Adaptive significance of amylase polymorphism in *Drosophila* XIII. Old World *obscura* species subgroup divergence according to biochemical properties of α -amylase

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Biochemical properties of enzyme α -amylase were surveyed in *Drosophila obscura* Old world group of species (*D. subobscura*, *D. ambigua*, *D. obscura* and *D. tristis*) sampled in the same habitat, with the aim to reveal some ecological and evolutionary aspects of amylase polymorphism, which has been studied extensively in *D. subobscura*, but not compared with other species in the group. The data obtained show that *D. subobscura* is distinct from the other three species regarding all biochemical amylase properties. Such a divergence also correlates with the niche breadth and relative abundance of these species in the same habitat.

Key words: *Drosophila* ecology, *obscura* species, enzyme activity, amylase

INTRODUCTION

The well known polymorphism of amylase locus (*Amy*) in several *Drosophila* species, shows intra- and interpopulation variability through a high degree of allozyme polymorphism, as well as through variability in tissue-specific expression of the enzyme in *Drosophila* midgut, and also in the enzyme specific activity, which are both related to polymorphism at the level of the regulation of gene expression (Milanovic and Andjelkovic, 1993). The differences in amylase enzymological features found in different species and among genotypes on different substrates (Powell, 1997) reflect an adaptive significance of the amylase enzyme system.

Studies were done with the aim to obtain more direct evidence for adaptive significance of amylase polymorphism and to evaluate the level of metabolic activity among *Drosophila* species with different ecology and evolutionary history (Milanovic et al., 1989; Shibata and Yamazaki, 1994). The present paper is a contribution to such surveys within the *obscura* group.

The Old World *obscura* subgroup is a cluster of the *obscura* group and such classification fits best the phylogenetic relationships, based on morphological, chromosomal, allozyme and molecular data (for the review see Powell, 1997). However, the relationships within the

subgroup are ambiguous, with the largest data set showing good support for the statement that there are two major clusters in the Old World *obscura* subgroup, one containing *D. subobscura* (with *D. guanche* and *D. maderiensis*) and the other containing *D. obscura*/*D. ambigua*/*D. tristis*. *D. tristis* has clustered together with *D. obscura* and *D. ambigua*, according to satellite DNA and mtDNA studies (Barrio et al 1994; Bachmann and Sperlich, 1993), with *D. subobscura* being distant from this triad.

In temperate woodlands of central Europe, *D. subobscura*, *D. obscura*, *D. ambigua* and *D. tristis* usually coexist, but their ratio differs significantly with habitat and season. While *D. subobscura* is abundant in numerous habitats, the latter three species vary in number, being very scarce under certain ecological conditions. The enzyme efficiency and resource utilization in particular habitats could be the way of adaptation to environmental changes.

In the present paper, we compare the biochemical properties of α -amylase in *D. subobscura*, *D. obscura*, *D. ambigua* and *D. tristis* sampled in the same habitat.

MATERIALS AND METHODS

The flies of *D. subobscura*, *D. obscura*, *D. ambigua* and *D. tristis* were collected at locality Ravniste (Serbia), in a pine-beech woodland, at 600m altitude. All four species were found at the same fruit baits but with different

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abundance. The isofemale lines were established from all four species, in uniform laboratory conditions (19°C, humidity of 60%, 12/12 hours light/dark regime and corn-meal-sugar-yeast food medium). *D. tristis* was found in lowest number and 27 lines were established and *D. subobscura* was the most abundant and 70 lines were set. *D. ambigua* and *D. obscura* were set in 50 isofemale lines each.

Individuals of both sexes, emerged F₁ progeny from the females caught in nature, were used for amylase assay. All assays were done with flies of the same generation for all species. From 25 random chosen isofemale lines of each species, one male and one female were sampled. Fifty adults of three to five days old flies were homogenized in 1mL of ice-cold distilled water and centrifuged (12000 × g, 10 min., at 4°C). The supernatant was assayed for enzyme activity by the 3,5-dinitrosalicylic acid (DNSA) method (Bernfeld, 1955), modified by Doane (1969). The buffer containing NaCl as an activator of α -amylase was introduced into reaction mixtures by the meaning of substrate solution. Mixtures were incubated for 60 minutes, at 35°C and the reaction stopped by addition of an equal volume of DNSA. After heating at 100°C for 5 min, the reddish-brown was read in a spectrophotometer at 550 nm. Quantification was based on a curve prepared with maltose standards. Parallel controls were run to test for the presence of some reducing sugars in extracts or in the starch-buffer solutions. Reducing values, if obtained, were subtracted from those determined for the respective reaction mixtures. All mixtures, stan-

dards and controls, were assayed in triplicate. The means of three measurements are presented. Activities of the enzyme were expressed in maltose units, one unit being defined as the amount of the enzyme that released one μ mol of maltose equivalent in 60min. Protein concentration in enzyme extracts was determined by a modification of the method of Lowry et al. (1951). Bovine serum albumin was used in standards.

The pH range was covered by three buffers: 0.05M citrate-phosphate (pH from 5.0 to 7.0), phosphate (pH from 7.0 to 8.0) and Tris-HCl (pH from 8.0 to 9.0). The amylase activity was measured over a range of temperatures between 15°C and 50°C at optimal pH for each species. The substrate used was soluble starch in the concentration range from 0.1% to 4% of the reaction mixture.

RESULTS

The enzyme specificities and kinetic properties of α -amylase, as a response to variations of pH, temperature, and substrate concentration, were compared among *D. subobscura*, *D. obscura*, *D. ambigua*, and *D. tristis*, samples of the F₁ progeny of the females caught at the same locality.

The response of α -amylase specific activity to pH variation is shown in Fig. 1. A similar curve shape was obtained for all four species, but with different pH optima between the species. A significantly higher activity was obtained for *D. subobscura* compared to the others, in the whole pH range, with the highest activity at pH

Fig. 1. The effect of pH on the amylase activity in four species of *obscura* subgroup

7.8. Generally, *D. ambigua* had the lowest enzyme activity, with peak at pH 7.2. The pH 7.0 is the optimum for *D. obscura* and *D. tristis*.

Amylase activity, measured over a temperature range,

is shown in Fig. 2. The maximal activities were obtained at 35°C for *D. subobscura* and *D. tristis* and at 30°C for *D. obscura* and *D. ambigua*. The activity at the optimum temperature were significantly higher in *D. subobscura*

Fig. 2. The effect of temperature on the amylase activity in four species of *obscura* subgroup.

Fig. 3. The effect of starch concentration on the amylase activity in four species of the *obscura* subgroup.

Fig. 4. The Lineweaver-Burk plot of the amylase activity versus substrate concentration in four species of *obscura* subgroup.

than in other species. The lowest was found in *D. tristis*. While the curve has a sharp peak at the optimum temperature for *D. ambigua*. *D. obscura* and particularly *D. subobscura*, the curve has no clear peak for *D. tristis*, and the optimum temperature range is from 30°C to 40°C.

The dependence of α -amylase activity on substrate concentration is shown in Fig. 3. One percent concentration of soluble starch was found to be sufficient to obtain the maximum activity of α -amylase in *D. subobscura* and *D. tristis*, 2% in *D. obscura* and 4% in *D. ambigua*. The enzyme activity in *D. subobscura*, at different substrate concentrations were the highest among the four species at all concentrations except at 4.0%.

The Lineweaver-Burk plot of these results gives the maximum of the catalytic activities ($V_{\max}$) and the Michaelis constant (K_m) of the enzyme-substrate activity (Fig. 4. and Table 1.). In both parameters there are dif-

ferences among the species analyzed, being the highest for *D. subobscura*. The $V_{\max}$ is lowest for *D. ambigua* and K_m is much the same among the species, being the lowest in *D. obscura*.

DISCUSSION

Among the Old World *obscura* group species, *D. subobscura* is the most studied in the laboratory and in the wild. It is clear that *D. subobscura* is the species with the broadest niche compared to other three species and it has been easily spread to another continent - America (Prevosti, 1985) without a reciprocal success from it's New World relatives (*D. pseudoobscura* and *D. virilis*). From what is known about the breeding sites of these temperate woodland species, *D. subobscura* has the broadest niche with regard to breeding and larval feeding, on a number of plants, fungi, fruits, fermenting sap, and rotting material (Shorrocks, 1982; Krimbas, 1993). Since α -amylase interacts directly with the environment through food substrates, the variability of this gene-enzyme system is of great interest in ecological genetics.

The differences in the optimal pH for α -amylase found between four *obscura* species in this study may partially be attributed to the adaptation of digestive enzymes of particular species and populations to the food in the natural environments where they live. The grouping of species according to pH optima is consistent with their

Table 1. Maximal activities ($V_{\max}$, given as specific activities) and Michaelis constants (K_m , given as % starch) of α -amylase in four species of *obscura* subgroup.

Species	$V_{\max}$	K_m
<i>D. subobscura</i>	3.70	0.19
<i>D. obscura</i>	3.03	0.10
<i>D. tristis</i>	2.38	0.13
<i>D. ambigua</i>	2.04	0.12

phylogeny, with *D. subobscura* being separate from the others, with the highest activity, at a different optimum pH. In the previous study (Milanovic et al., 1989) this species had the same pH optimum, but with the lowest activity if compared to *D. hydei*, *D. melanogaster* and *D. busckii*, phylogenetically distant species.

Populations of the *obscura* group species inhabit temperate orchards and woodlands with distinct seasons and often sharp temperature changes which may influence the synthesis, stability, and kinetic properties of enzymes. The curves obtained for temperature specificity of the α -amylase activity also appear to group according to shape. While the sharp curve for *D. subobscura* indicates its high sensitivity to temperature, the other two species show rather flat curves, particularly flat in *D. tristis*. Such a distinction was also obtained for *melanogaster* subgroup in the work of Shibata and Yamazaki (1994). Although *D. subobscura* amylase shows temperature sensitivity, the phenotypic plasticity of the amylase genotypes (the range of phenotypes produced by each genotype in different environments), in *D. subobscura* populations, could be of importance, but can not be revealed from this study. The significant source of variability could be the genotype-by-environment interactions associated with amylase, as well as with some other enzymes studied in this aspect (Stamenkovic-Radak, et al., 1999).

The dependence of amylase activity obtained on the starch concentration indicates that high concentrations of starch are inhibitory for *D. subobscura*. High starch concentrations in substrates lead to physiological stress in some *Drosophila* species, preventing an appropriate amylase response to an increased level of starch (Milanovic et al., 1989).

Functional differences between allelic allozymes are usually characterized by differences in kinetic parameters $V_{\max}$ (velocity) which reflects the enzyme catalytic efficiency and K_m (Michaelis-Menten constant) that represents the substrate concentration at which velocity is 50% of $V_{\max}$. Considerable discussion has centered on the evolutionary importance of $V_{\max}/K_m$ (Powers, et al 1991). The decreasing order of values observed for $V_{\max}$ in this paper is the same as of their respective amylase activities (*D. subobscura* > *D. obscura* > *D. tristis* > *D. ambigua*). High K_m values reflect weak affinities of the enzyme for the substrate, and vice versa. Amylase, as studied in the present paper has the weakest affinity for starch in *D. subobscura* and the strongest in *D. obscura*. The weak amylase activity obtained in *D. ambigua* may reflect an adaptation to some particular niche and the decline of abundance of this species in some habitats (Stamenkovic-Radak, unpublished data) is probably in correlation with habitat change and absence of primary breeding/feeding site. In other habitats, *D. ambigua* amylase activity may be increased if some plant/fruit component, advantageous for this species, is

present. An analogous situation was found in *D. erecta* (Shibata and Yamazaki, 1994). In the previous paper (Milanovic et al, 1989) *D. subobscura*, sampled from a different habitat, had significantly lower $V_{\max}$ if compared to the one obtained here, while K_m was the same. This suggests that $V_{\max}$ may be due to allozyme variability and specific to particular populations and environments, and may reflect the adaptability.

Our field observations in temperate woodlands (Stamenkovic-Radak, unpublished data) show that *D. subobscura* is a highly dominant species, over-numbering others in some seasons and even after very harsh climate changes (cold winters and/or very hot summers). The other three, especially *D. ambigua* and *D. tristis* are declining, being very scarce after habitat changes. One explanation for this could be in different enzyme efficiency and resource utilization in particular habitats and metabolic capacity to adapt to environmental changes. The number of breeding and feeding sites for these species (Shorroks, 1982) is smaller if compared to *D. obscura* and *D. subobscura*.

An interesting evidence is observed for *D. obscura* in our study: it exhibits the lowest value of K_m , but a higher $V_{\max}$ than *D. ambigua*. This is probably the reason why this species shows better adaptation when changing the niche if compared to *D. ambigua*, as the species with the weakest amylase activity becomes sparse after habitat change. Additional ecological and biochemical studies are necessary in order to obtain more evidence of this enzyme adaptation pattern in natural populations.

The results of Fujimoto et al. (1999) suggest that changing the regulation of amylase activity is important in adaptation to starch environment in different *Drosophila* species. The development profiles of amylase activity in different *Drosophila* species show that the level of activity differs among the species and between various dietary environments. The functional relationship between the structural and regulatory genes on the one hand and the variability of regulatory genes on the other may have a more important evolutionary role than the structural gene polymorphism itself. According to immunoelectrophoretic data, different levels of amylase activity are a consequence of different quantities of amylase protein, which is related to the regulation at transcriptional level. The differences in gene structure and regulation of the activity in terms of adaptation were studied through duplicated structure of the *Amy* gene in many *Drosophila* species (Matsuo et al, 1999). The *obscura* group is particularly interesting as there are both genes with and without intron (Da Lage et al., 1996), which may influence the enzyme activity regulation.

The divergence in amylase biochemical properties among species studied in the present paper may be the result of adaptation and evolution through complex and multiple interactions and changes in regulatory genes.

Further interspecific comparative approach to amylase activity as a quantitative character may give some insight into the source of existing variability and related phylogeny in the *obscura* subgroup.

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