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The class I α1,2-mannosidases of *Caenorhabditis elegans*

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Abstract During the biosynthesis of N-glycans in multicellular eukaryotes, glycans with the compositions $Man_{5\text{-}}GlcNAc_{2\text{-}3}$ are key intermediates. However, to reach this 'decision point', these N-glycans are first processed from $Glc_3Man_9GlcNAc_2$ through to $Man_5GlcNAc_2$ by a number of glycosidases, whereby up to four α1-2-linked mannose residues are removed by class I mannosidases (glycohydrolase family 47). Whereas in the yeast *Saccharomyces cerevisiae* there are maximally three members of this protein family, in higher organisms there are multiple class I mannosidases residing in the endoplasmic reticulum and Golgi apparatus. The genome of the model nematode *Caenorhabditis elegans* encodes seven members of this protein family, whereby four are predicted to be classical processing mannosidases and three are related proteins with roles in quality control. In this study, cDNAs encoding the four predicted mannosidases were cloned and expressed in *Pichia pastoris* and the activity of these enzymes, designated MANS-1, MANS-2, MANS-3 and MANS-4, was verified. The first two can, dependent on the incubation time, remove three to four residues from $Man_9GlcNAc_2$, whereas the action of the other two results in the appearance of the B isomer of $Man_8GlcNAc_2$; together the complementary activities of these enzymes result in processing to $Man_5GlcNAc_2$. With these data, another gap is closed in our understanding of the N-glycan biosynthesis pathway of the nematode worm.

Keywords Golgi mannosidase I · Endoplasmic reticulum mannosidase · Nematode

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Introduction

In recent years, major advances have been made in dissecting N-glycan structures and biosynthesis in various mode organisms such as the plant *Arabidopsis thaliana*, the fruitfly *Drosophila melanogaster* and the nematode worm *Caenorhabditis elegans*. Surprisingly, it is the N-glycome of the latter, which is probably most complex [1] and manipulation of the latter stages of its N-glycan biosynthetic pathway demonstrates the presence of many, sometimes unexpected, fates for the N-glycans of this organism [2–6]. In comparison, relatively little attention has been paid to the earlier stages of this pathway in the worm, although a recent RNAi screen of tunicamycin sensitivity is of interest regarding the enzymes required for biosynthesis of the dolichol-linked glycan precursors [7]. Additionally, there is almost no information regarding the enzymes catalysing the steps between the transfer of the $Glc_3Man_9GlcNAc_2$ precursor oligosaccharide to protein and the actions of *N*-acetylglucosaminyltransferase I (GlcNAc-TI) and mannosidase II (AMAN-2). In comparison to other multicellular organisms, it can be hypothesised that, after the action of glucosidases I and II, class I mannosidases may process $Man_{8\text{-}9}GlcNAc_2$ down to the substrate for GlcNAc-TI, $Man_5GlcNAc_2$ [8].

The first class I mannosidase to be molecularly characterised was the Mns1p endoplasmic reticulum mannosidase from *Saccharomyces cerevisiae* [9, 10], which cleaves a single α1-2-mannose residue to yield a specific isomer of $Man_8GlcNAc_2$ (Man8B, also known as Man8.1). In mammals, a semi-purified Ca^{2+}-dependent activity with similar function was found, which was inhibited by kifunensine, but not by swainsonine, and which was inactive towards aryl mannosides such as *p*-nitrophenyl-α-mannoside [11]. Later when the mammalian orthologues of yeast Mns1p were characterised recombinantly, these properties were confirmed [12, 13].

Whereas in yeast, $Man_8GlcNAc_2$ is a major substrate for subsequent hypermannosylation in the Golgi apparatus [14], the biosynthesis of complex and hybrid N-glycans in mammals requires the removal of a further three α1-2-mannose residues in the Golgi apparatus. Initially, a single Golgi mannosidase I activity was found in rat liver, but later the presence of a second related but distinguishable enzyme was surmised [15]. Finally, three genes encoding mammalian Golgi mannosidases 1A, 1B and 1C (also formerly known as Man_9-mannosidases) were identified and showed homology to the yeast Mns1p [16–19]; these sequences are grouped in the glycohydrolase family 47. Perhaps confusingly, the genetic nomenclature is such that these genes were christened *MAN1A1*, *MAN1A2* and *MAN1A3*, whereas the gene encoding endoplasmic reticulum mannosidase (sometimes referred to as endoplasmic reticulum mannosidase I) is named *MAN1B1*; mutations in this gene are associated with nonsyndromic autosomal-recessive intellectual disability in humans [20]. A kifunensine-insensitive enzyme referred to as 'endoplasmic reticulum mannosidase II' or 'ER/cytosolic mannosidase' [21] is actually unrelated to the α1,2-mannosidases and was later identified as a Co^{2+}-activated cytosolic class II mannosidase, a member of glycohydrolase family 38 encoded by the *MAN2C1* gene [22].

In addition to the α1,2-mannosidases whose activities have been proven with recombinant forms of these enzymes, there are other related proteins with roles in quality control in the endoplasmic reticulum. In particular, these proteins are important in the identification of misfolded proteins destined for degradation and are part of the ERAD (endoreticulum-associated degradation) system. Due to their role and their homologies, they have gained the name 'endoplasmic reticulum degradation enhancing α-mannosidase-like proteins' (EDEMs) [23]. These proteins at least accelerate trimming of specific mannose residues from misfolded proteins, a process, which was until recently only indirectly measured by examining glycans and glycoproteins from whole cells [24–26]; until now there is no published *in vitro* evidence for the enzymatic activity of mammalian EDEMs or of the related yeast YLR057W protein. This is potentially because misfolded proteins may be the only substrates and so typical *in vitro* assays with either the Manα1-2Man disaccharide or with oligomannosidic glycans will fail. Only for yeast Htm1p/Mnl1p was activity recently demonstrated [27, 28].

In eukaryotes other than mammals or yeast, there is relatively little literature regarding class I mannosidases or the related EDEM proteins. The *Drosophila* Golgi mannosidase I (*mas-1*) gene was identified some fifteen years ago [29], but the evidence for its activity as a recombinant protein was only mentioned as 'unpublished data'. The obvious role of *mas-1* in glycan processing was shown by examining the structures of oligosaccharides from relevant fruitfly mutants, but the data indicated that some processing beyond $Man_8GlcNAc_2$ was possible in *mas-1* mutants and so were suggestive of an alternative processing pathway [30]; indeed, homology searching indicates the presence of a second putative Golgi mannosidase I as well as a potential endoplasmic reticulum mannosidase in the fruitfly (Fig. 1). More recently, three class I mannosidases from *Arabidopsis thaliana* were characterised: MNS1 and MNS2 (also known as AtMANIa or AtMANIb) showed the typical activities of Golgi mannosidases I and MNS3 had the more restricted substrate specificity of endoplasmic reticulum mannosidase [31, 32].

In the case of *C. elegans*, there is even less information: only from commercially-oriented biotechnological studies on enzymes used to remodel glycosylation in the yeast *Pichia pastoris* can it be surmised that two class I mannosidases of *C. elegans* are actually active [33]. Therefore, as a complement to previous studies on the class II mannosidases of this organism [3], I set out to isolate cDNA fragments encoding the class I mannosidases of *C. elegans* and verify that recombinant forms of these mannosidases were enzymatically active. Indeed these data show, that seemingly unlike other organisms examined to date, *C. elegans* possesses two class I mannosidases with the typical 'endoplasmic reticulum' type of activity and two which can be predicted to reside in the Golgi apparatus.

Experimental procedures

Cloning of cDNA fragments and expression in yeast

In order to isolate cDNA fragments encoding putative class I mannosidases, the relevant genes were identified by homology. The alphanumeric predicted reading frame numbers are taken from Wormbase; alignments and phylogenetic analysis were performed using the Multalin server [34]. cDNA fragments encoding the putative lumenal domains of *Caenorhabditis elegans* class I mannosidases were then isolated by RT-PCR using Superscript III reverse transcriptase (Invitrogen) and Expand DNA polymerase (Roche). The following primer pairs were employed:

D2030.1	MANS-1/1/PstI: aaactgcagtatttccgagaagaaaactc
	MANS-1/2/XbaI: gctctagattaatgccgaatgcggaatg
C52E4.5	MANS-2/1/PstI: aaactgcaggaatctcataatgcagtagt
	MANS-2/2/XbaI: gctctagactaattagttaagactggaacc
T03G11.4	MANS-3/1/PstI: aaactgcagagagaacagccgatagttgaa
	MANS-3/2/XbaI: gctctagattaatgatcataaattggcaatg
ZC410.3	MANS-4/1/PstI: aaactgcaggaacttctcaatcctagaaga
	MANS-4/2/XbaI: gctctagatcaattgtaaattggaagtggg

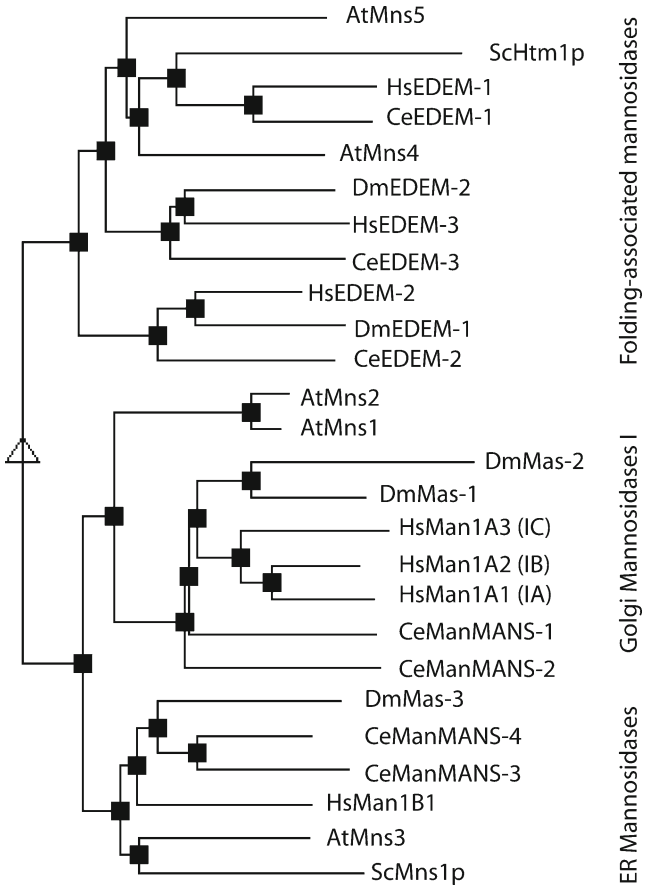

Fig. 1 A phylogeny of class I mannosidases. Selected mannosidases from *A. thaliana, C. elegans*, *D. melanogaster*, *H. sapiens* and *S. cerevisiae* are analysed using the Multalin server [34]. Three major groups are apparent: the folding-associated mannosidases/mannosidase-like proteins, the Golgi type I mannosidases and the endoplasmic reticulum mannosidases

PCR products were cloned into either the pGEM-T (Promega) or pSTBlue-1 (Novagen) vectors and selected clones sequenced. After restriction digestion of the plasmid DNAs with PstI and XbaI to release the mannosidase cDNA fragments, these were ligated into a form of the pPICZαC vector modified to encode a FLAG-tag following the secretion signal; competent *Pichia pastoris* GS115 cells were transformed with two clones encoding each mannosidase. Expression was induced with methanol at 16 °C for up to five days and culture supernatants were collected. Prior to SDS-PAGE and Western blotting, 100 μl aliquots of the culture supernatants were subject to precipitation with a five-fold volume of methanol and the pellets were dissolved in reducing SDS-PAGE buffer. Nitrocellulose membranes were incubated with anti-FLAG 1:10000 (Sigma-Aldrich) followed by alkaline phosphatase-conjugated anti-mouse IgG and chromogenic detection. Tryptic peptide mapping was performed by in-gel digestion of excised Coomassie-stained bands, prior to extraction with acetonitrile/water and MALDI-TOF MS using ACH as matrix.

Mannosidase assay and product analysis

Mannosidase activity was determined using Man9 ($Man_9GlcNAc_2$) as substrate; based on the relative expression levels as judged by Western blotting, the volumes of the supernatants of recombinant *Pichia* clones was varied between 0.5 and 2 μl. For each assay, 10 pmol of pyridylaminated Man9 (2 μM final concentration; Takara) was incubated with the supernatant in the presence of final concentrations of 80 mM MES, pH 7, and 10 mM $CaCl_2$ at room temperature. After the stated time, the incubations were directly injected onto a RP-HPLC column (Hypersil ODS, 5 μ, 4×250 mm) and the glycan products eluted with a linear gradient of 0.3 % methanol per minute (in 0.1 M ammonium acetate, pH 4); detection was by fluorescence (320/400 nm). Peaks were collected, lyophilised, redissolved in water and analysed using 6-aza-2-thiothymine (ATT) as matrix for MALDI-TOF MS (matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry) in positive mode using a Bruker Ultraflex equipped with a nitrogen laser.

Results and discussion

Phylogeny of class I mannosidases

As a preparation for cloning nematode members of the class I mannosidase (class 47 glycohydrolase) family, the 'family tree' of a number of class I mannosidases and 'mannosidase-like' proteins was examined (Fig. 1). Four 'classical' (*i.e.*, non-EDEM-type) *C. elegans* can be identified; these have the Wormbase reading frame numbers D2030.1, C52E4.5, T03G11.4 and ZC410.3. In the previous literature [35], one of the nematode Golgi mannosidase genes (reading frame D2030.1) was designated *mas-1*—in comparison to *Drosophila* mas-1 [29]; after consultation with the Wormbase curators, the nematode mannosidases are, however, hereby defined as being encoded by the *mans-1, mans-2*, *mans-3* and *mans-4* genes. RNAi-based knock-down of the worm *mans-1* gene is associated with a 9 % extension in mean lifespan [35].

As also proposed by others [36], such an analysis suggests that in the case of *C. elegans* two mannosidases are of the endoplasmic reticulum I type and two of the Golgi I type—this is in contrast to one endoplasmic reticulum type in many organisms such as *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Drosophila melanogaster* and *Homo sapiens* and to three Golgi type I mannosidases encoded by the human genome; the model

plant and the model fly have each two Golgi class I enzymes. In addition, three 'mannosidase-like' sequences from the worm can also be identified in the databases and are putative orthologues of mammalian EDEM-1, EDEM-2 and EDEM-3 (C47E12.5, F10C2.5 and ZC506.1); in the latter case, the nematode sequence lacks an ER-retrieval KDEL 'signal' present in the human form. Not only does the phylogenetic tree suggest a distinction between *C. elegans* MANS-1/-2 pair of proteins as opposed to MANS-3/-4: the arginine residue homologous to Arg_{273} of the *S. cerevisiae* Mns1p and important for determining is specificity [37, 38] is present in the sequences of MANS-3 and MANS-4. The corresponding residue in MANS-1 and MANS-2 is either Met or Leu as in other Golgi mannosidase I isoforms.

Cloning and expression of class I mannosidases

Partial reading frames encoding the putative lumenal domains of four 'classical' class I mannosidases from *Caenorhabditis elegans* were isolated, cloned into a yeast expression vector and sequenced prior to transformation into *Pichia pastoris*. Western blotting with anti-FLAG and tryptic peptide mapping using MALDI-TOF MS were used to verify the expression of the four proteins. Tests were performed on two yeast clones for each enzyme and SDS-PAGE was performed using a sample of the culture supernatant: thereby, apparent molecular masses of the major Coomassie-stained or anti-FLAG reactive bands in the range 60–70 kDa were observed (data not shown), in good agreement with the expected masses for the lumenal domains of 60–65 kDa excepting glycosylation; the identity of these bands as corresponding to the expected proteins was verified by tryptic peptide mapping.

Assay of recombinant class I mannosidases

As GH47 class I mannosidases, unlike their GH38 'cousins', cannot be simply assayed with aryl mannosides such as *p*-nitrophenyl-α-mannoside [11], a limited number of tests with specific glycan substrates were performed in order to ascertain the enzymatic function of the recombinant enzymes. The products of incubations with pyridylaminated $Man_9GlcNAc_2$ were examined by RP-HPLC in combination with MALDI-TOF MS, thereby gaining both isomeric information and product identification. Based also on the literature regarding class I mannosidases from other organisms [39], the incubations were performed at pH 7 in the

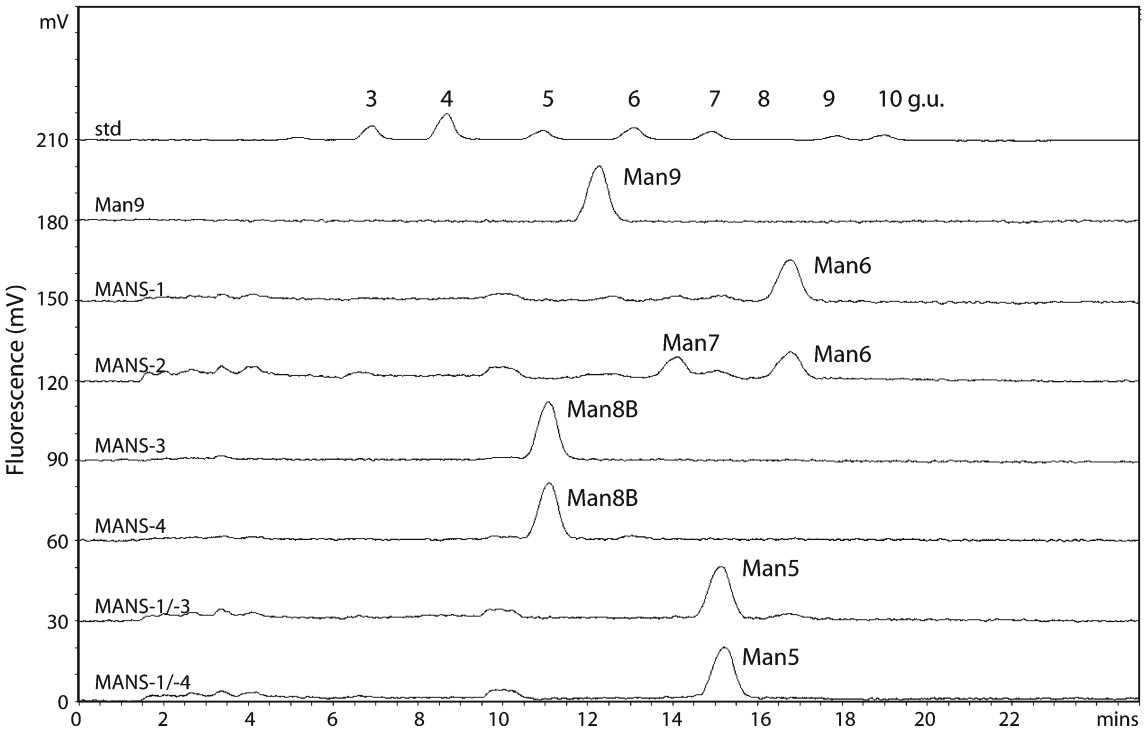

Fig. 2 Activity of *C. elegans* class I mannosidases. Supernatants of *Pichia* expressing MANS-1, MANS-2, MANS-3 and MANS-4 were incubated for 20 h with pyridylaminated $Man_9GlcNAc_2$ as substrate; also mixed assays of MANS-1 with either MANS-3 or MANS-4 were performed. The incubations were analysed by RP-HPLC and peaks were collected and analysed by MALDI-TOF MS; therefore, the major peaks are annotated with Man5, Man6, Man7, Man8 and Man9; chromatograms are also shown for the oligoglucose standard (3–10 glucose units) and 10 pmol of standard Man9

presence of 10 mM $CaCl_2$. The calcium dependency of nematode MANS-1 and MANS-3, as examples, was indeed verified as, in the absence of Ca(II) and presence of EDTA, no enzymatic activity was observed (data not shown). Assays with control *Pichia* supernatants showed no sign of degradation of the substrate.

As suggested by their phylogenetic relationships [40] and proven here apparently for the first time, *C. elegans* MANS-3 and MANS-4 remove a single mannose residue from $Man_9GlcNAc_2$; the shift from 5.5 g.u. to 5.0 g.u. is indicative that the isomer B of $Man_8GlcNAc_2$ (Man8B) is the product. The identity of the product as $Hex_8HexNAc_2$-PA was confirmed by its mass (Fig. 3). Conversion was complete within 4 h and no further products were obvious after 20 h (Fig. 2). In the case of *C. elegans* MANS-1 and MANS-2, a mixture of products was observed, depending on the length of incubation time. After 4 h, shifts to 5.8, 6.5 and ~8 g.u. were observed and mass spectrometric analysis indicated that these were, respectively, $Hex_8HexNAc_2$ (isomer 8.4, as defined by Tomiya *et al.* [41], also known as isomer A), $Hex_7HexNAc_2$ (isomer 7.4) and $Hex_6HexNAc_2$ (isomer 6.10). After 20 h (Fig. 2), the major product had an *m/z* of 1513 (as a potassium adduct; Fig. 3) and a retention time of ~8 g.u., indicative that $Man_6GlcNAc_2$ was the primary 'end' product of both MANS-1 and MANS-2. Within 20 h, a shift to $Man_5GlcNAc_2$ (7 g.u.) was only observed in combination with MANS-3 or MANS-4 (Fig. 2), whereas in extended incubations (5 days), significant digestion to $Man_5GlcNAc_2$ was observed in the case of MANS-1 (data not shown).

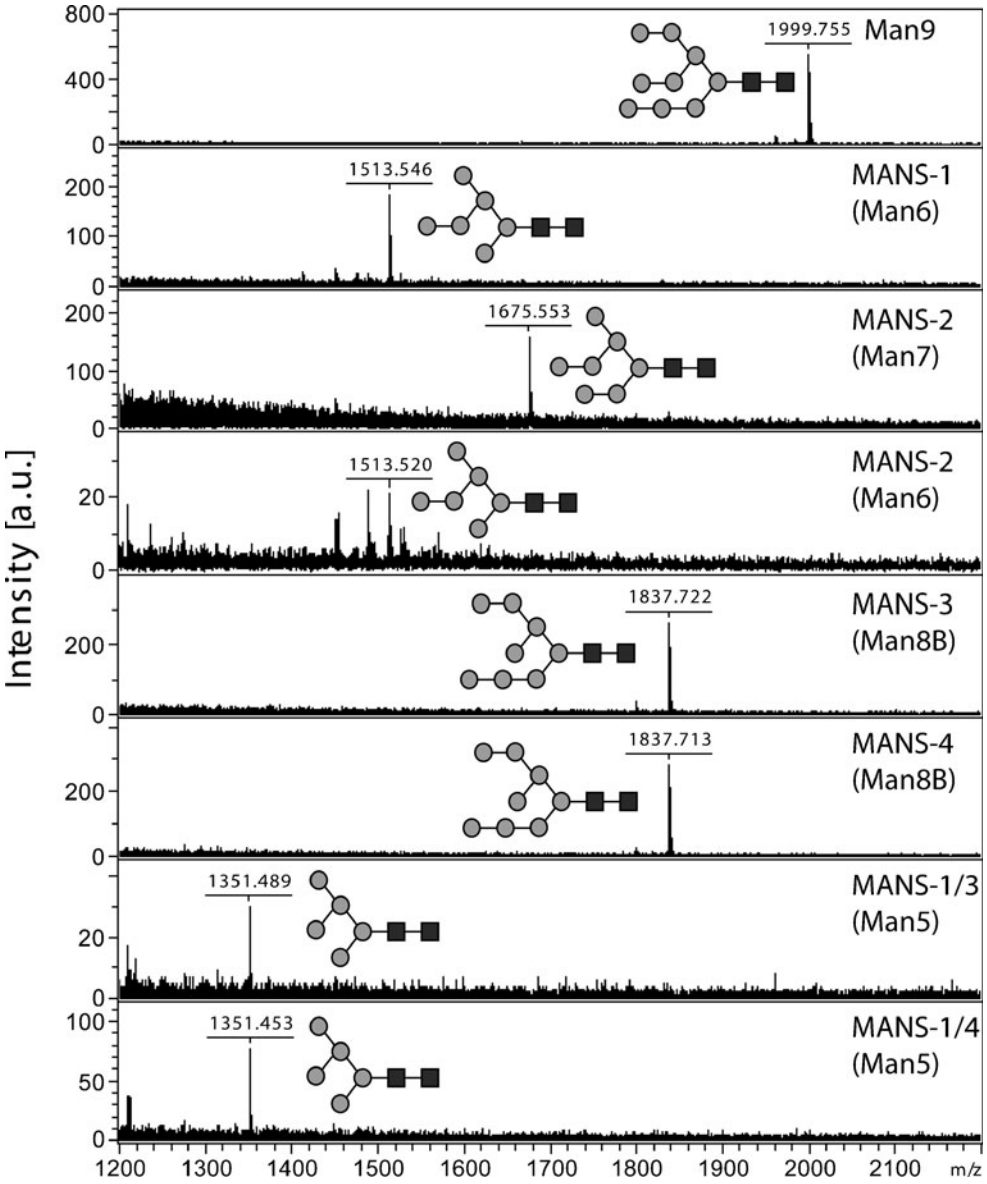

Fig. 3 Mass spectrometry of mannosidase digestion products. The major RP-HPLC peaks shown in Fig. 2 were subject to MALDI-TOF MS; the major $[M+K]^+$ species are annotated with *m/z* value. Based on the retention times, the isomer for each glycan can be proposed and is shown according to the nomenclature of the Consortium for Functional Glycomics (*squares*, GlcNAc; *circles*, Man)

Conclusion

My *in vitro* results suggest that *C. elegans* MANS-1 and MANS-2 do not efficiently remove the 'middlemost' terminal α1-2-linked mannose residue from $Man_9GlcNAc_2$, but first degrade pyridylaminated $Man_9GlcNAc_2$ *in vitro* to Man8A (removal of the terminal mannose on the 'lower' α1-3-antenna) and thereafter to the isomer of $Man_6GlcNAc_2$ in which the 'middlemost' mannose is retained. In contrast, MANS-3 and MANS-4 specifically cleave pyridylaminated $Man_9GlcNAc_2$ to Man8B; reports on mammalian endoplasmic reticulum mannosidase I indicate that this enzyme can indeed degrade $Man_9GlcNAc_2$ to species beyond Man8B, although the biological significance of such data was already questioned by the authors [42]. There is the concept that the endoplasmic reticulum mannosidase I produces specifically Man8B not only to allow export from the endoplasmic reticulum, but to prepare, *e.g.*, misfolded proteins for digestion of their glycans to $Man_7GlcNAc_2$ by EDEM mannosidases (such as the yeast Htm1p) and so aid their targeting for degradation [23]. However, it is probable that the actual proof of whether the three nematode EDEM homologues, not examined here, are true mannosidases (and not just lectins) will require careful experimentation with unfolded glycoprotein substrates complexed with other proteins.

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