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Drosophila melanogaster mutant *tan*.

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Drosophila melanogaster gene *tan* was originally discovered in the early 20th century as a mutant strain lacking the dark pigment pattern of wild-type (wt) flies and, therefore, showing a light yellowish brown color (McEwen, 1918). Flies lacking Tan function also exhibited abnormalities in vision (Benzer, 1967; Inoue *et al.*, 1988; True *et al.*, 2005), and *tan* males displayed an abnormal courtship behavior (Cook, 1980; Tomkins *et al.*, 1982). tan^1 (t^1) and tan^3 (t^3) alleles were found as spontaneous mutations, t^3 mutant being apparently lighter than t^1 (Brehme, 1941). *tan* is the structural gene for N-β-alanyldopamine hydrolase (NBAD-hydrolase or Tan protein), the enzyme that generates dopamine (DA) from NBAD (Wright, 1987; True *et al.*, 2005). Tan is expressed as a precursor protein of 43.7 kDa. This precursor is cleaved into two subunits of 29.9 and 13.8 kDa that apparently conform together a heterodimeric active protein (Wagner *et al.*, 2007).

The enzyme that generates NBAD from DA, the opposite reaction to the one catalyzed by Tan, is the NBAD-synthase or Ebony protein (Wright, 1987; Pérez *et al*., 1997), which is codified by the gene *ebony*. Since both Tan and Ebony are involved in cuticle tanning, carcinine regulation, and NBAD metabolism in nervous tissue (Wright, 1987; Pérez *et al.*, 1997, 2004; Hovemann *et al.*, 1998; Borycz *et al.*, 2002; True *et al.*, 2005), it has been suggested that they function together in a system regulating the levels of dopamine during cuticle sclerotization and histamine in the visual metabolism (Borycz *et al.*, 2002; Pérez *et al.*, 2010).

During the last few years, several publications appeared regarding NBAD-synthase (Wappner *et al*., 1996a, b; Pérez *et al*., 1997, 2002, 2004, 2010; Hovemann *et al.*, 1998; Borycz *et al.*, 2002; Wittkopp *et al.*, 2002; Schachter *et al*., 2007), but very little is known about *tan* (True *et al.*, 2005; Wagner *et al.*, 2007). Thus, it was important to further characterize the NBAD-hydrolase in *D. melanogaster* wt and in mutants t^1 and t^3.

Methods

All *Drosophila melanogaster* wt (*CS*) and mutant (t^1; t^3; $ebony^4$ (e^4); $white^{1118}$ (w^{1118})) strains were from Bloomington Stock Center. *Ceratitis capitata* wt (*Argentina-17*) was from INTA-Argentina Stock Center. To study NBAD-hydrolase activity we developed a heterologous coupled assay for sequential synthesis and hydrolysis of NBAD. We performed both assays in 50 mM Tris/ClH buffer, pH 7.5 at 22ºC. For NBAD synthesis the reaction mix contained dopamine (0.1

mM), $C^{[14]}$beta-alanine (5×10^4 cpm), Mg^{2+} (5 mM) and ATP (2 mM), which are essential requirements for NBAD-synthase (Pérez *et al.*, 2002). After 15 min of incubation with *C. capitata* protein extracts of 6 hours after onset of pupariation (Rabossi *et al.*, 1992), the reaction was stopped with 10 mM EDTA and the tube was boiled for 3 min. After centrifugation (10 min 14000 rpm), the supernatant was divided into two isovolumetric portions. The first one was set aside to quantify the synthesis of NBAD; the second moiety was used to develop the hydrolysis reaction (with the addition of 20 mM EDTA and *D. melanogaster* protein extract). The hydrolysis reaction was stopped after 10 minutes with 2.5% perchloric acid. Western blots were performed as described in Wittkopp *et al.* (2002). Rat Tan antibody was kindly provided by Dr. B. Hovemann, Ruhr University, Bochum, Germany. RNA was isolated from adult heads and then cDNA was synthesized using the SuperScript® II (Invitrogen) following the indications of the manufacturer. After cloning the cDNA into a T-easy vector (Promega), clones were sequenced in a 3130 Genetic Analyzer Applied Biosystem/Hitachi.

Results

We studied the activity of Tan in adults of *D. melanogaster* wt, t^1 and t^3 mutant strains in both epidermal and nervous tissues. We observed that the protein extracts from epidermis of wt, e^4 and w^{1118} flies hydrolyzed NBAD, while those from t^1 and t^3 extracts showed very low activity (Figure 1). When we studied NBAD-hydrolase activity in nervous tissue (heads), the results showed that the enzyme from wt, e^4 and w^{1118} strains hydrolyzed around 80% of total NBAD while, as expected, t^1 extracts were unable to hydrolyze NBAD (Figure 1). Surprisingly, t^3 head extracts were able to hydrolyze NBAD at almost the same rate than wt head extracts (Figure 1). We then performed western blots analysis of head and body extracts of wt, e^4, t^1 and t^3 strains (Figure 2). We found that Tan was synthesized in both epidermal and nervous tissues as precursor proteins of different sizes. The precursor protein of epidermal tissue exhibited an apparent MW of 43 kDa and the processed peptides behaved as species of 29 and 12 kDa apparent MW (Figure 2A, wt and e^4 strains). On the other hand, in nervous tissue the precursor protein had an apparent MW of 36 kDa and the processed fragments, 25 and 11 kDa, respectively (Figure 2B, wt and e^4 strains). The precursor protein was present in the protein extract of t^1, but no bands corresponding to the processed protein were found in both epidermal and nervous tissues (Figure 2A and 2B). Surprisingly, we found in epidermal extracts of t^3 the bands corresponding to both the precursor and the high MW processed peptide (Figure 2A). However, the low MW processed peptide band was not found (Figure 2A). In

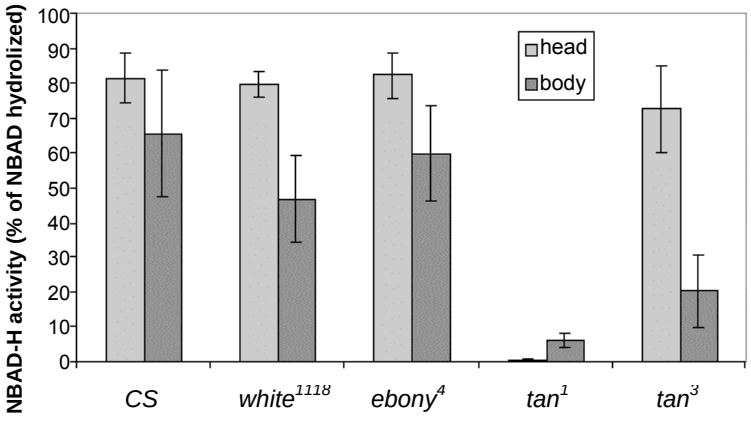

Figure 1. NBAD-hydrolase activity (% of NBAD hydrolized) in heads and bodies of wt (*CS*), w^{1118}, e^4, t^1 and t^3 strains. NBAD-hydrolase activity was normal in wt, w^{1118} and e^4; but residual in t^1 and surprisingly normal in heads of t^3 but weak in its bodies.

contrast, in head extracts of t^3 the three bands were found, although the precursor was very difficult to detect (Figure 2B).

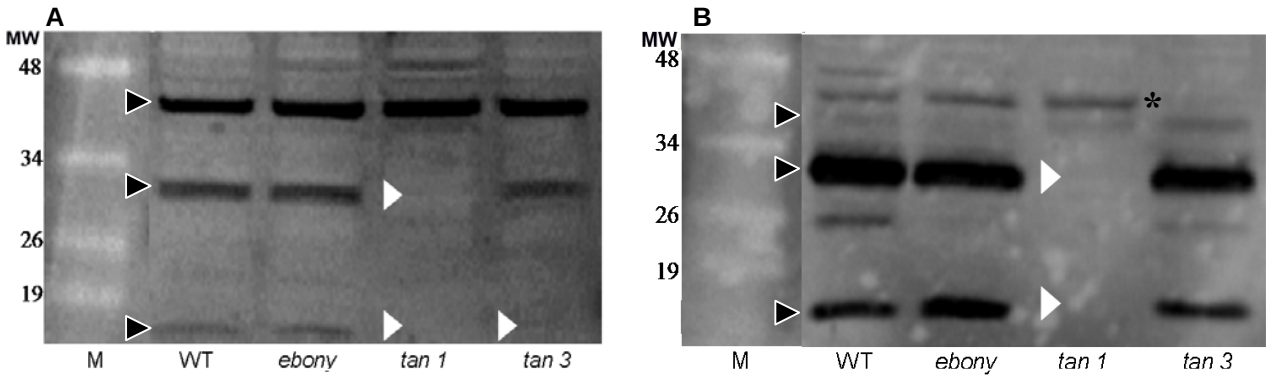

Figure 2. Western Blots analysis of bodies (A) and heads (B) extracts of wt, t^1 and t^3 strains using Tan antibody. The black arrow-heads point at the bands corresponding to the precursor protein and the processed peptides. The white arrow-heads point at the missing bands. In the left side are shown the MW (kDa) of the markers (M). Tan precursor from head epidermis was also detected (*).

In order to know the origin of the mutant defects, we isolated the coresponding mRNAs and synthesized the corresponding cDNAs. We then sequenced the cDNA of t^1 and t^3 mutants and found that t^1 had a point mutation resulting in the replacement of Arg_{217} for Pro_{217}. However, the cDNA sequence of t^3 was identical to that of wt (Pérez *et al.*, 2009).

Discussion

We developed a new method for measuring the NBAD-hydrolase activity, which consists in a coupled assay with heterologous homogenates, for sequential synthesis and hydrolysis of [^{14}C]NBAD. As far as we know, it was the first time that Tan activity was reported in adult flies, being a normally active protein in epidermal and nervous tissues. Western blot assays showed different apparent MWs for Tan in epidermis (43, 29 and 12 kDa) and nervous tissue (36, 25, and 11 kDa). This result suggests that Tan expression is regulated differentially in each tissue. As True *et al.* referred (2005), t^1 showed a point mutation, which we confirmed. We found that this mutation prevented Tan from being processed and hence from being active. Surprisingly, t^3 sequence showed no difference with wt cDNA, explaining the normal NBAD-hydrolase activity and western blot pattern in nervous tissue. Nevertheless, this mutant presented a weak NBAD-hydrolase activity in epidermis. This was in agreement with the western blot profile of t^3, where we were unable to observe the low MW processed peptide band. These results suggest that t^3 is a complex mutant with normal expression in nervous tissue and very scarce activity in epidermis.

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Preliminary list of the fauna of Drosophilidae from Užice, Serbia.

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During the past forty years, fauna of Drosophildae has been collected and investigated at more than 60 geographic localities within the territory of former Yugoslavia (for review see Kekić *et al*., 1999). A total of 59 species classified into 9 genera have so far been determined; the majority of them (as much as 37) belongs to the genus *Drosophila* (Kekić, 2002). However, Serbia is not yet analyzed sufficiently with respect to Drosophilidae fauna; to be more precise, the fauna of some regions is better explored, for example, in Vojvodina and the area along the Danube river (Kekić *et al*., 1999; Kekić, 2002, 2009), while in some parts of the country faunistical reasearches were not carried out.

Here we report the results of the first faunistical research conducted in west Serbia (Užice). Užice is the administrative center of western Serbia (Zlatibor District); it is placed in the ravine, along the banks of Đetinja River, surrounded by hills.

Investigation of Drosophilidae fauna was performed during August; flies were caught in the central part of the city, around the house, in garden and orchard. According to classification of Drosophilidae habitats (the main criterion for this classification was the estimated extend of human influence on habitat). This habitat belongs to semidomestic type (locations constantly under the