

Genetic characterization of a polymorphic dipeptidyl aminopeptidase of *Apis mellifera*

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Abstract – Dipeptidyl aminopeptidase (DAP) activity towards L-leucylglycine- β -naphthylamide (Leu-Gly NA) was characterized in pupae and adult extracts of *Apis mellifera*. Enzyme activity was more conspicuous in pupae than in adult extracts and it seemed to be concentrated in digestive tract tissues. Two genetically determined electrophoretic variants were observed in honeybee samples from the USA and Chile; in Brazilian Africanized bees, two additional variants were observed. Segregational analyses showed no close linkage between *Dap* and *Est-1*, *Lap D*, *Pgm-1*, *cd*, and *Ac* loci of *Apis mellifera*.

dipeptidyl aminopeptidase / genetic variation / developmental distribution / tissue distribution / *Apis mellifera*

1. INTRODUCTION

Endopeptidases represent the primary proteolytic enzymes in protein digestion in insect midgut lumen. Their function is to decrease the size of the protein molecules in food, generating products which can traverse the peritrophic membrane; only proteins of molecular weight < 50–100 kDa are able to pass through the membrane pores (Wolfersberger et al., 1986). However, endopeptidase activity alone is insufficient for complete digestion of dietary proteins in insects (Billingsley and Downe, 1985; Billingsley, 1990). Intermediate and final stages of protein digestion, which take place within the ectoperitrophic space and/or at the midgut microvillar surface, are effected by aminopeptidase and carboxypeptidase action.

Aminopeptidases (α -aminoacyl-peptide hydrolase EC 3.4.11) are used by a variety of insects during these stages of protein digestion. Aminopeptidases hydrolyze oligopeptides,

releasing free amino acids that are utilized by the insect. Aminopeptidase activities have been observed in both the soluble and membrane-bound fractions of insect guts (Ferreira and Terra, 1984, 1985; Lenz et al., 1991), with the latter being detected in specific regions of the insect midgut (Houseman and Downe, 1981; Espinoza-Fuentes et al., 1984).

Research over the past 30 years has demonstrated the presence in a variety of tissues of a distinct class of aminopeptidases which remove dipeptide moieties from the N termini of peptides. These enzymes have been designated as dipeptidyl aminopeptidases (DAP, EC 3.4.14). Five distinct activities have been characterized on the basis of their subcellular locations, substrate specificities, and inhibitor sensitivities (McDonald and Barrett, 1986; Hui, 1988).

Electrophoretic studies on aminopeptidase enzymes exist on a great number of bacteria,

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fungi, and plant and animal species. However, reports about developmental and tissue distribution, and genetic variation of enzymes with dipeptidyl aminopeptidase activity are absent from the literature. This paper characterizes a new enzyme of *Apis mellifera* L. that exhibits properties different from those of the aminopeptidases and peptidases identified so far in honeybees.

2. MATERIALS AND METHODS

Workers, drones, and queens of different ontogenetic developmental stages were obtained from either the apiary of the Department of Genetics, Faculty of Medicine of Ribeirão Preto (USP) or the Reserva Jataí, Luiz Antonio, the State of São Paulo, Brazil. Worker pupae from Pullman (WA, USA) or Coyhaique and Temuco (Chile) were also submitted to electrophoretic analysis.

Head-thorax or abdomen extracts from workers, queens, and drones were prepared separately in 0.2 mL of 0.2% 2-mercaptoethanol and centrifuged at 3500 g for 15 min at room temperature; the supernatants obtained were used for electrophoretic analysis.

Horizontal electrophoresis was carried out on 14% corn starch gel (Penetrose 30™, Corn Brazil Ltd) using tris-citrate-borate buffer (0.017 M tris + 0.0023 M citric acid, pH 8.0, and 0.3 M boric acid, pH 8.3, for the gel and electrodes, respectively). Similar results can be obtained with 11% potato starch gels. The gels were exposed to a constant current at 2 mA/cm for 4 h at 10 °C. In genetic linkage studies, esterase phenotypes were determined in phosphate-EDTA-MgCl₂, pH 6.7, buffer gels, while malate dehydrogenase (Mdh-1), hexokinase (Hk-1) and phosphoglucosmutase (Pgm-1) phenotypes were determined in tris-EDTA-maleate-MgCl₂, pH 7.4, buffer gels.

Dipeptidyl aminopeptidase activity was revealed by incubating the gels for 2 h at 37 °C in 100 mL tris-maleate buffer (0.1 M tris + 0.1 M maleic anhydride), pH 5.5, containing 30 mg L-leucylglycine-β-naphthylamide (Leu-Gly NA) or glycyglycine-β-naphthylamide (Gly-Gly NA) and 40 mg Fast Garnet GBC salt. For comparative purposes, in the present study aminopeptidase or peptidase activity was also detected by procedures described elsewhere (Del Lama et al., 2001).

The effect of EDTA and 1,10-phenanthroline on dipeptidyl aminopeptidase activity was investigated by first incubating the gel for 20 min in tris-maleate buffer, pH 5.5, containing 1mM EDTA or 1,10-phenanthroline. The enzymatic activity was then developed in the presence of these inhibitors. The

control consisted of another gel slice submitted to previous incubation in the absence of inhibitors and then stained with the reaction mixture.

Extracts from eggs, young and old larvae, and pupae and adults were prepared in a 0.2% 2-mercaptoethanol solution (10 mg/20 μL), centrifuged and submitted to electrophoretic analysis. Venom gland and venom sac extracts of newly-emerged and old workers, and extracts of integuments isolated from white-eyed and black-eyed pupae were also prepared in 0.2% 2-mercaptoethanol solution and electrophoretically analyzed.

The occurrence of DAP genetic variants was investigated in either worker pupae extracts of honeybees of European (Pullman, USA, and Coyhaique and Temuco, Chile) or Africanized origin (Ribeirão Preto, SP, Brazil) or abdomen extracts of Africanized adult drones from an apiary inside the Reserva Jataí, located near Luiz Antonio, SP, Brazil. Eight workers or six drones of each colony sampled were used to estimate gene frequencies averaged over all bees.

Drones produced by naturally inseminated doubly heterozygous queens for *Dap* loci and other genetic markers (*Est-1*, *Lap-D*, *Pgm-1*, *cordovan*, and *Ac*) were analyzed electrophoretically to detect genetic linkage between these loci. Expected segregational distribution was analyzed by the chi-square test.

The maternal origin of sampled colonies was determined based on their European (*carnica/ligustica* or *mellifera*) or African mitochondrial DNA patterns. Total DNA was extracted from the thorax of one worker per colony using a slightly modified method of Sheppard and McPherson (1991). Cytochrome B and cytochrome oxidase I loci were amplified by PCR, using primers and conditions described elsewhere (Sheppard et al., 1994). The PCR product was digested by *Bgl II* and *Hinc II* restriction enzymes, respectively, and the fragments generated were visualized in 7.5% polyacrylamide gels stained with silver.

3. RESULTS

The new enzyme was detected with L-leucylglycine-β-naphthylamide (Leu-Gly Na) as substrate; it was not revealed with L-leucyl-β-naphthylamide (Leu NA) or di and tripeptides, substrates usually employed to show aminopeptidase or peptidase activity, respectively. Therefore, this new enzyme is a dipeptidyl-aminopeptidase (DAP), unlike the aminopeptidases previously described for *Apis mellifera* (Fig. 1; see Del Lama et al., 2001). No enzyme

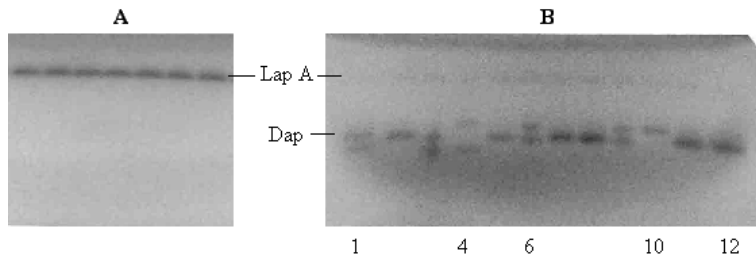


Figure 1. Starch gel stained to show differential aminopeptidase (Lap A - A) and dipeptidyl-aminopeptidase (DAP - B) activities in worker pupae extracts of *Apis mellifera*. Phenotypes 100/93 (sample 1), 104/93 (sample 4), 104/100 (sample 6), phenotype 104/104 (sample 10) and 100/100 (sample 12) are illustrated.

Table I. DAP allele frequencies in worker pupae (Pullman, Coyhaique, Temuco, and Ribeirão Preto samples) or in adult drones (Luiz Antonio) of *Apis mellifera*. (n = number of colonies).

Samples	n	DAP ¹⁰⁴	DAP ¹⁰⁰	DAP ⁹³	DAP ⁸⁸
Pullman (WA, USA)	19	0.247	0.753		
Coyhaique (Chile)	12	0.054	0.946		
Temuco (Chile)	23	0.014	0.986		
Ribeirão Preto (Brazil)	10	0.058	0.933		0.009
Luiz Antonio (Brazil)	46	0.023	0.964	0.003	0.010

activity was observed with glycylglycine- β -naphthylamide (Gly-Gly NA). Inhibition studies showed that DAP activity was completely inhibited in the presence of 1mM EDTA or 1,10-phenanthroline.

3.1. Enzyme distribution

DAP activity was detected in larvae, pupae, and adult extracts of drones, workers, and queens; only residual activity was detected in egg extracts. Enzyme activity seemed to be concentrated in the digestive tract. Comparatively, DAP activity was more intense in queen extracts than in drone or worker extracts at the same developmental stages. It was detected in neither venom gland nor venom sac extracts, and was absent in integument extracts of worker pupae. These homogenates exhibited LAP A activity (Del Lama et al., 2001), although a significant reduction in LAP A activity was observed in extracts of old pupae compared with young pupae.

3.2. Genetic variation and linkage

The occurrence of DAP variants genetically determined was investigated in worker pupae or drone abdomen extracts and the results appear in Table I. As shown, a new polymorphism was observed in both European (from EUA and Chile) and Brazilian Africanized bees (from Ribeirão Preto and Luiz Antonio). The double-banded pattern shown by the heterozygotes indicates that the new enzyme has a monomeric structure.

In the Pullman samples, two electrophoretic variants (DAP¹⁰⁰ and DAP¹⁰⁴) were observed, with frequencies close to 0.753 and 0.247, respectively. The observed genotype proportions agree with those expected according to the Hardy-Weinberg equilibrium model. Coyhaique and Temuco (Chile) samples also showed the variant DAP¹⁰⁴ but at lower frequencies than those observed in Pullman/USA bees. In Brazilian Africanized samples, another two variants were found, DAP⁹³ and DAP⁸⁸, at very low frequencies.

Table II. Segregational analysis of adult drone progenies of doubly heterozygous queens (A and B) to test genetic linkage between *DAP* and known genetic markers of *Apis mellifera*.

loci pair	queen	phenotypes				χ^2	<i>P</i>
<i>DAP</i> × <i>Est-1</i>	A	F / F	F / S	S / F	S / S	3.68	0.20 < <i>P</i> < 0.30
		35	24	22	26		
<i>DAP</i> × <i>LAP D</i>	A	F / F	F / S	S / F	S / S	6.64	0.05 < <i>P</i> < 0.10
		19	32	21	16		
		F / F	F / S	S / F	S / S	2.51	0.30 < <i>P</i> < 0.50
		12	06	07	08		
<i>DAP</i> × <i>Pgm-1</i>	A	F / F	F / S	S / F	S / S	4.51	0.20 < <i>P</i> < 0.30
		25	34	29	19		
<i>DAP</i> × <i>cd</i> *	A	F / +	F / <i>cd</i>	S / +	S / <i>cd</i>	0.24	0.50 < <i>P</i> < 0.70
		27	11	27	16		
<i>DAP</i> × <i>Ac</i>	B	F / <i>Ac</i>	F / <i>ac</i>	S / <i>Ac</i>	S / <i>ac</i>	2.03	0.50 < <i>P</i> < 0.70
		10	08	05	10		

* Intra-class (*cordovan* drones) linkage tests.

Segregational analysis of drone progenies from presumably heterozygous queens demonstrated that *DAP* locus is not closely linked to *Est-1*, *Lap D*, *Pgm-1*, *cd*, and *Ac* gene loci (Tab. II).

4. DISCUSSION

Enzyme activity patterns at developmental stages indicate that populational analyses must be done preferentially at the pupal stage when enzyme activity is highest. Genetic studies can also be conducted on abdomen extracts of adult drones and workers, but not on thorax extracts in which this activity is very low.

Higher *DAP* activity was verified in abdomen extracts of inseminated queens when compared to virgin queens. Results obtained were similar to those of other hydrolases such as *LAP A*, *Est-1*, and *Est-1a* (Del Lama et al., 2001; R vulo-Takasusuki et al., 1997). These findings indicate that further analyses are needed to understand completely the functional role of increased activity of some hydrolases in abdomen extracts of *A. mellifera* inseminated queens.

Although the *DAP* polymorphism was observed in European and Africanized samples, the exclusive presence of variants 93 and 88 in Africanized populations (all Brazilian colonies sampled exhibited an African mito-

chondrial haplotype) suggests that these alleles were originally introduced in Brazil in the 50s with the *Apis mellifera scutellata* queens. In the analyzed populations of European origin, the USA sample showed a higher heterozygosity than did the Chilean samples. This greater level cannot be attributed to a distinct racial composition of the samples, because all colonies from Coyhaique and sixteen of the twenty colonies from Temuco showed the same *carnicalligustica* mitochondrial DNA pattern identified in all Pullman colonies.

The physiological function of these enzymes is poorly understood. However, the first biological role identified in a member of this enzyme group was the activation of promelittin to melittin in honeybee venom glands (Kreil et al., 1980). A similar product conversion via a stepwise cleavage of dipeptides from the amino end was subsequently reported in the biosynthesis of the yeast α mating factor (Kurjan and Herskowitz, 1982; Brake et al., 1983) and in the conversion of caerulein and xenopsin from their precursors in frog skin (Mollay et al., 1986). In mammalian tissues, *DAP* enzymes are thought to be involved in the processing of peptide hormones rather than in general protein degradation (Lee and Snyder, 1982), and peptide hormones may be involved in mediating cell-cell intercommunication and controlling cell movements during morphogenesis in *Dictyostelium* (Chan et al., 1985).

Because of the biological roles described above for these enzymes, two tests were made to ascertain a possible function of the enzyme characterized in this study. Electrophoretic analyses of venom gland and venom sac extracts of young and old workers did not detect Leu-Gly NA activity, which suggests that the dipeptidyl aminopeptidase characterized in this work is not the enzyme responsible for that interconversion. In order to investigate some DAP function over integument enzymes (including prophenoloxidase, a key enzyme in the process of cuticular pigmentation), integument extracts isolated from young and old pupae were submitted to electrophoretic analysis. No DAP activity was shown, suggesting that this enzyme is not related to integument differentiation in *Apis mellifera*.

DAP activity was also detected in pupae extracts of other bee (*Euglossa* sp and *Melipona quadrifasciata*) and wasp species (*Polistes canadensis* and *Polistes versicolor*). A possibly ubiquitous presence and the important functions that these enzymes seem to carry out in different organisms indicate that genetically and biochemically well-characterized dipeptidyl aminopeptidases can be a useful model to studying how an enzyme family evolves.

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Résumé – Caractérisation génétique d'une aminopeptidase dipeptidyle d'*Apis mellifera*. Les aminopeptidases (α -aminoacyl-peptide hydrolase, EC 3.4.11) sont des peptidases qui hydrolysent les acides aminés uniques en position N-terminal de la chaîne polypeptidique. Les recherches ont montré la présence dans divers tissus d'une classe distincte d'aminopeptidases qui éliminent les dipeptides en position N-terminal des peptides. Ces enzymes ont été appelés aminopeptidases dipeptidyles (DAP, EC 3.4.14). Il existe de nombreux rapports sur la répartition au cours du développement et dans les tissus et sur la variation génétique des enzymes qui ont une activité d'aminopeptidase. Néanmoins la caractérisation génétique des DAP n'a jamais été relatée. L'activité de DAP envers L-leucylglycine- β -naphthylamide a été caractérisée par électrophorèse dans des extraits de nymphes et d'adultes d'*Apis*

mellifera. Les études d'inhibition ont montré que l'activité des DAP était complètement inhibée en présence de 1 mM d' EDTA ou de 1,10-phenanthroline. L'activité des DAP était plus évidente dans les extraits de nymphes que dans ceux d'adultes et elle semblait concentrée dans les tissus du tube digestif. Afin de tester une éventuelle fonction physiologique de cet enzyme, l'activité des DAP sur Leu-Gly NA a été étudiée dans des extraits de glande à venin ou dans des sacs à venin d'ouvrières adultes jeunes et âgées et dans les téguments de nymphes jeunes (aux yeux blancs) et âgées. Aucune activité de DAP n'a été mise en évidence dans ces extraits, ce qui suggère que l'enzyme n'est pas responsable de la transformation de la proméllittine en méllittine dans les glandes à venin de l'Abeille domestique et n'est pas liée à la différenciation du tégument d'*A. mellifera*. On a observé deux variants électrophorétiques déterminés génétiquement dans les échantillons d'abeilles des USA (Pullman, WA) et du Chili (Coyhaique and Temuco) ; chez les abeilles africanisées brésiliennes deux autres variants ont été observés. Des analyses de ségrégation n'ont montré aucune liaison entre le locus *Dap* et les locus *Est-1*, *Lap D*, *Pgm-1* et *Ac* d'*A. mellifera*.

Apis mellifera / aminopeptidase dipeptidyle / variation génétique / répartition dans les tissus / développement postembryonnaire

Zusammenfassung – Genetische Charakterisierung einer polymorphen Dipeptidyl-Aminopeptidase von *Apis mellifera*. Aminopeptidasen (α -Aminoacyl-Peptidhydrolase, EC 3.4.11) sind Peptidasen, die einzelne Aminosäuren vom N-Ende der Polypeptidkette hydrolysieren. Durch Untersuchungen wurde gezeigt, dass in einer Anzahl unterschiedlicher Gewebe eine bestimmte Klasse von Aminopeptidasen vorkommt, die Dipeptidhälften von den N-Enden der Peptide entfernen. Diese Enzyme wurden als Dipeptidyl-Aminopeptidasen bezeichnet (DAP, EC 3.4.14). Berichte über die Verteilung solcher Enzyme während der Entwicklung oder in den verschiedenen Geweben sowie über die genetische Variation der Enzyme mit Aminopeptidaseaktivität sind sehr verbreitet, es gibt aber bislang keine Untersuchung zur genetischen Charakterisierung der Dipeptyl-Aminopeptidasen. Die Aktivität der Dipeptidyl-Aminopeptidase gegen L-Leucylglycin- β -Naphthylamid wurde in Extrakten von Puppen oder adulten *Apis mellifera* elektrophoretisch charakterisiert. Untersuchungen zur Hemmung zeigten, dass DAP in Anwesenheit von 1mM EDTA oder 1,10-Phenanthrolin vollständig inaktiviert war. Die DAP-Aktivität war in Puppenextrakten auffälliger als in den Extrakten der adulten Bienen und war anscheinend in den Geweben des Verdauungstraktes konzentriert. Zur Untersuchung einer möglichen physiologischen Funktion dieses Enzyms wurde die DAP-Aktivität auf Leu-Gly NA in Extrakten der Giftdrüsen oder Giftsäcken junger und alter Arbeiterinnen und

in Extrakten des Integuments junger (weißäugiger) und alter Puppen untersucht. In diesen Extrakten trat keine Enzymaktivität auf. Dies weist darauf hin, dass das Enzym bei Honigbienen keine Rolle bei der Umwandlung von Promelittin in Melittin in den Giftdrüsen spielt und in keiner Beziehung zur Integumentdifferenzierung steht. In Honigbienenproben aus den USA (Pullman, WA) und Chile (Coyhaique und Temuco) wurden zwei genetisch determinierte elektrophoretische Varianten gefunden, bei brasilianischen afrikanisierten Bienen wurden zwei zusätzliche Varianten beobachtet. Kreuzungsanalysen zeigten, dass keine enge Koppelung zwischen *Dap* und *Est-1*, *Lap D*, *Pgm-1*, *cd* und *Ac Loci* von *Apis mellifera* vorliegt.

Dipeptidyl-Aminopeptidase / Genetische Variation / Verteilung während der Entwicklung / Verteilung im Gewebe / *Apis mellifera*

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