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The Amylases of Insects

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ABSTRACT: Alpha-amylases are major digestive enzymes that act in the first step of maltopolysaccharide digestion. In insects, these enzymes have long been studied for applied as well as purely scientific purposes. In many species, amylases are produced by multiple gene copies. Rare species are devoid of *Amy* gene. They are predominantly secreted in the midgut but salivary expression is also frequent, with extraoral activity. Enzymological parameters are quite variable among insects, with visible trends according to phylogeny: Coleopteran amylases have acidic optimum activity, whereas dipteran amylases have neutral preference and lepidopteran ones have clear alkaline preference. The enzyme structure shows interesting variations shaped by evolutionary convergences, such as the recurrent loss of a loop involved in substrate handling. Many works have focused on the action of plant amylase inhibitors on pest insect amylases, in the frame of crop protection by transgenesis. It appears that sensitivity or resistance to inhibitors is finely tuned and very specific and that amylases and their inhibitors have coevolved. The multicopy feature of insect amylases appears to allow tissue-specific or stage-specific regulation, but also to broaden enzymological abilities, such as pH range, and to overcome plant inhibitory defenses.

KEYWORDS: Amylase inhibitors, multigene families, diet, midgut, salivary gland

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Introduction

While conquering virtually all terrestrial and freshwater habitats, insects have evolved various feeding preferences. Many are phytophagous: They may consume seeds (eg, grain pests like weevils), stems (eg, lepidopteran stem borers like *Sesamia* species), roots (like the corn rootworm *Diabrotica virgifera*), or leaves (eg, leaf miner moths) or be sap feeders or nectar feeders. Other insects are carnivorous, saprophagous (eg, *Drosophila melanogaster*), or bloodsuckers. Particularly, phytophagous insects may be polyphagous or more specialized on a single or few host plants. Indeed even in the saprophagous drosophilids, some species are specialized on a single host plant (*Drosophila sechellia* on *Morinda citrifolia* fruit,¹ *Drosophila erecta* on *Pandanus* fruit²). These preliminary remarks are of importance because insects must harbor enzymatic tools devoted to their respective diets, for detoxifying or circumventing the plant defenses, and for metabolizing useful nutriment. Nutritional content is obviously different in plants and in animal flesh, or in blood, and insects have evolved to optimize energetic uptake from their food.

Here I will draw a quick and noncomprehensive picture of insect alpha-amylases. Alpha-amylases (EC 3.2.1.1) are glycosyl hydrolases that break down alpha-1,4 glycosidic bonds inside a maltopolysaccharide linear chain, mainly in starch and glycogen, resulting in maltose, maltotriose, and residual branched maltodextrins as final products. These molecules are in turn hydrolyzed into glucose by alpha-glucosidases. Starch granules may have various structures and composition, which are more or less resistant to amylase: It was shown that the amylases of the weevil *Sitophilus oryzae* were unable to attack raw starch

granules from potato, tapioca, wheat, or amylose-containing corn, but did degrade pea starch.³ In the bruchid *Zabrotes subfasciatus*, mastication seems a necessary process to damage starch granules and enable them susceptible to enzymatic degradation.⁴ In the living world, alpha-amylases (hereafter named simply amylases) are almost ubiquitous and are of utmost importance for nutrition of bacteria, plants, fungi, and animals, a lot of which having multiple copies of amylase genes, owing to gene duplications⁵ or horizontal transfer.⁶ The question of the evolutionary advantage for an organism to have several, sometimes diverged, amylase gene copies remains raised. Some enlightenment could be gained from insects, most of which rely on polysaccharides for their energy supply, and then depend on amylase activity.

One may acknowledge roughly two types of amylase studies on insects according to their focus: first, basic research dealing, for instance, with enzymology, genetics, evolution, and ecology; second, applied research that seeks to characterize digestive enzymes of insect of economical importance, such as crop pests or disease vectors. Intriguingly, it sometimes seems that these two research communities, ie, basic vs applied, are somewhat ignorant of each other.

Historically, insect amylases were widely studied early when electrophoresis techniques were developed, for it was easy and cheap. It allowed numerous studies on polymorphism when this unexpected kind of variation at the molecular level was evidenced,^{7–9} especially in *Drosophila*, raising questions about the functional or adaptive significance of the observed polymorphism, in terms of fitness and selection,^{10–15} or at the molecular level, in terms of catalytic activity or regulation.



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Amylase was then used as a gene model during the controversy between selectionists and neutralists.¹² For instance, researchers wondered if the various “isozymes” of amylases (ie, electrophoretic variants) had similar catalytic activities, similar heat sensitivities, similar tissular or temporal expression profiles^{9,16–23} Also, attempts were made to link some characteristics of amylases to the natural diets of their producers, eg, electric charge,²⁴ or catalytic activity.^{25,26}

A Multigene Family in Insects

Several amylase gene copies (*Amy*) were reported in many insect species. Table 1 shows the number of gene copies that were reported in literature or by search in databases. The copy number varies from only 1 (eg, in honeybees) to more than 12 (in some mosquitoes). Indeed, most species harbor several copies. The *Amy* family was well described in *Drosophila*, as soon as 1967 for *D melanogaster* when Bahn²⁸ discovered the *Amy* gene duplication, but importantly, like in many other insect groups, *Amy* duplications occurred largely independently in many *Drosophila* lineages.^{29–31,50–52} In this only genus, the number of gene copies vary from 1 (eg, *Drosophila virilis*)⁵³ to 6 (*Drosophila ananassae*),³⁰ not counting the paralog named *Amyrel* (*Amy*-related), a divergent copy (40% in amino acids) which is present throughout the drosophilids and is probably ancestral to Muscomorpha.^{54,55} Even more profound sequence divergence between *Amy* copies within genomes exists in most insect orders. Figure 1 shows a tree of selected amylase protein sequences of insects. Deep splitting of clusters is visible within Coleoptera, Lepidoptera, Hymenoptera, and Diptera, showing important divergences between paralogs. It is possible that intraspecific copy number variation occurs in species that have several *Amy* copies, like *D ananassae*, but there is no published report to my knowledge in insects, whereas it is well documented in humans⁵⁹ and dogs.⁶⁰ Classically, for a multigene family, sequence divergence³⁰ and concerted evolution among copies^{61,62} were reported. Note the absence of amylase gene in rare genomes, such as the bloodsucker louse *Pediculus humanus*, the sap-feeding aphid *Acyrtosiphon pisum*, and other aphidomorphs (Table 1). It is tempting to link this deficiency to their specific, specialized feeding habits. However, amylase activity was detected in some aphids,⁶³ although the genes were not identified, and a purely bloodsucking bug like *Rhodnius prolixus* does have an amylase gene. Nonetheless, exaptation of such an enzyme to another function linked to hematophagy is a possibility, because in this species an α -glucosidase was recruited for hemozoin formation from the heme of hemoglobin.⁶⁴ The number of *Amy* gene copies cannot be clearly related to the diet. For instance, the copy number may vary greatly between related species that share similar diets (*D virilis* vs *D melanogaster*; *Tenebrio molitor* vs *Tribolium castaneum*; *A pisum* vs *Bemisia tabaci*; Table 1). However, it has been proposed that several gene copies may increase dietary flexibility, for instance, in housefly³³ or in the soldier bug *Podisus maculiventris*.⁴⁸

Sequence and Enzymatic Characterization of Insect Amylases

Irrespective of the copy number, it is logically believed that phytophagous insects must have more active amylolytic enzymes than carnivorous insects⁶⁵ and that various vegetal diet may regulate amylase levels differentially (see below). In line with the fact that insect amylase studies are often devoted to crop pests, most enzymatic characterization of insect amylases are from such insects, like seed-feeding beetles, or lepidopteran stem borers. However, model insects like drosophilids were intensively investigated as well. Using purified recombinant amylases, Commin et al²⁵ attempted to evidence enzymological differences between amylases of the generalist *D melanogaster* and two specialists, *D sechellia* and *D erecta*. But more general and accurate comparisons between the specific activities (k_{cat}) of insect amylases from species differing in their diet are still wanting.

The *D melanogaster Amy* sequence was published in 1986.⁶⁶ Innumerable insect amylase sequences were published since then. Figure 1 is a small subset of what is available at GenBank and in various genome databases. These sequences allow comparative studies about the gene structure and protein evolution, regarding conserved or divergent parts of the protein. All insect amylases have about the same size, ie, coding sequences around 1500 nucleotides, corresponding to a mature protein weight around 50 to 55 kDa after removing the signal peptide, as amylase is secreted (Table 2). An exception is in some mosquitoes, where a long N-terminal domain of unknown function occurs in some copies.⁷¹ Accordingly, it is surprising that some amylase protein sizes reported in the literature are very different from this value.^{69,72} This may be in most cases due to migration artifacts arising from abnormal sodium dodecyl sulfate binding on proteins with very basic or acidic isoelectronic point or on glycosylated proteins, resulting in migration defects and false mass estimations. In such cases, amylase band excision from the gel and mass spectrometry analysis should provide more accurate results. The intron content of *Amy* genes is quite variable in insects, from no intron in *D melanogaster* to at least 6 in Lepidoptera⁷³ so that gene lengths may vary a lot. At least one case of alternative splicing was reported, in the beetle *Ips typographus*.⁴³ Importantly, insect amylases are overall quite similar to other animal amylases. They have been assigned to the GH13_15 subfamily of glycosyl hydrolases,⁷⁴ with other invertebrate amylases, whereas vertebrate amylases belong to GH13_24, a somewhat artificial division. All animal amylases (and beyond) are made of 3 major domains, named A, B and C and the structure requires a calcium ion.⁷⁵ The catalytic apparatus, in domain A, is conserved, but some interesting facts are to be noticed: An amino acid stretch named “flexible loop” with the motif GHGA, protruding near the catalytic cleft,⁷⁵ which is an ancestral feature, is missing in many insect amylase sequences.⁵ Figure 1 indicates the sequences lacking this motif. For example, the GHGA motif is deleted and the flexible loop is much shortened in most coleopteran amylase sequences,

Table 1. Number of reported *Amy* genes in insects from the literature or from genome database searches.

ORDER	SPECIES	NUMBER OF <i>AMY</i> COPIES	REFERENCE
Diptera	<i>Drosophila melanogaster</i>	2 + <i>Amyrel</i>	28, FlyBase
	<i>Drosophila ficusphila</i>	2 + <i>Amyrel</i>	FlyBase
	<i>Drosophila eugracilis</i>	2 + <i>Amyrel</i>	FlyBase
	<i>Drosophila biarmipes</i>	2 + <i>Amyrel</i>	FlyBase
	<i>Drosophila takahashii</i>	2 + <i>Amyrel</i>	FlyBase
	<i>Drosophila elegans</i>	3 + <i>Amyrel</i>	FlyBase
	<i>Drosophila rhopaloa</i>	2 + <i>Amyrel</i>	FlyBase
	<i>Drosophila kikkawai</i>	4 + <i>Amyrel</i>	29
	<i>Drosophila ananassae</i>	6 + <i>Amyrel</i>	30, FlyBase
	<i>Drosophila bipectinata</i>	2 + ψ + <i>Amyrel</i>	FlyBase
	<i>Drosophila pseudoobscura</i>	3 + <i>Amyrel</i>	31, FlyBase
	<i>Drosophila persimilis</i>	3 + <i>Amyrel</i>	FlyBase
	<i>Drosophila miranda</i>	2 + ψ + <i>Amyrel</i>	32
	<i>Drosophila willistoni</i>	2 + ψ + <i>Amyrel</i>	FlyBase
	<i>Drosophila mojavensis</i>	1 + <i>Amyrel</i>	FlyBase
	<i>Drosophila virilis</i>	1 + <i>Amyrel</i>	FlyBase
	<i>Drosophila albomicans</i>	2 + <i>Amyrel</i>	FlyBase
	<i>Drosophila grimshawi</i>	1 + <i>Amyrel</i>	FlyBase
	<i>Drosophila hydei</i>	1 + <i>Amyrel</i>	FlyBase
	<i>Ceratitis capitata</i>	2 + <i>Amyrel</i>	GenBank
	<i>Musca domestica</i>	5 + <i>Amyrel</i>	33, FlyBase
	<i>Glossina morsitans</i>	1 + ψ	FlyBase
	<i>Culex quinquefasciatus</i>	12	FlyBase
	<i>Aedes albopictus</i>	13	GenBank
	<i>Aedes aegypti</i>	9	GenBank
	<i>Anopheles darlingi</i>	5	FlyBase
	<i>Anopheles gambiae</i>	5	FlyBase
	<i>Mayetiola destructor</i>	1	FlyBase
	<i>Rhagoletis zephyria</i>	3-4 ?	GenBank
	<i>Bactrocera oleae</i>	4	GenBank
	<i>Lucilia cuprina</i>	4	GenBank
	<i>Zeugodacus cucurbitae</i>	4 + ψ ?	GenBank

(Continued)

Table 1. (Continued)

ORDER	SPECIES	NUMBER OF <i>AMY</i> COPIES	REFERENCE
Lepidoptera	<i>Bombyx mori</i>	3	34, FlyBase
	<i>Danaus plexippus</i>	4	FlyBase
	<i>Papilio machaon</i>	4	GenBank
	<i>Papilio polytes</i>	4	GenBank
	<i>Papilio xuthus</i>	4	GenBank
	<i>Epeestia kuehniella</i>	3	35
	<i>Spodoptera litura</i>	4	GenBank
	<i>Spodoptera frugiperda</i>	2	5
	<i>Helicoverpa armigera</i>	5	22,36, GenBank
	<i>Plutella xylostella</i>	4	GenBank
	<i>Chilo suppressalis</i>	2	Da Lage, unpubl.
	<i>Glyphodes pyloalis</i>	2	37
	<i>Bicyclus anynana</i>	3	GenBank
	<i>Pieris rapae</i>	3	GenBank
Coleoptera	<i>Tribolium castaneum</i>	7-9	Hickey, unpublished, 38, GenBank
	<i>Hypothenemus hampei</i>	2 ?	39
	<i>Blaps mucronata</i>	2	5
	<i>Sitophilus oryzae</i>	2	38,40
	<i>Tenebrio molitor</i>	1	38
	<i>Diabrotica virgifera</i>	2	41
	<i>Anthonomus grandis</i>	2	42
	<i>Ips typographus</i>	2	43
	<i>Acanthoscelides obtectus</i>	2	44
	<i>Zabrotes subfasciatus</i>	3 "isoforms"	45
	<i>Aethina tumida</i>	3	GenBank
	<i>Anoplophora glabripennis</i>	4	GenBank
	<i>Agrilus planipennis</i>	3	GenBank
	<i>Onthophagus taurus</i>	4	GenBank
Hymenoptera	<i>Apis mellifera</i>	1	46, Hymenoptera genome database
	<i>Apis florea</i>	1	Hymenoptera genome database
	<i>Apis dorsata</i>	1	Hymenoptera genome database
	<i>Bombus terrestris</i>	1	Hymenoptera genome database
	<i>Bombus impatiens</i>	1	Hymenoptera genome database
	<i>Megachile rotundata</i>	1	Hymenoptera genome database
	<i>Melipona quadrifasciata</i>	1	Hymenoptera genome database
	<i>Nasonia vitripennis</i>	5	Hymenoptera genome database
	<i>Acromyrmex echinatio</i>	2	Hymenoptera genome database

Table 1. (Continued)

ORDER	SPECIES	NUMBER OF AMY COPIES	REFERENCE
	<i>Atta cephalotes</i>	2	Hymenoptera genome database
	<i>Solenopsis invicta</i>	2	Hymenoptera genome database
	<i>Camponotus floridanus</i>	2	Hymenoptera genome database
Blattodea	<i>Blattella germanica</i>	3	GenBank
	<i>Periplaneta americana</i>	1	47
	<i>Zootermopsis nevadensis</i>	2	GenBank
	<i>Cryptotermes secundus</i>	3	GenBank
Phasmatodea	<i>Timema cristinae</i>	2	InsectBase
	<i>Sipyloidea sipyilus</i>	1	InsectBase
	<i>Meraudoidea extradentata</i>	1	InsectBase
	<i>Extatosoma tiaratum</i>	1	InsectBase
	<i>Aretaon asperrimus</i>	1	InsectBase
Orthoptera	<i>Locusta migratoria</i>	3	InsectBase
Hemiptera	<i>Rhodnius prolixus</i>	1	FlyBase
	<i>Cimex lectularius</i>	1	GenBank
	<i>Podisus maculiventris</i>	3 “isoforms”	48
	<i>Lygus lineolaris</i>	2	49
	<i>Halyomorpha halys</i>	2	GenBank
	<i>Nilaparvata lugens</i>	0	GenBank
	<i>Myzus persicae</i>	0*	GenBank
	<i>Diuraphis noxia</i>	0*	GenBank
	<i>Acyrtosiphon pisum</i>	0*	FlyBase
	<i>Bemisia tabaci</i>	5	GenBank
Phthiraptera	<i>Pediculus humanus</i>	0**	FlyBase
Siphonaptera	<i>Archaeopsylla erinacei</i>	1	InsectBase
Trichoptera	<i>Limnephilus lunatus</i>	8	InsectBase
Thysanoptera	<i>Frankliniella occidentalis</i>	1	InsectBase
Odonata	<i>Ladona fulva</i>	2?	InsectBase

BLAST search was performed on genome data in the indicated databases in June 2018, by BLASTP or TBLASTN²⁷ using *Drosophila melanogaster* Amy sequence BAB32511 as query. ψ : pseudogene. *: no amylase gene sequence found in Aphidomorpha, but amylase activity was reported in Aphididae species *Aphis fabae* and *Aphis gossypii*. **: the “putative amylase” with accession EEB15075 is an amino acid transport protein. FlyBase: flybase.org; GenBank: ncbi.nlm.nih.gov/protein/; Hymenoptera genome database: hymenopteragenome.org; InsectBase: insect-genome.com.

except, intriguingly in two of them. This is surprising because, otherwise, it would have been obvious that the GHGA motif was lost in the coleopteran ancestor. In Hymenoptera, two types exist, one gene group with the flexible loop, another group lacking the loop. This suggests that the two types have been coexisting ancestrally. In Muscomorpha flies, the Amyrel paralog also lacks the GHGA motif.^{54,55} These observations suggest recurrent losses of the flexible loop in the course of

evolution (convergences), due to selective constraints that remain to elucidate (see below). Another interesting feature is the substitution of a conserved arginine into a glutamine in some unrelated amylases, ie, another convergence. This arginine is involved in the fixation of an activating chloride ion which changes the protein conformation and without which a detrimental salt bridge interaction would form. The glutamine is found in all Lepidopteran amylases (studied in details by

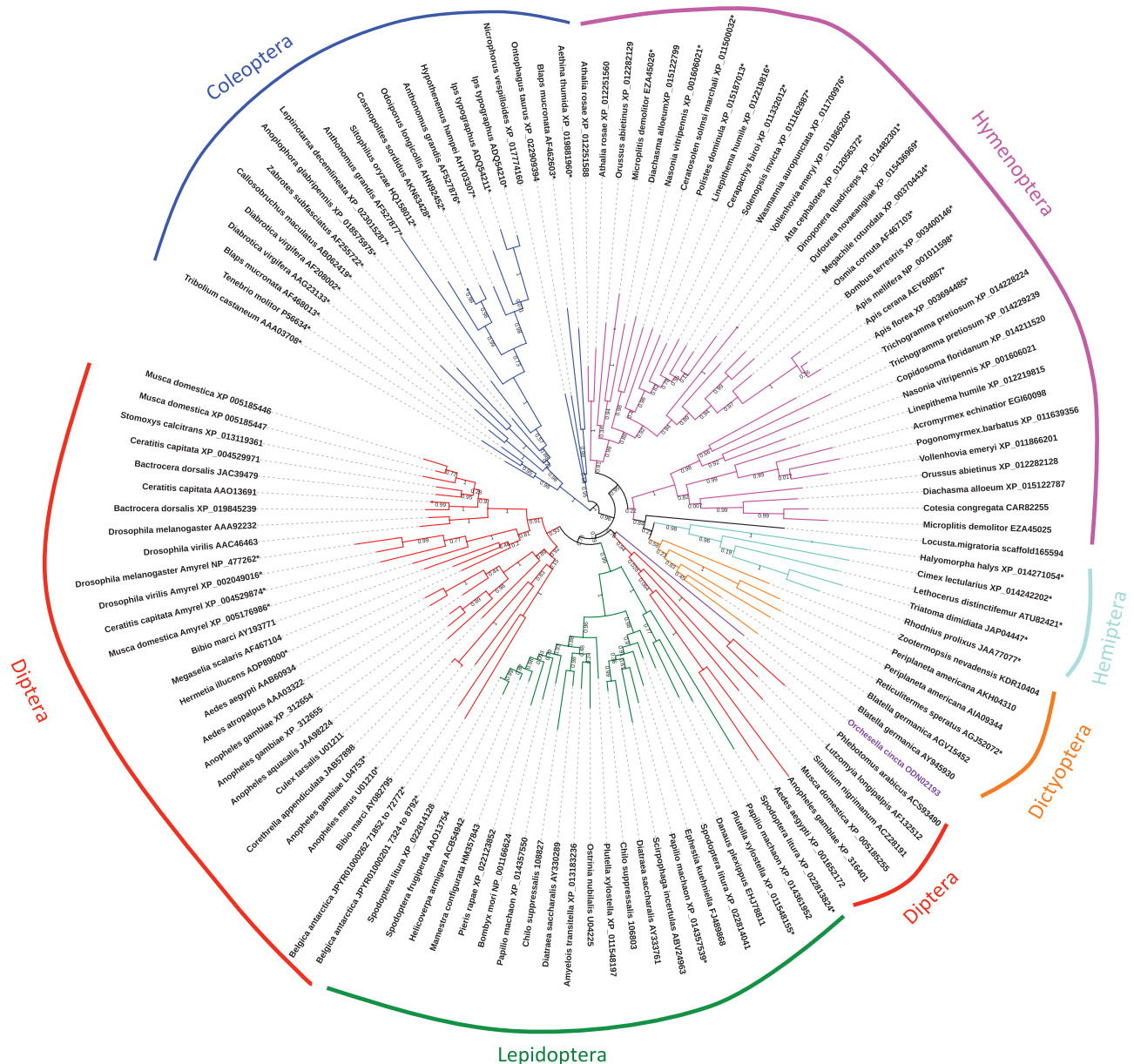


Figure 1. Unrooted tree of amylase protein sequences of insects. Sequences were aligned with MUSCLE,⁵⁶ and a Maximum Likelihood tree was built using the online server phylogeny.fr⁵⁷ with GBLOCKS curation and default parameters. The tree was drawn with iTOL.⁵⁸ Numbers along branches indicate posterior probabilities. Asterisks indicate the loss of the GHGA motif in proteins. Red: Diptera; green: Lepidoptera; dark blue: Coleoptera; pink: Hymenoptera; orange: Dictyoptera; light blue: Hemiptera; black: Orthoptera. Purple branch and label is a Collembola. Accession numbers or references follow the taxa names of the sequences.

Pytelková et al³⁵) and in the Amyrel protein of a part of drosophilids, eg, *D virilis*, but not *D melanogaster*.⁷⁶ Those glutamine-bearing amylases cannot bind the chloride ion but are nonetheless active, chloride independent,^{35,76} probably due to compensating mutations, because simply mutating to a glutamine when an arginine is normally present almost abolishes enzymatic activity.⁷⁶ It was proposed that the chloride independence would be an adaptation to an alkaline pH in the midgut.³⁵

The optimum pH of amylases generally corresponds to the pH values in the midgut lumen.⁷² The optimum pH of insect amylases varies greatly depending on the species. Table 3 shows

the optimum pH reported for some insect species. Coleoptera show mostly acidic optimum pH for amylase activity, whereas Lepidopteran amylases generally have alkaline preferences. Dipteran amylases have more neutral preference. Therefore, the hypothesis that chloride independence is adapted to high pH values does not hold in the case of Amyrel working at a neutral pH. In some species, several amylases are produced, with different pH optima, due to different tissue specificities^{37,108} or stage specificities.¹⁰⁴ Dow¹¹⁹ suggested that a high gut pH in insects, such as in Lepidoptera, could be an adaptation to feeding on tannin-rich plants, because high pH decreases the binding of tannins to nutritious proteins and thus enhances digestibility.

Table 2. Estimates of amylase molecular weights in some insects.

ORDER	SPECIES	MOLECULAR WEIGHT	METHOD	REFERENCE
Hemiptera	<i>Eurygaster integriceps</i>	49 kDa, 52 kDa	SDS-PAGE	67
Coleoptera	<i>Anthonomus grandis</i>	50870 Da, 52680 Da	Protein sequence	AF527876 AF527877
Coleoptera	<i>Sitophilus oryzae</i>	51318 Da 53 kDa 56 kDa	Protein sequence Sedimentation equilibrium centrifugation SDS-PAGE	HQ158012 38
Coleoptera	<i>Tenebrio molitor</i>	51240 Da 56 kDa	Protein sequence SDS-PAGE	P56634 38
Coleoptera	<i>Tribolium castaneum</i>	51568 Da 56 kDa	Protein sequence SDS-PAGE	AAA03708 38
Coleoptera	<i>Callosobruchus maculatus</i>	51768 Da	Protein sequence	AB062419
Coleoptera	<i>Zabrotes subfasciatus</i>	51438 Da	Protein sequence	AF255722
Coleoptera	<i>Diabrotica virgifera</i>	50910 Da 50517 Da	Protein sequence	AAG23133 AF208002
Coleoptera	<i>Morimus funereus</i>	31 kDa	FPLC column	68
Coleoptera	<i>Hypothenemus hampei</i>	51243 Da	Protein sequence	AHY03307
Diptera	<i>Drosophila melanogaster</i>	51915 Da	Protein sequence	AAA92232
Diptera	<i>Ceratitis capitata</i>	53010 Da	Protein sequence	XP_004529971
Diptera	<i>Aedes aegypti</i>	52386 Da	Protein sequence	AAB60934
Diptera	<i>Lutzomyia longipalpis</i>	54019 Da	Protein sequence	AF132512
Hymenoptera	<i>Apis mellifera</i>	53870 Da	Protein sequence	NP_001011598
Blattodea	<i>Blattella germanica</i>	53701 Da 53140 Da	Protein sequence	AGV15452 AY945930
Orthoptera	<i>Dociostaurus maroccanus</i>	73 kDa	SDS-PAGE	69
Lepidoptera	<i>Ostrinia nubilalis</i>	54292 Da	Protein sequence	U04225
Lepidoptera	<i>Pieris brassicae</i>	88 kDa	SDS-PAGE	70
Lepidoptera	<i>Ephestia kuehniella</i>	54442 Da	Protein sequence	FJ489868
Lepidoptera	<i>Helicoverpa armigera</i>	54043 Da	Protein sequence	ACB54942
Lepidoptera	<i>Bombyx mori</i>	54644 Da	Protein sequence	NP_001166624
Lepidoptera	<i>Chilo suppressalis</i>	55019 Da 54811 Da	Protein sequence	108827 106803
Lepidoptera	<i>Spodoptera frugiperda</i>	54007 Da	Protein sequence	AAO13754

Estimates through protein sequences used the accession numbers indicated in Figure 1, removing the peptide signal. Other estimates used biochemical methods and are given with literature references numbered in italics as in text. FPLC = fast protein liquid chromatography; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

The optimum temperatures reported are typical of mesophilic amylases for the insects studied to date. Note however that results may vary significantly according to purification and assay conditions. Indeed, the temperature for maximal activity is strongly dependent on the assay duration, because long incubation at high temperature accelerates the enzyme denaturation; raw extracts contain proteases that are also activated by increased temperature and therefore degrade proteins in the

sample, including amylases. This results in a lower apparent optimal temperature. For instance, optimum temperature for amylases of *D melanogaster*, *D sechellia*, and *D erecta* was estimated 37°C on raw extracts,²⁶ but rather 57°C to 60°C using purified enzymes produced in vitro.^{25,120} To avoid this drawback, addition of commercially available protease inhibitor cocktails to crude extracts is a good practice. It is supposed that species that experience sun exposure in open fields should have

Table 3. Optimum pH of insect amylases, from published studies, and inhibition or noninhibition by plant amylase inhibitors or other plant extracts.

ORDER	SPECIES	OPTIMUM PH	ACTIVE INHIBITORS	INACTIVE INHIBITORS	REFERENCE
Hemiptera	<i>Leptoglossus zonatus</i>	5.6			79
	<i>Podisus maculiventris</i>	6.0			48
	<i>Graphosoma lineatum</i>	6.0		α AI1	80
	<i>Eurygaster maura</i>	6-7			81
	<i>Eurygaster integriceps</i>	6.5			67
	<i>Aphis fabae</i>	7			63
Coleoptera	<i>Acanthoscelides obtectus</i>	7-7.5	0.19 WI, 0.53 WI, HI	α AI1, α AI2	80,82-85
	<i>Anthonomus grandis</i>	?	chimeric AI from α AI1 and α AI2, BIII from rye	α AI1, α AI2 0.19 WI, 0.53 WI	42,86
	<i>Prostephanus truncatus</i>	6.0	Amaranth AI	Maize AI <i>Phaseolus acutifolius</i> AI	87
	<i>Cryptolestes ferrugineus</i>	5.0-5.5	α AI1		80
	<i>Oryzaephilus surinamensis</i>	4.5-5.0	α AI1		80
	<i>Sitophilus granarius</i>	4.5	WI, HI, <i>Thymus vulgaris</i> extract	α AI1	80,82,88
	<i>Sitophilus oryzae</i>	5.0	WRP25, HI	Corn AI	3,38,82
	<i>Sitophilus zeamais</i>	5-7	WI		89
	<i>Tenebrio molitor</i>	5.4	WRP25, Corn AI, 0.28 WI, HI	Amaranth AI (weak effect) 0.19 WI, 0.53 WI	38,82,90,91
	<i>Tribolium castaneum</i>	4.5-5.0	α AI1, WRP25, HI, 0.28 WI, Corn AI Amaranth AI, <i>Withania somnifera</i> AI		38,80,82,92-94
	<i>Bruchus pisorum</i>	5.5	α AI1, α AI2		86,95
	<i>Rhyzopertha dominica</i>	7	WI	<i>Thymus vulgaris</i> extract, <i>Punica granatum</i> extract	82,88,96
	<i>Callosobruchus maculatus</i>	5.0	α AI, Amaranth AI, 0.19 WI, 0.53 WI, HI, <i>Punica granatum</i> extract, <i>Achyranthes aspera</i> AI	<i>Vigna unguiculata</i> AI	82,83,85,88,92,97-99
	<i>Zabrotes subfasciatus</i>	6-7	α AI2, WRP25, 0.19 WI, 0.53 WI	α AI1, WRP26	83,84,86,100,101
	<i>Diabrotica virgifera</i>	5.7	α AI1, wheat AI		41
	<i>Morimus funereus</i>	5.2			68
	<i>Hypothenemus hampei</i>	4.5-5.2	α AI1, Amaranth AI, <i>Ph. coccineus</i> AI	α AI2	38,97,102,103
	<i>Plagiodera versicolora</i>	4 ¹ , 8 ²			104
	<i>Alphitobius diaperinus</i>	5.0	α AI1		105

Table 3. (Continued)

ORDER	SPECIES	OPTIMUM PH	ACTIVE INHIBITORS	INACTIVE INHIBITORS	REFERENCE
Diptera	<i>Drosophila melanogaster</i>	7.4**	α AI1, WI		9,80
	<i>Ceratitis capitata</i>	8.0			106
	<i>Sarcophaga bullata</i>	7.0	α AI1		80
	<i>Aedes aegypti</i>	7.0	α AI1		80
	<i>Lutzomyia longipalpis</i>	8.5			107
Hymenoptera	<i>Monomorium pharaonis</i>	5.0-5.5	α AI1		80
	<i>Apis mellifera</i>	5.0-5.5	α AI1		80
	<i>Venturia canescens</i>	5.0-5.5	α AI1		80
Blattodea	<i>Blattella germanica</i>	6.0		α AI1	80
Orthoptera	<i>Acheta domesticus</i>	5.5-6.5		α AI1	80
	<i>Doclostaurus maroccanus</i>	6.0	α AI1, α AI2		108
	<i>Calliptamus italicus</i>	8			108
	<i>Grylodes sigillatus</i>	6.6-7.0			69
Lepidoptera	<i>Manduca sexta</i>	10		α AI1	80
	<i>Ostrinia nubilalis</i>	11		α AI1	80
	<i>Pieris brassicae</i>	8			70
	<i>Ephestia kuehniella</i>	9	WI1, WI3*	α AI1	35
	<i>Helicoverpa armigera</i>	9-11*	WI, <i>Achyranthes aspera</i> AI	Amaranth AI	36,92,99
	<i>Bombyx mori</i>	6.8 ³ -9.2 ⁴			108
	<i>Chilo suppressalis</i>	9			109
	<i>Acherontia atropos</i>	12			110
	<i>Lasiocampa quercus</i>	10.8			110
	<i>Lichnoptera felina</i>	10.8			110
	<i>Antheraea mylitta</i>	9.5			111
	<i>Spodoptera littoralis</i>	9.5			112
	<i>Mamestra brassicae</i>	9.5			113
	<i>Erinnyis ello</i>	9.8			114
	<i>Tecia solanivora</i>	9.0	Amaranth AI		115
	<i>Glyphodes pyloalis</i>	9 ⁵ -10 ⁴			37
	<i>Naranga aenescens</i>	10			116
	<i>Spodoptera frugiperda</i>	8.5-9.5	wheat tetrameric inhibitor		117
	<i>Tuta absoluta</i>	8.0	Amaranth AI, WI		118

AI: amylase inhibitor; 0.19, 0.28, 0.53 WI, WRP25, WRP26: wheat inhibitors; HI: barley inhibitor; *depending on the amylase paralog; **no amylase activity in the acidic mid-midgut⁷⁷; 1: larval amylase; 2: adult amylase; 3: hemolymph amylase; 4: digestive amylase; 5: salivary amylase. Another table of insect target/amylase inhibitor has been published elsewhere.⁷⁸

more thermal-resistant amylases than species living in cold areas. But therefore it is not easy to compare optimum temperatures among amylases from insects that have contrasted thermal preferences without using standardized enzyme purification and assay protocols.

Localization, Secretion, and Regulation of Amylases in Insects

In most species studied, amylase is secreted at least in the midgut. It seems that in a number of insect species, the enzyme is partly recovered from the residual undigested food, through endo-ectoperitrophic circulation.^{72,121} In *Drosophila*, *Amy* tissue-specific expression was studied in details; no clear expression was found outside larval or adult midgut, as can be seen in RNAseq data at FlyBase. In *D melanogaster*, compartmentalization was found along the midgut, with no expression in the acidic mid-midgut, and 3 areas in the anterior midgut and 2 areas in the posterior midgut, with various combinations depending on genotypes and diet.¹²² This tissue-specific expression was controlled by a putative trans-acting factor named “map” (midgut activity pattern),⁷⁷ located 2 cM downstream of the structural genes. However, the *map* gene was never identified until now in genome annotation. Similar complex midgut expression was found in other *Drosophila* species in larvae and adults.^{17,123} In *D ananassae*, different gene copies were expressed in different parts of the midgut.¹⁷ Whereas extraoral amylase activity was recognized in *Drosophila*,^{124,125} leading to a “social digestion,”¹²⁶ the enzyme is produced by the midgut and regurgitated, but not by the salivary glands. A fine picture of amylase secretion in adult *D melanogaster* was also published more recently.¹²⁷ In other Diptera, amylase expression may take place in salivary glands, as in the adult sand fly *Lutzomyia longipalpis*,¹⁰⁷ where it is downregulated after a blood meal. In *Aedes aegypti*, an amylase gene is specifically expressed in adult female salivary glands,⁷¹ showing that the occurrence of several *Amy* copies may serve fine regulation. A general review of midgut amylase secretion in insects was given in a rich review on digestive enzymes of insects by Terra and Ferreira,⁷² who studied the precise localization and secretion process, whether apocrine secretion (Lepidoptera, Coleoptera) or exocytosis (eg, Diptera). It appeared that the enzyme may be produced in the midgut and be moved forth to the foregut, where the first step of digestion, that involves cutting long polysaccharides by amylase, occurs. This is the case in Coleoptera, Dictyoptera, and Orthoptera.^{72,128,129}

In bugs, seed-feeding species have exclusively midgut-produced amylases, contrary to predatory species.⁷⁹ The predatory spined soldier bug *P maculiventris* injects a salivary amylase into its prey, performing an extraoral digestion,⁴⁸ like the Miridae *Lygus lineolaris*.⁴⁹ In the omnivorous Hemiptera *Apolygus lucorum*, amylase is produced mainly in salivary glands, and to a lesser extent in the midgut.¹³⁰ In Coleoptera, expression may be limited to the midgut like in *T molitor*¹²¹ but may take place also (or alternatively) in the foregut and hindgut,²²

or in the head in *I typographus*. In this species, the head-specific amylase is an unusually smaller protein due to alternative splicing.⁴³ Lepidoptera often produce amylase in their salivary glands in addition to midgut, which may be excreted through the mouth, eg, in *Sesamia nonagrioides* (Noctuidae) (Da Lage, unpublished), in the mulberry moth *Glyphodes pyloalis* (Pyrallidae) (possibly produced by different gene copies),³⁷ in *Chilo suppressalis* (Pyrallidae) with a tissular differentiation of the electromorphs,¹⁰⁹ or in *Helicoverpa armigera*.²² In *Bombyx mori*, amylase activity was also reported in hemolymph, although at a much lower level than in the digestive tract.¹⁰⁸ In the Tasar silkworm *Antheraea mylitta*, there is also a hemolymph activity.¹¹¹ In Hymenoptera, the ant *Acromyrmex subterraneus* shows amylase activity mostly in the midgut but also in labial glands.¹³¹ In the honeybee *Apis mellifera*, amylase activity is important in the hypopharyngeal gland of foragers, but not nurses.⁴⁶ Indeed, amylase is a component of honey. In *Blattella germanica* (Dictyoptera), an amylase named BGTG1 is active in the tergal gland and could play a nondigestive role by processing phagostimulating sugars that function as nuptial feeding stimulants.^{132,133}

Many studies have focused on the regulation of amylase secretion by food. At the genetic and molecular level, *Drosophila* has been the main model. *D melanogaster* larvae adapt amylase excretion to the hardness of food.¹²⁵ In the fruit fly, mostly downregulation by glucose or other sugars was reported,^{134–136} and also induction by starch, especially in larvae.¹³⁶ Glucose repression was largely dependent on the strain, therefore on the genotype.¹³⁵ Chng et al¹²⁷ have demonstrated the involvement of the transforming growth factor β /activin signaling pathway in this repression. Such regulation is classically interpreted by sparing resource when amylase is not necessary.¹⁶ In a selection experiment, it was shown that genotypes favoring low amylase activity were favored in glucose-rich environments, and that natural populations of *D melanogaster* were adapted to a sugar-rich (but variable) environment.¹⁶ This is not the case in housefly, which seems insensitive to dietary glucose, and may secrete amylase constitutively due to its polyphagous diet.³³ In the omnivorous bug *A lucorum*, amylase production is induced by vegetal food, whereas proteases are induced by animal food.¹³⁰ In the moth *H armigera*, amylase expression depends on food richness in starch and saccharose. Higher levels of sugars occurring in the natural host plant lower *H. armigera* amylase gene expression.²² In the Western corn rootworm *D virgifera* (Chrysomelidae), there is much more amylase produced on maize seedlings than on an artificial diet.⁴¹ Interestingly, amylase secretion may be upregulated in the presence of an inhibitor in *Ephestia kuehniella* as a compensation for loss of activity.³⁵ Also, amylase is upregulated upon insecticidal treatment in the cockroach *Periplaneta americana*.¹²⁹

Amylase regulation is also stage specific. Larvae and adults may have very different feeding habits; in some species adults do not feed at all. In Lepidoptera and Coleoptera, most studies were done on larvae, the stages which damage crops. *Amy* genes

may be differentially expressed in larvae or in adults, in the sense that not only the same gene may be differentially expressed,¹³⁷ but also different gene copies.⁸⁰ In Muscomorpha, *Amyrel* is expressed only in larvae.⁵⁴ In *D. ananassae*, which has six *Amy* copies, some are active in larvae but not in adults.¹⁷ In *D. serrata* and *D. lebanonensis*, larvae also express different amylase variants from those of adult flies.¹³⁶ In the bug *P. maculiventris*, the 3 isoforms show specific temporal expression.⁴⁸

Insect Amylases and Their Inhibitors

One of the most fascinating aspects of insect amylases is their relationships with plant defenses directed toward them, ie, amylase inhibitors. Phytophagous insects face inhibitory molecules produced by plants as defenses against their feeding on them. Many studies were devoted to the sensitivity or resistance to plant extracts, mainly proteinaceous inhibitors, which are abundant in cereals and leguminosae, with the goal of making transgenic plants resistant to their own pests. As shown in Table 3, a given insect amylase may be insensitive to a plant inhibitor, and sensitive to an inhibitor from another plant, and reciprocally, a given inhibitor may inhibit strongly one insect amylase but have no action on a related species. Kluh et al⁸⁰ have compared various insect species for their amylase sensitivity toward the α AI1 inhibitor from *Phaseolus vulgaris*, the most studied proteinaceous inhibitor. Importantly, there is a pH dependence in amylase/inhibitor interaction, so that the study must be done at the relevant biological pH at which interaction forms.^{80,101} There was a general trend among insect orders regarding their sensitivities (in fact more between legume feeders and the others), and at a lower taxonomic level, there were contrasted results too. For instance, *Acanthoscelides obtectus* amylase was tolerant to α AI1, but the one of *T. castaneum* was very sensitive. It is related to the fact that *A. obtectus* feeds on legume seeds. Experiments were carried out using 1% α AI1 in the food, a realistic value. Also, amylase paralogs in a species may have contrasted sensitivities toward inhibitors, and this is another adaptive response to overcome plant defenses.^{35,80,138} The pea weevil *Bruchus pisorum* is sensitive to the bean inhibitor α AI1, but not to the pea inhibitor. This led to design transgenic peas expressing the bean inhibitor in their developing seeds, yielding a high larval mortality (93%) in the pests.⁹⁵ Unfortunately, immunogenicity was reported for transgenic amylase inhibitors likely because of minor changes in molecular architecture of the transferred protein.¹³⁹ Similar experiments were done on another grain legume culture, the cowpea *Vigna unguiculata*, transgenized with the same α AI1. It became resistant to its pests *Callosobruchus maculatus* and *C. chinensis*.¹⁴⁰ Coffee plants were also transformed with α AI1 and became resistant to the coffee berry borer *Hypothenemus hampei*.¹⁴¹ α AI1 has a paralog in some wild accessions of *P. vulgaris*, named α AI2. They share 78% identity⁸⁶ but have different inhibitory properties on insects⁸³ (Table 3). None of them are able to inhibit the *Anthonomus grandis* amylase, but chimeric proteins made from

pieces of both inhibitors were able to show inhibition.⁸⁶ Note that another way to overcome plant inhibitors is to produce a lot of amylase.⁸⁴

At the molecular level, interactions between amylases and their inhibitors were studied, in part to elucidate why closely related amylases may exhibit so contrasted sensitivities. The tridimensional structure of the *T. molitor* amylase (TMA) was published, in interaction with different inhibitors.^{91,142} Compared with mammal amylases, a striking feature of TMA is the lack of the flexible loop. It was supposed to explain differences in sensitivities between mammals and insects toward some inhibitors, because in porcine amylase the existing loop is pushed away in the presence of inhibitor instead of moving toward the saccharide.⁹⁴ However, many insects have the loop, and this may not be the reason.^{143,144} Loops protruding from the inhibitors interact with the catalytic cleft of the enzyme through ionic and hydrogen bonds⁹⁸ and may also block the sugar-binding “subsites”⁹¹; the formation of the complex depends on a large number of amino acids at the interface of the two proteins.¹⁰² The sensitivity or resistance to an inhibitor depends rather from multiple incompatible structural changes rather than a single crucial mutation.⁸⁰ Importantly, the efficiency of inhibitors also depends on their own natural resistance to the insect proteases encountered in the gut.^{84,102}

Concluding Remarks

Most insects are strongly dependent on their amylases for development and survival. In this review, I have shown that the presence of several gene copies can be of interest in different ways: for more enzyme production, for fine developmental and tissue-specific expression, for broadening pH and substrate range, for overcoming the natural defenses of plants. The coevolution between insect amylases and proteinaceous plant inhibitors is a passionating adaptation paradigm and would deserve more basic studies. In this respect, more structures of insect amylases would be needed, but only the one of *T. molitor* is publicly available to date.

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JLDL designed the review, analyzed online data and wrote the article.

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