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Enzymatic characterization of recombinant α-amylase in the *Drosophila melanogaster* species subgroup: is there an effect of specialization on digestive enzyme?

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We performed a comparative study on the enzymological features of purified recombinant α-amylase of three species belonging to the *Drosophila melanogaster* species subgroup: *D. melanogaster*, *D. erecta* and *D. sechellia*. *D. erecta* and *D. sechellia* are specialist species, with host plant *Pandanus candelabrum* (Pandanaceae) and *Morinda citrifolia* (Rubiaceae), respectively. The temperature optima were around 57–60°C for the three species. The pH optima were 7.2 for *D. melanogaster*, 8.2 for *D. erecta* and 8.5 for *D. sechellia*. The k_{cat} and K_m were also estimated for each species with different substrates. The specialist species *D. erecta* and *D. sechellia* display a higher affinity for starch than *D. melanogaster*. α-Amylase activity is higher on starch than on glycogen in all species. α-Amylases of *D. erecta* and *D. sechellia* have a higher activity on maltooligosaccharides (G6 and G7) than on starch, contrary to *D. melanogaster*. Such differences in the enzymological features between the species might reflect adaptation to different ecological niches and feeding habits.

Key words: α-amylase, adaptation, *Drosophila erecta*, *Drosophila melanogaster*, *Drosophila sechellia*, microcalorimetry, specialization

INTRODUCTION

The α-amylase (AMY) is one of the most studied enzymes in *Drosophila*. It hydrolyzes starch from food and is encoded by the *Amy* genes. Because AMY is required to interact with changes in the food environments in natural populations, *Amy* is a candidate gene under natural selection. In fact, several laboratory experiments using *Drosophila* have indicated that *Drosophila* with high AMY enzyme activity have a selective advantage in starch-rich environments (De Jong and Scharloo, 1976; Hickey, 1977; Powell and Andjelkovic, 1983; Araki et al., 2005).

In natural populations of *Drosophila melanogaster*, α-amylase, hereafter named amylase, is highly polymorphic both in the number of isozymes and in terms of activities (Abe, 1958; Kikkawa, 1964; Hickey, 1979; Yamazaki and Matsuo, 1984). The *Amy* region of *D. melanogaster* consists of a reverse duplication and the products of the two copies of the gene *Amy* are enzymatically active (Levy et al., 1985, but see Klarenberg and Scharloo, 1986). The gene is composed of a single exon of 1482 bp that is translated into a polypeptide of 494 amino acids. All species of the subgroup *D. melanogaster* have this duplication, which thus most likely occurred before the radiation of the nine species of the subgroup (Daïnou et al., 1987).

Shibata and Yamazaki (1995) conducted a study on the molecular evolution of the *Amy* gene in several species of the subgroup *D. melanogaster*. Examining nucleotide sequences, they highlighted high sequence similarity between the two copies of the gene *Amy* within each species. They also showed an excess of non-synonymous substitutions by comparing interspecific nucleotide sequences, especially in *D. erecta* where all fixed substitutions were non-synonymous. They therefore concluded

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that adaptive evolution of proteins played a role in *D. erecta* which is a specialist species that uses a single species (*Pandanus candelabrum*) as a host plant. This dependence to a specific host plant occurred during or after the speciation of *D. erecta*. As the alteration of an ecological niche also has an impact on natural selection, the change of host plant used as a feeding and breeding site is probably very important for the evolution of digestive enzymes, such as α-amylase. It is likely that the adaptive evolution of proteins plays a role in the process of specialization to a host plant.

To determine how mechanisms of adaptation and constraints change with ecological shift, we are focusing on *Drosophila* α-amylases as related to feeding habit variation. The *D. melanogaster* subgroup contains nine species (Lemeunier et al., 1986; Lachaise et al., 2000), two of which, *D. erecta* and *D. sechellia*, are called specialists, as they breed on specific host plants.

D. erecta (Tsacas and Lachaise, 1974) behaves as a specialist as soon as fruits of *Pandanus candelabrum* (Pandanaceae) appear; this is called seasonal specialization. Therefore, only generations that have access to the fruits of the host plant are strictly specialists, so next generations that do not have access to these fruits revert more or less generalists. We must also point out that when *D. erecta* has no opportunity to exploit the fruits of *Pandanus*, populations seem to decrease, which is explained by the fact that the cost seems higher when they have to find a new nesting site (Rio et al., 1983).

D. sechellia (Tsacas and Bächli, 1981) is endemic to the Seychelles Islands (Lachaise et al., 1988, 2004; Dean and Ballard, 2004). This is a specialist species that feeds and reproduces only on *Morinda citrifolia* (Rubiaceae), a plant toxic to all other *Drosophila* (R'Kha et al., 1991). The distal copy of *D. sechellia* is inactivated by a 35 bp deletion and 4 bp insertion causing a frameshift (Shibata and Yamazaki, 1995).

To obtain biochemical clues of adaptation in this enzyme, we performed a comparative study of the biochemical properties of α-amylase of three species of subgroup *D. melanogaster*. We were interested in the characteristics of the α-amylase enzyme of two specialist species: *D. erecta* and *D. sechellia* that we compared to the α-amylase of a closely related, generalist and cosmopolitan species *D. melanogaster*. This is, to our knowledge, the first report on biochemical properties of a digestive enzyme in *D. sechellia*.

Biochemical features of α-amylases from different species and among *D. melanogaster* have been studied generally from crude extracts (Doane, 1969; Hoorn and Scharloo, 1978; Milanovic et al., 1989; Milanovic and Andjelkovic, 1992; Shibata and Yamazaki, 1994). In this study, we performed enzymatic assays on α-amylase produced in yeast. Compared with crude fly protein extracts, it has the advantage that a large amount of enzyme with good purity may be produced, without further interference with other fly molecules, and importantly, the enzyme concentration may be readily and accurately determined for kinetic parameter calculation.

We looked for effect of pH and temperature, and estimated the kinetic parameters k_{cat} and K_m and the reaction products on different substrates.

MATERIALS AND METHODS

Production and purification of proteins The amylase proteins were produced in the yeast *Pichia pastoris*. The α-amylases genes of *D. melanogaster*, *D. erecta* and *D. sechellia* were cloned into the expression plasmid pPIC3.5K (Invitrogen™) and integrated to the genome of *P. pastoris* strain GS115 by electroporation. The *Amy* sequences of *D. melanogaster*, *D. erecta* and *D. sechellia* that were used in this study have GenBank accessions BAB32511, D17728 and D17732, respectively. In *D. erecta* the known protein sequences (proximal D17727 and distal D17728) are identical. For *D. melanogaster*, sometimes only one copy is active or both copies give the same protein or different proteins (Klarenberg and Scharloo, 1986). The sequence we used here belongs to the widespread electrophoretic class AMY1 (Inomata et al., 1995; Araki et al., 2001), and differs from the most divergent AMY6 electrophoretic class by 5-6 amino acid substitutions (Inomata et al., 1995). Doane (1969) showed that biochemical properties of the α-amylases from eleven homozygous *Amy* strains were very similar in temperature stability, pH optimum and substrate specificity. All were activated by chloride ions and showed a pH optimum of about 7.4. For *D. sechellia*, only one copy is active, as the distal copy is a pseudogene.

Recombinant *P. pastoris* clones were grown at 30°C in BMGY (Buffered Glycerol-complex Medium) until a A_{600} absorbance ~ 5. The cells were then induced by methanol in BMMY (Buffered Methanol-complex Medium). After 24 h of induction at 28°C in baffled flasks under strong shaking, the supernatant was recovered and filtered. The purification of the amylase comprised a step of precipitation with ammonium sulfate (80% saturation), followed by dialysis of the pellets against 20 mM Hepes buffer containing 20 mM NaCl and 1 mM $CaCl_2$ (pH 7.5) followed by a glycogen precipitation step in alcoholic condition using cold ethanol (40% final concentration), followed by a second dialysis. Sample concentration was determined by analyzing a Coomassie-stained SDS-PAGE with an imager Odyssey® FC (LI-COR Biosciences) with a bovine serum albumin (BSA) mass scale. Concentrations were estimated as the average value of three samples. The following molecular weights were used for calculations of concentrations: *D. melanogaster* (51,899 Da), *D. erecta* (51,895 Da) and *D. sechellia* (51,879 Da).

Determination of the enzymatic activity The activity of α-amylase was determined in two ways: the dinitrosalicylic acid (DNS) method (Bernfeld, 1955; D'Amico et al., 2001) and microcalorimetry (Todd and Gomez, 2001; Haq, 2002; D'Amico et al., 2006). The DNS method was used for the characterization of pH and temperature curves using starch, and microcalorimetry for measuring enzyme kinetics (k_{cat} and K_m) on soluble starch from potato (ref 569379 Merck©), glycogen from oyster (MP Biomedicals, LLC) and maltooligosaccharides maltotetraose (G4) to maltoheptaose (G7) (Sigma-Aldrich®).

The activity of the enzyme as a function of the temperature and the pH was determined in triplicate using the DNS method under standard conditions.

Activity-based temperature

The temperature range for the measurement of the activity spanned from 5 to 80°C (by 5°C steps). The enzyme and the substrate (1% starch) were set to temperature by incubation for 5 min.

Activity-based pH:

Various buffers were prepared to obtain a pH ranging from 4 to 10, 25 mM acetate (pH 2 to 5.5), MES (pH 6, 6.5), HEPES (pH 7, 7.5), Tris base (pH 8–10) and 20 mM NaCl and 1 mM $CaCl_2$. The starch was then added to the different solutions in order to obtain a 1% solution.

Determination of kinetic constants

Microcalorimetry records the heat generated by the hydrolysis of glycosidic bonds.

Different substrates were prepared in 20 mM Hepes, 20 mM NaCl, 1 mM CaCl2 (pH 7.5) at a concentration of 20 g/l for starch and 5 mM for maltooligosaccharides.

The results were recorded on a VP-ITC microcalorimeter (MicroCal, Northampton, MA) at 25°C and analyzed with MicroCal Origin software (version 7).

Measuring the catalytic constant k_{cat}

This measure consists of a single injection (in triplicate) of the enzyme in substrate. The concentration of α-amylase was adjusted to produce about 1 µcal/sec under saturating substrate concentration. The stirring speed was set to 310 rpm. Injection of 5 µl enzyme was programmed 1 min after thermal equilibration of the device to see if there was any change in the baseline. A five minutes delay was allowed between injections. The activity was calculated in µcal/sec, in order to record the difference between the power generated by the ITC immediately after injection of the enzyme and the power of the base line in the steady state.

To transform the activity (µcal/sec) in the number of α-1,4 glycosidic bonds broken per unit time (k_{cat}), we need to know the reaction enthalpy (ΔHr). This has been accurately measured and is available on the website of the National Institute of Standards and Technology Databank (http://xpdb.nist.gov/enzyme_thermodynamics/enzyme_thermodynamics_data.html). Here, we used the value of –1083 cal/mol, which corresponds to the hydrolysis of a α-1,4 linkage at 25°C. The catalytic constant k_{cat} (s^{-1}) was calculated according to the following equation (D'Amico et al., 2006):

$$k_{cat}=(Pr-Pi)M/\Delta HrCV$$

where $(Pr–Pi)$ is the energy of reaction in µcal/sec, M the mass of the enzyme in Da, ΔHr is the bond enthalpy (in calories), C the concentration of the enzyme in the syringe (mg/ml) and V the volume of injection (µl).

Measurement of affinity constant K_m

This measure consists of multiple injections of substrate in an enzyme solution. The syringe was filled with different saturating substrates, the cell by an enzyme solution at concentrations depending on their catalytic activity and the reference cell with the buffer. The stirring speed was 310 rpm. After equilibration of the microcalorimeter, 25 injections of 10 µl spaced 5 min were scheduled. At each injection, the baseline stabilized at a power level lower than before the injection, which corresponds to the heat generated by the enzymatic reaction. By knowing the concentration of the substrate, the volume of the cell and the volume of injection, it is possible to calculate the concentration of substrate in the cell after each injection. It is then necessary to determine dQ/dt, which is the difference between the value of the baseline before and after injection for each concentration. Once the measures dQ/dt and injection concentration were recorded, we can then create the Michaelis-Menten curve using the software Origin 7. This software also allows to directly calculating the affinity constant K_m and V_{max} with a hyperbolic function $y=x*P1/(P2+x)$ with $P1=V_{max}$ and $P2=K_m$ (Todd and Gomez, 2001; Haq, 2002).

Thin-layer chromatography The reaction products for starch and maltooligosaccharides (G3 to G7) produced by α-amylase reaction were identified by thin-layer chromatography (TLC). The products were spotted on a precoated silica gel plate (Sigma-Aldrich® Silica gel on TLC Al foils) and developed with a solvent of chloroform: acetic acid: water (5:7:1). The sugars developed on the plate were stained by spraying 10% (v/v) sulfuric acid in ethanol followed by heating at 130°C for 10 min (adapted from Kumagai et al., 2013)

Alignment of primary structures Amino acid sequences and translated nucleotide sequences of α-amylases were extracted from the GenBank database. A multiple sequence alignment, after removal of the signal peptides, was generated by the MUSCLE program (Edgar, 2004).

RESULTS AND DISCUSSION

Temperature dependence The temperature dependence of the α-amylase activity was determined by the DNS method (Fig. 1). For each of the three enzymes studied the optimum temperature is around 57–60°C. The three species have quite similar spectra of activity as a function of the temperature. They retain 90% activity between 45°C and 70°C. We can still notice a slight shift of *D. melanogaster* curve to the right, which might suggest that the *D. melanogaster* enzyme would be a little more stable. These results are not consistent with those found in Shibata and Yamazaki (1994); they found an optimum temperature of about 37°C but they conducted the measures on whole protein extracts which were obtained from homogenized adult flies. Indeed, the temperature for maximal activity is an apparent value and is strongly dependent of the assay conditions. This temperature results from a compromise between thermal activation (left limb of the bell-shaped curve) and heat inactivation (right limb). Accordingly, methods using short incubation time (as reported in our paper) disfavor heat inactivation and increase the apparent temperature for maximal activity, whereas methods using longer incubation time decrease this temperature (if reaction linearity has not been checked). Furthermore, in a raw extract, proteases are expected to interfere with the assay: as temperature is raised, their activity increases and reduces the amount of active amylase by proteolysis. This results in a lower apparent temperature (Beynon and Bond, 2001). Such effect is absent when using a purified enzyme. Prigent et al. (1998) found relatively low maximal temperatures in partially purified *D. virilis* and *D. repleta* amylases, but their buffer lacked calcium ions, which was further chelated by the phosphate of their buffer, and the incubation times were long, which strongly affected stability. Our results are more consistent with those found by Cipolla et al. (2012) who used purified enzyme with the same protein sequence as ours, produced in recombinant *Escherichia coli* (i.e. non glycosylated), and the same method (DNS). They estimated the temperature optimum of *D. melanogaster* at 54°C. Actually this range of temperature do not seem to match the usual environmental conditions of the *Drosophila*, but the measurements done here correspond to the biochemical parameters of the enzyme only. Note that the pH curve and K_m and k_{cat} estimates were obtained at 25°C, a realistic and physiological temperature for those species.

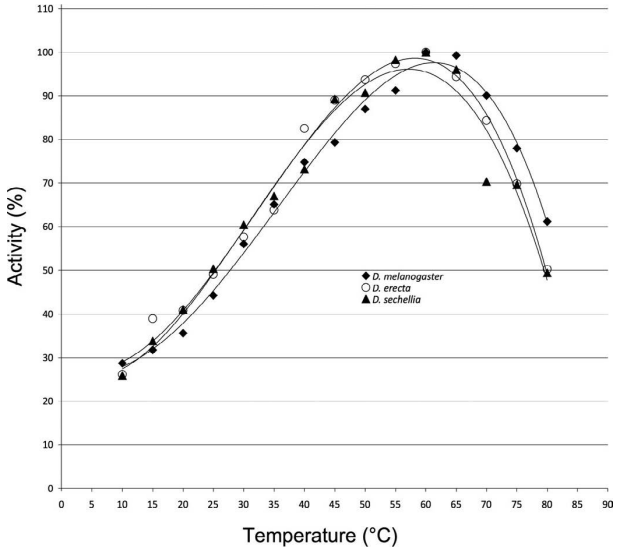

Fig. 1. The effect of the temperature on the amylase activity in three sibling species of *Drosophila*. The specific activity was determined by the DNS method in which 20 µl of enzyme was added to 200 µl of 1% starch in Hepes buffer (pH7.5) and the mixture was incubated for 5 min. The activity is shown as percentage of its maximum value.

pH dependence

The effect of pH on α-amylase activity was also determined by the DNS method and is shown in Fig. 2. The three species showed similar pH dependence curves. The curves of *D. erecta* and *D. sechellia* show a slight shift to the basic compared to *D. melanogaster*. The amylase of *D. melanogaster* has an optimum activity around pH 7.2 while *D. erecta* and *D. sechellia* have a pH optimum of 8.2 and 8.5 respectively. The value for *D. melanogaster* is well within the range of pH optimum (7.2–7.8) already found for different Drosophila species and consistent with previous reports in *D. melanogaster* (Doane, 1969; Hoorn and Scharloo, 1978; Milanovic et al., 1989; Shibata and Yamazaki, 1994; Prigent et al., 1998). Although the difference between the three species is not large, it might be significant of an adaptive change. More striking differ-

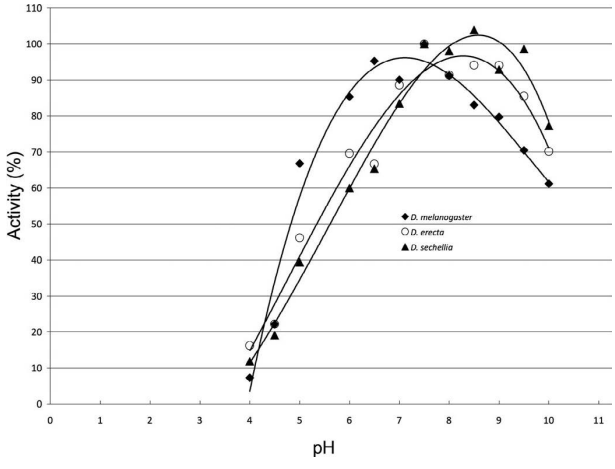

Fig. 2. The effect of the pH on the amylase activity in three sibling species of *Drosophila*. The specific activity was determined by the DNS method in which 20 µl of enzyme was added to 200 µl of 1% starch in buffer and the mixture was incubated for 5 min. The activity is shown as percentage of its maximum value.

ences have been reported in other insect, either toward more acidic (pH 4.5–5 for the weevil *Sitophilus oryzae*, Kluh et al., 2005) or more basic α-amylases (pH 9.2–10 for the silkworm *Bombyx mori* or the noctuid *Ephestia kuehniella*, Pytelkova et al., 2009).

The pH of the fruit of *Morinda citrifolia* is between 4.15 and 4.36 and the pH of the fruit of *Pandanus* is between 4.9 and 5.2 (Rosalizan et al., 2010; Miller et al., 1956). The optimum pH of α-amylases does not correspond with the pH found in the fruits of host plants, which suggests that a buffered, slightly basic pH is maintained in the midgut lumen of larvae. However, the pH of the digestive tract of *D. erecta* and *D. sechellia* is not currently known. On the other hand, we know that, in *D. melanogaster*, the luminal content of the anterior segment and the anterior part of the posterior segment is between neutral to mild alkalinity (pH>7 and <8), the middle segment is highly acidic (pH<3.0) and the posterior part of the posterior segment is highly alkaline (pH>10) (Shanbhag and Tripathi, 2009). The greatest amylolytic activity is found at the end of the anterior midgut and the beginning of the posterior midgut, which is consistent with the optimum pH (Doane, 1969). We can assume that the higher pH values found in *D. erecta* and *D. sechellia* correspond to a more basic pH of the digestive tract of these species. This is what has been shown in *Ephestia kuehniella* (Lepidoptera) which has an alkaline digestive tract (pH 9.8–11.2) containing alkaline amylases with an optimum pH between 9 and 10 (Pytelkova et al., 2009). In fact, high midgut pH has been shown in such insects that feed on plant materials rich in tannins (Chapman, 1998).

Degradation products produced by α-amylase from *D. melanogaster*, *D. erecta* and *D. sechellia*

Degradation products of maltooligosaccharides and starch were analyzed by TLC. For this experiment, the substrates (G3 to G7) were of equal mass concentration (5 mg/ml), and the aliquots for chromatography were taken at approximately the same extent of reaction. As shown in Fig. 3a, there is no qualitative or quantitative difference across species. The patterns are similar for each oligosaccharide and for starch too. The three enzymes could degrade maltooligosaccharides larger than maltotriose producing mainly maltose and maltotriose as expected. Interestingly, it also shows that the cleavages of maltotetraose (G4) and maltohexaose (G6) produce a larger amount of maltose than maltotriose. For G4, this was expected, since cleavage should theoretically not occur between glucose 1 and glucose 2 or between glucose 3 and glucose 4. For maltohexaose, this suggests that asymmetric accommodation of G6 (G4+G2) into the subsites of the catalytic cleft is more efficient than the "symmetric" (G3+G3). This result is consistent with Robyt and French (1970) who found that the distribution of bond cleavage of porcine pancreatic α-amylase on G4 and G6 was 30% on bond 1 and 70% on bond 2; and 67% on bond 2 and 32% on bond 3, respectively. But in contrast to the same study, no hydrolysis occurred on maltotriose. Ramasubbu et al. (2003) found a somewhat different result in human salivary amylase. Fig. 3b shows the degradation products of starch by the α-amylases of the different species, the three enzymes similarly degraded starch producing maltose (G2), maltotriose (G3) and to a lesser extent maltohexaose (G6), maltoheptaose (G7) and glucose (G1).

Kinetics parameters for starch and oligosaccharide hydrolysis

P. pastoris, as a yeast, is known to perform glycosylation of proteins, although the glycans are shorter than in *Saccharomeces cerevisiae* (Grinna and Tschopp, 1989). Indeed, the natural *D. melanogaster* amylase is not glycosylated (Vanderborre et al., 2011). We have checked that the main enzymological parameters k_{cat} and K_m were not strongly affected by glycosylation. For this purpose, we compared in exactly the same conditions the enzyme

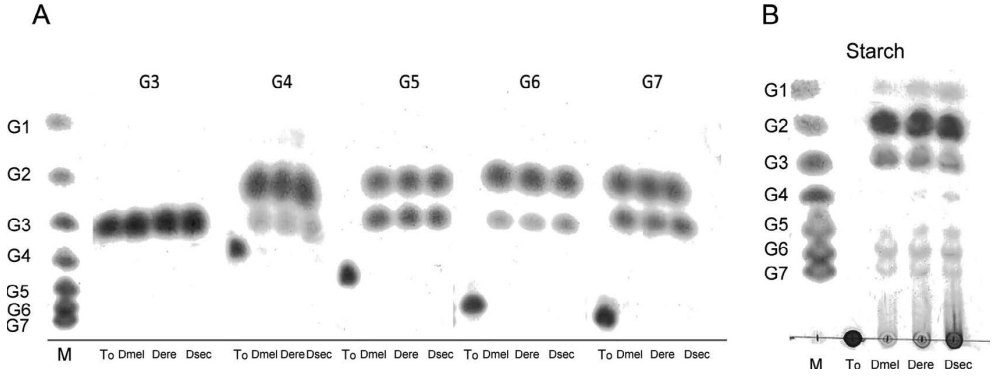

Fig. 3. Thin-layer chromatography for the degradation products of maltooligosaccharides **(a)** and starch **(b)** produced by amylases from *D. melanogaster* (Dmel), *D. erecta* (Dere) and *D. sechellia* (Dsec). 5 mg/ml maltooligosaccharides and 2 mg/ml starch in 20 mM Hepes Buffer (pH 7.5) were digested with α-amylases at 25°C for 2 h. M: marker sugars comprising G1–G7. T_o: Undigested sugar.

Table 1. Kinetics parameters for the hydrolysis of starch and maltooligosaccharides at 25°C given as means ± SD

	D. melanogaster			*D. erecta*			*D. sechellia*		
Substrat	k_{cat} (sec^{-1})	K_m (mg l^{-1})	k_{cat}/K_m (s^{-1} mg^{-1} l)	k_{cat} (sec^{-1})	K_m (mg l^{-1})	k_{cat}/K_m (s^{-1} mg^{-1} l)	k_{cat} (sec^{-1})	K_m (mg l^{-1})	k_{cat}/K_m (s^{-1} mg^{-1} l)
Starch	306 ± 9	543 ± 21	0,6	153 ± 2	103 ± 4	1,5	176 ± 15	255 ± 22	0,7
G4	13 ± 1	109 ± 2	0,1	30 ± 3	114 ± 8	0,3	18 ± 6	111 ± 8	0,2
G5	159 ± 2	82 ± 6	1,9	113 ± 3	35 ± 2	3,2	67 ± 12	123 ± 6	0,5
G6	268 ± 6	72 ± 7	3,7	211 ± 5	20 ± 1	10,8	239 ± 49	67 ± 8	3,6
G7	245 ± 1	88 ± 8	2,8	173 ± 6	56 ± 4	3,1	207 ± 33	107 ± 12	1,9

produced in *P. pastoris* with the same enzyme (100% identical sequence), but non glycosylated, produced in *E. coli* (Cipolla et al., 2012). The k_{cat} differed by about 10% on starch (measured by microcalorimetry at 25°C) and 6% on an artificial oligosaccharide Ethylidene-pNP-G7 at 37°C (not shown). The K_m values for starch were of the same order, i.e. 0,41 g/l and 0,27 g/l, respectively, at 25°C (Supplementary Fig. S1), and therefore the efficiencies k_{cat}/K_m were similar. Thus, we are confident that glycosylation does not strongly affect the results. This has been also found on human recombinant amylase produced in *P. pastoris* (Rydberg et al., 1999).

Table 1 gives the kinetic parameters for the hydrolysis of the substrates tested. *D. erecta* displays low K_m values for starch, whereas *D. melanogaster* and *D. sechellia* exhibit 2–5 times larger K_m values. For a maximal α-amylase activity, *D. melanogaster* needed 0.5% starch, whereas 0.1% was sufficient in *D. erecta* and 0.25% in *D. sechellia*. The low and high K_m values reflect the strong and weak affinity of the enzyme to the substrate, respectively. This means that amylase in *D.erecta* and to a lesser extent in *D. sechellia* maintains a sufficient affinity for starch compared to *D. melanogaster* showing large K_m value. *D. erecta* displays also a relatively weak activity on starch, but combined with a strong affinity, *D. erecta* has the highest efficiency (k_{cat}/K_m) overall. This relatively weak activity and strong affinity may imply adaptive protein evolution of *Amy* in *D. erecta*, as already suggested by Shibata and Yamazaki (1994).

In the previous report by Shibata and Yamazaki (1994), the activity of *D. melanogaster* on starch was five times greater than the activity of *D. erecta*, but the amylase content of their samples was not determined. Our study on purified protein showed only a factor of 2 for the k_{cat} (Table 1). This can be explained by the fact that the amount of enzyme produced is more abundant in *D. melanogaster*. It has already been shown that difference in specific activity between strains of *D. melanogaster* is due to change in transcriptional regulation (Yamate and Yamazaki, 1999). Indeed, selection may act upon promoters, as well as on the protein sequence (Araki et al., 2005). In *D. sechellia*, the loss of activity in the distal copy *Amy-d* due to a deletion may also have an impact on the amount of enzyme molecules produced, which could explain the reduced activity in this species. Moreover, the *D. sechellia* strain Rob3c used in the genome sequencing (www.flybase.org) harbors deletions in both *Amy* copies, suggesting that this species may no longer need amylase activity. However, the extent of complete loss of amylase activity through pseudogenization in this species in not currently known.

Table 2. Relative activity of the α-amylases on starch, glycogen and maltooligosaccharides at 25°C (starch = 100%)

	Relative activity (%)		
Substrat	*D. melanogaster*	*D. erecta*	*D. sechellia*
Starch	100	100	100
Glycogen	39	32	32
G4	4	20	10
G5	52	74	38
G6	88	138	136
G7	80	113	117

Table 2 gives the relative activities of the generalist and specialist α-amylases using the natural substrate (starch) as a reference in order to compare enzymes. No clear differences were observed on glycogen between species, which all show a residual activity between 30 and 40%. It is worth mentioning that the generalist α-amylase is more active on starch but less active on maltooligosaccharides than the specialists α-amylases. For instance, *D. erecta* and *D. sechellia* display higher relative activity on maltohexaose (G6) and maltoheptaose (G7) than starch, unlike *D. melanogaster*. Specialist species, in contrast, have a higher activity on shorter sugars (G6 and G7). From the ecological point of view, it is important to note that in natural conditions, insects are faced to raw starch, which is insoluble, contrary to the gelatinized starch usually used in laboratory assays. *D. melanogaster*, as a generalist, may find various and suitable sources of starch, whereas the specialists are limited to their host plants, which may exhibit starch grains less accessible to enzymatic attack, as found for example in sorghum, whose starch granules are known to be hardly degraded by α-amylase (Rooney and Pflugfelder, 1986). Therefore, for those species, it may be advantageous to be able to use efficiently maltooligosaccharides, for instance

the ones released by the microbial community from the fruit's starch. Interestingly, an analysis of polysaccharides from the *Morinda* fruit juice shows that starch might be absent or rare (Bui et al., 2006). Indeed, microorganisms present on nutrient resources may have a major effect on the digestion of carbohydrates. We know that the composition of gut bacterial communities of *Drosophila* is determined by diet and physiology of the host (Chandler et al., 2011). Further study on the actual effects of the microbiome on the use of carbohydrates should tell us more about the adaptive significance of the α-amylase.

It is challenging to assume whether changes in K_m or k_{cat} would be favored by selection. This issue has been discussed in general (Benner, 1989; Bar-Even et al., 2011), suggesting that K_m should be selected to correlate to the actual substrate concentration. Diverse alternatives in kinetic efficiency of amylases have been described among species of *Drosophila* (Milanovic et al., 1989; Milanovic and Andjelkovic, 1992). However, our data strongly suggest that α-amylase from *D. melanogaster* has been selected for an increase of its k_{cat} on macromolecular polysaccharides such as starch while a higher activity on maltooligosaccharides (G6 and G7) would have been favored in *D. erecta* and *D. sechellia*, the specialist species.

Hoorn and Scharloo (1978) noted that the K_m on starch has only a selective value for individuals that feed on a poor starch diet and the k_{cat} for those on a rich starch diet. Our results are consistent with this and suggest that selective pressure from environment cannot act simultaneously on K_m and k_{cat}, which can only have antagonistic evolution (Somero, 2004).

Protein alignments From protein alignments performed on the species of *melanogaster* subgroup (Table 3), we can conclude that the residues involved in the binding sites of chloride and calcium, the non-catalytic triad, the four disulfide bridges, the flexible loop and the catalytic cavity are strictly preserved. Substitutions are mostly conservative or semi-conservative. It should be noted, however, that residues 138, 145, 255, 266 and 333, which differ across species, are part of or are close to "variable loops" that line the active site. In the pig pancreatic amylase (PPA), the crystal structure reveals that residues 51–57, 104–113, 138–146, 148–153, 268–273, 344–356 form six variable loops bordering the active site. In PPA, the loops 51–57 in domain A and 138–146 in domain B are involved in saccharide binding (Larson et al., 1994; Qian et al., 1995). The size and sequence variability of these loops could modulate the accessibility of the substrate, resulting in a different measure of the affinity between amylases depending on the origin and size of the substrate. This might explain the differences in affinity found between *D. melanogaster* and *D. erecta*. Indeed, *D. erecta* has, in general, the best affinity whatsoever on starch or oligosaccharides (see Table 1).

Table 3. Analysis of multiple sequences alignment of α-amylase from *D. melanogaster* species subgroup (*D. melanogaster*: BAB32511, L22728, L22729, L22730, L22731, L22733 and L22734; *D. simulans*: D17734; *D. mauritiana*: D17730; *D. yakuba*: D17738; *D. teissieri*: D17736; *D. sechellia*: D17732; *D. erecta*: D17728). The Major Residue column displays the most common residue in the *melanogaster* subgroup. The lines in grey represent the residues that are close to or inside the variable loops. Numbering is that of the mature *D. melanogaster* α-amylase

Position	Major Residue	*D. melanogaster*	*D. sechellia*	*D. erecta*
37	Y	Y	F	F
68	E	E	E	D
75	E	E	Q	Q
82	K	K	K	R
91	T	T	I	I
96	V	V	I	V
120	S	T	S	S
136	A	A	A	G
138	S	S	N	S
145	E	E	Q	Q
165	Q	Q	Q	R
240	L	L	V	L
255	V	V	V	A
266	T	T	T	R
333	T	T	S	S
351	I	I	V	V
356	N	N	N	H
383	S	S	L	S
385	A	E	A	A
398	S	S	S	A
445	K	K	K	R
448	T	T	S	S
458	N	N	Y	S
459	I	I	I	V
461	S	S	S	R

Little is known about the nutrient characteristics of *Morinda citrifolia* and *Pandanus candelabrum*. Rosalizan et al. (2010) studied the physico-chemical characteristics of the fruit of *Morinda citrifolia*, and showed that the fruit was mature 15–16 weeks after fruit set and carbohydrate composition was at its maximum 12 weeks after fruit set. Carbohydrate contents increases up to the 12th week (4.34 g/100 g), then decreases, but, as we mentioned above, the starch content seems to be low. Carbohydrate composition of the edible portion of fresh *Pandanus* is about 14 to 18 percent, which is equivalent to the sugar content of apples or pears (Miller et al., 1956). The fresh samples contain starch granules which

were easily stained blue with iodine.

The differences between specialist and generalist species provide supporting argument for the hypothesis of adaptive protein evolution. Evidence for adaptive evolution could theoretically be also gained from sequence polymorphism analysis. However, *D. sechellia* shows little overall genetic diversity (Legrand et al., 2009) and few strains or populations are currently available for *D. erecta*. To better understand the impact of specialization on the action of a digestive enzyme such as amylase, we should have a clearer picture of the composition of polysaccharides and oligosaccharides of the host plants *Morinda citrifolia* and *Pandanus candelabrum* and test characteristics of amylase from other specialist species.

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