A comparative study of the enzymological features of α -amylase in the *Drosophila melanogaster* species subgroup

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ABSTRACT

A comparative study was done on the enzymological features of α -amylase among six sibling species belonging to the *Drosophila melanogaster* species subgroup. The pH optima were 7.6 for *D. melanogaster*, *D. mauritiana* and *D. simulans* and 7.4 for *D. erecta*, *D. teissieri* and *D. yakuba*. The temperature optima were commonly 37 C in all species studied. However, there are two types of temperature dependence; a strict one as observed in *D. melanogaster*, *D. mauritiana* and *D. simulans*, and a flat one as observed in *D. erecta*, *D. teissieri* and *D. yakuba*. These separations are consistent with the general phylogeny within this species subgroup. The substrate dependence was almost the same in all the species studied. The $V_{\max}$ and K_m were also estimated for each species. We found clear differences in the enzymological features between the species. Thus these differences might reflect an adaptation of the amylase enzyme system to speciation in this species subgroup.

1. INTRODUCTION

Recently, departures from neutrality (Kimura and Ohta, 1971) have been observed in nucleotide sequence data (McDonald and Kreitman, 1991; Eanes, Kirchner and Yoon, 1993; Shibata and Yamazaki, 1994). This has caused a controversy as to the explanation for this departure. The two most likely causes are either adaptive evolution or stochastic fixation of slightly deleterious mutations such that observed in the bottle neck effect. McDonald and Kreitman (1991) and Eanes, Kirchner and Yoon (1993) attributed the departure observed in *Drosophila* species at the *Adh* and *G6pd* loci, respectively, to adaptive evolution, because this explanation is simpler than the alternative explanation. Ohta (1993) attributed the departure observed at the *Adh* locus in Hawaiian *Drosophila* to their small population size which helps to fix slightly deleterious mutations. In our previous report (Shibata and Yamazaki, 1994), we showed an excess of nonsynonymous substitutions at the duplicated *Amy* locus in the *D. melanogaster* species subgroup, especially in the lineage of *D. erecta*. Therefore, we concluded that this departure from neutrality was caused by adaptive evolution for the

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following three reasons: (1) *D. erecta* is a highly specialized species adapted to a unique host plant, *Pandanus* (Rio et al., 1983). (2) All fixed substitutions (seven) observed in *D. erecta* were replacements and no fixed synonymous substitution was found. (3) In *D. erecta*, the departure observed in *Amy* was not observed at the *Adh* locus (Jeffs, Holmes and Ashburner, 1994). In order to obtain more direct evidence for such adaptive evolution, in addition to the sequence data, in this study we decided to make a comparative study of the enzymological features of α -amylase protein among the six species belonging to the *D. melanogaster* species subgroup; *D. melanogaster*, *D. erecta*, *D. mauritiana*, *D. simulans*, *D. teissieri* and *D. yakuba*. We examined each for specificity of pH, temperature and substrate, and also estimated the kinetic parameters ($V_{\max}$ and K_m). All these properties were found to differ clearly among the species. In particular, the lowest activity of amylase in all the conditions studied was observed in *D. erecta*.

2. MATERIALS AND METHODS

Fly stocks

The TN329 (*Amy*¹) strain of *D. melanogaster* in Inomata et al. (1994) was used as the standard. Each strain used for the five sibling species; *D. simulans*, *D. mauritiana*, *D. erecta*, *D. teissieri* and *D. yakuba* is the same as those used previously in Shibata And Yamazaki (1994). All flies were reared on a corn meal-molasses medium at 25 C.

Biochemical assays

Amylase activity was determined by two different methods: the starch-iodine method and the DNSA method (Doane, 1969). The starch-iodine method was used in the survey of biochemical specificities (substrate, pH and temperature). The enzyme extracts were obtained from ten adult male flies homogenized in 300 ml of dH₂O. After centrifugation at 10,000 rpm for 5 minutes, 50 ml of supernatant was incubated 60 minutes in 1 ml of a reaction solution containing 0.05% substrate, 0.05 M NaCl and 0.05 M buffer. Staining solution (Smith and Roe, 1949) was added to the mixture after the incubation. The activity was then measured as the loss in OD resulting from substrate hydrolysis. The DNSA method was performed in the estimation of K_m and $V_{\max}$ according to Doane (1969) and Hoorn and Scharloo (1978). The total protein of enzyme extract was determined by the BCA method using the commercially available BCA protein assay kit (Takara). For the precision determinations in each assay were done at least three times.

3. RESULTS AND DISCUSSION

We compared the following enzyme specificities of α -amylase: pH, temperature and substrate. In addition we estimated the kinetic parameters ($V_{\max}$ and K_m) using crude fly extracts. Note that we estimated only the "pseudo- $V_{\max}$ " and "pseudo- K_m " because of the variability of starch as a substrate. Each starch source (e.g. potato, rice, corn) has a different amylose to amylopectin ratio which affects the spectrophotometric estimates of α -amylase activity. However, in this study for each carbohydrate we used only one source, thus we were able to compare our estimates with one another.

pH specificity

The pH specificity of α -amylase activity determined by the starch-iodine method is shown in Fig. 1. *D. melanogaster* showed the strongest activity in all the pH ranges studied, while *D. erecta* showed the weakest activity. Curves of the same shape were obtained for all the species. However, pH optima appear to be different. Maximum activity of α -amylase in *D. melanogaster*, *D. mauritiana* and *D. simulans* was at pH 7.6, whereas in *D. erecta*, *D. teissieri* and *D. yakuba* it was at pH 7.4. Although this difference is slight, the separation of species is consistent with their phylogeny, with the former group matching the *D. melanogaster* complex and the latter group matching the *D. erecta* complex and *D. yakuba* complex (Lachaise et al., 1988). In previous reports where the DNSA method was used (Doane, 1969; Hoorn and Scharloo, 1978; Milanovic, Andjelkovic

Fig. 1. The effect of pH on the amylase activity in six sibling species of *Drosophila*. The specific activity was determined by the starch-iodine method in which 50 μ l of enzyme extract was added to 1 ml of 0.05% starch in buffer and the mixture was incubated at 37 C for 60 min. The pH range was covered with three different buffers: 0.1 M sodium acetate + 0.1 M NaCl, pH 5.0–6.0; 0.1 M sodium phosphate + 0.1 M NaCl, pH 6.0–8.0; 0.1 M Tris-HCl pH 7.0–10.0. mel = *D. melanogaster*; ere = *D. erecta*; mau = *D. mauritiana*; sim = *D. simulans*; tei = *D. teissieri*; yak = *D. yakuba*.

and Stamenkovic-Bojic, 1989), the optimum pH in *D. melanogaster* was pH 7.4. This difference may well be due to the difference in the assay methodology.

Temperature specificity

The temperature specificity of the α -amylase activity was also determined by the starch-iodine method and is shown in Fig. 2. For each of the six species studied the temperature optima approximate 37 C. These results are consistent with the previous reports (Hoorn and Scharloo, 1978; Milanovic, Andjelkovic and Stamenkovic-Bojic, 1989). The determined curves appear to fall into two different shapes. The curves of *D. melanogaster*, *D. mauritiana* and *D. simulans* are sharp, whereas those of *D. erecta*, *D. teissieri* and *D. yakuba* are flat. This means that the α -amylase activities of *D. melanogaster*, *D. mauritiana* and *D. simulans* are more sensitive to temperature than those of the other three species. This grouping parallels the one obtained from pH dependence and is also consistent with the phylogeny.

Fig. 2. The effect of the temperature on the amylase activity in six sibling species of *Drosophila*. The specific activity was determined by the starch-iodine method in which 50 μ l of enzyme extract was added to 1 ml of 0.05% starch in Tris-HCl buffer (pH 7.5) and the mixture was incubated for 60 min.

Substrate specificity

Substrate specificity of α -amylase was also determined by the starch-iodine method with alternative substrates as shown in Fig. 3. According to Doane (1969) who used the DNSA method, the order for substrate specificity is starch > amylopectin > glycogen > amylose. The DNSA method gives absolute activity values, but is influenced by other sugar-producing enzymes. The starch-iodine method used here gives only relative activity values, because the specific activity was determined as the quantity of substrate digested and each substrate has its

Fig. 3. Substrate specificity on the amylase activity in six sibling species of *Drosophila*. The specific activity was determined by a modified starch-iodine method in which 50 μ l of enzyme extract was added to 1 ml of 0.05% substrate in Tris-HCl buffer (pH 7.5) and then the mixture was incubated at 37 C for 60 min. The specific activity was expressed as the weight of the digested substrate. (A) for starch (B) for amylopectin, amylose and glycogen.

own molecular weight. However, this method is not influenced by other sugar-producing enzymes since only the reduction in substrate is observed. As shown in Fig. 3 the specificity order is starch > glycogen > amylose > amylopectin, and this pattern of substrate dependence is common to all six species studied. This different order from the one for *D. melanogaster* in Doane (1969) may be due to the difference in the assay methodology.

Substrate concentration

The dependence of α -amylase activity on the substrate concentration determined by the DNSA method is shown in Fig. 4. One percent starch was observed to suffice for maximal α -amylase activity in *D. melanogaster* and *D. simulans*, whereas 0.5% was sufficient in *D. mauritiana* and *D. erecta*. However, the concentration sufficient for *D. teissieri* and *D. yakuba* appears to be greater than 1%. The Lineweaver-Burk plot (Fig. 5) gives the maximal activities ($V_{\max}$) and the Michaelis constants (K_m) (Table 1). The observed values for *D. melanogaster* ($V_{\max} = 4.74$, $K_m = 0.104$) are consistent with the values reported by Milanovic, Andjelkovic and Stamenkovic-Bojic (1989) ($V_{\max} = 5.3$, $K_m = 0.2$). The observed values of $V_{\max}$ approximate each species activity except *D. mauritiana* whose observed value of $V_{\max}$ (1.87) is smaller than that expected from the

Fig. 4. The effect of substrate concentration on the amylase activity in six sibling species of *Drosophila*. The specific activity was determined by the DNSA method.

Fig. 5. The Lineweaver-Burk plot of the amylase activity versus substrate concentration in six sibling species of *Drosophila*.

Table 1. Maximal activities ($V_{\max}$) and Michaelis constants (K_m) of α -amylase in six sibling species of *Drosophila*

	$V_{\max}$	K_m
<i>D. melanogaster</i>	4.74	0.104
<i>D. erecta</i>	0.95	0.077
<i>D. mauritiana</i>	1.87	0.028
<i>D. simulans</i>	2.22	0.320
<i>D. teissieri</i>	2.40	0.305
<i>D. yakuba</i>	1.84	0.294

$V_{\max}$ is given as specific activity.

K_m is given as the % starch.

order of species arranged according to their amylase activity of *D. melanogaster* > *D. mauritiana* > *D. simulans* > *D. teissieri* > *D. yakuba* > *D. erecta* (see Fig. 3). The low and high K_m values reflect the strong and weak affinity of the enzyme and substrate, respectively. The lowest value (0.028) was observed in *D. mauritiana*. This means that the amylase enzyme of *D. mauritiana* has the strongest affinity for starch among the six species studied. A low value of K_m (0.077) was also observed in *D. erecta*. This means that amylase in *D. erecta* maintains a sufficient affinity for starch compared to the other species showing large K_m values. Therefore, the weak amylase activity in *D. erecta* is due either to the small $V_{\max}$ itself or to the small quantity of amylase protein. The relatively weak activity and strong affinity may imply adaptive protein evolution of *Amy* in *D. erecta*.

Here we found several clear differences in the enzyme properties among the six *Drosophila* species. In particular, *D. erecta* showed the lowest activity of amylase in all the comparisons, although these differences alone are not sufficient to provide direct evidence for adaptive evolution as suggested by the nucleotide sequence analysis in the *D. melanogaster* species subgroup, especially *D. erecta* (Shibata and Yamazaki 1994). We are now trying to identify the cause for this low activity with the RNA transcript assay and somatic transformation. This weak amylase activity may reflect the adaptation of the amylase enzyme system and thus somehow may be advantageous to *D. erecta* in its ecological niche. It is also possible that some component of either fresh or rotten *Pandanus* fruit, which is the only known host plant of *D. erecta*, may activate amylase activity. At any rate, further detailed ecological and biochemical studies (e.g. a chemical analysis of the fresh or rotten fruit of the host plants, and the effect of those components on the amylase activity) are still necessary in order to obtain direct evidence of adaptation in this enzyme system.

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