Apidologie (1997) 28, 259–267

Original article

Genetic characterization of a new *Apis mellifera* esterase

MCC Ruvolo-Takasusuki[1], MA Del Lama[1*], AEE Soares[2]

[1]*Departamento de Genética e Evolução, Universidade Federal de São Carlos, Rodovia Washington Luiz, km 235, Caixa Postal 676-13565.905 - São Carlos, SP;*
[2]*Departamento de Genética, Faculdade de Medicina de Ribeirão Preto (USP), Av Bandeirantes s/n - 14049.900 - Ribeirão Preto, SP, Brazil*

(Received 28 January 1997; accepted 18 April 1997)

Summary — A new esterase, denoted esterase-1a (Est-1a), was first identified by starch gel electrophoresis of abdomen extracts from adult drones of *Apis mellifera*. This enzyme was developed only with 4-methylumbelliferyl esters and its inhibition properties suggest that it is an arylesterase. It does not contain reactive sulfhydryl groups and has a monomeric structure. A genetic variant (*Est-1a*^{92}) was detected at an average frequency of 4.2% in drones collected from five apiaries. Genetic linkage studies showed no close linkage between the *Est-1a* locus and the genetic markers *Est-6*, *Mdh-1* and *Hk-1*. Esterase-1a activity was observed in the reproductive and digestive tracts of sexually mature drones, queens and egg-laying workers, suggesting its association with sexual maturation. In view of its presence in the egg and its restricted localization in the middle portion of the digestive tract (midgut), this enzyme may play a proteolytic role in early embryonic development.

esterases / Africanized honeybees / genetic characterization / tissue expression / development expression

INTRODUCTION

The carboxylesterases represent a large and diverse group of enzymes that exhibits wide and overlapping substrate specificities and inhibition patterns. They show universal distribution in different groups of animals and plants, occurring in a large number of forms determined by distinct gene loci, showing tissue distribution and temporal differences and a high degree of genetic variability. Because of these characteristics, this family of enzymes has been the subject of much biochemical and genetic research in

* Correspondence and reprints
Fax: (55) 16 274 8306; e-mail: dmdl@power.ufscar.br

terms of their catalytic mechanism, molecular evolution and regulatory expression. However, the physiological functions of esterases are known in only a few cases despite the relative ease with which esterases can be demonstrated. Juvenile hormone esterases constitute the group of esterases whose role is best understood.

Apis mellifera esterases (EC311) have been studied using different zone electrophoresis procedures in order to determine their electrophoretic profile (Bitondi and Mestriner, 1983), to detect genetic polymorphisms (Del Lama et al, 1985a; Biasiolo and Comparini, 1990), to estimate the frequencies of the known variants in European (Badino et al, 1982, 1984, 1985; Sheppard and Berlocher, 1984, 1985; Sheppard and McPheron, 1986) and Africanized honeybee populations (Lobo et al, 1989; Del Lama et al, 1990), to detect genetic linkage between the esterase loci and the biochemical markers known in *A mellifera* (Del Lama et al, 1985b, 1993), and to quantify the esterase activity in worker and drone honeybee extracts (Del Lama et al, 1984; Del Lama and Del Lama, 1987).

Six regions of esterase activity were described in homogenized pupae of *A mellifera* after starch gel electrophoresis and using substrates derived from 4-methylumbelliferyl esters. These esterases were numerically ordered, with esterase-1 being the enzyme of most anodal mobility (Bitondi and Mestriner, 1983).

Electrophoretic analyses of drone reproductive tract homogenates led to the detection of a new esterase (Ruvolo and Del Lama, 1991; Ruvolo-Takasusuki et al, 1994). Owing to its possible association with the reproductive biology of *A mellifera*, this study was conducted in order to characterize this esterase based on substrate preference and inhibition patterns, and to describe differences in the expression of this esterase among tissues, life stages, sexes and castes.

MATERIAL AND METHODS

Samples

Africanized honeybee *A mellifera* eggs, larvae, pupae, adult workers, queens and drones were provided by the Department of Genetics, Faculty of Medicine of Ribeirão Preto, SP, Brazil. Adult drone samples from Ribeirão Preto/SP (20 hives), Santa Rosa do Viterbo/SP (14 hives), Luis Antônio I/SP (11 hives), Luis Antônio II/SP (61 hives) and Viçosa/MG (23 hives) were electrophoretically analyzed in order to detect genetic variants of *Est-1a*.

Electrophoretic analysis

Eggs, larvae, pupae and different tissues of adult workers, queens and drones were homogenized with a glass rod in a volume of 0.2% aqueous solution of 2-mercaptoethanol (1 mg/2 μL) and centrifuged at 2400 *g* for 15 min at room temperature. Horizontal electrophoresis was carried out on 14% corn starch gels (Penetrose 30^{TM}, Refinações de Milho Brasil S/A) using phosphate–EDTA–$MgCl_2$ buffer (bridge buffer: Na_2HPO4 0.1 M + NaH_2PO_4 0.1 M + EDTA 3 mM + $MgCl_2$ 5 mM; gel buffer: 10 × dilution), pH 6.7. Electrophoresis was carried out at 8 °C for 5 h 30 min at a constant voltage (9.0 V/cm).

Substrates and inhibitors

4-Methylumbelliferyl esters, fluorescein diacetate and α- and β-naphthyl esters were used to study the substrate preferences of esterase-1a. 4-Methylumbelliferyl esters (acetate, butyrate, propionate and palmitate), fluorescein diacetate at a concentration of 0.01% and α-naphthyl (acetate, butyrate and propionate) and β-naphthyl (acetate, butyrate, propionate and laurate) esters were tested according to the procedures described by Bitondi and Mestriner (1983).

To characterize the inhibition properties of the enzyme, the following inhibitors were employed in aqueous solution: 1 mM p-cloromercuribenzoate (PCMB), 1 mM eserine sulfate, 1 mM mercuric chloride, 200 mM sodium fluoride, 1 mM acetazolamide, 1 mM cupric sulfate and 0.1 mM phenylmethylsulphonyl fluoride (PMSF). Commercial organophosphates such as ethion, polytrin and malathion, or the carbamate

thiodan, in 0.1% aqueous solution, were also used. The usual procedure was to preincubate the gel in a buffer solution containing an appropriate amount of the inhibitor for 20 min at 37 °C prior to staining with a solution of 4-methylumbelliferyl propionate also containing the inhibitor to be tested (Bitondi and Mestriner, 1983).

To investigate the presence of reactive sulfhydryl groups in the enzyme, extracts of adult drone abdomens were first treated with different concentrations of cystamine (0.1–20 mM) as described by Bitondi and Mestriner (1985).

Thermal stability

The thermal stability of esterase-1a was tested in extracts of adult drone abdomens preincubated for 0–10 min at 50, 55 and 60 °C. The extracts were then submitted to electrophoretic analysis according to the procedures described above.

Genetic variants and linkage analysis

Abdomen extracts from drones collected from the five localities described previously were analyzed. Except for the Luis Antonio II population (four drones/hive), 12 drones from each hive were used for electrophoretic analysis.

Drones produced by naturally inseminated doubly heterozygous queens for the biochemical markers *Est-1a*, *Est-6*, *Mdh-1* and *Hk-1* were analyzed electrophoretically to detect genetic linkage between these loci. Expected segregational distribution was analyzed by the chi-square test.

Developmental and tissue distribution

Eggs (24 and 48 h), larvae and pupae of different ages were homogenized and electrophoresed as described above. Adult drones, queens and workers were dissected under a stereomicroscope and the digestive and reproductive tracts of adult drones (0–28 days old), virgin queens (0–12 days old), just mated and laying queens, and egg-laying workers were isolated, homogenized and submitted to electrophoresis. The digestive systems were separated into three regions (foregut, midgut and hindgut), which were washed, homogenized and then analyzed electrophoretically.

RESULTS AND DISCUSSION

Biochemical characterization

According to its relative electrophoretic mobility and considering the nomenclature proposed by Bitondi and Mestriner (1983), the new esterase was designated esterase-1a (fig 1).

Substrate preference studies showed that esterase-1a was visualized only when 4-methylumbelliferyl propionate or butyrate was used as substrate. Esterase-1a was not observed with 4-methylumbelliferyl acetate or palmitate, fluorescein diacetate and α- and β-naphthyl esters. This enzyme shares with esterase-1 the characteristic of activity toward 4-methylumbelliferyl esters but not naphthyl esters; the other esterases of *A mellifera* are detected with esters of both 4-methylumbelliferone and naphthol.

Inhibition studies showed that esterase-1a is completely inhibited by PCMB, PMSF and mercuric chloride. Its activity was partially inhibited with eserine sulfate. No inhibition was observed when sodium fluoride, cupric sulfate, acetazolamide, organophosphates or carbamates were used.

Spackman et al (1994) classified esterases in *Drosophila melanogaster* and *Lucilia cuprina* based on their inhibitor specificities. Acetylesterases are insensitives to sulfhydryl reagents (PCMB), organophosphate insecticides (OPs) and eserine sulfate, arylesterases are inhibited by sulfhydryl reagents but not by OPs or eserine sulfate, carboxylesterases are inhibited by OPs and resistant to sulfhydryl reagents and eserine sulfate, and cholinesterases are inhibited by OPs and eserine sulfate. According to these criteria, esterase-1a should be classified as an arylesterase.

Treatment with cystamine produced no apparent change in esterase-1a activity or electrophoretic mobility and the results obtained suggest that this enzyme does not contain reactive sulfhydryl groups. The

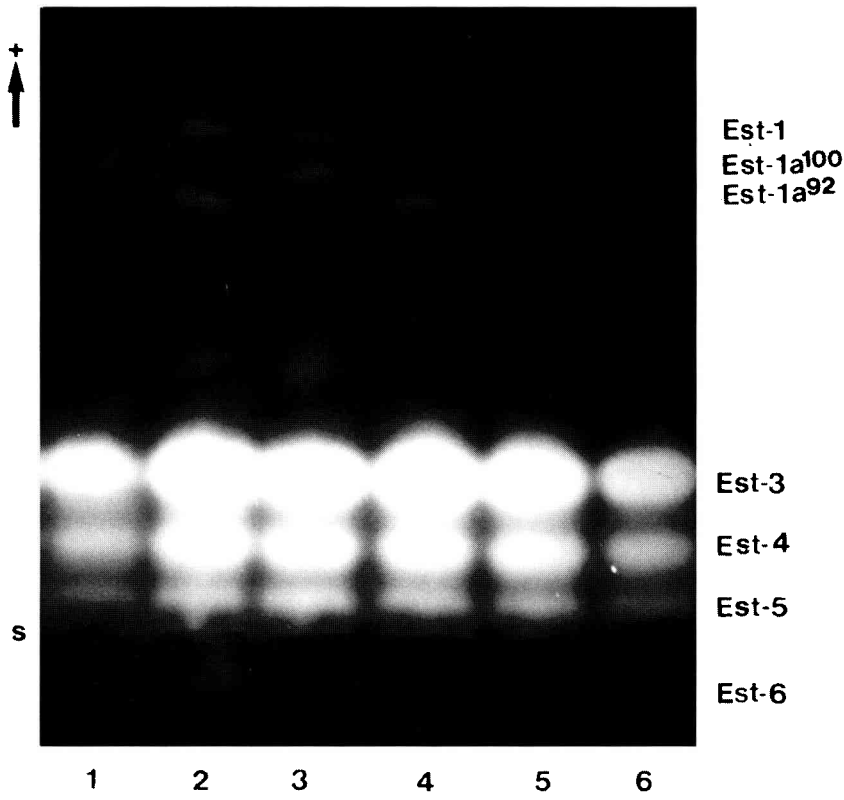

Fig 1. Starch gel electrophoretic profile of esterase activity of abdomen extracts from adult drones of *A mellifera* stained with 4-methylumbelliferyl propionate showing the two variants of esterase-1a.

absence of such radicals in esterase-1a raises the question of inhibition of this enzyme by PCMB, PMSF and mercuric cloride. This apparent discrepancy can be explained by assuming that the -SH group reactive with the mercuric group is occupying part of the enzyme catalytic site and is not accessible to cystamine.

Esterase-1a activity was not detected by electrophoresis when abdominal extracts were previously incubated at 50 °C or higher for 4 min. These findings demonstrate that esterase-1a has a lower thermal stability than the other esterases of *A mellifera* (unpublishing data).

Genetic variants and linkage analysis

Electrophoretic studies of abdominal extracts from adult drones showed two phenotypes of esterase-1a, each represented by a single band pattern (fig 1). Analysis of drones produced by queens heterozygous for the *Est-1a* locus agreed with the genetic hypothesis of one locus (*Est-1a*) with two alleles, *Est-1a*^{100} and *Est-1a*^{92} (table I). Queen homogenates exhibited a third, two-banded

pattern, interpreted as the heterozygous condition. This pattern suggests a monomeric structure for this esterase.

Drone analysis revealed a genetic variant, *Est-1a*^{92}, occurring at a weighted average frequency of 4.2% (range 0–5%) in the five drone samples analyzed (table II). The chi-square test for homogeneity revealed that the frequency of this allele is not significantly different among these populations ($\chi^2 = 7.77$; $0.10 < P < 0.20$). The degree of polymorphism exhibited by the *Est-1a* locus is consistent with the low levels of heterozygosity detected for the other esterase loci in Africanized honeybees in South and Central America (Lobo et al, 1989; Del Lama et al, 1990), with the exception of *Est-6* in *A m ligustica* (Biasiolo and Comparini, 1990).

Results of linkage studies for the *Est-1a*, *Est-6*, *Mdh-1* and *Hk-1* loci are seen in table III. The chi-square values for the expected segregations suggest that there is no close linkage between the loci studied. Analysis of gene linkage between *Est-1a* and *Est-6* presented in table III represents the total number of phenotypes observed in the offspring of two queens heterozygous for these loci. Homogeneity of the results was determined by the chi-square test ($\chi^2_{(3)} = 0.67$; $0.95 < P < 0.98$). Although not all of the two-point tests have been performed, studies have demonstrated that there is no close genetic linkage between the other esterase loci and other biochemical and morphological markers of *Apis* (Del Lama et al, 1985b, 1993).

Table I. Segregation analysis in adult drones of *A mellifera* descending from heterozygous queens for the determination of inheritance for the *esterase-1a* locus.

Queen	*Drone progeny*		χ^2
Est-1a^{100} / *Est-1a*^{92}	100	92	
1	39	29	1.47
2	27	22	0.51
3	13	07	1.80

$\alpha = 0.05$; $\chi^2_{(1)} = 3.48$.

Developmental and tissue distribution

Esterase-1a was detected in the embryonic phase before hatching. During the larval and pupal stages, this enzyme was not detected, but activity was observed again in the adult phase. Esterase-1a was detected in the mucus gland and seminal vesicle of adult drones from emergence to 25 days of age. This esterase was visualized in adult drone testes from 2 days of age. Its activity was

Table II. Phenotypes observed in drones of *A mellifera* and frequency of the *Est-1a* variants (*n* = number of colonies analyzed).

Locality	*n*	*Drone*			
		Phenotype		*Frequency*	
		100	*92*	*100*	*92*
Ribeirão Preto (SP)	20	228	12	0.950	0.050
Luis Antonio I (SP)	11	132	00	1.000	0.000
Luis Antonio II (SP)	61	235	09	0.963	0.037
Santa Rosa Viterbo (SP)	14	164	04	0.976	0.024
Viçosa (MG)	23	263	13	0.953	0.047

Table III. Segregation analysis of *A mellifera* adult drones produced by double heterozygous queens in order to verify genetic linkage.

Gene pair	*Queen phenotype*	*Drone progeny*				χ^2
Est-1a x *Est-6*	92/100; S/M	92; S	92; M	100; S	100; M	
		39	41	37	42	0.37
Est-1a x *Hk-1*	92/100; S/F	92; S	92; F	100; S	100; F	
		37	30	28	33	1.44
Est-1a x *Mdh-1*	92/100; A/B	92; A	92; B	100; A	100; B	
		27	40	37	24	5.56

$\alpha = 0.05, \chi^2_{(3)} = 7.81$.

not detected in semen or mucus, although these tissues showed all the other esterases.

Electrophoretic analysis of extracts from the ovaries and spermathecae of virgin queens (0–12 days old), of mated non-laying queens and laying queens revealed the presence of esterase-1a activity in these organs. Esterase-1a was also detected in ovarial extracts from laying workers, but was not seen in homogenized ovaries from non-laying workers.

Esterase-1a was detected in the digestive tract of drones from 10 days of age and in laying queens. Electrophoretic analysis of the foregut, midgut and hindgut of adult drones showed that esterase-1a activity is restricted to the midgut.

Extracts obtained from the digestive tract of the adult workers sometimes showed esterase-1a activity. This finding suggests the presence of a protease in the digestive tract of the worker that attacks the enzyme. This assumption is corroborated by the fact that extracts from drone abdomens do not exhibit esterase-1a activity when they are previously incubated with worker abdomen homogenates. It is important to point out that, if this protease exists, it is not present in the digestive tract of drones and queens.

The esterase-6 of *Drosophila* constitutes an esterase the functional role of which is well characterized. This enzyme is expressed primarily in the male reproductive system and is transferred to the female reproductive organs during mating (Manning, 1962; Gilbert et al, 1981; Ludwig et al, 1993).

Although esterase-1a of *A mellifera* was also found in the male reproductive tract, this enzyme was not detected in male reproductive secretions; in addition, esterase-1a activity was also found in the female reproductive tract. As a result of these facts and the significant differences in the reproductive biology of these two species, we belive that there is no functional homology between esterase-1a of *A mellifera* and esterase-6 of *Drosophila*.

We suggest that esterase-1a is associated with the sexual maturity of males and females and must be transferred by the female to the eggs during vitellogenesis. Its restricted localization in the midgut suggests a potencial proteolytic activity of this enzyme. On this basis, we cannot rule out the possibility that esterase-1a is one of the proteases responsible for the hydrolysis of protein reserves during early embryo development.

Regulatory mutant

Esterase-1a activity was observed in extracts from pupae and from the thorax of adult workers from a Chilean hive. We suggest that these insects show a regulatory mutation resulting in the presence of this enzyme in all growth phases and in various tissues. First analyses demonstrated the presence of the enzyme in 50% of the worker thoraces; a month ago, only around 5% of the workers exhibited the enzyme, indicating that this mutation was of paternal origin. The marked decrease in segregation and the death of the queen a month later prevented further analyses.

CONCLUSIONS

Esterase-1a can be associated with sexual maturity of males and females and must be transferred by the female to the egg during vitellogenesis. Therefore, the presence of esterase-1a activity in the eggs constitutes an example of a maternal gene used at the beginning of embryo development in this species. The preferential localization in the midgut of adults suggests that esterase-1a may have a proteolytic activity. On this basis, it is possible to consider the assumption that the enzyme can be used as one of the proteases responsible for the hydrolysis of protein reserves during early embryo development.

Although the results obtained point towards a possible association between esterase-1a and sexual maturity in *A mellifera*, they are not sufficient to postulate this biological role. Subsequent studies are necessary to establish the functional role of this esterase in the tissues and in the developmental phases.

ACKNOWLEDGMENTS

We are grateful to MI Casale, IC Godoy, A Penatti, MA Bezerra and J de Souza for technical assistance. We also thank LA de Oliveira Campos and D Message for providing the bees from the apiary of Universidade Federal de Viçosa. This research was supported by a grant from FAPESP (No 91/2500-0) and CNPq (No 300054/95-5).

Résumé — Caractérisation génétique d'une nouvelle estérase chez l'abeille *Apis mellifera* L. Une nouvelle estérase, nommée estérase-1a, a été identifiée pour la première fois par électrophorèse sur gel d'amidon dans des extraits d'abdomen de mâles adultes d'*A mellifera*. Parce qu'elle est susceptible d'être liée à la biologie de la reproduction, cette estérase a été caractérisée d'après les préférences pour le substrat, les profils d'inhibition et les différences dans l'expression en fonction des tissus, des stades de développement, du sexe et de la caste. Des œufs, des larves, des nymphes et divers tissus d'abeilles adultes (reines, ouvrières et mâles) ont été soumis à une électrophorèse horizontale sur des gels à 14 % d'amidon. Le système tampon utilisé était du phosphate-EDTA-$MgCl_2$ à pH 6,7. Des esters de 4-méthylumbelliféryle, du diacétate de fluorescéine et des esters d'α- et β-naphtyle ont été utilisés pour étudier les préférences de l'estérase pour les substrats. Pour caractériser les propriétés inhibitrices de l'enzyme, les inhibiteurs suivants ont été employés : p-chloromercuribenzoate (PCMB), sulfate d'ésérine, chlorure de mercure, fluorure de sodium, acétazolamide, sulfate de cuivre et fluorure de phénylméthylsulphonyle (PMSF), des organophosphorés du commerce tels qu'éthion, polytrine et malathion et le carbamate thiodan. En raison de sa relative mobilité électrophorétique et en se basant sur la nomenclature proposée par Bitondi et Mestriner (1983), la nouvelle estérase a été nommée estérase-1a (fig 1). L'enzyme ne s'est développée qu'avec les esters de 4-méthylumbelliféryle. Les propriétés inhibitrices suggèrent que l'estérase-1a est une arylestérase selon la classification de Spackman et al

(1994). Elle ne contient pas de groupes sulfhydryle réactifs et possède une structure monomérique. Un variant génétique (*Est-1a*^{92}) a été détecté à la fréquence moyenne pondérée de 4,2 % chez des mâles prélevés dans cinq ruchers. Les études de liaison génétique n'ont pas montré de liaison entre le locus *Est-1a* et les marqueurs génétiques *Est-6, Mdh-1* et *Hk-1*. L'activité de l'estérase-1a a été observée dans les appareils reproducteur et digestif des mâles sexuellement mûrs, des reines et des ouvrières pondeuses, ce qui suggère une association avec la maturation sexuelle. En raison de sa présence dans l'œuf et de sa localisation restreinte dans la portion moyenne du tube digestif, il est possible que cette enzyme ait un rôle protéolytique au cours du développement embryonnaire précoce.

abeille africanisée / estérase / caractérisation génétique / développement embryonnaire / expression tissulaire / biologie reproduction

Zusamenfassung — Genetische Charakterisierung einer neuen *Apis mellifera* Esterase. Eine neue Esterase, bezeichnet als Esterase-1a, wurde mit Stärkegelelektrophorese aus Abdominalextrakten von adulten Drohnen von *Apis mellifera* erstmals identifiziert. Wegen einer möglichen Verbindung dieses Enzyms zur Reproduktionsbiologie der Honigbiene wurde es anhand von Substratpräferenz, Inhibitionsmustern und Unterschieden in der Expression in Geweben, Lebensstadien, Geschlechtern und Kasten charakterisiert. Hierzu wurde eine horizontale Elektrophorese mit 14% Stärkegel von Eiern, Larven, Puppen und verschiedenen Geweben adulter Arbeiterinnen, Königinnen und Drohnen durchgeführt. Als Puffersystem wurde Phosphat-EDTA-$MgCl_2$, pH 6.7 benutzt. Die Substratpräferenz von Esterase-1a wurde mit 4-Methylumbelliferylester, Fluoresceindiacetat und α- und β-Naphtylester untersucht. Zur Bestimmung der Inhibitionseigenschaften des Enzyms wurden folgende Inhibitoren eingesetzt: p-Chloromercuribenzoat (PCMB), Eserinsulfat, Quecksilberchlorid, Natriumfluorid, Azetazolamid, Kupfersulfat und Phenylmethylsulphonylfluorid (PMSF); kommerzielle Organophosphate (Phosphatsäureester) wie Ethion, Polytrin und Malathion, und Carbaminatthiodan.

Auf Grund seiner relativen elektrophoretischen Beweglichkeit und bei Berücksichtigung der von Bitondi und Mestriner (1983) vorgeschlagene Nomenklatur wurde die neue Esterase als Esterase- 1a bezeichnet (Abb 1). Dieses Enzym war nur bei 4-Methylumbelliferylestern aktiv. Die Inhibitionseigenschaften lassen vemuten, daß Esterase- 1a nach der Esteraseklassifikation von Spackman et al (1994) eine Arylesterase ist. Es enthält keine reaktive Sulfhydrylgruppen und hat eine monomere Struktur. Eine genetische Variante (*Est-1a*^{92}) wurde mit einer gewogenen mittleren Frequenz von 4,2% bei Drohnen von fünf Bienenständen entdeckt. Genetische Kopplungsuntersuchungen zeigten keine enge Kopplung zwischen dem *Est-1a* Locus und den genetischen Markern *Est-6*, *Mdh-1* und *Hk-1*. Eine Aktivität der Esterase-1a wurde in dem reproduktiven System und dem Verdauungssystem von sexuell reifen Drohnen, von Königinnen und von eierlegenden Arbeiterinnen nachgewiesen, was eine Verbindung zur sexuellen Reifung nahelegt. Wegen seines Vorkommens in Eiern und des begrenzten Vorkommen im mittleren Teil des Verdauungstraktes ist es möglich, daß dieses Enzym eine proteolytische Rolle während der embryonalen Entwicklung spielt.

Esterase / Afrikanisierte Honigbienen / genetische Charakterisierung / Gewebeexpression / ontologische Expression

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