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Proteomic and molecular analyses of esterases associated with monocrotophos resistance in *Helicoverpa armigera*

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ABSTRACT

We have obtained a *Helicoverpa armigera* strain (MonoR) showing high level resistance to monocrotophos by 15 generations of selection on a weakly resistant field-caught strain. Bioassays using various synergists show a major role for esterases in the resistance, and little if any role for cytochrome P450s or glutathione *S*-transferases. Biochemical data also show elevated levels of esterase activity in the resistant line. There were also minor differences among our strains in the kinetics of acetylcholinesterase activity and in its susceptibility to monocrotophos inhibition, and sequence comparisons revealed one amino acid substitution in AChE1 but none in AChE2 in the MonoR strain. However the AChE1 substitution does not match any others linked to resistance in this or other species and would be located well away from the enzyme active site, so it may not be causally involved with resistance. Native PAGE shows several individual esterase isozymes are more intensely staining in the resistant line and native Western analysis with an antibody against Clade 1 esterases show that several of these belong to Clade 1 and that their greater staining intensity in MonoR is due to greater amounts of the respective enzymes. Proteomic analysis of gel slices also matches the overexpressed bands with up to six Clade 1 enzymes, with some involvement also from esterases in three other Clades. Two of the isozymes that are overexpressed in MonoR are also overexpressed in a fenvalerate resistant line and proteomic analysis of that line bears out previous proteomics of another fenvalerate resistant line which also implicates Clade 1 isozymes.

1. Introduction

Organophosphate (OP) insecticides have been an important element of control strategies for the cotton bollworm, *Helicorverpa armigera* (Hübner) in many parts of the world for the last 40 years [1–3]. They were often the insecticides of choice in the 1970s and were again important in the late 1980s to manage growing problems with resistance to the then-new synthetic pyrethoids (SPs). They have also often been used over the last 15 years to manage the limited late season efficacy of transgenic Bt-cotton crops. Although *H. armigera* larvae were initially quite sensitive to OPs, resistance to OPs such as monocrotophos, quinalphos and phoxim is now quite common, at least in China and the subcontinent [2–6].

Bioassays of *H. armigera* testing for synergistic effects of specific inhibitors have generally shown a strong role for carboxylesterases in metabolic resistance to OPs, plus a variable but usually smaller role for cytochrome P450s, with little or no contribution from glutathione *S*-transferases (GSTs) [2,6–11]. Work using acetylcholinesterase (AChE) inhibition assays generally implicates some target site resistance as well, although the effect does not seem to be strong [6,8,9,12–14].

Little is known about the biochemical/molecular basis for any of these mechanisms. The esterase-based metabolic resistances have consistently been associated with elevated levels of *in vitro* esterase activities in larval homogenates [2,6–8,10,15] and two studies have reported associations between this and heavier staining of specific esterase isozymes, albeit issues with overlapping resistance phenotypes limit the interpretation of the latter. Thus Srinivas et al. [9] found that an Indian strain resistant to OPs, synthetic pyrethroids (SPs) and endosulfan yielded more intense staining of specific esterase isozymes at both the fast and slow moving extremes of the esterase isozyme profile. And Kranthi et al. (cited in [6]), also working on Indian lines, likewise found heavier staining in the fastest migrating isozyme (in both their OP and carbamate resistant material), as well as in one with relatively slow mobility (specific to OP resistance) and one with intermediate mobility (shared with carbamate resistance). Another study has identified two amino acid polymorphisms in the AChE2 enzyme that are correlated with resistance [12,13], albeit it is AChE1 rather than AChE2 that has proven to be the cholinergic target site for OPs in all other insect species with two AChE systems characterized to date [16,17].

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In this study, we have taken a field-caught Chinese strain of *H. armigera* that was initially weakly resistant to monocrotophos and selected it to high level resistance in the laboratory. AChE inhibition assays suggest that weak target site resistance may be operating in the selected strain and we find one amino acid substitution in AChE1, but none in AChE2, that is correlated with resistance. Synergism bioassays with specific inhibitors suggest that most of the resistance is due to esterase-based metabolic resistance. Larval extracts from the resistant strain also show higher *in vitro* esterase activities than do extracts of a susceptible strain or the original, weakly resistant progenitor of the selected strain, and native PAGE shows that this activity difference is due to the greater intensities of several specific esterase isozymes. Notwithstanding methodological differences among the studies, these isozymes appear largely dissimilar to those associated with OP/SP/endosulfan resistance in Srinivas et al. [9] or OP/carbamate resistance in Kranthi et al. (cited in [6]). However there is some overlap with bands associated with resistance to the pyrethroid fenvalerate in two other Chinese strains [18]. Native Western analysis using an antibody raised against a large clade of presumptively detoxifying esterases identified in the Expressed Sequence Tag and proteomic analyses of Teese et al. [19] suggests that several of the bands implicated in monocrotophos resistance belong to this Clade and proteomic analysis confirms their matches to this Clade, albeit also implicating representatives of three other Clades.

2. Materials and methods

2.1. Strains

Four strains of *H. armigera* were studied in this research. The fully susceptible SCD strain has been reared in the laboratory without exposure to pesticides for about 30 years [18]. The weakly resistant Anyang strain (AY) was obtained from a cotton field in Anyang, Henan Province, China in 2005 and then reared in the laboratory for more than two years without any pesticide pressure. The Anyang resistant strain (MonoR) was derived from AY by selection with monocrotophos (mortality at 50–70%) for 15 generations. The FenR strain, which was essentially susceptible to OPs but resistant to the pyrethroid fenvalerate, was obtained by selecting another Chinese field caught strain (collected from Anyang province in 2009) for fenvalerate resistance in the laboratory, reaching about 1000 fold resistance before its use herein compared with the SCD strain. AY was lost before the detailed electrophoresis and Western blot analysis while FenR was only used in these latter analyses.

2.2. Chemicals

Monocrotophos was purchased from Qingdao Pesticide Plant (Qingdao, China), and piperonyl butoxide (PBO) from Endura (Italy). Diethyl maleate (DEM), *S,S,S,*-tributylphosphorotrithioate (DEF), Fast blue RR salt, 1-chloro-2, 4-dinitrobenzene (CDNB), *p*-nitroanisole (*p*-NA), acetylthiocholine iodide (ATChI), 1-naphthyl acetate (α-NA) and 5,5′-dithio-bis(2-nitrobenzonic acid) (DTNB) were purchased from Sigma (USA) and 1,2-dichloro-4-nitrobenzene (DCNB) and methoxyresorufin (MR) from Fluka (USA). Reduced glutathione (GSH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Amresco (USA). All chemicals were AR grade.

2.3. Bioassays and synergism tests

Bioassays of monocrotophos resistance were carried out on individual third instar larvae as per the methods of Yang et al. [20]. A 0.2 μl drop of monocrotophos in acetone was applied to the dorsal prothorax of each larva and mortality was recorded after 48 h. As per Yang et al. [20], synergism tests involved applying DEF, PBO or DEM to the dorsal prothorax of individual larvae 1 h before monocrotophos treatment, at a dose of 8 μg/larva. All resistance/synergism tests were replicated five times for each strain, with 10 larvae per replicate. Control larvae were treated with acetone or the synergists alone.

2.4. Metabolic enzyme assays

All enzyme assays were carried out on homogenates of final instar larvae aged two days since their last moult. The homogenates were prepared at 4 °C as follows. Dissected midguts without gut contents were washed in 0.1 M sodium phosphate buffer (pH 7.6). The midguts from five larvae were then pooled and homogenized in 1200 μl of 0.1 M sodium phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, 1 mM PMSF and 20% glycerol. The homogenates were centrifuged at 10,000*g* for 20 min and the resultant supernatants filtered through glass wool and centrifuged again at 10,000*g* for 20 min. The latter supernatants were then stored at 4 °C and assayed as soon as practical (<0.5 h).

Non-specific esterase activity was measured with α-NA as substrate by the method of Van Asperen [21], with the following modifications. The assay mixture contained 250 μl of substrate solution (0.2 M sodium phosphate buffer containing 10 mM α-NA and 1 mM Fast Blue RR salt, pH 6.0) and 50 μl of the diluted enzyme solution (25-fold in 0.1 M pH 7.6 sodium phosphate buffer). The assays were conducted in 96-well microplates and absorbance measured by a microplate reader (Versa Max, USA) at 450 nm, 27 °C for 10 min.

Microsomal O-demethylase activity with *p*-NA as substrate was determined according to the method of Rose et al. [22], with the following modifications. Ninety microliters of enzyme solution and 100 μl of 2 mM *p*-NA were mixed and incubated at 30 °C for 3 min and then 10 μl of 9.6 mM NADPH was added. The assays were conducted at 30 °C in 96-well microplates and absorbance was measured by a microplate reader (Versa Max) at 405 nm for 20 min.

Microsomal O-demethylase activity with MR as substrate was determined using a modification of the procedure of Mayer et al. [23]. Fifty microliters of enzyme solution and 100 μl of 0.01 mM MR were mixed and incubated at 30 °C for 3 min. After incubation, 10 μl of 9.6 mM NADPH was added. The assays were conducted at 30 °C in 96-well microplates and luminescence was measured by a luminescence microplate reader (Spectra Max Gemini XS, USA) at an excitation wavelength of 530 nm and emission wavelength of 585 nm for 20 min.

GST activity was measured with CDNB or DCNB as substrate following the methods of Yang et al. [20]. For CDNB, the 210 μl reaction mixture consisted of 10 μl of the diluted enzyme solution (10-fold in 0.1 M pH 7.6 sodium phosphate buffer), 100 μl of 1.2 mM CDNB, and 100 μl of 6 mM GSH. For DCNB, the 225 μl reaction mixture consisted of 25 μl of enzyme solution, 100 μl of 1.2 mM DCNB, and 100 μl of 6 mM GSH. The assays were conducted in 96-well microplates and absorbance measured by a microplate reader (Versa Max) at 340 nm and 30 °C for 20 min.

Protein concentrations were determined by the method of Bradford [24] with bovine serum albumin as a standard. All enzyme activities were calculated with SOFTmax software (Molecular Devices, USA).

2.5. AChE enzyme assays

Five third instar larvae from each of the SCD, AY and MonoR strains were homogenized in 1.5 ml of 0.02 M sodium phosphate buffer pH 7.5 containing 0.05% Triton X-100 on ice. The homoge-