Genetics and Molecular Research

Identification and functional characterization of esterases in *Euschistus heros* (Hemiptera, Pentatomidae) and their relationship with thiamethoxam and lambda-cyhalothrin

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Genet. Mol. Res. 14 (3): 11079-11088 (2015)
Received February 17, 2015
Accepted June 8, 2015
Published September 22, 2015
DOI http://dx.doi.org/10.4238/2015.September.22.1

ABSTRACT. The brown stink bug *Euschistus heros* is the most abundant species of the soybean-sucking bugs, and causes large economic losses. Applying different chemical groups of organosynthetic insecticides for its control increases the potential for resistance. Esterases are a group of enzymes that play a variety of roles in insects, and some of them are related to the metabolism of xenobiotics. The aim of this study was to analyze the esterase isoenzyme system of this species and investigate its response to Engeo™ Pleno (thiamethoxam and lambda-cyhalothrin), which is the most widely used pesticide in soybean crops. Two strains were analyzed: the EB strain, which had been free of insecticides for several generations; and the MA strain, which was collected in a

location exposed to agrochemicals. By analyzing the polyacrylamide gel electrophoresis profile, seven different esterases in adults and nymphs of both strains were found. Eight gene loci were responsible for the synthesis of these enzymes. The differences in esterases between the two strains and enzyme changes in insects exposed to Engeo™ Pleno suggest that EST-2 and EST-4 are related to the metabolism of the agrochemical used and are mechanisms of resistance.

Key words: Engeo™ Pleno; Esterase pattern; Soybean pest

INTRODUCTION

The soybean is one of the most important elements of the Brazilian economy (Yorinori, 1996; Klahold et al., 2006), and is vulnerable to attack by pests from emergence to maturity (Panizzi, 2006). Several species of bugs are major pests of soybean crops in many countries (Panizzi, 2000); *Euschistus heros* (F.), *Piezodorus guildinii* (West.), and *Nezara viridula* (L.) are the most abundant in Brazil (Corrêa-Ferreira and Peres, 2003). They feed on different plant structures, and can cause significant losses in yield, quality, and germination potential of the soybean (Corrêa-Ferreira and Azevedo, 2002).

E. heros is particularly problematic in regions with high average temperatures, and is common in the north and west of Paraná State in Brazil, and incorporate at lower latitudes. It is the most abundant soybean pest in Brazil, and, depending on its population level, can cause irreversible damage to crops by reducing bean size and causing wrinkled, hollow, or dark-colored beans. It also delays maturation (green stem and leaf retention), reduces oil content, increases the percentage of protein and free fatty acids in the seeds, and transmits pathogens (Panizzi et al., 1979; Villas-Boas et al., 1990; Sosa-Gómez and Moscardi, 1995; Corrêa-Ferreira and Panizzi, 1999; Boethel et al., 2000).

The application of different chemical groups of organosynthetic insecticides is the main method of controlling *E. heros*. Currently, neonicotinoid and pyrethroid insecticides are used for soybean, and blending the active ingredients of these two groups is common (AGROFIT, http://www.agricultura.gov.br/servicos-e-sistemas/sistemas/agrofit). Of these mixtures, thiamethoxam plus lambda-cyhalothrin (Engeo™ Pleno) is frequently used in the control of bugs in soybean.

Since the introduction of Engeo™ Pleno in 2010, the susceptibility of populations of *E. heros* to thiamethoxam plus lambda-cyhalothrin has decreased (Husch and Sosa-Gómez, 2014). Among the mechanisms of response insects have to insecticides, three groups of enzymes are particularly important: the cytochrome p450 complex, glutathione S-transferases, and esterases. Esterases are a broad group of isoenzymes that have a high rate of polymorphism, and exhibit multifunctional hydrolytic activity and catalyze the hydrolysis of a large number of ester bonds, by which they increase esterase resistance to a wide range of insecticides. Resistance to insecticides that contain ester bonds may occur by metabolic detoxification via ester hydrolysis or insecticide sequestration (Wheelock et al., 2004). Esterases have different functions, such as the control of juvenile hormone levels (Coundron et al., 1981; Yamamoto et al., 1988; Bownes, 1989), the regulation of reproductive mechanisms (Vermut et al., 1998; Mikhailov and Torrado, 2000), and in digestive processes (Healy et al., 1991).

The study of esterase patterns and the identification of enzymes involved in the detoxification of insecticides in pest insects increase our understanding of resistance mechanisms to these compounds, and consequently contribute to pest control. Therefore, this study examined the esterase enzyme system in the development of *E. heros*, in order to identify the genetic loci and alleles involved and to functionally classify these enzymes. In addition, the responses of the esterases to Engeo™ Pleno were investigated.

MATERIAL AND METHODS

Biological material

Two strains of *E. heros* (EB and MA) that were provided by EMBRAPA (Brazilian Agricultural Research Corporation) were used. The EB strain had been kept free of insecticides in the laboratory for at least 25 generations, and the MA strain was collected from soybean crops in Londrina, Parana, Brazil, where chemical pest control was conducted. The insects were maintained in a chamber at 26° ± 2°C, with 70% humidity and a 12-h photoperiod. Feeding was performed with green beans and peanuts that had been sanitized. EB nymphs were used for the standardization of the esterases.

Identification of *E. heros* esterases

Vertical electrophoresis was performed using polyacrylamide gel electrophoresis (PAGE) at a 12% concentration and stacking gels at a 4% concentration (Davis, 1964; Laemmli, 1970). For each sample, an individual bug was homogenized without wings, legs, or antennae in 115 µL 0.1% β-mercaptoethanol buffer and 10 µL carbon tetrachloride at a temperature of 0°C. The samples were centrifuged for 10 min at 11,600 *g* at 4°C in a refrigerated centrifuge. A volume of 10 µL supernatant was used as a sample. The electrophoretic run lasted for 5 h and 30 min with a constant voltage of 150 V, and 0.1 M Tris-glycine, pH 8.3, was used to fill the upper and lower compartments of the tank. For the esterase identification, the gels were pre-incubated in 50 mL 0.1 M sodium phosphate buffer, pH 6.2, for 30 min. A staining solution containing 50 mL 0.1 M phosphate buffer, pH 6.2, 5 mL n-propanol, 0.06 g Fast Blue RR Salt, and 0.03 g α-naphthyl acetate and 0.02 g β-naphthyl acetate that had been solubilized in 1 mL acetone was then added. The solution was incubated for approximately 0.5 h at room temperature in darkness.

For the specificity analysis, 0.05 g of the substrates α-naphthyl acetate and β-naphthyl were used separately, and simultaneously a control gel was prepared with the usual concentrations of substrates.

Tests with different inhibitors of enzymatic activity were conducted in order to classify the esterases. Two gels containing the same samples were made simultaneously; staining for one of the gels was performed in the presence of inhibitors, and the control gel followed the usual staining protocol. The inhibitors used were 0.27 mM p-chloromercuribenzoate (pCMB), 1 mM eserine sulfate, and 1 mM malathion. The gels were stained and pre-incubated in the presence of the inhibitors, which were dissolved in 0.1 mM sodium phosphate buffer, pH 6.2. Esterase classification was performed according to the method used by Healy et al. (1991).

Insect exposure to insecticides and mortality analysis

The insects were subjected to sublethal doses of Engeo™ Pleno. The lethal concentration for 50% of the insects (LC_{50}) was estimated for the EB strain to determine the dosage. The sublethal doses and concentrations used in the experiment are presented in Table 1.

Adult insects (sex unknown) were placed on Petri dishes that contained filter paper (150 mm in diameter) and food that had been impregnated with 2 mL insecticide solution. Only water (2 mL) was used in the control. Four runs with 10 insects were conducted. The Engeo™ Pleno concentrations were 0.005, 0.01, 0.02, and 0.03% (Table 1). For the MA strain, the tested concentrations were 0.01 and 0.02%.

Table 1. Concentrations of thiamethoxan and lambda-cyhalothrin used in the experiments in 2 mL (g/L).

Engeo™ Pleno	Thiamethoxan (g/L)	Lambda-cyhalothrin (g/L)
0.1%	1.41	1.06
0.005%	1.41×10^{-5}	1.06×10^{-5}
0.01%	2.82×10^{-5}	2.12×10^{-5}
0.02%	5.64×10^{-5}	4.25×10^{-5}
0.03%	8.46×10^{-5}	6.36×10^{-5}

Mortality was assessed after a 24-h exposure to the insecticide. Insects that did not exhibit any voluntary movements were considered dead; survivors were frozen and subjected to electrophoresis for esterase analysis. The LC_{50} was estimated for the EB strain using the Probit method in the SPSS 17.0 software (SPSS, Chicago, IL, USA).

RESULTS

E. heros esterase patterns

Analysis of the electrophoretic profiles revealed 18 bands of esterases in adults and nymphs of different stages of *E. heros* (Figure 1). Approximately 300 insects were analyzed to determine these esterase patterns. The bands were grouped into six types of esterase, based on their migration speed on the gel, their affinity to the substrates, and their sensitivity to the different inhibitors.

The esterases EST-1 and EST-6 exhibited different patterns between the larval and adult stages. Only one isoform (band) of EST-6 was expressed in the nymphal stages, while five appeared in the adult isoforms. Therefore, at least two loci were expressed for EST-6: one that was expressed throughout development and the other(s) only expressed in adults. EST-1 was absent in the nymph stages, and only appeared in adults.

All of the loci identified for the esterases were represented by at least two bands (polymorphic). Analysis of the heterozygous individuals identified these enzymes as being monomeric (Table 2), with the exception of isoforms of EST-6 in adults that did not exhibit a known pattern, making it impossible to classify the quaternary structure of this esterase.

Adult MA strain exhibited different esterase patterns compared to adult EB strain. These differences were found in EST-1, EST-2, and EST-4. EST-1 was not detected in the MA strain (Figure 2), except in 0.5% of individuals, which was considered residual activity. The relative activity of EST-2 and EST-4 increased, and EST-4 was present in only 10% of the EB individuals (Figure 3).

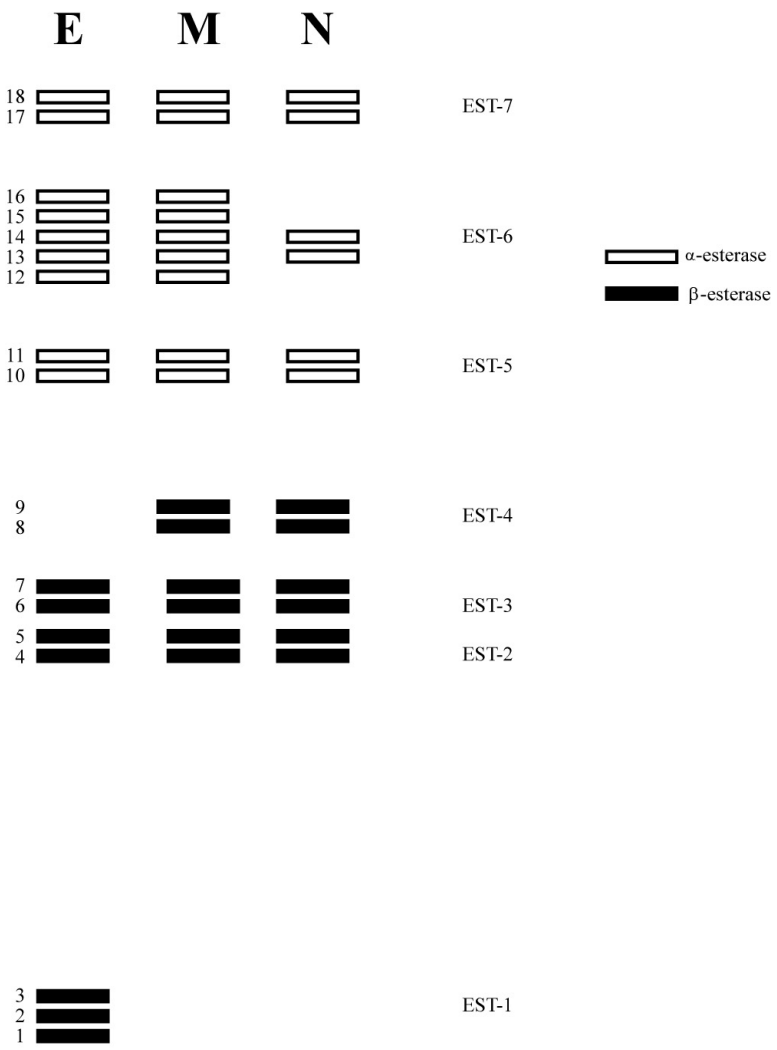

Figure 1. Esterase zymogram pattern detected in adults of EB lineage (E), adults of MA (M) lineage and EB stages of nymphs (N) of *Euschistus heros*.

Table 2. Classification and characterization of esterases in *Euschistus heros*.

Esterase	Malathion	Eserine sulfate	pCMB[1]	Classification	α / β
EST-1	-	-	-	Acetylesterase	β
EST-2	+	++	-	Cholinesterase	β
EST-3	+	++	-	Cholinesterase	β
EST-4	+++	++	-	Cholinesterase	β
EST-5	-	-	-	Acetylesterase	α
EST-6	++	++	-	Cholinesterase	α
EST-7	++	-	-	Carboxylesterase	α

α = α-esterase; β = β-esterase; ++ = partial inhibition; +++ = total inhibition; - = absence of inhibition; pCMB = p-chloromercuribenzoate.

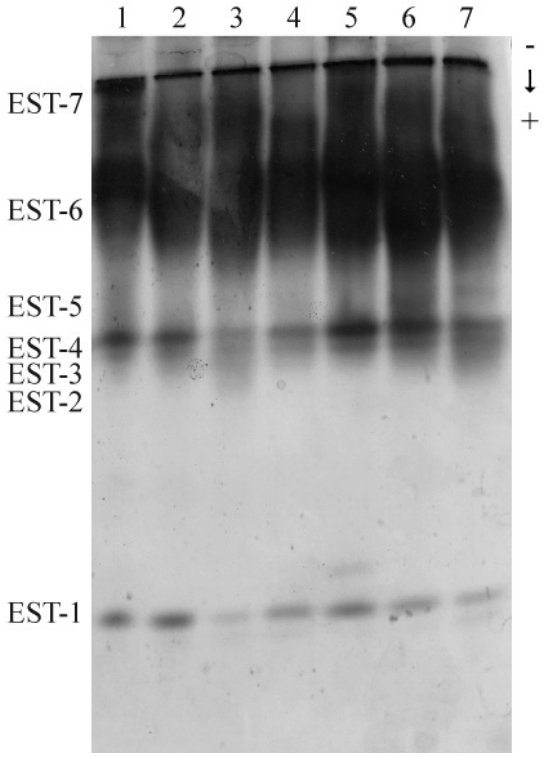

Figure 2. Esterases in adults of EB lineage of *Euschitus heros*. *Lanes 1* to *7* = esterases in adults of EB lineage of *Euschitus heros*.

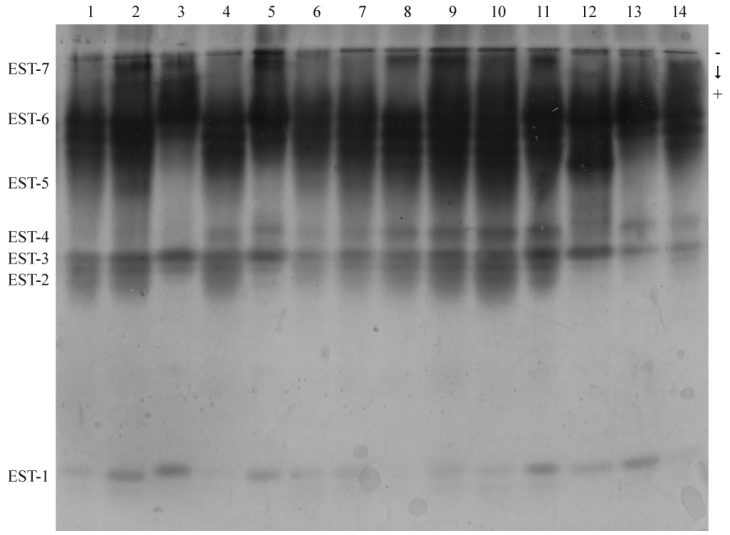

Figure 3. Esterases in adults of MA lineage of *Euschitus heros*. *Lanes 1* to *14* = esterases in adults of MA lineage of *Euschitus heros*.

Analysis of substrate specificity and inhibition of esterase activity

The separate use of the substrates α-naphthyl acetate and β-naphthyl acetate allowed the classification of EST-5, EST-6, and EST-7 as preferred α-esterases, and EST-1, EST-2, EST-3, and EST-4 as preferred β-esterases (Table 2). No specific esterase substrate was observed.

The esterases were characterized by their sensitivities to the inhibitors malathion, eserine sulfate, and pCMB. These inhibitors allowed the classification of the esterases according to the criteria of Holmes and Master (1967) into four classes: cholinesterases, carboxylesterases, arylesterases, and acetyl esterases. According to the inhibition tests, EST-2, EST-3, EST-4, and EST-6 were inhibited by eserine sulfate and malathion, and were characterized as cholinesterases. EST-7 was only inhibited by malathion, so it was characterized as a carboxylesterase. EST-1 and EST-5 were not inhibited by any of the inhibitors, and were classified as acetyl esterases. The pCMB did not inhibit any of the esterases tested (Table 2).

Insecticide exposure and esterase analysis

The different concentrations of Engeo™ Pleno were based on the results of the LC_{50} test, which were estimated for the EB strain in 0.017% of 4.794×10^{-5} g/L thiamethoxam and 3.604×10^{-5} g/L lambda-cyhalothrin. EB insects that were subjected to Engeo™ Pleno at a concentration of 0.005% did not exhibit any changes in their esterase patterns. At concentrations of 0.01, 0.02, and 0.03%, EB-strain individuals exhibited relative increases in the activity of all of the esterases, with EST-4 and EST-2 exhibiting the greatest activity. EST-4 exhibited residual activity in insects of this strain, even when not exposed to Engeo™ Pleno (Figure 4).

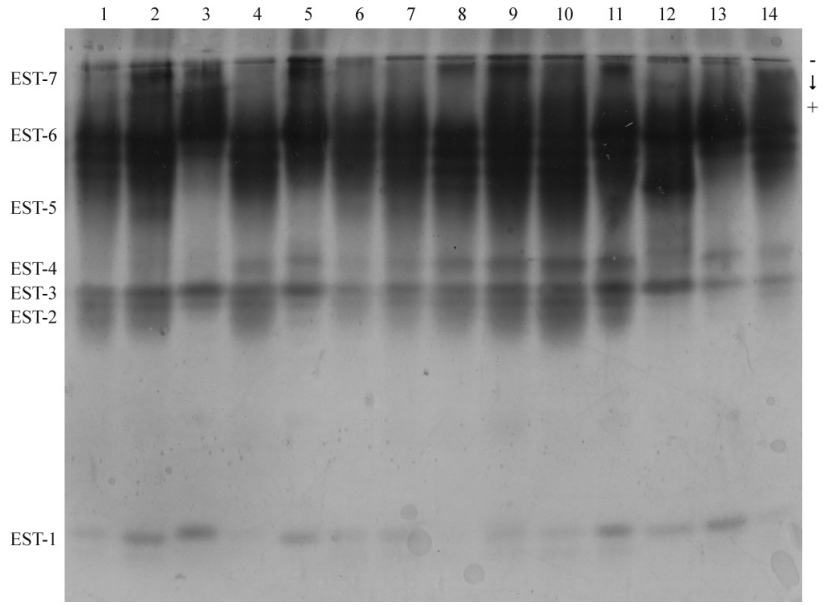

Figure 4. Esterases in adults of EB lineage of *Euschitus heros*. *Lanes 1* to *7* = exposed to a concentration of 0.01% in g/L. *Lanes 8* to *14* = exposed to a concentration of 0.02% in g/L.

When exposed to Engeo™ Pleno at concentrations of 0.01 and 0.02%, insects of the MA strain exhibited no difference in esterase activity. These concentrations were based on the LC_{50} results of the EB strain.

DISCUSSION

Esterases have been identified and characterized in several species of insect by PAGE, which is a rapid and efficient technique to indirectly verify the genes for this isoenzyme system, and to detect qualitative and quantitative differences between these isoenzymes in different populations or species (Rossiter et al., 2001). In this study, 18 bands were identified and grouped into seven types of esterase. Each esterase was characterized by its migration speed, substrate specificity, and sensitivity to different inhibitors.

The esterase isoenzyme system consists of four classes of enzyme: carboxylesterases (EC 3.1.1.1); cholinesterases, including acetylcholinesterases (EC 3.1.1.7) and pseudocholinesterases (EC 3.1.1.8); acetyl esterases (EC 3.1.1.6); and arylesterases (EC 3.1.1.2). Of the seven esterases identified in this study, one was classified as a carboxylesterase (EST-7) and four as cholinesterases (EST-2, EST-3, EST-4, and EST-6). In many insect species, carboxylesterases and cholinesterases are involved in insecticide resistance (Conyers et al., 1998). These esterases may be involved in metabolic detoxification, a mechanism by which pesticides are modified into less toxic forms to the insect or are rapidly eliminated, preventing their action at the target site (Beckel et al., 2006).

There are no studies in the literature regarding the esterase patterns of bugs of the Pentatomidae family. Sosa-Gómez et al. (2009) evaluated the activity of esterases in populations of *E. heros* in the Brazilian states of Paraná and São Paulo using α-naphthyl acetate as a substrate, and found significant variation in esterase activity in these populations. The present study also found six different types of esterase in these insects that are capable of hydrolyzing α-naphthyl acetate.

The analysis of enzymatic expression at different stages of development revealed differences in esterase patterns between nymphs and adults involving EST-1 and EST-6. Differences in the synthesis of esterases during developmental phases may be due to regulatory mechanisms that adjust enzyme production according to bodily requirements (Lucena et al., 2012).

The exposure of insects to an insecticide for consecutive generations makes this compound an important selective agent by selecting resistant genotypes. The genes for enzymes related to the metabolism of these compounds are targets of this selection, including esterase genes. Silva and Lapenta (2011) demonstrated that genetic variability in esterases is related to insecticide resistance in the pests *Oryzaephilus surinamensis* and *O. mercator*. Therefore, a comparative study of esterase patterns between exposed and non-exposed strains to insecticides will enhance our knowledge of esterase involvement in the metabolism of these compounds, and find a possible mechanism of resistance to them.

The differences in esterase patterns between the two analyzed strains and the changes in this enzyme in exposed insects suggest that EST-2, and particularly EST-4, may be related to insecticide metabolism and possibly a resistance mechanism. EST-2 was highly active in MA-strain insects and EST-4 was not detected in most insects of the EB strain, but was present in MA-strain insects. EB-strain insects that had been exposed to Engeo™ Pleno exhibited an increase in the relative activity of EST-4, indicating an increase in the expression of the gene responsible for this enzyme upon exposure to Engeo™ Pleno.

Tests with specific inhibitors classified EST-4 as a cholinesterase; according to Conyers et al. (1998) and Silva and Lapenta (2011), cholinesterases constitute one of the classes that may be involved in insecticide resistance. Cholinesterases have been found in other taxa, such as EST-9 in *Drosophila melanogaster* (Healy et al., 1991). When comparing different strains of *O. mercator*, Silva and Lapenta (2011) found that EST-1 only occurred in the most resistant strain, which suggests that EST-1 is a non-cholinergic cholinesterase and plays an important role in resistance.

The present study found at least six different esterases that were present throughout the development of *E. heros*, and revealed the possible involvement of the cholinesterase EST-4 as an enzyme that participates in the detoxification. The characterization of a large number of factors that are involved in the development of resistance should be part of management strategies that aim to prevent, delay, or reverse resistance to agrochemicals.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

To Embrapa Soja and to Program Coordination of Higher Education Personnel Improvement (CAPES) for granting study scholarship.

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