGT) in azinphosmethyl-resistant AChE, could cause a significant alteration in the location of the αE'1 helical structure and modify both the esteratic subsite of the catalytic center as well as the peripheral anionic site using the three-dimensional structure AChE of *Torpedo*.

An enlarged esteratic subsite of the catalytic center of AChE may better fit OPs and carbamates with larger alkyl groups such as propaphos and N-propyl carbofuran. At the same time, this enlarged esteratic subsite loses its capacity to interact effectively with methoxy-substituted OPs and N-methyl-substituted carbamates, historically found to be the more potent inhibitors of native AChE. In fact, native AChE shows poor binding capacity for such propyl-substituted inhibitors and the azinphosmethyl-resistant AChE has become a catalytically inefficient AChE, which hydrolyzes acetylcholine poorly but now is able to hydrolyze butyrylcholinesterase more efficiently (Zhu and Clark, 1995a).

Initially the S291G mutation was thought to be the only contributing factor in AChE insensitivity to azinphosmethyl. However, a second mutation, R30K, which is only found in AZ-R CPB (Zhu *et al.*, 1996a), appears also to be important. The lack of detection of this mutation in the SS and BERTS strains, which are not resistant to azinphosmethyl, seemed more than coincidental.

A theory can be proposed based on the mutational evidence from the BERTS substrains as well as that found previously in the AZ-R strain (Fig. 10, Table 8). It is possible that the S291G mutation reduces the binding of insecticides with small alkyl groups by causing the aforementioned conformational alterations, which make binding of larger insecticides, such as the N-propyl carbamates more efficient. The presence of the S291G mutation in all CPB from the AZ-R and BERTS strains supports the contention that this is a major site of modification in AChEs insensitive to OP and carbamate inhibition.

The BERTS strain elicits substantially lower resistance to azinphosmethyl compared to the AZ-R strain, possibly due to the absence of the R30K mutation in the BERTS strain (Tables 4 and 8). The SS strain, which is susceptible to both N-methyl carbofuran and azinphosmethyl, has neither the R30K nor the S291G mutations. The sorting of the BERTS strain into BERTS-R and BERTS-S substrains was essential for the identification and functional location of a third mutation, I392T. Co-expression of the I392T mutation appears to nullify the effects of the S291G mutation, allowing the BERTS-S AChE to behave like the SS AChE. This mutation may have evolved after azinphosmethyl and N-methyl carbofuran ceased to be used in the field. Argentine (1989) showed a fitness disadvantage linked to the altered acetylcholinesterase of AZ-R

CPB. In the absence of selection pressure, there would by evolutionary pressure against the S291G mutation to eliminate it from the populations or to nullify it by a second mutation.

The I392T mutation site is predicted to reside within the aromatic gorge, which in *Torpedo* AChE, is at a location (P 337) where butyrylcholinesterase activity is modified (Sussman *et al.*, 1991). Given, the drastic difference between the chemical make-up and hydrophobicity of isoleucine versus threonine, this substitution is likely to cause a conformational change in the secondary structure of the BERTS-S AChE and may result in reduced buyrylcholinesterase activity and susceptibility to azinphosmethyl-oxon and carbofuran.

A summary of the proposed effects of the nonsilent mutations from our mutational analysis is given in Table 9: An azinphosmethyl- and carbofuran- susceptible AChE occurs either when none of the mutations are present (seen in SS CPB) or when the S291G and I392T mutations are co-expressed. An altered AChE for azinphosmethyl resistance and increased susceptibility to OP insecticides with larger alkyl groups, such as that seen in the AZ-R strain, occurs with both the R30K and S291G mutations present. An altered AChE for N-methyl carbofuran resistance with greatly increased susceptibility to N-propyl carbamates as seen in the BERTS-R substrain, occurs when only S291G mutation is present.

Proposed Mutational Analysis for the I392T and R30K Point Mutations

Development of DNA diagnostic techniques that rapidly and easily identify insecticide resistance mutations would greatly facilitate the monitoring of pest

populations and resistance management strategies. If the method is rugged, high-throughput, cost efficient and rapid, it could be used to assess insecticide resistance in the field at the genotypic level, thereby allowing growers to make appropriate spray decisions (choice of insecticides and application regime). DNA-based diagnostic techniques based on point mutations have been previously developed for various types of insecticide resistance including the cyclodienes (*Rdl)*, A302S/G, azinphosmethyl, S291G, and permethrin (*kdr*), L1014F. Such techniques include Single-Stranded Conformation Polymorphisms (SSCP), competitive PCR Amplification of Specific Alleles (cPASA), and minisequencing (Coustau *et al.*, 1995; Zhu and Clark, 1996; Zhang *et al.*, 1999). The sequence information of the AChE cDNA of the BERTS-S and BERTS-R strains of CPB presented in this study is applicable to the development of diagnostic techniques for the detection of the I392T mutation that is associated with susceptibility to N-methyl carbofuran in the presence of the S291G mutation associated with azinphosmethyl/carbofuran resistance. Also, the development of a diagnostic techniques for the R30K mutation associated with azinphosmethyl resistance in the AZ-R strain is proposed, as all three mutations are necessary for a complete profile of azinphosmethyl/carbofuran resistance.

It has previously been determined that it is most efficient to use both the SSCP and minisequencing methods in conjunction with each other for detection of alleles associated with resistance (Zhang *et al.*, 1999). SSCP is a rugged, efficient, and cheap way to identify both known and unknown mutations, including point mutations as well as additions and deletions in amplified DNA fragments. The proposed SSCP methods involve the amplification of a 110 bp fragment from genomic DNA that includes the nt

1284 mutation site I392T, and amplification of a 101 bp fragment that includes the nt 198 mutation site R30K. Expected results would be a different diagnostic banding pattern of single-stranded DNA fragments from of each the BERTS-R and BERTS-S AChE gene, due to the T→C nucleotide change at nt 1284 after the PCR products are resolved on an polyacrylamide gel. DNA fragments from both BERTS-S and BERTS-R substrains would be expected to show the same banding patterns for the 101 bp fragment containing nt 198. A SSCP method was successful in the previous studies including detection of the S291G mutation done on SS and AZ-R CPB for an amplified 163 bp fragment of the AChE gene. SSCP also was successfully used to detect the *kdr*-type of resistance associated with the insecticide permethrin in CPB (Clark *et al.*, 1999). Nevertheless, SSCP is not a direct sequencing method and its diagnostic ability can be reduced by the presence of silent mutations or nonsilent mutations not associated with resistance.

The tentative findings of the SSCP analysis can be validated by minisequencing, a more precise method for determining the presence of point mutations by direct DNA sequencing. The same PCR amplification method is used with the substitution of a biotinylated PCR primer against the DNA template strand to be sequenced. In the case of the 110 bp amplification for the nt 1284 I392T mutation, a biotinylated reverse primer would be used. A biotinylated forward primer would be used for the 101 bp amplification containing the nt 198 R30K mutation site. The proposed methods are outlined in figures 12 and 13. This technique is based on the binding affinity of a digoxigenin-labeled dideoxy-nucleotide to normal or mutated allele. Presently, only two digoxigenin-labeled dideoxy-nucleotides are available, adenine (dig-ATP) and uracil (dig-UTP), which cannot be used to detect a cytosine to guanine or a guanine to cytosine

mutation. However, we can use this technique to detect both the R30K and I392T mutations, which is the result of a guanine to adenine and thymine to cytosine point mutation, respectively.

In the case of I392T detection, an amplified 110 bp double-stranded DNA fragment, biotinylated on the 5‘ end of the nonsense strand, is bound to a streptavidin-coated microplate. The DNA is denatured and the plate is rinsed, such that only the annealed nonsense strand remains. A detection primer is annealed to the fragment such that its 3’ end is adjacent to the mutation site at nt 1284, the location of the I392T point mutation. Next a typical DNA sequence extension reaction takes place in the presence of one of the digoxigenin-labeled dideoxy-nucleotides and Taq polymerase. The addition of the nucleotide is dependent on the template DNA.

In the case of the I392T mutation, it is expected the BERTS-S CPB single-stranded DNA template would be unable to incorporate either the dig-UTP or the dig-ATP, given it has a guanine at position nt 1284 of the nonsense strand. However, the BERTS-R CPB single-stranded DNA template has an adenine (complement of the thymine in sense strand), and would therefore incorporate the dig-dUTP during minisequencing. Incorporation of the dideoxynucleotide would terminate the reaction since there is no oxygen available on the 3’ end that is required for further extension. The method is coupled to an immunoassay involving subsequent binding of digoxigenin-specific antibodies conjugated with peroxidase and a color change upon addition of TMB/$H_2O_2$. A color change, signifying the binding of the dig-NTP, is measured on a UV spectrophotometer microplate reader at 450nm (Fig. 12).

In the case of detection of the R30K mutation, the amplified 101 bp double-stranded DNA fragment is biotinylated on the 5‘ end of the sense strand for binding to the streptavidin-coated microplate. Denaturation of DNA and subsequent washing of the plate results in only the sense strand remaining bound. A detection primer is annealed to the fragment such that it is adjacent to the mutation site at nt 980, the location of the R30K point mutation. A DNA sequence extension reaction takes place in the presence of either dig-UTP or dig-ATP.

In this case, it is expected neither the SS, BERTS-S nor BERTS-R CPB single-stranded DNA templates would be unable to incorporate either the dig-UTP or the dig-ATP, given it has a guanine in position 980 of the nonsense strand. However, an AZ-R single-stranded DNA CPB template would have an adenine at position 980 and incorporate the dig-UTP during minisequencing, resulting in detection (Fig. 13). Proposed methods for SSCP and minisequencing analysis of the R30K and I392T mutations are outline in the appendix.

Minisequencing has been used to discriminate SS and AZ-R CPB AChE based on the S291G point mutation associated with azinphosmethyl resistance (Zhang *et al.*, 1999). In the case of the S291G mutation, the single-stranded DNA template was the nonsense strand. The biotinylated SS CPB single-stranded DNA template at nt 980 has a thymine and therefore could bind the dig-ATP. This binding is reflected in an increase in absorbance seen at 450 nm. The alternative minisequencing reaction using dig-UTP with the SS template shows an absorbance averaging less than 0.1 OD, a significant decrease from the >0.3 OD absorbance in the reaction with dig-ATP. The difference is due to the lack of binding of the dig-UTP to the SS template. The level of absorbance seen is most

likely to be due to the inability to rinse out 100% of the unbound dig-NTP during the procedure and also to a small amount of mismatched binding. In the case of the AZ-R template, the nucleotide at position 980 is a cytosine and should not bind to either the dig-UTP or the dig-ATP. The absorbance readings at 450nm are both less than 0.1 OD (Table 10).

In the case of the S291G mutation, the normal allele was detected in the SS CPB. It can be concluded from the results that the SS CPB had an adenine at nt 980. The results in the AZ-R CPB show that the nucleotide at 980 is not an adenine, since it did not bind the dig-UTP, nor a thymine, since it also did not bind the dig-ATP. It is therefore a cytosine or guanine, and is likely a guanine based on information known of the mutation. This method has also been successfully used for detection of the *kdr* mutation in the sodium channel thought to confer resistance to the pyrethroid insecticide, permethrin (Clark *et al.*, 1999). In this case, the mutation, a leucine (susceptible, C̲TT) to phenylalanine (resistant, T̲TT), allows for the mutated allele to be detected.

It is proposed that both SSCP and minisequencing would be useful methods for detection of the I392T and R30K mutations. The eventual use of this method in the field would involve simultaneous minisequencing reactions used for all three mutations, R30K, S291G and I392T to create a proper profile. It is crucial to check all three of the mutation sites for determination of resistance type as summarized in Table 9.

Throughput of both methods is increasing steadily with developments in technologies such as multi-channel liquid handlers and microtitre plate formats. PCR amplifications can be easily done in 384-well microtitre plate formats. Minisequencing is

currently done in a 96-well format. High throughput would allow for large samples to be taken from field populations for more accurate assessments.

Resistance Monitoring and Management

The information generated in the present study on CPB, in congruency to similar works being done with other insecticides on CPB and other pests, is useful in the development of novel resistance management techniques. Excessive and indiscriminate use of non-selective insecticides in the past has brought about a present crisis in pest control. The arsenal of available and effective insecticides is rapidly being reduced due to increased awareness of adverse effects that some of these chemicals have had on the environment, increased regulations, and increased numbers and levels of resistant insects. Also, the availability of new insecticides is decreasing because of high cost of research necessary for approval to market as well as the increased specificity of the new biosafe insecticides. It is crucial to salvage the options left, and to try to use what is available judiciously.

Novel-acting insecticides such as *Bacillus thuringiensis*, imidacloprid, cyromazine and avermectins have been shown to control CPB. However, CPB has historically developed resistance to every insecticide employed for its control. CPB resistance to abamectin and imidacloprid has already been reported (Clark *et al.*, 1992; Grafius and Bishop., 1996). It is inevitable that CPB will eventually develop resistance to these novel insecticides. Development of resistance is rapid when a particular insecticide is over-used. Application schemes, including rotation and use of mixtures of insecticides, are essential means to delay the development of resistance from developing.

Efficacious spray decisions to delay the development of resistance and to provide pest control can be made through the use of specific and reliable monitoring techniques.

Molecular techniques have shown a proficiency for the detection of point mutations that lead to insecticide resistance. It is plausible that a large number of insect pests could be screened for a wide variety of insecticide resistance within a time frame that would not delay the timing of a spray. The information would allow the most efficient and environmentally benign course of spraying.

Specifically, this study illustrates the necessity of screening for all three mutations R30K, S291G, and I392T, for the determination of organophosphate and carbamate resistance in CPB. Although resistance to OPs and carbamates is wide-spread in CPB, it is not complete. Data presented here for various field strains of CPB show variability in the types and levels of resistance from site to site (Table 2). Therefore, methoxy-OPs and N-methyl carbamates may still be a viable choice of control for some farmers.

This study also proposes the possible effectiveness of alternative insecticides. The evidence presented suggests that propyl carbamates such as N-propyl carbofuran are practical means of control for CPB that are resistant to the methyl carbamates and can be applied as resistance-breaking insecticides. Fortuitously, N-propyl carbamates are not good inhibitors on native AChEs, and have little toxicity to humans and other non-target organisms, including beneficial insects and biological control organisms. This characteristic makes propyl carbamates an ideal complement to biological control agents, such as nematodes and predatory mites. Propyl carbamates have been used regularly in other countries, such as Japan, for the control of GRLH in combination with the more typical methyl carbamates or alone when the level of methyl carbamate resistance is high

(Yamamoto, 1993). Results from this study are supportive of a similar resistance management scheme, which would employ the use of various combinations of insecticides to control resistance in CPB (i.e. methyl paraoxon / ethyl paraoxon / propaphos combination, or N-methyl carbofuran / N-propyl carbofuran combination, etc.).

Proper choice of insecticides, application schemes, and timing of sprays, are becoming essential techniques for anyone currently involved in pest control. Insects greatly impact our society in important areas such as food and disease. Increased knowledge of the modes of action, mechanisms of resistance, and environmental fate of insecticides are of utmost importance as the population of the world continues to grow in the 21$^{st}$ century.

Table 8
Summary of mutations found in the SS, AZ-R, BERTS-S and BERTS-R strains of CPB.

| Mutation location | CPB strains | | | |
|---|---|---|---|---|
| | SS | AZ-R | BERTS-S | BERTS-R |
| R30K[a] | - | + | - | - |
| S291G[b] | - | + | + | + |
| I392T[c] | - | - | + | - |

[a] The nt 198 mutation is G→A, conferring a change of an arginine (AGA) in normal (-) AChE to lysine (AAA) in mutated (+) AChE.

[b] The nt 980 mutation is A→G, conferring a change of a serine (AGT) in normal (-) AChE to glycine (GGT) in mutated (+) AChE.

[c] The nt 1284 mutation is T→C, conferring a change of a isoleucine (ATC) in normal (-) AChE to threonine (ACC) in mutated (+) AChE.

Table 9
Determination of resistance type based on presence of mutations found via minisequencing analysis.

| Resistance type | | Presence of mutations R30K | S291G | I392T |
|---|---|---|---|---|
| susceptible to azinphosmethyl | | No | No | No |
| susceptible to N-methyl carbofuran | (1)[a] | No | No | No |
| | (2)[b] | No | Yes | Yes |
| resistant to azinphosmethyl | | Yes | Yes | No |
| resistant to N-methyl carbofuran | | No | Yes | No |

[a] CPB of the SS strain are susceptible to N-methyl carbofuran. Sequencing of the AChE cDNA of the SS strain show no mutations.

[b] CPB of the BERTS-S strain are susceptible to N-methyl carbofuran. Sequencing of the AChE cDNA of the BERTS-S strain reveal presence of both S291G and I392T mutations.

Table 10

Minisequencing results (as measured by optical density, $O.D._{450}$) obtained from digoxigenin-labeled dATP or dUTP reactions with amplified genomic DNA templates containing the S291G point mutation site of the AChE gene from SS and AZ-R strains of CPB[a].

| | | Mean $O.D._{450}$ (± S.D.) | |
|---|---|---|---|
| CPB strains | n | dATP | dUTP |
| SS | 4 | 0.338 (± 0.035) | 0.076 (± 0.029) |
| AZ-R | 3 | 0.028 (± 0.021) | 0.070 (± 0.018) |

[a]Data taken from Zhang *et al.*, 1999.

Figure 12: Schematic of the minisequencing reaction for the detection of the I392T mutation in BERTS-S and BERTS-R CPB.

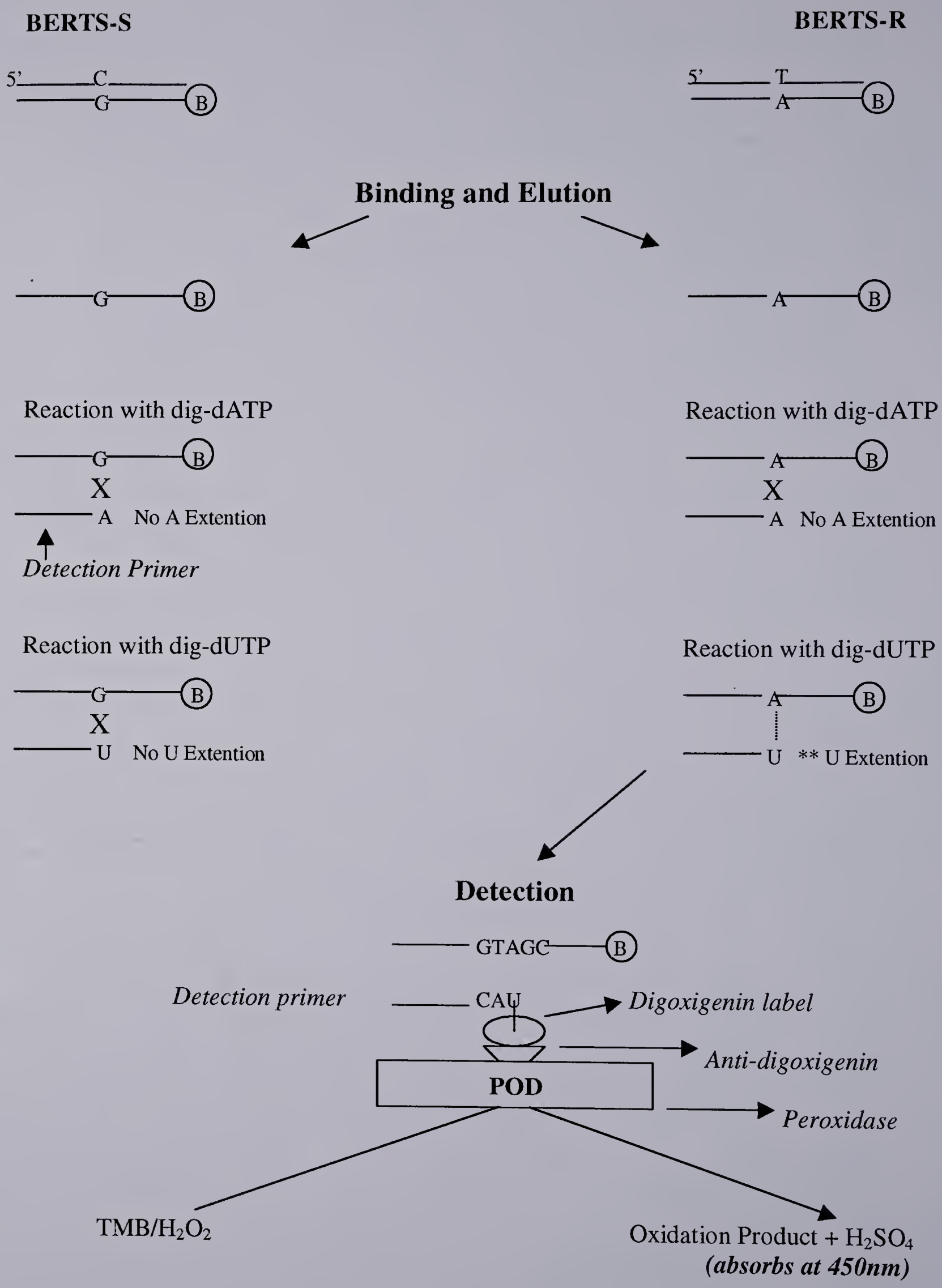
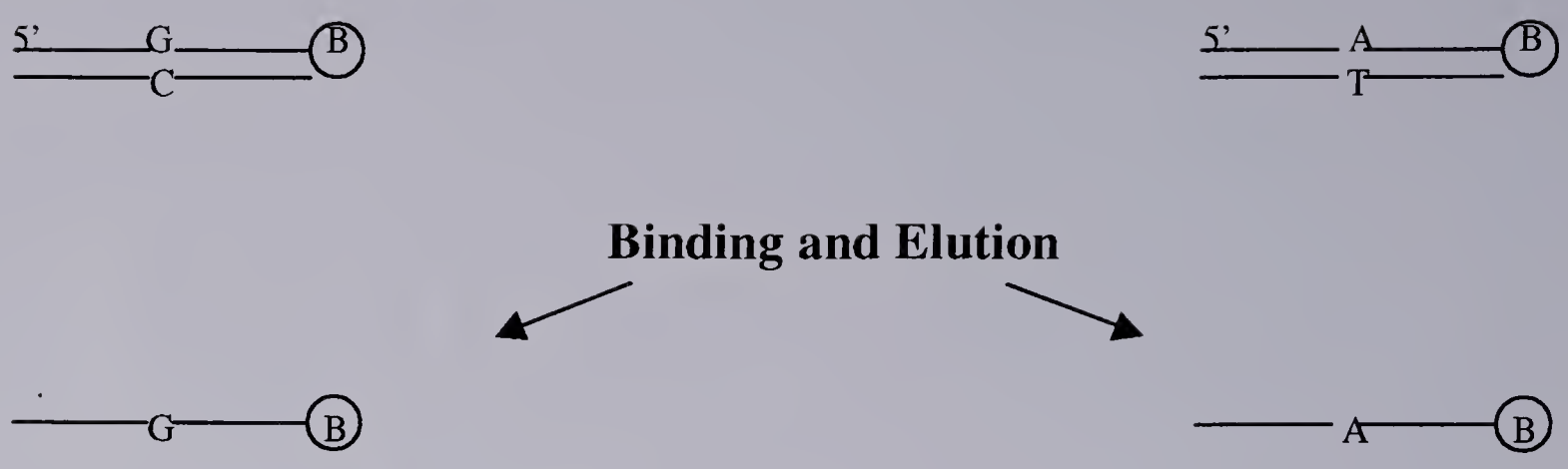

BERTS-S
5' C
G
B
BERTS-R
5' T
A
B
Binding and Elution
G
B
A
B
Reaction with dig-dATP
G
B
X
A No A Extention
Detection Primer
Reaction with dig-dATP
A
B
X
A No A Extention
Reaction with dig-dUTP
G
B
X
U No U Extention
Reaction with dig-dUTP
A
B
U ** U Extention
Detection
GTAGC
B
Detection primer
CAU
Digoxigenin label
Anti-digoxigenin
POD
Peroxidase
TMB/H2O2
Oxidation Product + H2SO4
(absorbs at 450nm)

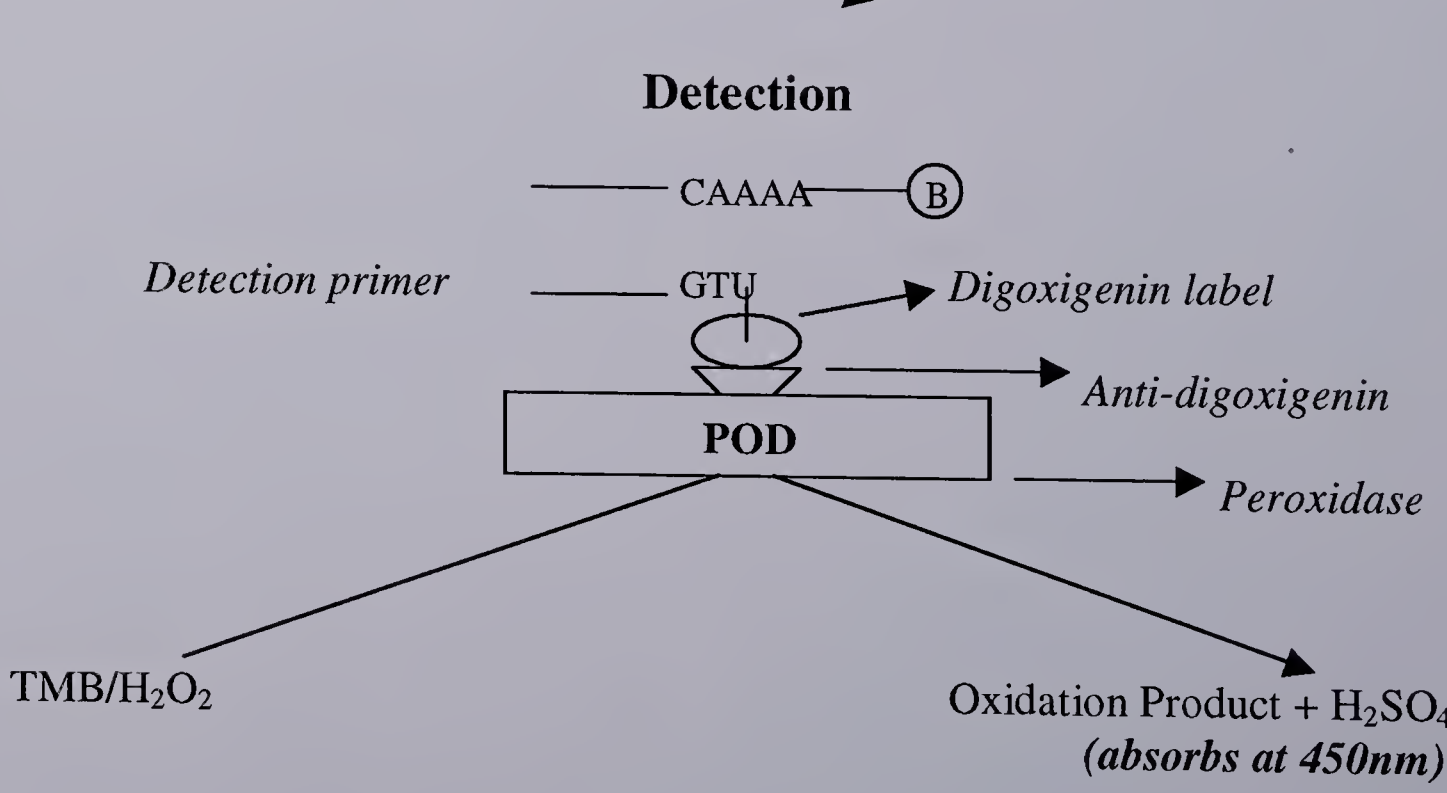
Figure 13: Schematic of the minisequencing reaction for the detection of the R30K mutation in SS, BERTS-S, BERTS-R and AZ-R CPB.

**SS, BERTS-S, BERTS-R**

**AZ-R**







# APPENDIX

# DNA DIAGNOSTIC PROCEDURES FOR THE DETECTION OF POINT MUTATIONS IN THE CDNA OF THE BERTS STRAIN

## DNA Diagnostic Technique #1: Single-Strand Conformation Polymorphisms (SSCP)

*I392T mutation:* A DNA fragment (110 bp) that included the point mutation site at nt 1285 of the AChE cDNA will be amplified using genomic DNA of CPB as template in a final volume of 50 µl reaction mixture containing; 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (each), 15 pmol of PCR forward primer (20-mer, 5' GATTACGAAGATATGGAAAT 3'), 15 pmol of PCR reverse primer (20-mer, 5' TGGAGAAAGCTAGGGCCGTC 3'), and 1 U of AmpliTaq DNA polymerase. After denaturation of DNA at 94°C for 30 sec in the thermal cycler, 35 cycles of PCR will be completed with each cycle consisting of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min. Samples will be cooled at 4°C after extension for 10 min at 72°C.

*R30K mutation:* This 101 bp DNA fragment, including the nt 980 point mutation site, will be amplified using the same method as previously described for I392T, with the exception of the primers. The forward primer (20-mer) will be the oligonucleotide, 5' ACTCGGTGAATCGCCCTTTC 3'. The reverse primer (20-mer) will be the oligonucleotide, 5' TGTTTCGACGACTAGAGGGT 3'.

Single-stranded DNA will be generated by combining 5 µl of PCR product (10 ng/ul) with an equal volume of loading buffer (95% formamide, 20 mM EDTA, 0.05%

bromophenol blue, 0.05% xylene cyanol), heating to 95°C and immediately loading onto an electrophoresis gel (12% polyacrylamide with 1X TBE containing 0.1% ammonium persulfate and 0.1% TEMED). Gels will be prepared at 1.0 mm thickness. Electrophoresis will be performed at 5W for 10 h at a range of temperatures (4°C to room temperature) until an optimum temperature is found for unique and reproducible DNA strand separation. Following electrophoresis, the gel will be visualized via silver staining.

DNA Diagnostic Technique #2: Minisequencing

In this method, a minisequencing reaction is coupled to a 96-well microplate immunoassay for the detection of a point mutations associated with carbofuran and azinphosmethyl resistance.

*I392T mutation:* Two aliquots of PCR amplified fragments from genomic DNA (=110 bp allele non-specific product from SSCP assay using a 5'biotinylated reverse primer) will be adhered to a streptavidin-coated microplate (Boehringer Mannheim). Each well of the microplate will contain 45 µl of the binding buffer (25 mM Tris HCl (pH 7.5), 125 mM NaCl, 5 mM EDTA, 1.0 g/L bovine serum albumin, 1.0 g/L Ficoll 400, 1.0 g/L polyvinyl pyrrolidone and 1.0 g/L Tween 20). The plate will be shaken for 15 min, 50 µl of 0.1 M NaOH in 300 mM NaCl will be added to each well. After 2 min, the plate will be rinsed thoroughly with buffer (250 mM Tris HCl (pH 7.5), 125 mM NaCl, 2 mM $MgCl_2$ and 3.0 g/L Tween 20). Minisequencing reaction will be carried out in the presence of the detection primer (primer immediately adjacent to the point mutation, 20-mer, 5' TTTCCTTCTATACGATTTCA3'), 10 mM Tris HCl (pH 8.3), 50 mM KCL, 1 mM

$MgCl_2$, 10 pmol digoxigenin-labeled dATP, or alternatively 0.5 pmol digoxigenin-labeled dUTP and 0.5 U AmpliTaq DNA in a volume of 50 µl for 30 min in an oven at 55°C. The plate will be washed thoroughly with wash buffer prior to the addition of 7.5 units of peroxidase conjugated digoxigenin antibodies. Plate will be incubated at room temperature for 30 min and washed thoroughly. A 100 ul aliquot of 3,3',5,5'-tetramethyl-benzidine (6mg/ml)/$H_2O_2$ (3%) will be added to each well and incubated at room temperature until a noticeable color change is observed. Color change will be quantitated at 450 nm on a microplate reader (Molecular Devices) after the addition of 50 µl of 4 M $H_2SO_4$. A color change would indicate the genomic DNA has a mutation that is complimentary to the digoxigenin-labeled NTP.

*R30K mutation:* The same method will be employed for detection of the point mutation at nt 198. In the case, the 101 bp allele non-specific product from the SSCP assay will be generated using a 5'biotinylated forward primer. This product will be adhered to the streptavidin-coated microplate. The detection primer used for the minisequencing reaction will be the 20-mer oligonucleotide, 5' AGTCTTTGAATTGCGAGGGT 3'.

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