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CHARACTERIZATION OF ESTERASES IN *Tetragonisca angustula* AND *Tetragona clavipes* (HYMENOPTERA; MELIPONINAE)

Maria Claudia Colla Ruvolo-Takasusuki[1*], Luiz Henrique de Oliveira Viana[1], Tatiane Vicente Baitala[1], Kely Cristina Nicolin[1] and Vagner de Alencar Arnaut de Toledo[2]

[1]Department of Cellular Biology and Genetics, and
[2]Department of Zootechnology, Maringá State University (UEM), Maringá, PR, Brazil.

ABSTRACT

Esterases form a large, diverse group of enzymes with wide, overlapping substrate specificities and patterns of inhibition. These enzymes occur in a large variety of isoforms encoded by distinct gene loci with a high genetic variability and temporal differences in expression that make them appropriate for studying population structure. In this work, we investigated the substrate specificity and pattern of esterase expression in body parts of the stingless bees *Tetragonisca angustula* and *Tetragona clavipes*. Fourteen hives of *T. angustula* were collected in Cianorte and *T. clavipes* were collected from three colonies in Maringá, two cities in the southern Brazilian state of Paraná. The esterase electrophoretic patterns were determined using polyacrylamide gels. Seven bands of esterase activity were detected in *T. clavipes* (EST-1 to EST-7) and two bands in *T. angustula* (EST-1 and EST-2). There was variation in the tissue esterase activity of *T. clavipes*, with EST-6 occurring in the abdomen of workers and EST-7 occurring in cephalic/thoracic extracts. The differences in the number of esterase bands and substrate specificity were attributed to the number of esterase loci involved in each species, and/or variation in substrates specificities. The variation seen here should be useful for determining the role of esterases in intermediate metabolism in the Trigonini, as well as to use esterases as a genetic marker for this stingless bee.

Key words: Esterases, genetic characterization, *Tetragonisca angustula*, *Tetragona clavipes*, tissue expression

INTRODUCTION

The stingless social bees of Brazil belong to the superfamily Apoidea, with the subfamily Meliponinae (highly eusocial stingless bees) containing more than 200 species [20]. The Meliponinae contains two tribes, the Meliponini and Trigonini [19], with most species of the Trigonini occurring in the Neotropics [3]. The Trigonini constitutes a diversified group, with many species building their nests in living or dead tree trunks [20]. Brazil contains a variety of ecosystems that provide diverse habitats for numerous meliponine species, with these bees being important pollinators throughout the Atlantic forest [15]. *Tetragonisca angustula* (Latreille, 1811), locally known as "jataí", and *Tetragona clavipes* (Fabricus, 1804), locally known as "borá", are important meliponine species in this region. Increased environmental degradation, with consequent habitat (forest) destruction, poses a serious threat to the Meliponinae and can result in the isolation of subpopulations. Hence, a knowledge of the genetic structure and genetic variability of stingless bees is essential for the control and preservation of these species.

Isozyme analysis provides an important source of information for the genetic characterization of bee populations. Esterases are one of the most extensively studied isoenzyme systems, and represent a large, diverse group of enzymes with wide, overlapping substrate specificities and patterns of inhibition. Esterases occur in numerous isoforms expressed by distinct gene loci that generally have a high degree of genetic variability. The tissue distribution and temporal differences in the expression of esterases, as well as allozyme variability, have been studied in the Apidae [1,2,6-8,11,12,17,22,23]. However, only a few studies have investigated allozyme variability in Brazilian meliponine bees. Isoenzyme

Correspondence to: Dr. Maria Claudia Colla Ruvolo-Takasusuki
Departamento de Biologia Celular e Genética, Universidade Estadual de Maringá (UEM), Av. Colombo, 5790, CEP 87020-900, Maringá, PR, Brazil. E-mail: mccrtakasusuki@uem.br

studies in the Meliponinae have been done using esterases, phosphoglycomutase, glycerol-3-phosphate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase, hexokinase, aminopeptidase A, hydroxybutyrate dehydrogenase, α-glycerophosphate dehydrogenase and total proteins [4,8,11,12,17,18]. Lima and Mestriner [17] studied the electrophoretic patterns of esterases and nonspecific proteins in 11 species of the Meliponini and described variants in esterase 6 (Est-6) of *Melipona marginata* and esterase 4 (Est-4) of *M. nigra*. Falcão and Contel [11] described the esterase isozyme patterns for 10 species of Brazilian stingless bees, with genetic variability in esterase expression being observed in only four of these species. Aidar *et al*. [1] reported polymorphism in one esterase locus in *M. quadrifasciata*, whereas Contel and Mestriner [7] detected monomorphism for this enzyme in this same species. In contrast to the Meliponini, little is known about the variability of esterases in the Trigonini.

In view of the importance of esterases in intermediate metabolism in bees, in this study, we examined the substrate preferences of esterases in two trigonine species, *T. angustula* and *T. clavipes*, and assessed the expression of these enzymes in different body parts.

MATERIAL AND METHODS

Bee samples

Workers of *T. angustula* and *T. clavipes* were collected from two areas of northwestern Paraná state. Fourteen hives of *T. angustula* were collected in two meliponaries from Cianorte (23º 39' 48" S, 52º 36' 18" W), with 10 workers from each hives being analyzed. *Tetragona clavipes* were collected from three colonies in Maringá (23º 25' 31" S, 51º 56' 19" W), with 20 workers from each colony being analyzed. Workers bees were collected at the entrance of each hive or colony, sacrificed and maintained at -20ºC until isozyme analysis.

The head and thorax were subsequently separated from the abdomen using entomological scissors and the samples (head/thorax and abdomen) were homogenized in centrifuge tubes in 40 μL of a 0.1% β-mercaptoethanol in 10% glycerol solution and then centrifuged at 14,000 rpm for 30 min at 3ºC.

Esterase electrophoretic analysis

Polyacrylamide gels (8% for *T. angustula* and 5.5% for *T. clavipes*) were prepared in 0.375 M Tris-HCl, pH 8.8 [C.R. Ceron, PhD thesis, University of São Paulo, São Paulo, Brazil; 24] and 20 μL of supernatant from each sample was separated by electrophoresis (constant voltage of 200 V for 5 h at 4ºC). The running buffer was 0.1 M Tris-glycine, pH 8.3. The staining for esterase activity was done as modified by Ceron [Ceron CR, PhD thesis, University of São Paulo, São Paulo, Brazil; 24]. The gels were soaked for 30 min in 50 mL 0.1 M sodium phosphate, pH 6.2, at room temperature. Esterase activity was visualized by incubating the gels for 1 h in a solution containing 50 mL of sodium phosphate, 20 mg of β-naphthyl acetate, 30 mg of α-naphthyl acetate, 60 mg of Fast Blue RR salt and 5 mL of N-propanol. The specificity to the substrate was verified by staining the gels only with α-naphthyl acetate or β-naphthyl acetate separately and then comparing the result with that of gels stained with a mixture of α- and β-naphthyl acetate. For long-term storage, the gels were soaked for 1 h at room temperature in a mixture of 7.5% acetic acid and 10% glycerol containing 5% gelatin [5,16], after which they were placed between two tightly stretched sheets of wet cellophane paper and dried for 24-48 h.

RESULTS

The esterases were designated numerically according to their electrophoretic mobilities, starting from the cathode (Figs. 1 and 2). Staining for enzyme activity revealed two esterase loci (*Est-1* and *Est-2*) in adult workers of *T. angustula*. Esterases produced by *Est-1* were specific for β-naphthyl acetate (β-esterase) whereas those produced by *Est*-2 utilized α- and β-naphthyl acetates (αβ-esterase) to equal extents. The expression profiles of these esterases were the same in the head/torax and abdomen.

The quaternary structure of enzymes can be often be inferred from their electrophoretic profile based on the number of bands seen in heterozygotes. The electrophoretic analysis of extracts from *T. angustula* heterozygous for EST-2 showed a single two-band pattern (Fig. 1), with one band produced by each of the two EST-2 alleles. This finding suggested that these enzymes have a quaternary monomeric structure, i.e., they consist of a single polypeptide subunit with catalytic activity. The frequency of allele $Est\text{-}2^{A}$ was 78.6% and that of $Est\text{-}2^{B}$ was 21.3% in the 14 hives analyzed.

Seven regions of activity (EST-1 to EST-7) were recognized in each extract of *T. clavipes*. Esterases produced by the loci *Est-1*, *Est-2*, *Est-3*, *Est-4* and *Est*-5 preferentially cleaved α-naphthyl acetate, whereas esterases produced by *Est-6* and *Est-7* loci preferentially hydrolyzed β-naphthyl acetate. With the exception of the esterase produced by *Est-2*, for which only one allele was identified, each *T. clavipes*

esterase presented two alleles. The electrophoretic analysis of extracts from *T. clavipes* specimens heterozygous for esterases showed a single two-band pattern (Fig. 2), with one band produced by each of the two alleles at each locus. This finding indicated that in *T. clavipes* those enzymes also have a monomeric quaternary structure. The frequency of esterase alleles in *T. clavipes* was not estimated because only three colonies were analyzed.

Analysis of the tissue distribution of *T. clavipes* esterases showed that EST-6 occurred in the abdomen whereas EST-7 was present in the head/thorax (Fig. 2). Overall, there were differences in the number of zones of esterase activity and in the substrate specificity between the two species analyzed, with *T. clavipes* showing seven zones of activity whereas *T. angustula* showed only two (Figs. 1 and 2).

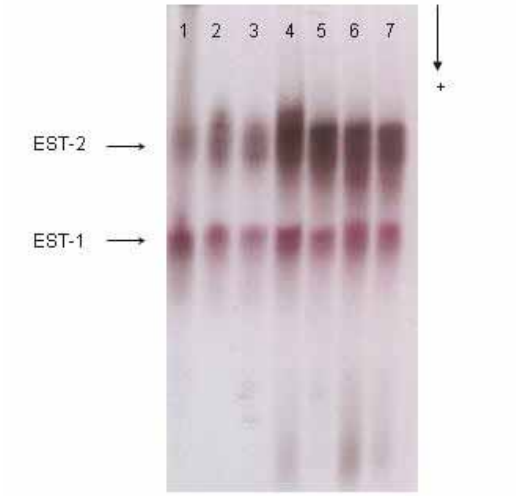

Figure 1. Polyacrylamide gel electrophoretic profile of esterases detected in cephalic/thoracic (1-3) and abdominal (4-7) extracts from *Tetragonisca angustula* using the substrates α- and β-naphthyl acetate.

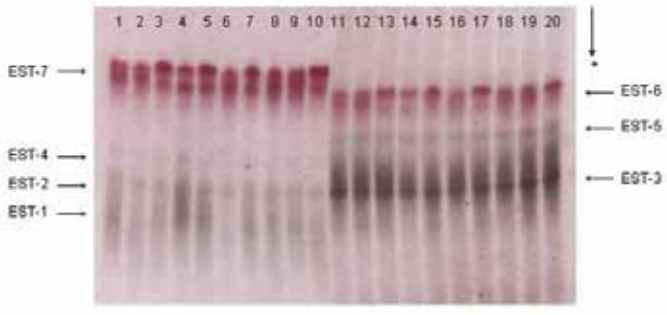

Figure 2. Polyacrylamide gel electrophoretic profile of esterases detected in cephalic/thoracic (1-10) and abdominal (11-20) extracts from *Tetragona clavipes* using the substrates α- and β-naphthyl acetate.

DISCUSSION

The difference in the number of zones of esterase activity and in the substrate specificity in the two trigonine species analyzed here reflects variation in the number of loci involved in each species and variation in the enzymatic properties of the enzymes. In contrast to Falcão and Contel [11], who did not detect esterase in *T. angustula*, our results showed two bands of activity in this species and seven bands in *T. clavipes*. The latter finding was similar to that of Bitondi and Mestriner [2], who reported six regions of esterase activity for *A. mellifera*, with Ruvolo-Takasusuki *et al.* [22] describing an additional band (to give a total of seven). Aidar *et al.* [1] reported polymorphism in one esterase locus in *M. quadrifasciata*.

Variation in the number of isozymes among organs and tissues has been reported for Africanized honeybees (*Apis mellifera* L). Thus, esterase-1a activity was observed in the reproductive and digestive tracts of sexually mature drones, queens and egg-laying workers, which suggested its association with sexual maturation [22]. For aminopeptidases, Lap-G was concentrated in the midgut and was associated with food in the digestive tract, whereas Lap-D was more prominent in the reproductive tract of adult drones [9]. Dipeptidyl aminopeptidase (DAP) activity towards L-leucylglycine-β-naphthylamide (Leu-Gly NA) was present in pupal and adult extracts of *A. mellifera* [10], with enzyme activity being more conspicuous in pupal than in adult extracts, particularly in digestive tract tissue.

The tissue-specific variation of β-esterases detected in *T. clavipes* may be related to the duplication of genes that codify for this isozyme and to mutations that have altered the quaternary structure, thereby influencing the pattern of electrophoretic migration. The relationship between esterase gene duplication and resistance to insecticides has been studied in mosquitoes [14], *Culex pipiens* [13] and the homopteran *Schiazaphis graminum* [21]. The tissue distribution of esterases is expected to be intimately associated with the physiological roles of these enzymes [22]. However, additional studies will be necessary to determine how these isoenzymes are involved in the ontogenetic development and intermediate metabolism of *T. clavipes*. Future studies will also need to address the usefulness of these enzymes as genetic markers for this group of insects.

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