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Relation between Dopa Decarboxylase activity and paralytic activity in *Tenebrio molitor* and *Neobellieria bullata*

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ABSTRACT. Paralysins are endogenous compounds in immature insects that cause paralysis or death in adult individuals after injection into the thorax. We have proven the universal effect of paralysins by injection of paralysins from *Neobellieria bullata* into adult *Tenebrio molitor* and vice versa. The toxic effect of the tested, 30% acetonitrile fraction from whole body homogenates depends on the stage of the insect from which the extraction was made. The activity of the paralysins shows a temporal distribution with the highest level at pupation and a second, smaller effect at eclosion.

The dopa decarboxylase (DDC) activity, measured by using a radioactive labeled substrate, in developing *N. bullata* and *T. molitor*, peaks at the most important moments in the development of the insect: at pupation but especially at eclosion.

The DDC enzyme could play an important role in the toxicity of paralysins. Relating the temporal distribution of toxic activity in both species to their correlated distribution of DDC activity shows the same pattern: DDC activity increases after stages that show high paralytic activity. Injection of β-alanine-L-tyrosine (BAY, a known paralysin of *Neobellieria bullata*) into the thorax of adult flies did not induce the DDC activity. So, this could be the key to the toxic effect of BAY, because injection of BAY into the thorax of juvenile (pupae) *Tenebrio molitor* did cause an induction of DDC activity.

KEY WORDS: dopa decarboxylase, paralysins, insects.

INTRODUCTION

Paralysins recently discovered in our lab are a new class of endogenous toxic substances found in juvenile insects that cause instant paralysis or death after injection of physiological concentrations into adults (CHIOU et al., 1998a). From *Neobellieria bullata* Parker, 1916, two paralysins were purified by means of HPLC. By means of Fast Atom Bombardment Mass Spectrometry and Nuclear Magnetic Resonance spectroscopy these substances were identified as β-alanine-L-tyrosine (BAY) and 3-OH-kynurenine (3HK). The first paralysin, BAY, (CHIOU et al., 1998b) is a dipeptide with a modified N-terminal amino acid. This paralysin was known long before but in other physiological circumstances (LEVENBOOK et al., 1969). It was named sarcophagine because it was the predominant non-protein ninhydrin-positive material in fully-grown larvae of *Neobellieria* (=*Sarcophaga*) *bullata*. Sarcophagine was found to be synthesised in the fat body and to accumulate in the larval hemolymph up to the moment of the formation of the white puparium. Thereafter, its concentration drops dramatically to almost undetectable levels. The reason for this decline is that at the moment of pupariation, hydrolases from the fat body degrade the dipeptide into the amino acids β-alanine and tyrosine, which are subsequently incorporated in the cuticle, to play a role in sclerotisation (BODNARYK & LEVENBOOK, 1969; DUNN et al., 1977). This is where DDC is implicated as this enzyme is responsible for the formation of products needed in sclerotisation. The enzyme displays a high substrate specificity in arthropods (LUNAN & MITCHELL, 1969) in contradiction to the homologous enzyme in mammals (FELLMAN, 1959; CHRISTENSON et al., 1970) where the enzyme carboxylases several aromatic amino acids. CHEN & HODGETTS (1976) studied the biochemical

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properties of the enzyme in *N. bullata*. N-acetyl dopamine acts as negative feed back for the DDC (FRAGOULIS & SEKERIS, 1975). We will refer to negative feed back mechanism on enzymes by down stream products in the discussion. In 1974, CHEN & HODGETTS tested the enzymatic activity in *N. bullata* in imaginal wing discs and whole body homogenates by trapping radioactive CO_2 released by the enzyme's activity. The enzyme activity peaks twice during postembryonic development: first at pupariation and second at eclosion, respectively correlated with sclerotisation of the puparium and the adult cuticle. Besides these two major peaks, there was a third smaller peak 5.5 to 6.5 days after pupariation. This peak could be responsible for the formation of the prothoracal spiracula of the pharate adult. Using a more accurate radiometric assay MARSH & WRIGHT (1980) determined the pattern of DDC expression throughout the entire development of *Drosophila*. Five defined peaks of DDC activity were recorded at embryonic hatching, the two larval moults, pupation and eclosion with the last two being the greatest. TURNBULL & HOWELLS (1980) tested the enzyme activity from *Lucilia cuprina* Wiedemann, 1830 using whole body homogenates and also found an increase of activity at pupariation and eclosion.

In this study we were looking for a temporal relation between the DDC activity and the paralytic activity during development. A relation can give us new insights in the toxicity mechanisms of paralysins.

MATERIAL AND METHODS

Animals

The fleshfly, *N. bullata* was obtained from our own breeding program at the laboratory. Individuals were kept in cages in a climate controlled room with a constant temperature of 23°C-25°C and a relative humidity of 60%-70%. There was a long day – short night cycle respectively 16h-8h. The adults were fed sugar and water until day 4. Thereafter they were also fed bovine liver, which is necessary for development of the eggs. Larvae also feed on bovine liver, and for detailed descriptions see HUYBRECHTS & DE LOOF (1977). Larvae of *Tenebrio molitor* were obtained from a local pet shop (Squamata, Herent). The larvae were kept in plastic containers in a climate controlled room with a constant temperature of 32°C and a relative humidity of 40 %. They were subjected to the same long day – short night cycle. The containers were filled (4 cm) with oatmeal and small amounts of milk powder and brewer's yeast. Pupae were collected every morning and placed in dated petri dishes. Adults were separated and also kept in dated petri dishes containing only a small amount of oatmeal.

Preparation of the solutions needed for the enzyme assay

The assay was modified after HIRUMA & RIDDIFORD (1985). Solution A: 0.5 M $NaH_2PO_4.H_2O$; solution B: 0.5 M Na_2HPO_4; homogenisation buffer: 66.0 ml A + 134 ml B (up to 1000 ml, pH: 7.1) containing 0.034 g phenylthiourea and 102.6 g sucrose. Wash buffer: 39.0 ml A + 61.0 ml B, (up to 1000 ml, pH: 7.0). Reaction buffer: 15.6 ml A + 24.4 ml B (up to 200 ml, pH: 7.0) containing 0.006 g phenylthiourea dissolved in 200 µl ethanol. L-dopa solution: 0.00986 g L-dopa dissolved in 50 ml reaction buffer. PLP solution: 0.00247 g Pyridoxal-5-L-phosphate in 50 ml reaction buffer. DEHPA solution: 1.360 ml di-(2-ethylhexyl)phosphate and 48.640 ml chloroform. Labeled L-dopa solution: 0.5 ml L-dopa solution + 0.5 ml PLP solution + 10 µl L-3,4-dihydroxyphenyl(3-^{14}C)alanine solution.

Preparation of the whole body homogenates

We respectively used two and three ml homogenisation buffer for two equivalents (one equivalent means one animal of a given developmental stage) of *N. bullata* and *T. molitor*. We prepared three samples of each stage. The homogenisation was performed with the glass homogeniser of Potter and Elvehjem (544S, 2 ml, B. Braun Melsungen AG, the pestle was driven by a boring machine: 400R electronic, AEG SBE). This was done on ice to reduce overheating from the rotating apparatus. The homogenate was centrifuged twice (15000 g, 17 min, 4°C; Beckman Optima LE-80K Ultracentrifuge) and the supernatant was stored in several portions at -80°C.

Measurement of the DDC activity

The DDC activity was measured three times per sample (3x3 measurements/stage), therefore we added 10 µl of the labeled L-dopa solution to 3 µl of sample and incubated during 30 minutes at 38°C. Three hundred µl wash buffer was added to stop the reaction (until now, steps were performed on ice). One hundred µl DEHPA solution was added and shaken well. All samples were centrifuged (11000 g, 2 minutes; Eppendorf centrifuge 5415 Belgolabo) to separate the organic layer, containing dopamine, and the aqueous layer, containing L-dopa. Two hundred µl of the aqueous layer was removed and again 300 µl of wash buffer was added, shaken and centrifuged. To 50 µl of the organic layer 4 ml of scintillation fluid was added and counted during 5 minutes. Before each measurement series a calibration of the instrument was done. We included a blank vial (3µl of sample replaced by 3µl of distilled water); a background vial (scintillation fluid only) and a positive control standard. For this standard we used white pupae of the fleshfly since this stage is easy to select and has a high DDC activity. All control pupae were collected and prepared (on the same day) for the assay using the same sample preparation as described above. The supernatant was pooled and divided into 75 Eppendorf 1.5 ml tubes and immediately stored at -80°C, until assessment of its DDC activity and subsequent scintillation count. Afterwards, that control was recalculated to the predetermined mean of the control standards

(400 dpm) and the experimental counts were then adjusted accordingly. The counts are a measure for the quantity of dopamine, so it is a measure for the enzymatic activity. Because we wanted to know the specific enzymatic activity we also determined the protein content for each sample by using the Bradford method.

For the induction experiment we used a Hamilton 100 µl syringe (2 µl per injection) for the flies. We injected anaesthetised flies (CO_2) in the thorax under the wing, more precisely under the squama. For *Tenebrio molitor*, we used glass needles pulled from capillaries (length: 75 mm, diameter 1.4-1.75) using a vertical pipette puller. The pupae were immobilised with ice and the glass needle was inserted into the thorax between the last thoracic segment and the first abdominal segment. The injected product was a sublethal dose of BAY, the paralysin discovered in *N. bullata*, 50 µg/10 µl distilled water in *N. bullata* and 50 µg/6 µl distilled water in *T. molitor*.

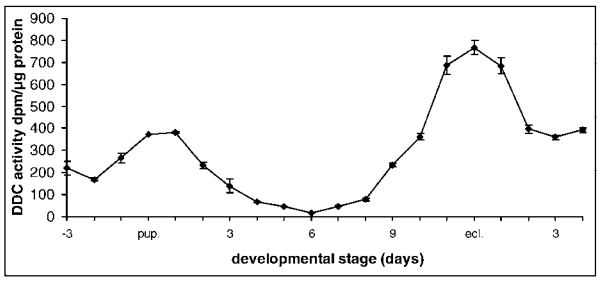
RESULTS

Determinations of the DDC activity in whole body extracts of the fleshfly and the mealworm

In *N. bullata*, the DDC activity was determined from approximately 3 days before pupation to 4 days after eclosion (Fig. 1). We noticed a slight increase of activity at pupation (371 dpm/mg protein). After this peak, the enzyme activity decreased reaching a minimum on day 6 (16 dpm/mg protein). After this temporary period of low activity the DDC activity rose again to reach a maximum at eclosion (767 dpm/mg protein). This high activity was maintained for 24 hours. After 24 hours the DDC activity dropped again and remained constant (± 400 dpm/mg protein) for the following days. In *T. molitor* we measured the DDC activity starting from approximately 3 days before pupation to 6 days after eclosion (Fig. 2). Generally the DDC activity was lower than in *N. bullata*. A peak at pupation was almost absent (65 dpm/mg protein), but was followed by a decrease, and the lowest DDC activity was reached 3 days after pupation (26 dpm/mg protein). The DDC activity peaked at eclosion (306 dpm/mg protein) and again there was a lower, but stable level of activity maintained after eclosion (100 dpm/mg protein).

Comparison between dopa decarboxylase activity and paralytic activity

This was possible as Chiou et al. (1998a) established the paralytic activity during development in the fleshfly. Thus, the corresponding data (paralytic activity and DDC activity of corresponding stages) was incorporated into one graph (Fig. 3). The same comparison was done for the mealworm. In the present study we established the paralytic profile, according to Chiou et al. (1998a). Fig. 4. shows the relation between the DDC activity and the paralytic activity in both species. An increase in toxicity (lower LD50 values) is followed by an increase of DDC activity.



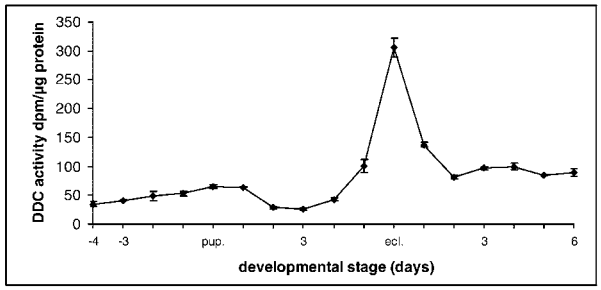


Fig. 1. – Distribution of DDC activity (presented in dpm/µg protein) during post embryonic development of *Neobellieria bullata* using whole body homogenates. Each data point represents the mean of three independent tests (three different samples of the same developmental stage) and each test concludes three different counts of the same sample. Vertical lines represents standard error.

Fig. 2. – Distribution of DDC activity (presented in dpm/µg protein) during post embryonic development of *Tenebrio molitor* using whole body homogenates. Each data point represents the mean of three independent tests (this is three different samples of the same developmental stage), each test concludes three different counts of the same sample. Vertical lines represents standard error.

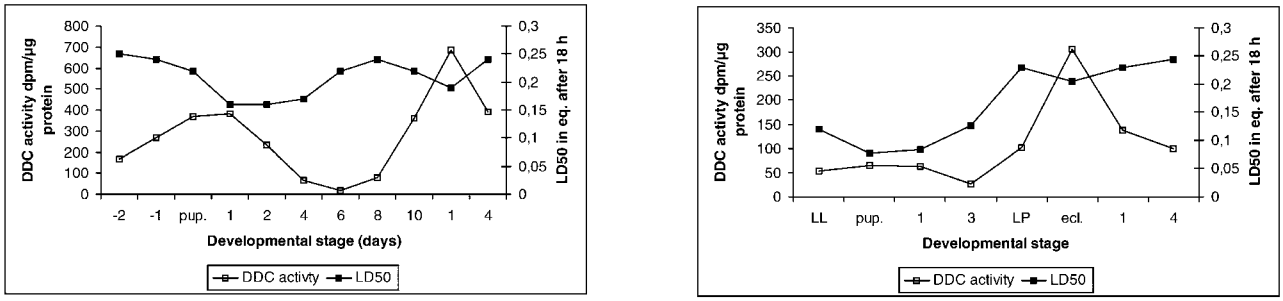


Fig. 3. – Relation between DDC activity and paralytic activity in developing *Neobellieria bullata*. DDC data is copied from Fig. 1. The LD50 values are represented in equivalents of the injected 30% ACN/0.1% TFA extract (extracts from developing *Neobellieria bullata* into adult *Neobellieria bullata*). Each data point represents the mean of three independent tests, and every test included ten flies. The standard deviation is never greater than 10 %. Injection of controls with distilled water had no effect. The LD50 values are obtained from Chiou et al. (1998a).

Fig. 4. – Relation between DDC activity and paralytic activity in developing *Tenebrio molitor*. DDC data are copied from Fig. 2. The LD50 values are represented in equivalents of the injected 30% ACN/0.1% TFA extract (extracts from developing *Tenebrio molitor* into adult *Neobellieria bullata*). Each data point represents the mean of three independent tests, and every test included ten flies. The standard deviation is never greater than 10%. Injection of controls with distilled water had no effect. The LD50 values are obtained from unpublished results.

Induction experiments

Fig. 5 shows the DDC activity in whole body homogenates of adult flies injected with 50 µg BAY into the thorax. All flies were injected at day 2 (this is 2 days after eclosion). One day after injection, there was no difference in activity between our experimental conditions (50 µg BAY dissolved in 10 µl distilled water) and the two controls (untreated flies and flies that were injected with 10 µl distilled water). Two days after the injection, the DDC activity of flies under the experimental condition decreased but remained close to both controls. Fig. 6 shows the DDC activity in whole body extracts of pupae of *T. molitor* injected with 50 µg BAY into the thorax. All pupae were injected on day 1 (this is 1 day after pupation). One day after injection, there was already a distinct difference in activity between our experimental condition (50 µg BAY dissolved in 6 µl distilled water) and both our controls (pupae without injection, pupae injected with an equal volume of solvent). The DDC activity had more than doubled from the first day values and it continued to rise on the second day while the controls further decreased.

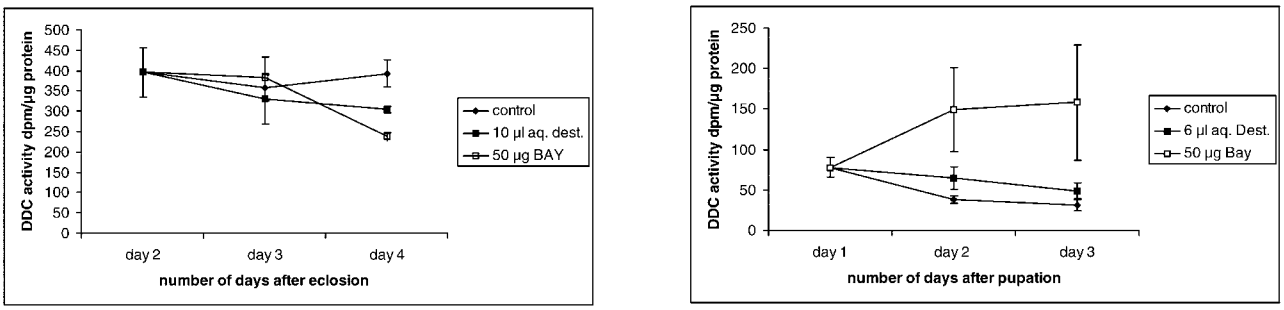


Fig. 5. – Influence of BAY on the DDC activity in adult *Neobellieria bullata*. Controls were left untreated or injected with 10 µl distilled water. The experimental condition was 50 µg BAY dissolved in 10 µl distilled water. The determination and calculation of DDC activity were the same as described in Fig. 1.

Fig. 6. – Influence of BAY on the DDC activity in juvenile *Tenebrio molitor*. Controls were left untreated or injected with 6 µl distilled water. The experimental condition was 50 µg BAY dissolved in 6 µl distilled water. The determination and calculation of DDC activity were the same as described in Fig. 1.

DISCUSSION

Dopa decarboxylase assay (Figs 1-2)

As previously stated, DDC plays a very important role in the metabolism of insects. It is necessary for sclerotisation and this is reflected in the high activity at pupation and eclosion in both species. In *T. molitor*, we see a slight increase of DDC activity in whole body extracts at pupation but the highest activity we measured at eclosion (six times higher than at pupation). These are in relation to the hardening and colouring of the pupal and adult cuticle. The pupae (pupa libra) stay relatively soft and light coloured whereas adult beetles develop a very hard and dark exoskeleton over a time period of two days. The overall activity compared to *N. bullata* is four times lower, which may be related to the intensity of sclerotisation.

In *N. bullata*, there is significant increase in enzymatic activity at pupation in contrast with *T. molitor*. This high activity is correlated with the formation of the puparium. This puparium is the larval skin that turns very hard and very dark to protect the developing insect inside. The highest DDC activity, at eclosion, is correlated with the formation of the adult cuticle. The fact that the activity starts to rise before eclosion is related to the mobilisation of products needed for sclerotisation and pigmentation. A remarkable fact is that CHEN & HODGETTS (1974) had measured a third but small activity peak at the moment we measured the lowest DDC activity (day 6). The temporal relation between dopa decarboxylase activity and paralytic activity is remarkably similar in both species (Figs 3-4). It seems that an increase in toxicity is followed by an increase of enzymatic activity. During the transition larval – pupal we see a gradual increase of toxicity associated with a gradual increase of enzymatic activity. After pupation we see the inverse effect.

Experimental data from the injection experiments suggest that we are able to induce DDC activity in juvenile *T. molitor* by injection of BAY (BAY was never identified in *T. molitor* as a paralysin, and we assume that there is a similar small peptide for storage of tyrosine in the beetle.). This is not possible in adult *N. bullata*. Here, we may find the key to the toxicity of BAY in adult flies. Consider the natural pathway from BAY to dopamine in the opposite direction (Fig. 7). Accumulation of L-dopa then leads to an accumulation of L-tyrosine because of a negative feedback of tyrosine-hydroxylase. The accumulation of L-tyrosine inhibits the dipeptidase responsible for the mobilisation of BAY. The BAY itself accumulates to toxic concentrations.

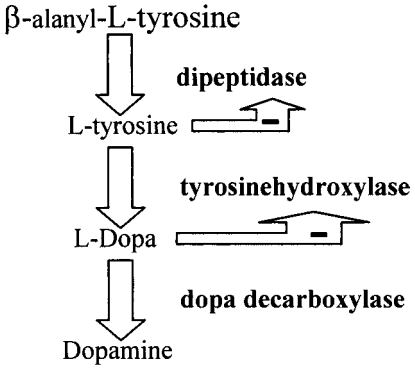


Fig. 7. – The followed pathway of BAY to dopamine and the involved enzymes (bold). Straight arrows indicate the formation of products, bent arrows with "-" indicate the negative feedback. If DDC is induced as in juveniles, dopamine will be formed. If DDC is not induced as in adults, L-dopa accumulates and is responsible for the start of the negative feed back.

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