Glycoconjugate Journal (1995) **12**: 150–155

Processing of asparagine-linked oligosaccharides in insect cells: evidence for α-mannosidase II

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Received 13 October 1994, revised 17 November 1994

The occurrence of α-D-mannosidase II activity in insect cells was studied using pyridylaminated oligosaccharides as substrates and two-dimensional HPLC and glycosidase digestion for the analysis of products. $GlcNAcMan_5GlcNAc_2$ was converted to $GlcNAcMan_3GlcNAc_2$ by each of the three cell lines investigated (Bm-N, Sf-21, and Mb-0503). The respective activity was highest in Bm-N cells which were used for further experiments. $Man_5GlcNAc_2$ was not degraded by the Bm-N cell homogenate. Thus, this α-mannosidase essentially exhibits the same substrate specificity as mammalian and plant Golgi α-mannosidase II. The α-mannosidase II-like activity from Bm-N cells exhibits a pH optimum of 6.0–6.5, has no requirement for divalent metal ions, and is highly sensitive to swainsonine. The α1,6-linked mannosyl residue is removed first as deduced from the elution time on reversed phase HPLC of the intermediate product. The same branch preference was found with α-mannosidase II from mung bean seedlings and *Xenopus* liver. Upon ultracentrifugation of Bm-N cell homogenate, 72% of the mannosidase acting on the $GlcNAcMan_5GlcNAc_2$ substrate was found in the microsomal pellet indicating the enzyme to be membrane-bound.

Keywords: Insect cells, α-mannosidase, N-linked oligosaccharides, glycoprotein processing

Abbreviations: Endo H (Endo D), endo-β-*N*-acetylglucosaminidase H (D); GlcNAc, *N*-acetylglucosamine; M3, M5, M5Gn etc., oligosaccharides – for an explanation see Table 1; -PA, -pyridylamine.

Introduction

Recent years have seen a considerable growth of our knowledge about protein-N-glycosylation in insects and insect cells [for a review see reference 1]. With the recent discovery of *N*-acetylglucosaminyl (GlcNAc) transferases I and II in insect cells, it became apparent that insects harbour at least several of the enzymes required for the synthesis of complex type sugar chains [2–4]. The frequent occurrence of 'paucimannose' structures ($Man_3GlcNAc_2$) suggests the presence of an enzyme which removes the α1,3- and α1,6-linked mannosyl residues from the high-mannose type structure $Man_5GlcNAc_2$ (M5) [5]. In mammals and plants this is carried out by a single enzyme which is strictly dependent on the prior action of GlcNAc-transferaseI [6–8]. This hydrolase, termed α-D-mannosidase II, usually resides in the Golgi and is, in contrast to α-mannosidase I, strongly inhibited by swainsonine [7, 9].

In this study evidence for the occurrence of α-mannosidase II in homogenates of cultured insect cells will be presented. Measurement of enzyme activity and characterization of enzyme specificity and properties were performed with pyridylaminated, non-radioactive oligosaccharides.

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Materials and methods

Insect cells, other specimens and microsome preparation
Cells of the strains Bm-N, IPLB-Sf21AE and Mb-0503 were grown, harvested and disrupted with a Dounce homogenizer as described [3]. If not otherwise stated, unfractionated cell homogenate was used as the enzyme source. For the preparation of microsomes, about 80 mg of washed Bm-N cells (stored frozen until use) were suspended in 2 ml of 3 mM imidazole/HCl buffer at pH 7.25 containing 0.25 M sucrose. Cells were disruptured by ultrasound using a microprobe at 30 W for 3 × 8 s. The microsomal pellet was obtained by centrifugation at 36 000 rpm in a Beckman SW60 rotor for 60 min and suspended in 2 ml of the same buffer.

Mung beans were soaked in tap water overnight and germinated at 37°C using a dedicated ceramic apparatus. *Xenopus laevis* liver was kindly provided by the Institut Für Klinische Pharmakologie (AKH Wien). Both mung bean seedlings and frog liver were homogenized by three 10 s bursts of an ultra-thurax homogenizer using the sucrose-imidazole buffer which, in the case of the bean seedlings, contained 0.5 mM 2-mercaptoethanol. Separation of membrane-bound and soluble protein was performed as above.

Enzyme substrates and other oligosaccharides All oligosaccharides were used as pyridylaminated derivatives. The structures of the glycans dealt with in this study are depicted in Table 1. $Man_5GlcNAc_2$-PA (M5) was prepared from fungal α-amylase as described [3]. M5 was converted to M5Gn by the use of partially purified rabbit liver GlcNAc-transferase I, which was obtained and used as previously described [3, 10]. M3 and M3Gn were available from an earlier study [3]. $Man_4GlcNAc_2$ from bee venom phospholipase A_2 (fraction **5-B**), which has recently been shown by methylation analysis to have the isomeric structure M4.1 (Table 1) [13], was used as a reference compound. Further reference oligosaccharides were prepared by exploiting the known substrate specificity of jack bean α-mannosidase [14, 15]. Digestion of M5Gn-PA with jack bean α-mannosidase (Sigma) yielded M4.1Gn-PA which was isolated by HPLC on MicroPak AX5. Further digestion of this compound with jack bean β-*N*-acetylhexosaminidase (Sigma) yielded M4.1-PA. All three possible M4 isomers were obtained by limited hydrolysis with HCl of underivatized $Man_5GlcNAc_2$ [15]. Upon evaporation of the acid, the mixture of mono- and oligosaccharides was pyridylaminated and size-fractionated on MicroPak AX5. The M4-PA fraction was collected, checked for purity on MicroPak AX5 and finally submitted to reversed phase HPLC.

Assay for α-mannosidase II activity Two nmol of M5Gn-PA or M5-PA were incubated with insect cell homogenate containing 0 – 38 μg of protein in a total volume of 20 μl for 16 h at 37 °C. The standard assay mixture contained 0.1 M MES-HCl at pH 6.3, 0.5% Triton X-100 and 0.02% sodium azide. Other additives, e.g. swainsonine (Sigma), were included as indicated in the Results section. The enzymatic reaction was terminated by boiling for 5 min. The samples were analysed by size fractionation on MicroPak AX-5 [11, 12]. For this purpose the samples were diluted with 80 μl starting buffer and a 50 μl aliquot was injected. Some samples and isolated peaks were additionally subjected to reversed phase HPLC on an octadecylsilica column which was performed essentially as described [13] except that the gradient was already started at 3% methanol in order to shorten analysis time. Both columns were calibrated with pyridylaminated isomalto-oligosaccharides [13]. Aryl-mannosidase activity was determined photometrically using the same incubation conditions and 5mM 4-nitrophenyl α-mannopyranoside as the substrate. GlcNAc-transferase I was measured by HPLC as described [3].

Glycosidase digestion The two products **A** and **B** obtained by digestion of M5Gn-PA with Bm-N cell extract were isolated by HPLC on MicroPak AX-5 and dried in a SpeedVac concentrator. Approximately 0.2 nmol of each sample was incubated with 2 mU of jack bean β-*N*-acetylhexosaminidase (Sigma) in 0.1 M citrate-phosphate buffer at pH 5.0 for 20 h at 37°C. Another aliquot was treated with 1 mU endo-β-*N*-acetylglucosaminidase H (Endo H; Boehringer Mannheim) under the same conditions. For digestion with endo-β-*N*-acetylglucosaminidase D (Endo D; Boehringer Mannheim), samples were placed in 0.1 M citrate-phosphate buffer, pH 6.0, and likewise incubated for 20 h at 37°C.

Table 1. Structures of oligosaccharides

M5	R = H	Manα1-6 Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc (-PA) R -2Manα1-3
M5Gn	R = GlcNAc	
M4.1	R = H	Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc (-PA) R -2Manα1-3
M4.1Gn	R = GlcNAc	
M4.6		Manα1-6 Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc (-PA) Manα1-3
M4.7		Manα1-6 Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc (-PA)
M3	R = H	Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc (-PA) R-2Manα1-3
M3Gn	R = GlcNAc	

Isomers are designated according to [14]. In a previous publication, M3 and M3Gn were termed MM and MGn [3].

Results

Degradation of M5Gn by Bm-N homogenate – analysis of products Incubation of M5Gn-PA with cell homogenate of the insect cell line Bm-N led to two compounds (products **A** and **B**) smaller than the substrate by one and two Glc units when analysed on the sizing column (Fig. 1, Table 2). The MicroPak AX-5 column was used for quantitative determination of enzyme activity, since on reversed phase the two products could not be separated. However, reversed phase HPLC was used for the structural identification of the products by glycosidase degradation. Both peaks were sensitive to β-*N*-acetylhexosaminidase, which effected an increase in retention time on reversed phase of 0.4–0.5 Glc-units (Table 3) as expected for the removal of a GlcNAc residue linked to the α1,3-arm [3]. Thus, the reaction catalysed by the cell homogenate must have been the successive removal of two mannosyl residues and the two products **A** and **B** were M4Gn and M3Gn. Most experiments were performed with 3 μg of cell protein resulting in the formation of primarily M4Gn and some M3Gn (Fig. 1, Table 2). Increasing the amount of added cell homogenate led to a successive growth of the M3Gn peak (Fig. 1), ultimately resulting in the complete conversion of M5Gn to M3Gn. The formation of products with various amounts of enzyme is depicted in Fig. 2. However, as can be seen from Fig. 1, M3Gn was now subject to further degradation by a β-*N*-acetylhexosaminidase present in the cell homogenate (F. Altmann, unpublished results), since this final product **C** did not contain terminal GlcNAc and exhibited the chromatographic characteristics of M3.

When M5-PA was offered as the substrate, absolutely no degradation was observed even with as much as 38 μg of cell protein, an amount which would have left nothing of the M5Gn-PA substrate. Thus, the mannosidase responsible for the conversion of $Man_5GlcNAc_2$ to $Man_3GlcNAc_2$ in insect cells relies on the prior action of *N*-acetylglucosaminyl transferase I – just like the α-mannosidases II of mammals and plants [7, 8].

The intermediate product **A** (M4Gn) gave a single peak on the reversed phase column (Fig. 3). According to the results reported by Tomiya *et al.* [14] and Oku *et al.* [15] it appears possible to determine the isomeric structure of product **A** from its elution time on reversed phase. These literature data were carefully verified by two approaches to avoid any misinterpretations caused by possible differences in chromatographic selectivity.

By comparing the elution times of reference compounds M5, M4.1 (either prepared from phospholipase A_2 or by

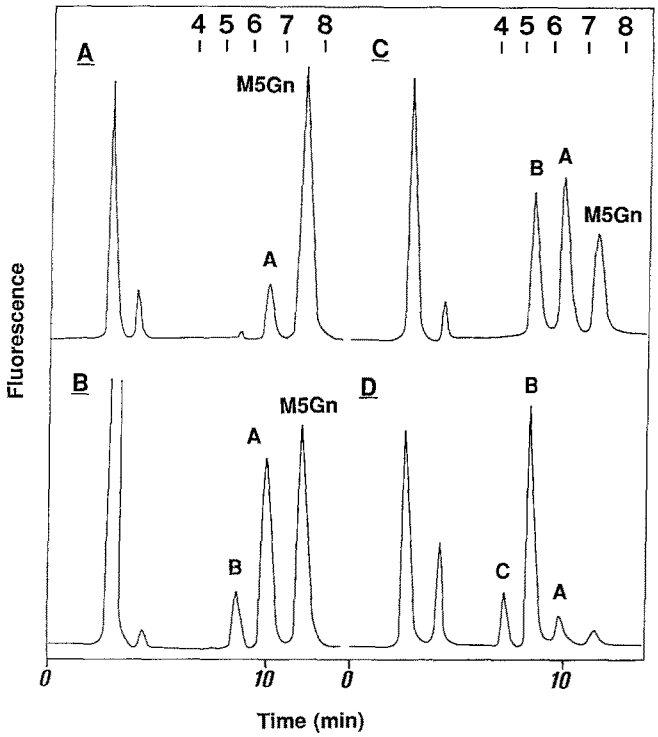

Figure 1. HPLC analysis on MicroPak AX-5 of digests of M5Gn with Bm-N cells. The substrate was incubated for 20 h with 0.75 μg, 3.0, 7.5, and 30 μg of cell protein (panels A to D, respectively). Peaks A, B and C refer to products **A**, **B**, and **C**. Numbers at the top of the figure indicate the elution positions of isomalto-oligosaccharides of the respective degree of polymerization. The large peak at 2–3 min is a chromatographic artefact.

Table 2. Elution values of pyridylaminated substrates, products and reference oligosaccharides on HPLC.

PA-glycan	*Elution values in Glc units on*	
	MP[a]	*ODS*[b]
M5Gn (substrate)	7.4	7.4
Product **A**	6.4	7.9
Product **B**	5.4	7.8
Product **C**	4.6	8.2
Reference compounds		
M4.1Gn	6.4	7.9
M3Gn	5.4	7.8
M5	6.7	7.8
M4.1	5.5[c]	8.4[d]
M4.6	5.5[c]	7.65
M4.7	5.5[c]	8.25
M3	4.6	8.2

[a] MicroPak AX-5 column
[b] Octadecylsilica reversed phase column
[c] Elution position of a mixture of the three isomers.
[d] This value was obtained with three different preparations of M4.1 as detailed in the Methods section.

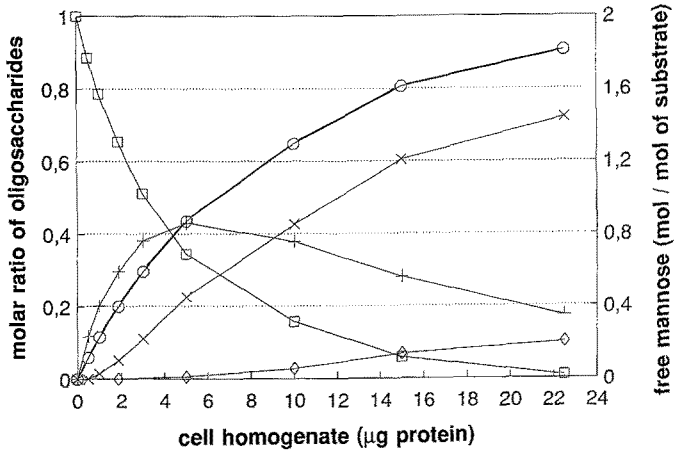

Figure 2. Enzyme amount and product formation: Varying amounts of a homogenate of Bm-N cells were incubated with M5Gn as described in the Methods section. The graphs depict the consumption of substrate (-□-□-), the formation of products **A** = M4Gn (-+-+-), **B** = M3Gn (-×-×-), and **C** = MM (-◇-◇-), and the generation of free mannose (-○-○-) as calculated from the amounts of products.

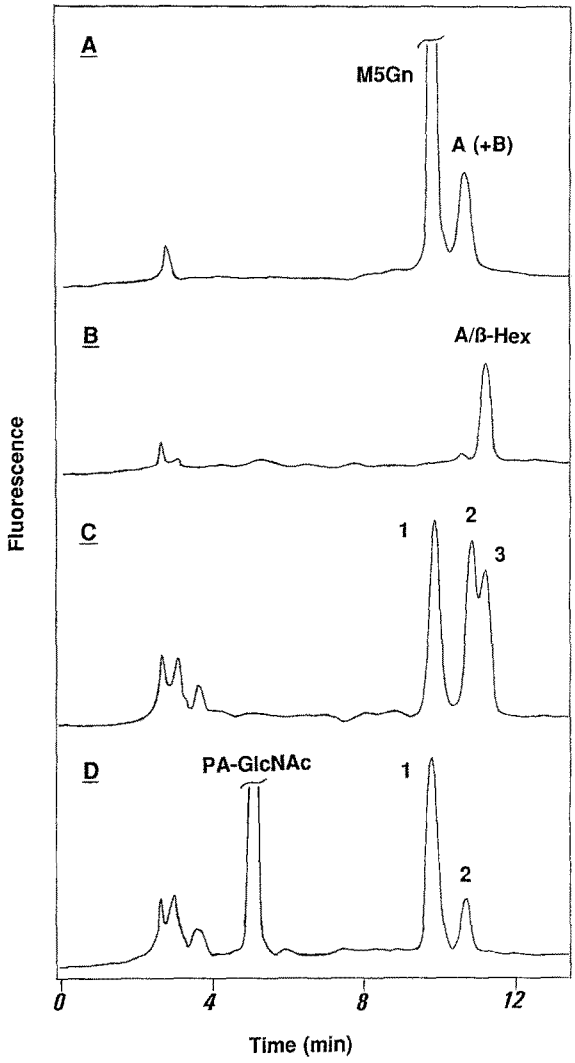

Figure 3. Determination by reversed phase HPLC of the isomeric structure of product **A** (M4Gn). Panel A: M5Gn digested with Bm-N cell homogenate (0.75 µg protein), product **B** is not separated from the dominating product **A**. Panel B: Product **A** isolated on the sizing column and digested with jack bean *β-N*-acetylhexosaminidase. Panel C: Mixture of M4 isomers obtained by limited acid hydrolysis of M5; Peaks 1, 2 and 3 represent structures M4.6, M4.7 and M4.1, respectively, as explained in the text. Panel D: Analysis of the M4 mixture after treatment with Endo H. The small peaks eluting after about 3 min are artefacts.

partial degradation of M5Gn by jack bean *α*-mannosidase followed by jack bean *β-N*-acetylhexosaminidase) and M3 on the one hand and M5Gn, M4.1Gn and M3Gn on the other hand, we have determined unit contributions of –0.4 to –0.5 Glc units for the *α*1,6-linked and 0–0.2 Glc units for the *α*1, 3-linked mannosyl residue. This result corresponds with the reported values of –0.24 and +0.1 Glc units, respectively [14]. The retention times of M5Gn, product **A** and product **B** show that the mannosyl residue which was removed first by Bm-N mannosidase II had a unit contribution of –0.5 Glc units, whereas the second mannose had 0.1 Glc units only.

In a second approach, the elution position of *β-N*-acetylhexosaminidase-treated product **A** was compared with those of a mixture of M4 isomers obtained by limited acid hydrolysis of M5. The homogeneous M4 pool from the MicroPak AX5 fractionation of the hydrolysate gave three peaks on reversed phase. The elution order of the isomers has been reported to be M4.6, M4.7 and M4.1 [15] and this was corroborated by co-injection of reference compound M4.1 and by treatment with endoglycosidases as follows. Isomers M4.1 and M4.7 are

Table 3. Oligosaccharide analysis by reversed phase HPLC upon incubation with glycosidases. Jack bean β-N-acetylglucosaminidase and endo-β-N-acetylglucosaminidases H and D were used to probe the presence of terminal GlcNAc and of substituents on the α1,6-arm, respectively.

PA-glycan	*β-N-acetylglucosaminidase*		*Endo H*	*Endo D*
	Digested	*Effect*[a]	*digested*[b]	*digested*[b]
M5Gn (substrate)	Yes	7.4 → 7.8 (M5)	Yes	
Product **A**	Yes	7.9 → 8.4 (M4.1)	Yes	
Product **B**	Yes	7.8 → 8.2 (M3)	No	
Reference compounds				
M4.1 (peak 3 in Fig. 3C)			Yes	Yes
M4.6 (peak 1 in Fig. 3C)			No	Yes
M4.7 (peak 2 in Fig. 3C)			Partially	No

[a] Elution positions of glycosidase action products before and after digestion on an octadecylsilica column expressed in Glc units. The structures of the products are given in brackets and were deduced on the basis of the data shown in Table 2 and Fig. 3.
[b] The product of Endo H or D digestion had an elution position of 3.9 Glc units corresponding with authentic PA-GlcNAc [3].

known to be susceptible to Endo H [16]. In contrast, isomer M4.6 is said to be resistent to Endo H [8]. In fact, while the third peak which coeluted with the M4.1 reference, was completely hydrolysed by Endo H, the second peak was at least partially (i.e. more slowly) degraded, and the first peak representing M4.6 was resistant to Endo H (Table 3, Fig. 3). Endo D (which relies on the presence of Manα1-3Manβ1-) hydrolysed peaks one and three, but not the second peak. Thus, the reported chromatographic selectivity also applied to our column and solvent system. Taken together, these observations permit us unambiguously to assign structure M4.1Gn to product A.

Properties of the enzyme The activity of Bm-N cell α-mannosidase II was highest at pH 6.0–6.5 and decreased rapidly outside this range. The effects of divalent metal ions and EDTA were investigated at a concentration of 20 mM. Mg^{2+}, Ca^{2+}, Mn^{2+} had no influence, EDTA reduced the activity by 20% and Cu^{2+} by 90%. Under the conditions employed, 30 nM swainsonine inhibited the mannosidase by 85%. The effect of detergent was studied with microsomes. Between 0.2 and 1% the concentration of Triton X-100 had no effect on measured enzyme activity, but omission of Triton resulted in a 40% decrease of activity.

Upon ultracentrifugation at an average centrifugal force of 120 000 ×**g**, most of the α-mannosidase activity II-like activity was found in the microsomal pellet (Table 4). Similar values were obtained for GlcNAc-transferase I (Table 4). The high amount of activity found in the supernatant may in part arise from solubilized enzyme. However, experiments at a lower speed (100 000 × **g**, 30 min) and re-centrifugation of supernatant indicate incomplete sedimentation of vesicles. The mannosidase acting upon M5Gn appears to be membrane-bound or at least membrane-associated.

The centrifugation pellet also exhibited activity towards 4-nitrophenyl α-mannoside. The relative rate of mannose release from 4-nitrophenyl α-mannoside was 3.1 times higher than from M5Gn-PA under the conditions used. A nearly identical ratio of 3.75 was found for rat liver α-mannosidase II [7]. However, most of the aryl-mannosidase activity of insect cells was found in the supernatant (Table 4) of which only 23% was accounted for by the α-mannosidase II activity, if we assume the same relative hydrolysis rates for sedimented and non-sedimented α-mannosidase II. This indicates that Bm-N cells contain yet another α-mannosidase acting upon 4-nitrophenyl α-mannoside but not on the complex substrate M5Gn-PA.

Other cell lines and tissues In addition to Bm-N cells, the cell lines Sf-21 and Mb-0503 were examined for α-mannosidase II activity. Each of the three cell lines exhibited the same product pattern. The rates of product formation and the α-mannosidase activity calculated therefrom are listed in Table 5. It is to be noted that the human cell line HepG2 exhibited a very similar rate of α-mannosidase II trimming (data not shown).

Table 4. Distribution of enzyme activities between ultracentrifugation pellet and supernatant. Enzyme activities were determined after centrifugation of homogenized insect cells (for details see text). However, the experiments with NaCl and Triton X-100 were performed with suspended microsomes which were treated with the respective agent for 1 h prior to centrifugation.

Measured enzyme / conditions	*Amount of enzyme activity found in (%)*	
	Pellet	*Supernatant*
α-mannosidase II like activity	72.6	27.4
Aryl-mannosidase	19.8	80.2
N-acetylglucosaminyltransferase I	70.1	29.9
α-mannosidase from microsomes treated with:		
1 M NaCl	77.0[a]	23.0
1% Triton	36.6	63.4
Control	86.5	13.5

[a] Value probably too low because pellet could not be completely suspended after centrifugation.

Table 5. Formation of products from M5Gn-PA by homogenates of Bm-N, Sf-21, and Mb-0503 cells. Two nmol M5Gn-PA was incubated for 20 h at 37°C with various amounts of cell protein. Enzyme activity was calculated as the amount of released mannose which was deduced from the amounts of products considering that two mannosyl residues have been removed in the case of M3Gn.

Cell line dase	*Protein (μg)*	*M5Gn*	*M4Gn (%)*	*M3Gn*	*α-mannosi- activity ($nmol \times mg^{-1} \times h^{-1}$)*
Bm-N	1.9	65	30	5	26
Mb-0503	3	64	32	4	13
Sf-21	7.5	68	25	7	5

For comparison, the trimming of Man5Gn by mannosidase II from microsomal preparations of mung bean seedlings and *Xenopus laevis* liver was investigated. In both cases, the M4Gn intermediates gave one peak on the reversed phase column with retention times corresponding to M4.1Gn-PA and, upon treatment with β-*N*-acetylhexosaminidase, to M4.1-PA. The M4Gn-fraction generated by mung beans contained a small (8%) second peak at the putative elution position of M4.6Gn. This compound, however, was apparently insensitive to β-*N*-acetylhexosaminidase and could therefore not be positively identified as M4.6Gn. However, the trimming of M5Gn by the frog and bean mannosidase II starts preferentially, if not exclusively, with the α1,6-linked mannosyl residue.

Discussion

With regard to substrate specificity, pH-dependence, requirement for metal ions, sensitivity to swainsonine, branch specificity and membrane association, the α-D-mannosidase activity in insect cells closely resembles the Golgi α-mannosidase II from mammals and plants [7–9]. Together with other recently presented findings, such as the demonstration of GlcNAc-transferase I and II activity and the specificity of the core-fucosyltransferases [3], this means that insects and vertebrates not only have in common the initial steps of N-glycosylation leading to high-mannose structures, but also the subsequent steps which in mammals initiate the attachment of polylactosamine antennae. While it is not yet possible to perform interspecies comparisons of protein sequences of the enzymes involved, the biochemical evidence leads us to assume that even these early steps of complex N-glycan biosynthesis trace back to the common ancestors of animals and plants. Insects, the largest group within the phylum arthropoda, do not appear to have digressed substantially from this line of evolutionary heritage.

Acknowledgements

This work was supported by a grant from the Austrian Bundesministerium für Wissenschaft und Forschung. The help of Mr Christian Sodl and Dipl Ing Herwig Schwihla, Zentrum für Angewandte Genetik der Universtität für Bodenkultur Wien, in cell culture and the excellent technical assistance of Mr Thomas Dalik are gratefully acknowledged. In particular, we thank Dr Erika Staudacher for the donation of GlcNAc-transferase I.

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