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Characterisation of class I and II α-mannosidases from *Drosophila melanogaster*

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Abstract Homology searches indicated that up to five class I α-mannosidases (glycohydrolase family 47) and eight class II α-mannosidases (glycohydrolase family 38) are encoded by the fruitfly (*Drosophila melanogaster*) genome. Selected example mannosidases were expressed in secreted form using the yeast *Pichia pastoris.* A number of characteristics of these enzymes were determined with *p*-nitrophenyl-α-mannoside as substrate; particularly striking were the low optima (pH 5) of three class II mannosidases most closely related to known lysosomal mannosidases and the distinct Co(II)-requirement of a mannosidase previously named ManIIb. Some of the recombinant mannosidases were demonstrably active towards oligomannosidic glycans, specifically, the Co(II)-requiring ManIIb, two 'acidic' mannosidases and the class I *mas-1* mannosidase. Other than previous characterisations of the well-known Golgi mannosidase II, this is the first study summarising various properties of recombinant mannosidases from the fruitfly.

Keywords Mannosidase · N-glycans · Insect

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Introduction

The metabolism of oligosaccharides involves both biosynthesis and degradation; thus, organisms require both glycosyltransferases to form glycosidic bonds and glycosidases to degrade them, the latter necessary either for the correct processing of glycans so that they attain their final structure or for their catabolism at the end of their 'life'. The sequences of the glycosyltransferases and glycosidases can be classified into a large number of protein families as listed in the CAZy database [1]. However, sequence homology alone is not necessarily a guide to the actual substrate specificity of these enzymes; therefore, a major effort in recent years in glycobiology has been the cloning and recombinant expression of genes and cDNAs encoding enzymes required for oligosaccharide metabolism.

α-Mannosidases are enzymes which, as the name suggests, remove non-reducing terminal mannose residues from glycoconjugates [2]; based on homology, there are two major classes of the α-mannosidases [3]. The class I enzymes (family 47) are present in the endoplasmic reticulum and Golgi and are responsible for the initial trimming of N-glycans to yield $Man_{5-9}GlcNAc_2$ structures; the class II enzymes (family 38) are more heterogeneous and encompass Golgi enzymes involved in processing of hybrid N-glycans to result in $Man_3GlcNAc_3$ (MGn) as well lysosomal and cytosolic enzymes involved in catabolism of glycoproteins. Defects in lysosomal mannosidases are associated with diseases in mammals [4–6], whereas a double knockout of the two Golgi mannosidase II isoforms in mice results in embryonic lethality [7]. Class II mannosidases are also found in many archaea and bacteria, whereas the class I mannosidase family appears to have one characterised bacterial member. Class I mannosidases are also related to proteins involved in protein folding in the endoplasmic reticulum (so-called EDEMs or ER degradation-enhancing α-mannosidases), which may have specificity for oligomannosidic glycans attached to misfolded proteins [8].

In the case of the fruitfly *Drosophila melanogaster*, there are only limited data regarding its mannosidases. Mutants of only one class I mannosidase (*mas-1*) are known, which display some subtle neural and eye defects [9] as well as some impact on life-span [10]. There are some effects of *mas-1* mutations on the N-glycosylation profile, including reduced levels of $Man_5GlcNAc_2$ and an accumulation of $Man_8GlcNAc_2$ [11]; the processing of N-glycans is most likely not completely abolished since there are four other homologues of class I mannosidases in the fly. However, other than a brief mention of 'unpublished data' [9], there has been no report to date regarding the activity of proteins encoded by the fruitfly *mas-1* or any other class I mannosidase gene.

As regards class II mannosidases, the *Drosophila* Golgi mannosidase II [12] has been intensively investigated in terms of its intracellular localisation, 3D-crystal structure, natural substrate specificity and interaction with inhibitors [13–20], as it plays a key role in the processing of products of *N*-acetylglucosaminyltransferase I (GlcNAc-TI). In contrast, there is a general lack of biochemical data on the other seven class II mannosidases from the fly. In terms of genetics, a gene-trap line affecting the closest homologue in the *Drosophila* genome to Sf9 α-mannosidase III (CG4606; α-Man-IIb) has a recessive lethal phenotype [21], whereas overexpression of the fruitfly α-mannosidase II (CG18802) results in a 'rough eye' phenotype and increased life span [22]. The effects of ablation and overexpression on the glycosylation profile have, however, not been examined for either mannosidase.

Recently, some of us have characterised the class I and II mannosidases of *Caenorhabditis elegans* [23, 24] and so it was of interest to examine examples of those mannosidases from the fruitfly which remained uncharacterised and thereby expand on our recent preliminary work on inhibition and crystallisation of a lysosomal mannosidase from *Drosophila* [25, 26]. Using an artificial substrate, key optima and sensitivities of the class II mannosidases were determined; also, potential natural substrates of class I and class II mannosidases were tested in HPLC-based assays. Thereby, we could prove the enzymatic functionality of a number of the α-mannosidase homologues in the fruit fly.

Material and methods

Database and homology searching: By searching of *D. melanogaster* database using the protein sequence of bovine lysosomal α-mannosidase (Genbank L31373.1) as the query, six putative lysosomal mannosidases were retrieved that are classified as glycosyl hydrolase family 38 (GH38) members in the CAZy database (Carbohydrate Active Enzymes; www.cazy.org): CG5322 (NP_609407.1) designated here LManI, CG6206 (NP_609408.1 or LM408) designated here LManII, CG9463 (NP_609250.2) designated here LManIII, CG9465 (NP_609251.1) designated here LManIV, CG9466 (NP_609252.1) designated here LManV, CG9468 (NP_609253.1) designated here LManVI. Three representatives of these were selected for further work; for comparison, one class II enzyme (ManIIb; CG4606) and two class I enzymes of the glycosyl hydrolase family 47 (Mas-1 and Mas-2) were also examined in this study. Phylogenies were examined using the Multiple Sequence Alignment server (http://www.ebi.ac.uk/Tools/msa/clustalo/) [27, 28], before visualisation using TreeView Software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) [29].

Cloning of the *Drosophila melanogaster* mannosidase cDNAs

The cDNA encoding the soluble domain of the LManII protein (*i.e.*, lacking first 33 amino acids corresponding to signal peptide of bovine lysosomal mannosidase Man2B1) was amplified by RT-PCR using total RNA from *Drosophila melanogaster* type CS embryos. The embryos (0.6 g) were homogenized in mortar with liquid nitrogen and kept at −80 °C. Total RNA from 50 mg of homogenized embryos was isolated by CV Total RNA Isolation System (Promega). The quality of RNA was checked by gel electrophoresis and concentration measured at 260 nm. The cDNA was transcribed using ImProm II™ Reverse Transcription System (Promega) and oligo dT_{18} primer. PCR amplification was performed using Expand High Fidelity PCR System (Roche Applied Sciences) as described by the manufacturer with dNTPs (200 μM each) and specific pairs of primers (0.2 μM) as listed in Table 1. The other cDNAs were cloned following RT-PCR of RNA prepared by Trizol extraction of *Drosophila*

Table 1 Primers used in this study

dLManI/for/PstI: aactgcagacaacctgtggattcgag
dLManI/rev/XbaI: gctctagatcatgatggaatatacttttgttg
dLManII/for/KpnI: ggggtacctgcggctatcagagctgc
dLManII/rev/KpnI: ggggtacctcacgctggtgttaggta
dLManV/for/AclI: ccggaacgtttcaagctaagtcagccca
dLManV/rev/KpnI: ggggtaccaattattcatgtttaatgatgaa
dManIIb/for/EcoRI: gagaattctcctcggaaacaaagtcg
dManIIb/rev/KpnI: ctgggtacccagccaccttacacctc
dMas-1/for/PstI: aaactgcagtgccgtgcaatgatg
dMas-1/rev/XbaI: gctctagagtttacgatgcgttcgaaacc
dMas-2/for/PstI: aaactgcagtgccgtgcaatgatg
dMas-2/rev/XbaI: gctctagagtttagttcgatgagttatgct
dMas-3/for/PstI: aaactgcagagtggcgatcagctgctg
dMas-3/rev/XbaI: gctctagagttaagtcttgagcggatag

CS or w^- embryos with subsequent reverse transcription using SuperScript III (Invitrogen) and Expand polymerase.

The amplification was performed in two steps: first, preincubation at 94 °C (3 min) was followed by a temperature step cycle of 94 °C (30 s), 38 °C (30 s) and 72 °C (4 min) for 15 cycles; thereafter, the annealing temperature was increased to 57 °C (30 s) for 30 cycles in order to decrease the amplification of unspecific PCR products. PCR products were gel purified using Wizard SV Gel and PCR Clean-Up System (Promega) or using a GFX kit (GE Healthcare) and cloned into the pPICZαFLAGC3 vector, adapted from the commercial plasmid [30]. The cloning was performed by direct ligation of *Kpn*I digested PCR product into *Kpn*I digested vector or *via* subcloning of PCR products into the pGEM-T Easy Vector System (Promega); positive clones were confirmed by sequencing. Additionally, the cDNA of LManII was also subsequently subcloned into pPICZαHisFLAG, another re-engineered vector [30], which encodes a hexahistidine sequence facilitating affinity purification (see Supplementary Figure 1).

Expression and purification of the fusion proteins

Plasmids were isolated using QIAGEN Plasmid Midi Kit (Qiagen), linearized by *Pme*I digestion and transformed into the *Pichia pastoris* host strain GS 115 by electroporation as described in the *Pichia* expression manual (Invitrogen). Transformants were selected on YPDS plates supplemented with Zeocin (100 μg/ml) and screened for expression of the recombinant mannosidase in a small scale experiment using methanol-containing MMYC medium (1 % methanol) at 18 °C with vigorous shaking. Samples were collected on the second, third, fourth, fifth and sixth day of induction and α-mannosidase activity determined in the supernatants. Control assays were performed using the supernatant of yeast transformed with an empty vector. The *Pichia* transformants expressing the highest level of extracellular α-mannosidase activity were used in a large-scale experiment. The optimal expression was achieved in MMYC media at 18 °C with methanol induction over 5 days. The recombinant protein was concentrated and partially purified by ammonium sulphate step precipitation with increasing amounts of ammonium sulphate (up to 75 %). The protein fraction containing major α-mannosidase activity was re-suspended in 50 mM HEPES, pH 7.5, 150 mM NaCl and dialyzed against the same buffer to remove residual ammonium sulphate and assessed by enzyme activity assay and SDS-PAGE analysis. Glycerol for protein stabilisation was added to the sample at a final concentration of 30 % and stored at −20 °C. For more detailed studies, the LManII-His-Flag protein was further affinity purified on an Ni–NTA agarose column (Qiagen) using elution with imidazole [26].

Enzyme assay and characterisation of recombinant α-mannosidases

Mannosidase activity of enzyme preparations were measured using *p*-nitrophenyl-α-D-mannopyranoside (pNP-Man; Sigma) as a substrate at a concentration of 2 mM (diluted from a 100 mM stock in dimethylsulphoxide) in 100 mM acetate buffer pH 5.2 (unless indicated otherwise) and 10–20 μl (original medium) or 1–5 μl (concentrated or purified samples), in a total volume of 50 μl for 1–2 h at 37 °C. The reactions were terminated with ten volumes (0.5 ml) of 100 mM sodium carbonate and the production of *p*-nitrophenol was measured at 410 nm using a spectrophotometer (Beckmann). The control values without substrate and using supernatant of *Pichia* transformed with empty vector (not expressing recombinant mannosidase) were subtracted. One unit of lysosomal α-mannosidase activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol in 1 min at 37 °C. All enzyme characteristics were performed under the same assay conditions in triplicates. Apparent Michaelis-Menten constants (K_m) were determined from Lineweaver–Burk double reciprocal plots [31] of assays performed at optimal pH with pNP-Man at the indicated concentration (1–5 mM). The amount of enzyme added was optimized to keep the reaction in the linear range.

To examine the effect of metal cations on enzyme activity, the enzymes were preincubated with 1 mM concentrations of either $CoCl_2$, $CdCl_2$, $MnCl_2$, $ZnCl_2$, $CuSO_4$ or EDTA in acetate buffer pH 5.2 at 37 °C for 15 min. The pNP-Man was added to a final concentration of 2 mM, and the mixture was then incubated at 37 °C for 1 h. To determine the influence of pH on enzyme activity, enzyme assays were performed using 100 mM of either McIlvaine or acetate buffers at the indicated pH ranges. The temperature optimum of enzyme reaction of each recombinant mannosidase was determined at the optimal pH in temperature range 30–55 °C. Inhibition tests to determine the half maximal inhibitory concentration (IC_{50}) and K_i-value were performed with swainsonine (Sigma) and mannostatin A (Calbiochem) at concentrations of 5–400 nM and 1–30 μM, respectively. The IC_{50} values were calculated from dose–response curves and K_i values from Dixon semi-reciprocal plots [32].

Assays with natural substrates

For assays with the pyridylaminated oligomannosidic glycans $Man_9GlcNAc_2$ (Man9-PA), $Man_8GlcNAc_2$ (Man8B-PA) or $Man_5GlcNAc_2$ (Man5-PA; Takara), 0.1 nmol substrate was incubated with 1 μl of unconcentrated enzyme preparation and 2 μl 0.4 M 2-morpholinoethanesulphonic acid buffer (pH 6) in a final volume of 10 μl in a PCR tube at 30 °C for 14 h. ManIIb was assayed in the presence of 10 mM $CoCl_2$ and Mas-1 in the presence of 10 mM $CaCl_2$. The products

were analysed by RP-HPLC (gradient of 0.3 % methanol per minute with fluorescence detection) and MALDI-TOF MS of HPLC fractions (using 6-aza-2-thiothymine as matrix and a Bruker Ultraflex II mass spectrometer) as recently described for *C. elegans* class I mannosidases [23].

Results and discussion

Identification of mannosidase homologues from the fruitfly

Class I and class II mannosidases have been studied from a number of species; in a recent study [23], a family tree of class I mannosidases was proposed and showed that possibly five homologues are encoded by the fruitfly genome. Mas-1 and Mas-2 are putative Golgi class I mannosidases and Mas-3 is closest to those resident in the endoplasmic reticulum (ER); these numbers are the same as in *Arabidopsis thaliana,* whereas *C. elegans* possesses two ER and two Golgi and humans one ER and three Golgi class I mannosidases. Two further class I mannosidase-like genes are predicted to encode EDEM-type proteins involved in ER quality control (as compared to three in both *C. elegans* and *H. sapiens*). It was attempted to clone open reading frames (suitable for secreted protein production) of the first three; both *mas-1* and *mas-2* clones could be isolated, but none of *mas-3*.

For the class II mannosidases, we reassessed a phylogeny previously shown by others [33]; this was in part necessary since the predicted open reading frames in the databases for some of the sequences lacked the regions encoding a typical secretion signal. We also performed standard tBLASTn searches using known class II mannosidase sequences. The sequences of two predicted mannosidases were corrected *in silico* by manually examining the 5′ genomic region. In total, eight fruitfly class II mannosidases were included in the analysis, alongside other insect, nematode and mammalian homologues. The results (Fig. 1) suggest that there are six *D. melanogaster* mannosidases most closely related to lysosomal 'general' α-mannosidases from mammals (LAMAN; MAN2B1) and the putative lysosomal mannosidase AMAN-1 from *C. elegans*, but appeared to be distinct from the lysosomal 'epididymal' α1,6-mannosidase (MAN2B2). These six fruitfly sequences were designated as LManI through to LManVI. Four of these (LManIII-VI; CG9463, CG9465, CG9466 and CG9468) are present as a tandem in the same genomic region (within 15 kbp; 29 F1 on the cytological map), whereas the CG5322 (LManI) and CG6206 (LManII) genes are organised 'head-to-tail' in the region 31E5. In case of LManII (previously designated LM408 in two previous reports [25, 26]) there are two transcripts, whose predicted reading frames differ by 22 amino acids in a non-conserved region (residues 276 to 320); the existence of two transcripts is apparently due to alternative splicing (utilisation of either exon 5 or exon 6). In total, the LManII gene has 11 exons, whereas LManI has seven exons and the other four genes possess six exons each. All six putative mannosidases exhibit

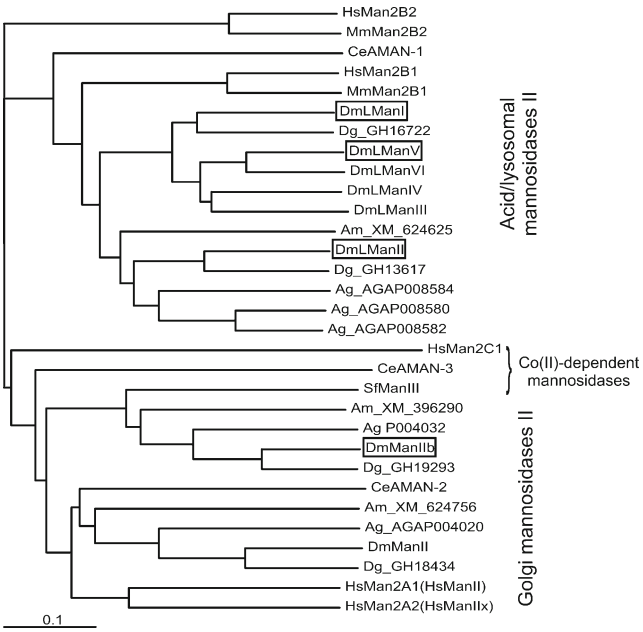
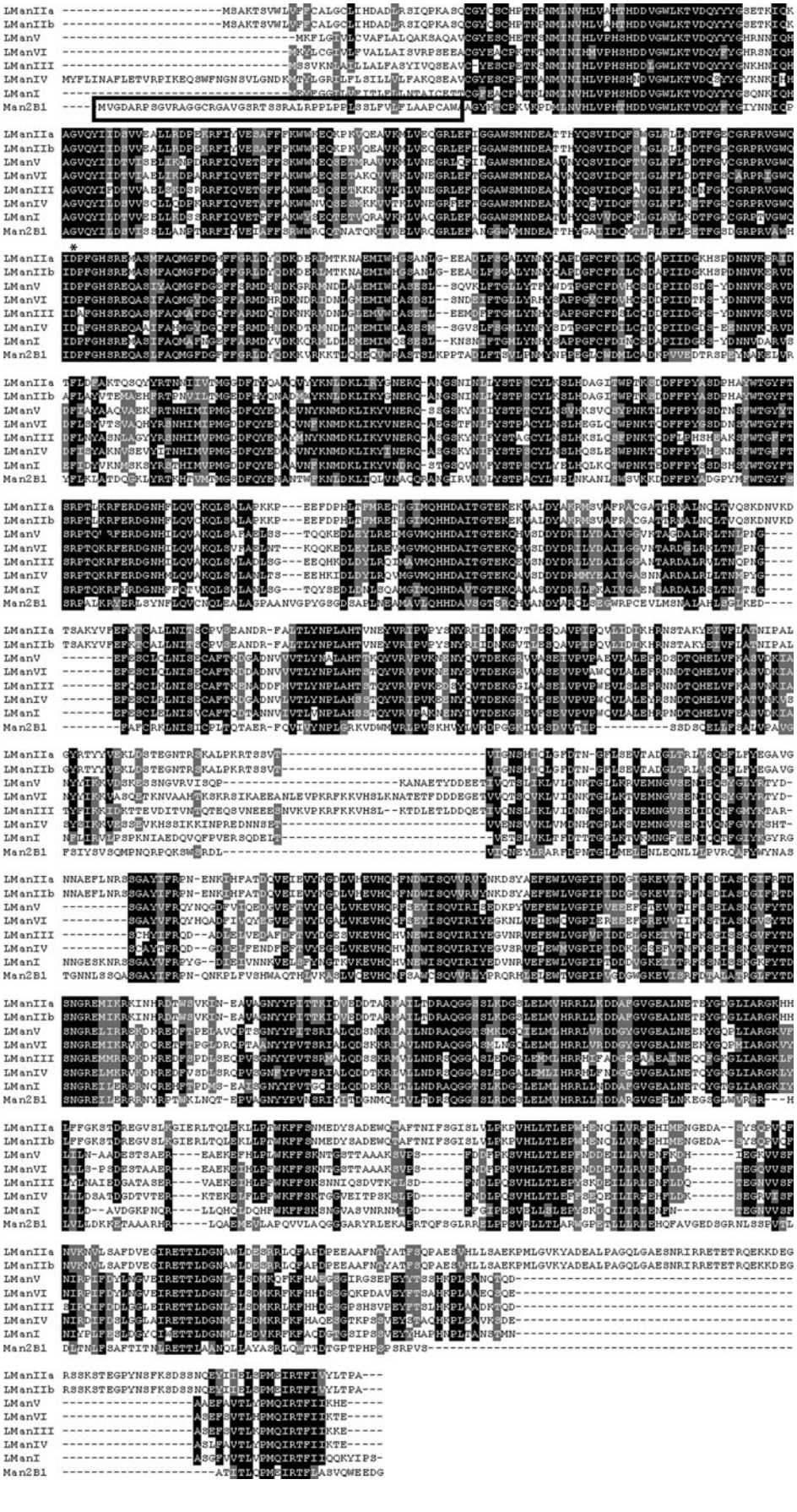


Fig. 1 A phylogeny of class II mannosidases. Selected mannosidases of GH38 family were analysed using the Multiple Sequence Alignment server (http://www.ebi.ac.uk/Tools/msa/clustalo/) and the resulting phylogenetic data visualised using TreeView Software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The abbreviations for the respective organism are as follows: *Hs*, *Homo sapiens; Mm, Mus musculus; Dm*, *Drosophila melanogaster; Ce*, *Caenorhabditis elegans; Ag*, *Anopheles gambiae; Am, Apis mellifera; Dg, Drosophila grimshawi.* The names of the latter three protein sequences include GenBank accession numbers in their names. Other protein names were taken from the CAZy database (http://www.cazy.org/GH38_eukaryota.html), where Man2A1(ManII)/Man2A2(ManII^{x}) are the two mammalian Golgi mannosidases, Man2B1/Man2B2 are the two mammalian lysosomal mannosidases (one general mannosidase and one α1,6-specific mannosidase), Man2C1 is the mammalian cytosolic mannosidase and AMAN-1, AMAN-2 and AMAN-3 are, respectively, acidic, Golgi and Co(II)-dependent mannosidases from *C. elegans. Drosophila* class II mannosidases were designated as follows: ManII (CG18474), ManIIb (CG4606), LManI (CG5322), LManII (CG6206), LManIII (CG9463), LManIV (CG9465), LManV (CG9466) and LManVI (CG9468). From other insects, sequences are included for the Sf9 mannosidase III as well as mannosidases from *Anopheles gambiae*, *Apis mellifera* and *Drosophila grimshawi*. The four class II mannosidases examined in this study are indicated by *boxes*

Fig. 2 Sequence alignments of lysosomal mannosidases. Multiple ▶ sequence alignment of the predicted protein sequences of *Drosophila* potential lysosomal mannosidase as compared to the bovine lysosomal mannosidase Man2B1 using ClustalW Multiple Sequence alignment program (http://www.genome.jp/tools/clustalw/) and Protein Box Shade program (http://www.fr33.net/boxshadeprotein.php). The signal sequence of Man2B1 (1–50 amino acids) is framed. Essential amino acid residues of Man2B1 including nucleophile D197 of the active site (marked with an asterisk) as well as metal binding residues H73, D75, D197 and H448 are well conserved in all *Drosophila* ORFs


LManIIa
LManIIb
LManV
LManVI
LManIII
LManIV
LManI
Man2B1

similar identity to bovine lysosomal mannosidase (38 % to 46 %; see also Fig. 2) and all contain the aspartate residue identified as the catalytic nucleophile in bovine lysosomal mannosidase [34].

Of the other two fruitfly class II mannosidases, one is the well-characterised Golgi mannosidase II (designated ManII, CG18474) [13] and also closest to the *C. elegans* AMAN-2 enzyme, whereas the other (designated ManIIb, CG4606) is potentially related to the Co(II)-activated *C. elegans* AMAN-3 [24] and is also 'close' to the so-called mannosidase III from the Sf9 lepidopteran cell line [35]. Interestingly, according to tBLASTn of whole genome shotgun sequences, the mosquito *Anopheles gambiae* has three putative lysosomal mannosidase genes (in the same genomic region), *Drosophila*

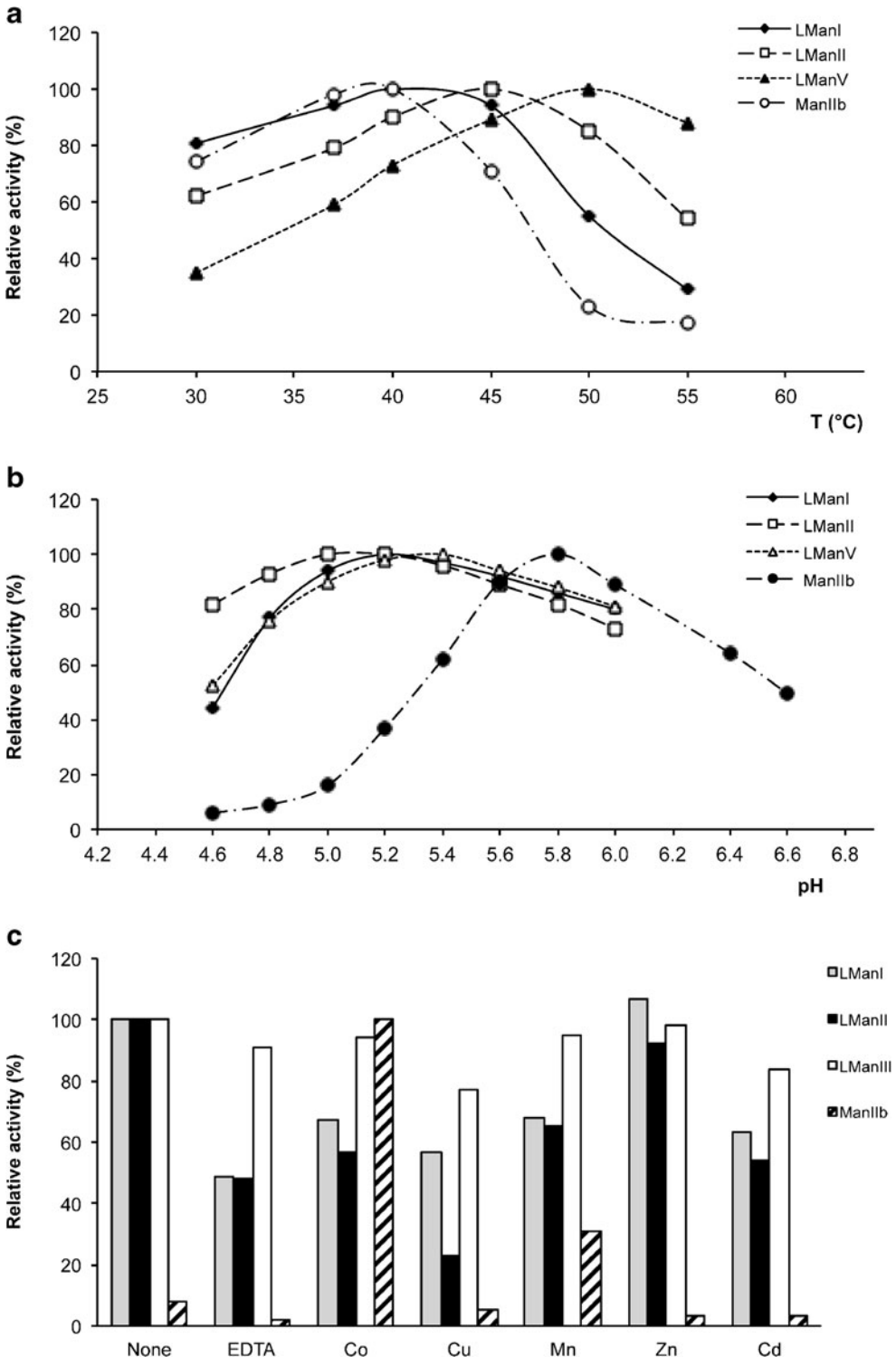


Fig. 3 Characterisation of *Drosophila* class II mannosidases. Ammonium sulphate fractions of media of yeast expressing *Drosophila* mannosidases LManI, II and V or ManIIb were assayed in triplicate with *p*-nitrophenyl-α-mannoside for 1–3 h using citrate buffers at different temperatures (**a**), with differing pH values (**b**) and in the presence of 1 mM of various cations or EDTA (**c**). The amount of enzyme activity was expressed in relative activity calculated after subtracting the values of incubations lacking substrate from the observed absorbance data at 410 nm (A_{410}). The conditions resulting in maximal activity are normalised to be 100 % for the temperature and pH optima; for the cation dependency, the activity of the LMan enzymes (*grey, black* and *white bars*) is normalised to be 100 % in the absence of added cations and the activity of ManIIb (*striped bars*) is normalised to 100 % in the presence of Co(II)

grimshawi has two (at different loci) and the honeybee *Apis mellifera* only one such homologue, in addition to one homologue each of mannosidase II and mannosidase IIb; these sequences are included in the phylogenetic analysis (Fig. 2). Other than *D. grimshawi,* sequenced *Drosophila* species have the same number of putative lysosomal mannosidases as *D. melanogaster*, which suggests expansion of this gene family during evolution of *Drosophila* species, but after their divergence from other insects.

Expression of recombinant fruitfly mannosidases in yeast

As *Pichia pastoris* has been used successfully to express human and nematode mannosidases in the past, we used this host for expression of selected homologues from the fruitfly. Both fruitfly class I mannosidases (Mas-1 and Mas-2) were expressed as judged by either Coomassie staining or anti-FLAG Western blotting of media of yeast transformed with the relevant constructs. For the fruitfly class II mannosidases, ManIIb as well as a selected three of the six 'lysosomal' mannosidases were expressed (three other highly-homologous 'lysosomal' mannosidases were not cloned, whereas the Golgi mannosidase II could not be successfully expressed in yeast). The activity of the four recombinant class II mannosidases (ManIIb, LManI, LManII and LManV) was initially tested in media using *p*-nitrophenyl-α-mannoside as substrate. Due to its sequence similarity to Co(II)-activated enzymes, ManIIb activity was screened in the presence and absence of Co(II); indeed, the activity was far higher in the presence of this cation. The two class I enzymes were only tested with an oligomannosidic glycan (see below), as it is known that this type of mannosidase does not accept the aryl monomannoside substrate. In general, in the subsequent studies ammonium sulphate fractions of the *Pichia* supernantant were used as an enzyme source. However, LManII was also prepared as a His-tag purified protein, whose dimerisation, proteolytic processing and preliminary crystallisation have already been reported [26]; the dimerisation of LManII under non-reducing conditions is similar to that observed with, *e.g.*, the native bovine mannosidase [36] and the recombinant form of the acidic mannosidase from tomato [37].

Characteristics of fruitfly class II mannosidases

After initial optimisation, the effects of temperature on enzyme activity were tested at pH 5.2 for the lysosomal enzymes and at pH 5.8 (in the presence of Co(II)) in the case of ManIIb; the optimum for the latter was at 37 °C, whereas the three lysosomal enzymes were most active in the range 40–50 °C (Fig. 3a). A typical hallmark of lysosomal enzymes is an acidic pH optimum, whereas Golgi enzymes tend to have an optimum of around pH 6. The assays indeed indicated that the three putatively lysosomal enzymes had pH optima of around pH 5, whereas ManIIb had one of pH 5.8 (Fig. 3b). The effects of metal ions also distinguished the 'acidic' mannosidases from ManIIb. Except for Zn(II), which is present in the active site of Golgi mannosidase II and bovine lysosomal mannosidase, all other cations tended to have negative effects on the activity of these mannosidases, whereas ManIIb was 13-fold more active in the presence of Co(II) as compared to the control with no added cations. Only Mn(II) also had a positive effect on the activity of this enzyme (Fig. 3c). This pattern of activation and the pH optimum of ManIIb are highly reminiscent of the properties of mannosidase III from Sf9 cells [35]; also, Co(II) activation to varying extents has been reported for the cytosolic mammalian MAN2C1 mannosidase [38], human lysosomal α1,6-mannosidase [39] and a Gingko mannosidase [40].

In terms of kinetic characteristics, the K_m values for all enzymes towards the simple substrate were in the millimolar range (1.2–2.5 mM; Table 2 and Supplementary Figure 2). In terms of the use of known mannosidase inhibitors, screening with at least three concentrations of swainsonine [41] and mannostatin A [42] indicated that all four enzymes were sensitive to these reagents. The K_i values for the two inhibitors were determined using Dixon plots and varied in the range 2.9–71 nM for swainsonine and 0.075–13 μM for mannostatin A (Table 2 and Supplementary Figure 3). These values in the nM-μM range are similar to those obtained for, *e.g.*, *Drosophila* and human Golgi mannosidase II and human lysosomal mannosidases [13, 15, 17, 39, 43]. The IC_{50} values presented here for LManII and ManIIb are also similar to those previously published [25].

Activity towards natural N-glycan substrates

The activities of fruitfly class I and class II mannosidases were assessed using pyridylaminated oligomannosidic glycans and enzyme incubations were analysed by RP-HPLC in

Table 2 Kinetic parameters of recombinant *Drosophila* class II mannosidases. The pH value represents the optimal pH at which measurements were performed. K_m is Michaelis constant for pNP-Man as substrate; rounded values are shown for K_m, IC_{50} and K_i. The values for *Drosophila* Golgi mannosidase II (ManII) from the literature [13, 15, 17, 49] were added for comparison (* K_m was determined for 2,4-dinitrophenyl α-mannoside)

Enzyme	pH	K_m (mM)	Swainsonine (nM)		Mannostatin (μM)	
			IC_{50}	K_i	IC_{50}	K_i
LManI	5.2	2.1	95	65	1.8	1.1
LManII	5.2	2.3	12	7.1	3.3	3.1
LManV	5.2	1.2	130	71	15	12
ManIIb	5.8	2.5	4.0	2.7	0.15	0.1
ManII	5.7[13]	*5.5[49]	17[13]	20[17]	n.d	0.036[15]

combination with MALDI-TOF MS in order to determine the isomeric status and mass of the products; the class I mannosidases were assayed in the presence of Ca(II) as this has been previously shown to be essential for such enzymes [44], whereas ManIIb was assayed in the presence of Co(II). The class I mannosidase Mas-1 was active towards $Man_9GlcNAc_2$-PA and, as expected for a Golgi resident member of this enzyme family, it primarily processed the glycan to an isomer of $Man_6GlcNAc_2$ (~8 g.u.) with an intact 'B' antenna (*i.e.*, the 'middlemost' α1,2-mannose normally processed by ER mannosidases; Fig. 4a) as well as, when incubations were performed for extended periods of time, to $Man_5GlcNAc_2$ (7 g.u.; data not shown); this would indicate that this enzyme can remove all α1,2-mannose residues from $Man_9GlcNAc_2$. Yeast transformed with clones of Mas-2 isolated from two different fly strains did not display any activity towards this substrate (Fig. 4a), despite confirming the expression of the protein (data not shown). This may be due to a different substrate specificity or to the lack of a conserved sequence in the polypeptide; indeed, a part of the helix α8 including a conserved aspartate residue, close to the Ca(II)-binding site observed in the crystal structure of a mammalian class I mannosidase [45], is not present in the predicted Mas-2 protein sequence.

Of the four class II mannosidases examined, three were able to remove mannose residues to various extents. When incubated with $Man_9GlcNAc_2$, LManI and ManIIb trimmed the substrate to $Man_8GlcNAc_2$ and, to a lesser extent, to $Man_7GlcNAc_2$, whereas LManII produced a number of products ranging from $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ (Fig. 4b; see also Supplementary Figure 4); based on the known RP-HPLC retention times of pyridylaminated oligomannosidic glycans [46], it is concluded that the Man8C isomer is the dominant first product of these three enzymes (Man8A would elute at ~5.7 g.u. and Man8B at ~4.8 g.u., as compared to Man8C at ~6.4 g.u.). Similarly when $Man_8GlcNAc_2$ was used as a substrate, LManII removed a number of mannose residues, whereas LManI and ManIIb apparently acted inefficiently (Fig. 4c). In the case of $Man_5GlcNAc_2$, only with ManIIb were $Man_{3-4}GlcNAc_2$ digestion products observed (Fig. 4d). LManV was not observed to digest any oligomannosidic glycans under the conditions employed; this result is akin to the apparent lack of activity towards natural substrates of recombinant *C. elegans* AMAN-1 [24].

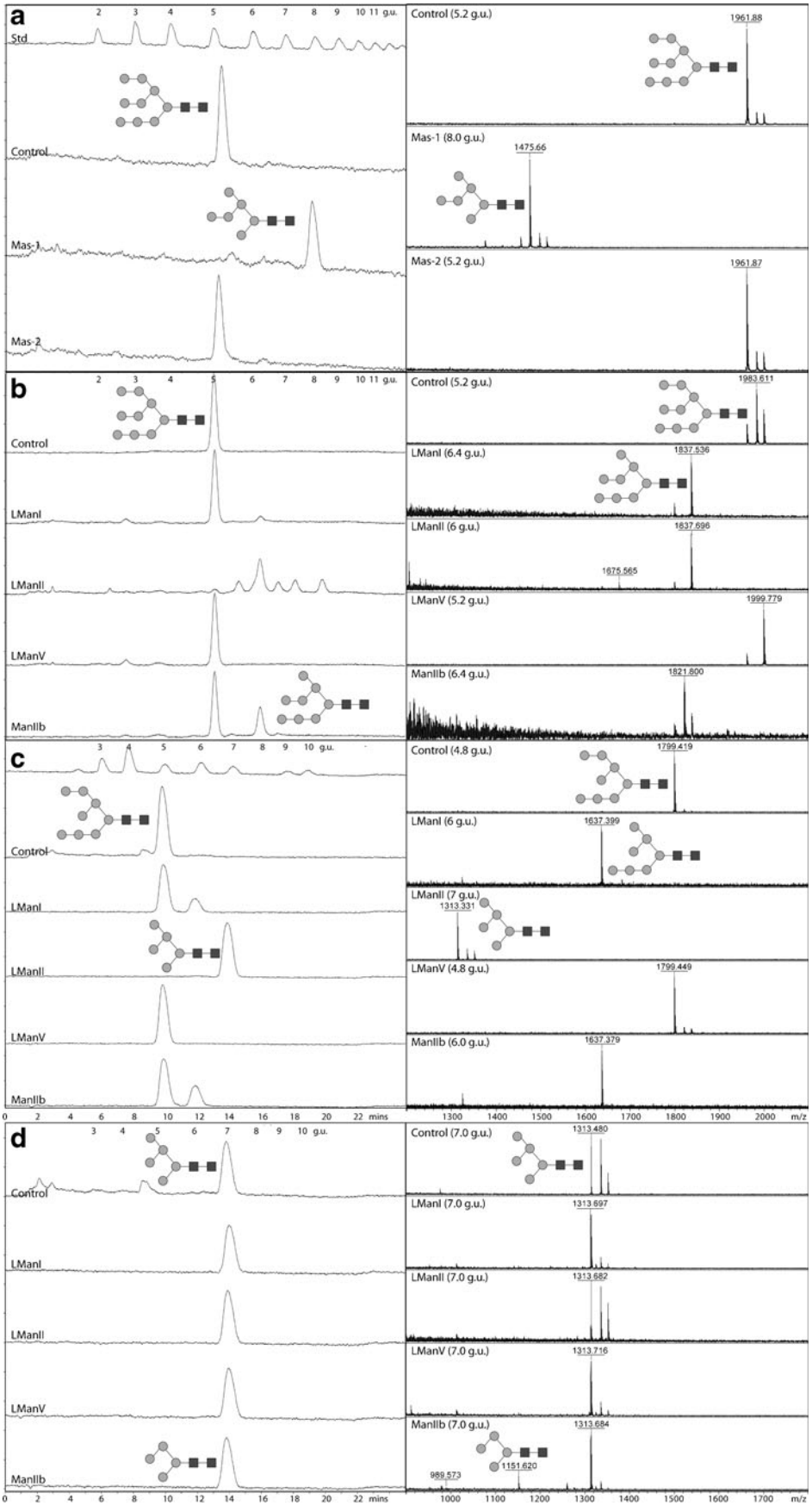
It is known that some acidic mannosidases have narrowly-defined substrate requirements; for instance, the second mammalian lysosomal mannosidase (also known as an epididymal mannosidase) specifically acts as an α1,6-mannosidase to remove the last α-linked mannose from $Man_2GlcNAc_2$. On the other hand, there are contrasting data as to whether the major lysosomal mannosidase requires Zn(II) ions for the digestion of $Man_5GlcNAc_1$ [47, 48]. The acidic mannosidases (LManI, II and V) examined here appear to be members of the main lysosomal mannosidase clade and not be closely related to the 'epididymal' α1,6-mannosidase [33] and the processing of $Man_9GlcNAc_2$ by LManI and LManII *via* Man8C is also similar to that of both jack bean and native human lysosomal mannosidases [46, 47].

The specificity of the Co(II)-dependent ManIIb is somewhat similar to that of the Sf9 mannosidase III; the latter enzyme tends also to only remove single mannose residues when 'challenged' with larger oligomannosidic glycans. ManIIb could also digest $Man_5GlcNAc_2$ to $Man_{3-4}GlcNAc_2$, which raises the question as to whether ManIIb may, as proposed for mannosidase III [35], be active in an alternative processing pathway in the Golgi bypassing class I mannosidases or the GlcNAc-TI-dependent Golgi mannosidase II. As ManIIb apparently has a signal sequence, it is probably not cytosolically located like its Co(II)-activated mammalian 'cousin' MAN2C1 [38], which is involved in catabolism of glycan chains from glycoproteins exported into the cytosol as a part of the ER associated degradation machinery. Indeed, it would appear that cytosolic mannosidases are absent from nematodes and from the fruit fly.

Fig. 4 Analysis of incubations of *Drosophila* mannosidases with natural substrates. Reaction products of pyridylaminated oligomannosidic N-glycans with *Drosophila* class I (**a**) and class II (**b**, **c** and **d**) were examined by RP-HPLC with fluorescence detection (*left*); fractions were collected and analysed by MALDI-TOF MS with the spectra for one selected major fraction from each chromatogram being shown (*right*). RP-HPLC columns were calibrated with a pyridylaminated dextran partial hydrolysate of either 2–20 or 3–10 glucose units (g.u.). Incubations of class I mannosidases Mas-1 and Mas-2 with $Man_9GlcNAc_2$ (**a**) and of class II mannosidases (LManI, LManII, LManV and ManIIb) with $Man_9GlcNAc_2$ (**b**), $Man_8GlcNAc_2$ (**c**) and $Man_5GlcNAc_2$ (**d**) show diagnostic shifts in retention time, which can be correlated with the *m/z* values for the isolated glycans detected either as $[M+H]^+$, $[M+Na]^+$ and/or $[M+K]^+$. Selected spectra and chromatograms are annotated with example glycans depicted according to the nomenclature of the Consortium for Functional Glycomics (mannose, circles; *N*-acetylglucosamine, squares). Spectra of four major products of LManII digestion of $Man_9GlcNAc_2$ are shown in Supplementary Figure 4

Conclusion

In this study, we have examined a number of characteristics of a number of α-mannosidases from *D. melanogaster*. As expected from its homology, the class I mannosidase encoded by the *mas-1* gene was shown to remove all α(1-2)-mannose residues from an oligomannosidic glycan; one of the acidic class II mannosidases (LManII) could also similarly digest such glycans. The activity of another class II mannosidase (ManIIb) was dependent on the presence of Co(II) ions and its pH optimum is suggestive of a Golgi localisation. Remarkable is the number of potential lysosomal mannosidases in this


a
Std
Control
Mas-1
Mas-2
2 3 4 5 6 7 8 9 10 11 g.u.
Control (5.2 g.u.)
1961.88
Mas-1 (8.0 g.u.)
1475.66
Mas-2 (5.2 g.u.)
1961.87
b
Control
LManI
LManII
LManV
ManIIb
Control (5.2 g.u.)
1983.611
LManI (6.4 g.u.)
1837.536
LManII (6 g.u.)
1837.696
1675.565
LManV (5.2 g.u.)
1999.779
ManIIb (6.4 g.u.)
1821.800
c
Control
LManI
LManII
LManV
ManIIb
3 4 5 6 7 8 9 10 g.u.
0 2 4 6 8 10 12 14 16 18 20 22 mins
Control (4.8 g.u.)
1799.419
LManI (6 g.u.)
1637.399
LManII (7 g.u.)
1313.331
LManV (4.8 g.u.)
1799.449
ManIIb (6.0 g.u.)
1637.379
1300 1400 1500 1600 1700 1800 1900 2000 m/z
d
Control
LManI
LManII
LManV
ManIIb
3 4 5 6 7 8 9 10 g.u.
0 2 4 6 8 10 12 14 16 18 20 22 mins
Control (7.0 g.u.)
1313.480
LManI (7.0 g.u.)
1313.697
LManII (7.0 g.u.)
1313.682
LManV (7.0 g.u.)
1313.716
ManIIb (7.0 g.u.)
1313.684
1151.620
989.573
1000 1100 1200 1300 1400 1500 1600 1700 m/z

organism whose genetic loci are on two chromosomal regions suggesting expansion of the class II mannosidase gene family during *Drosophila* evolution; three of these six mannosidases are shown here to have acidic pH optima supporting their putative intracellular localisation. In contrast, *D. grimshawi* and *A. gambiae* apparently possess only two or three lysosomal mannosidase genes, compatible with their phylogenetic distance to *D. melanogaster*. Thereby, the repertoire of α-mannosidases in *D. melanogaster* shows some distinct differences in comparison to other invertebrates and vertebrates; the biological significance of the apparent absence of a cytosolic α-mannosidase, the presence of a GlcNAc-TI-independent Golgi mannosidase and the abundance of acidic mannosidases in the fruit fly remains to be assessed.

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