Glycoconj J (2007) 24:475–489
DOI 10.1007/s10719-007-9040-5

Neutral N-glycan patterns of the gastropods *Limax maximus*, *Cepaea hortensis*, *Planorbarius corneus*, *Arianta arbustorum* and *Achatina fulica*

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Received: 27 December 2006 / Revised: 27 February 2007 / Accepted: 11 April 2007 / Published online: 22 May 2007

Abstract The N-glycosylation potentials of *Limax maximus*, *Cepaea hortensis*, *Planorbarius corneus*, *Arianta arbustorum* and *Achatina fulica* were analysed by investigation of the N-glycan structures of the skin and viscera glycoproteins by a combination of HPLC and mass-spectrometry methods. It is one of the first steps to enlarge the knowledge on the glycosylation abilities of gastropods, which may help to establish new cell culture systems, to uncover new means for pest control for some species, and to identify carbohydrate-epitopes which may be relevant for immune response. All snails analysed contained mainly oligomannosidic and small paucimannosidic structures, often terminated with 3-*O*-methylated mannoses. The truncated structures carried modifications by β1-2-linked xylose to the β-mannose residue, and/or an α-fucosylation, mainly α1,6-linked to the innermost *N*-acetylglucosaminyl residue of the core. Many of these structures were missing the terminal *N*-acetylglucosamine, which has been shown to be a prerequisite for processing to complex N-glycans in the Golgi. In some species (*Planorbarius corneus* and *Achatina fulica*) traces of large structures, terminated by 3-*O*-methylated galactoses and carrying xylose and/or fucose residues, were also detected. In *Planorbarius* viscera low amounts of terminal α1-2-fucosylation were determined. Combining these results, gastropods seem to be capable to produce all kinds of structures ranging from those typical in mammals through to structures similar to those found in plants, insects or nematodes. The detailed knowledge of this very complex glycosylation system of the gastropods will be a valuable tool to understand the principle rules of glycosylation in all organisms.

Keywords Glycosylation · N-glycans · Gastropod · Snail

Abbreviations

Endoglycosidase H	endo-β-*N*-acetylglucosaminidase H (E.C. 3.2.1.96)
peptide: N glycanase A	peptide-*N*4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (E.C. 3.5.1.52)
HRP	horseradish peroxidase

Introduction

Gastropods are a class of hundreds of known and for sure at least the same amount of unknown species. Snails are intermediate hosts of human and animal parasites, which cause infections of more than 200 million people worldwide and are also a recognized veterinary problem in Africa and Asia [1, 2]. The glycans seem to play a role in immunopathology and immunomodulation [3, 4]. Only a few gastropodian molecules have been investigated in detail and are in biotechnological use so far. Since the early seventies a number of lectins have been purified from gastropodian sources, have been cloned now, and are sometimes used in biochemical research today. A component of the venom of the marine snail *Conus magus* seems to be a highly potent drug, which is able to block neuronal N-type voltage-sensitive calcium channels and which therefore may be a

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painkiller in human medicine in the near future [5]. An already established medical application is the use of keyhole limpet hemagglutinin from *Megathura crenulata* for diagnosis of schistosomiasis and to differentiate serologically between acute and chronic infection [6, 7]. The hemagglutinin of the snail has been shown to share carbohydrate epitopes with *Schistosoma mansoni* [8], which have been analysed in detail [9–12]. Furthermore it is described as an immune stimulant and already in use in cancer therapy conjugated to therapeutics [13, 14].

Only a few data exist on gastropodian N-glycosylation abilities. N-glycans from the hemocyanins of the snails *Helix pomatia*, *Lymnaea stagnalis*, *Rapana venosa*, the keyhole limpet *Megathura crenulata* and a structure linked to the major soluble protein of the organic shell matrix of *Biomphalaria glabrata* have been analysed [15–20]. In a previous paper we published the complete N-glycan spectrum of *Arion lusitanicus* [21]. This slug contains an enormous potential for generating a large set of structural elements commonly found in eukaryotic N-glycosylation: they sialylate [22], they carry α1-6-linked as well as α1-3-linked fucose as shown for some insects, nematodes and trematodes, β1-2-linked xylose, as found in plants and trematodes, and they are able to methylate terminal sugars (mannose and galactose) as found in nematodes. Thus they combine structural features from mammals, plants, insects, nematodes and trematodes.

The knowledge on structural aspects of N-glycosylation in snails until 2003 was summarized in detail in Gutternigg *et al*. [21]. Additional information is now available from a number of studies published during the last three years.

There are some publications how *Biomphalaria glabrata* snails are, or are not, infected by the blood fluke *Schistosoma mansoni* [23], how hemocytes of resistant snails handle the challenge of infection [24] and about qualitative and quantitative changes of hemocyte proteins of *Biomphalaria alexandrina* snails in the course of infection [25]. Recently, those N-glycans of *Biomphalaria glabrata* which cross-react with *Schistosoma mansoni* glycoconjugates were enriched and completely determined [26].

Also some more information is given on the structural elements of *Rapana venosa* hemocyanin carbohydrates [27] and its low-molecular mass fragments [28].

Some information is given on gastropod enzymes which are involved in gastropod glycan biosynthesis, mostly specificity *in vitro* and biochemical parameters were determined. GlcNAc-transferase I, GlcNAc-transferase II, xylosyltransferase, β1,4-GalNAc-transferase, β1,3-galactosyltransferase, β1,4-GlcNAc-transferase, β1,4-glucosyltransferase, α1,2-fucosyltransferase, α1,3-fucosyltransferase forming the Lewis^x -structure and another α1,3-fucosyltransferase able to transfer fucose to the GlcNAc-residue linked to the asparagine were found in various organs and tissues of *Lymnaea stagnalis* [29–37], however, the corresponding structures to some of these enzymes have not been found so far.

An α1,2- L-galactosyltransferase, found in *Helix pomatia* [38], seems not to be involved in the N-glycan biosyntheses and also the so far described exoglycosidases seem to be part of the degradation and recycling processes of the cells and not be involved in the N-glycosylation pathway.

In this study we sought to extend the knowledge about neutral N-glycans of gastropods by performing a detailed analysis of five species (*Limax maximus*, *Cepaea hortensis*, *Planorbarius corneus*, *Arianta arbustorum* and *Achatina fulica*) of land- and water-living gastropods, with and without shell, to give an overview on the gastropod glycosylation abilities. Analysing the complete set of N-glycan structures of a species yields important insights into its biosynthetic capacity for glycosylation. It is the first step for the detection and identification of enzymes which may have a new specificity which is of interest for further use in research or for the recombinant production of glycans for diagnosis or therapy.

Materials and methods

Materials

Limax maximus, *Cepea hortensis* and *Arianta arbustorum* were collected by the authors under the supervision of Dr. Manfred Pintar (Department of Integrative Biology and Biodiversity Research, Institute for Zoology, University of Natural Resources and Applied Life Sciences, Vienna) in areas close to Vienna. *Planorbarius corneus* and *Achatina fulica* were bred in the laboratory at room temperature. All animals were frozen at −80°C immediately after collection.

Sephadex G25 fine and Sephadex G15 were purchased from Amersham Biosciences, Dowex 50W×2 was from Fluka. Peptide:*N*-glycosidase A from almonds was from Roche. HPLC-columns: Hypersil ODS column (0.4× 250 mm, 5 μ) from Forschungszentrum Seibersdorf, Austria, and Palpak type N column (4.6×250 mm) from Takara, Japan. Standard pyridylaminated glycans were prepared in the course of previous studies [39, 40]. All other materials purchased were of the highest quality available from Merck or Sigma.

Preparation of proteins

Thawed slugs were washed to remove the extraneous mucous components and dissected into three fractions; the skin and inner organs (viscera) were lyophilised separately, while the intestinal tract was discarded.

The lyophilised material was homogenised in 0.05 M Tris/HCl buffer, pH 7.5 by an IKA Ultra Turrax T25 (IKA

Labortechnik, Janke und Kunkel GmbH, Staufen, Germany) at 15,000 rpm for 2×20 s and centrifuged at 5,000×*g* for 15 min to remove not soluble material. For each species at least three independent preparations were carried out, each containing 2–3 individuals for *Achatina* and *Limax* or 6–8 individuals for the smaller species.

Electrophoresis and lectin blots

The skin and viscera preparations were separated on SDS-polyacrylamide gel electrophoresis using a Bio-Rad Mini-Protean II Cell with gels containing 12.5% acrylamide and 1% bisacrylamide and electroblotted onto nitrocellulose [41]. The nitrocellulose sheets were stained either with amido black or were blocked with bovine serum albumin 0.5% in buffer (100 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.01% Tween 20) and subsequently incubated for 1 h with the biotinylated lectins or antibodies diluted to optimal concentration in blocking solution buffer. After washing the nitrocellulose membrane three times with buffer, the biotinylated lectins were detected by streptavidin-alkaline-phosphatase conjugate (Boehringer Mannheim, Germany) followed by colour detection using Fast BCIP/NPT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, Sigma). Fucosylated and xylosylated structures were detected by horseradish peroxidase antibodies from rabbit, further recognized by alkaline phosphatase conjugated anti-rabbit antibodies from goat. Appropriate positive and negative controls were run in parallel on all gels and blots.

Preparation and analysis of 2-aminopyridine labelled N-glycans

Proteolytic digests with proteases (either thermolysine or pepsin), release of N-glycans from the glycopeptides by peptide:N-glycanase A, labelling of the released N-glycans with 2-aminopyridine and fractionation of these labelled glycans on HPLC by size (Palpak type N) and by hydrophobicity (reverse-phase) was carried out as described in detail from Gutternigg *et al*. [21].

Columns were calibrated in terms of glucose units with a pyridylaminated partial dextran hydrolysate (3–11 glucose units). Peaks from either size fractionation or reverse-phase chromatographies were analysed by MALDI-TOF and subjected to exo- or endoglycosidase digestions.

Analysis of monosaccharides

Monosaccharide analysis was carried out by hydrolysis of the glycans with 4 M trifluoroacetic acid at 100°C followed by labelling with anthranilic acid and reverse-phase HPLC with fluorescence detection [42] or by conversion of the monosaccharides into their corresponding alditol acetates which were analysed by gas chromatography/mass spectrometry as described [43].

MALDI-TOF analysis

MALDI-TOF MS was carried out as previously described [44]. The sample (1 μl 0.2–0.8 pmol) was spotted onto a target and dried, followed by the addition of 0.8 μl of matrix (2% 2,5-dihydroxybenzoic acid in water containing 30% acetonitrile). The plate was immediately transferred to a desiccator and vacuum was applied until all solvent had evaporated. Spectra were recorded on a DYNAMO linear MALDI-TOF mass spectrometer (Thermo BioAnalysis, Hemel Hempstead, UK) operated with a dynamic extraction setting of 0.1. External mass calibration was performed with pyridylaminated N-glycan standards derived from bovine fibrin. About 20 individual laser shots were summed.

In some cases on-target digestions with exoglycosidases were carried out using 6-aza-2-thiothymine [0.5% (*w/v*) in water] as the matrix [45].

LC-ESI tandem MS/MS experiments

The LC-ESI-MS/MS experiments were carried out using a Q-TOF Ultima Global mass spectrometer (Micromass, Manchester, UK) equipped with an atmospheric pressure ionization electrospray interface and an upstream Micromass CapLC. The pre-column was a Thermo Aquastar 30×0.32 mm and the separation column was a Thermo Hypercarb 100×0.32 mm. The flow rate was 4 μl/min, starting with 95% solvent A (aqueous 0.1% formic acid) and 5% solvent B (acetonitrile containing 0.1% formic acid); a separating gradient from 5–40% B was applied from 5–40 min.

The MS instrument was calibrated with [Glu1]-Fibrinopeptide B in the mass range of 72–1,285 amu. Derivatized oligosaccharides were dissolved in water. The sampling cone potential was 80 V and the capillary voltage 3.0 kV. The electrospray source temperature was 60°C, and the desolvation temperature 120°C. Mass spectra were scanned over the range m/z 100–1900.

Exo- and endoglycosidase digests

Endoglycosidase H (recombinant from *E.coli*, Roche) was used at a concentration of 2 mU in 150 mM citrate-phosphate buffer, pH 5.0 containing 0.1 M NaCl; α-mannosidase (jack bean, Sigma) at 2 mU in 50 mM sodium acetate pH 4.5 containing 0.2 mM $ZnCl_2$; α-fucosidase (bovine kidney, Sigma) at 2 mU in 50 mM sodium citrate pH 4.5; α1,2-fucosidase (recombinant, Sigma) at 0.2 mU in 50 mM sodium phosphate pH 5.0; β-galactosidase (bovine testis,

Roche) at 1.6 mU in 50 mM sodium citrate pH 5.0, β-hexosaminidase (bovine kidney, Sigma) at 25 mU in 20 μl of 0.1 M sodium citrate, pH 5.0 and β-xylosidase (own preparation [46]) at 2 mU in 50 mM sodium citrate, pH 4.0.

Incubations were carried out in 20 μl of appropriate buffer at 37°C overnight.

In some cases, in order to retain a mixture of the original glycan and all intermediate products, a reduced amount of enzyme was employed.

For chemical release of fucose α1-3-linked to the inner GlcNAc-residue the dry sample was incubated for 48 h at 0°C with 20 μl of 48% (*v/v*) hydrofluoric acid. The acid was then removed under a stream of nitrogen [47].

Results

Analytical strategy

In order to get an overview on the structures to expect in the different snail species, the whole protein-fractions of viscera and skin preparations were analysed for their monosaccharide composition and subjected to a number of lectin- and antibody-blots (Fig. 1).

From protein backbone released neutral N-glycans were separated on reverse phase HPLC after fluorescent labelling with 2-aminopyridine. We then adopted a similar analytical strategy as in our previous paper using a reverse phase chromatography [21]. The neutral N-glycans can be divided there into four regions with characteristic structures eluting in each of them, thus facilitating a simplification of the structural spectrum in a distinct area from all possible variations to the most probable variations (Fig. 2). The single structures were determined in detail by MALDI-TOF, two dimensional HPLC (reversed phase and size exclusion) with and without exoglycosidase digests, endoglycosidase digest or chemical release as described before [21].

Glycan structures carrying the same number and type of sugar residues in different positions were distinguished by their elution behaviour on reverse phase HPLC and by comparing their retention times with standard oligosaccharides. For example the different variations of M_7 can be separated clearly: the glycan with the seventh mannose on the α1-6-mannose of the α1-6-antenna (M_7 D1) elutes first, next comes the structure with the additional mannose linked to the α1-3-antenna (M_7 D3), finally elutes the structure with the mannose linked to the α1-3-mannose to the α1-6-antenna (M_7 D2).

The determination of the position of fucose residues was carried out by comparing the retention times of the glycans on reverse phase HPLC before and after an α-fucosidase digest with the α1,2-fucosidase for terminal fucosylation, the fucosidase from bovine kidney for core α1-6-fucosylation or after a treatment with hydrofluoric acid for core α1,3-fucosylation with standards from earlier studies [48].

Methylation of terminal hexoses prevent these sugars from a digest by corresponding exoglycosidases. Therefore, in the case of incomplete methylation, the position of the unmethylated antenna can be determined by its sensitivity to exoglycosidase digest.

For *Achatina fulica* also a more detailed analysis on LC-MS/MS was carried out.

To confirm the presence of 3-*O*-methylmannose and 3-*O*-methylgalactose residues a carbohydrate composition analysis by gas chromatography/mass spectrometry was performed (data not shown). For details of the analytical strategy see

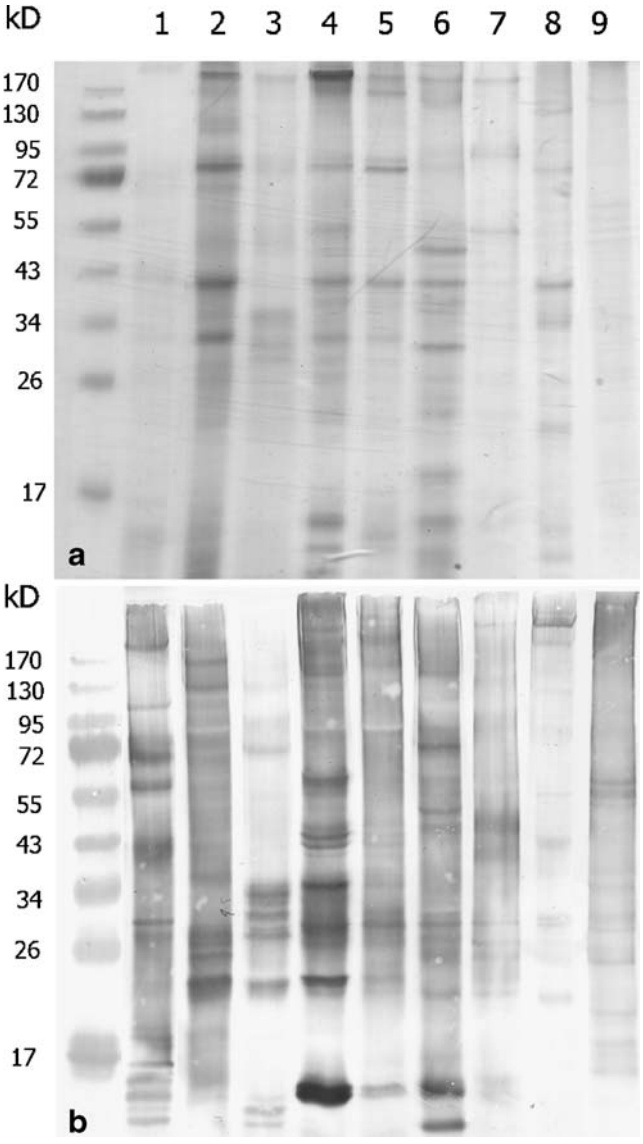

Fig. 1 Snail proteins on **a** SDS-gel electrophoresis stained with Coommassie blue and **b** the corresponding anti-HRP blots. (*1*) *Achatina fulica* muscle (*2*) *Arianta arbustorum* skin (*3*) *Arianta arbustorum* viscera (*4*) *Cepaea hortensis* skin (*5*) *Cepaea hortensis* viscera (*6*) *Limax maximus* skin (*7*) *Limax maximus* viscera (*8*) *Planorbarius corneus* skin (*9*) *Planorbarius corneus* viscera

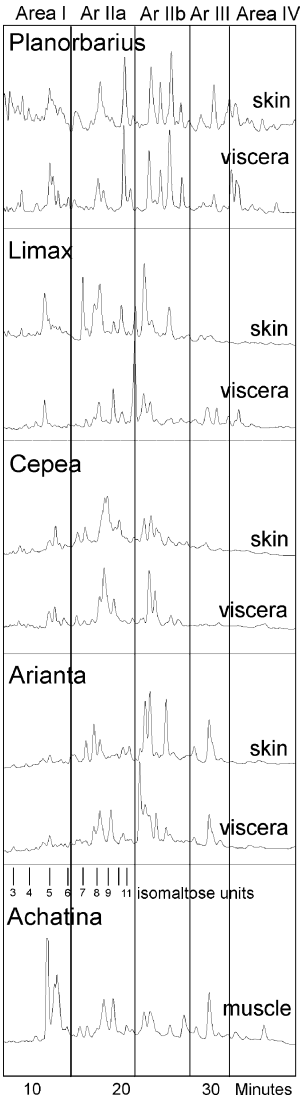

Fig. 2 HPLC-analysis of pyridylaminated neutral N-glycans of *Planorbarius corneus*, *Limax maximus*, *Cepaea hortensis*, *Arianta arbustorum* and *Achatina fulica* on a reverse-phase column. For estimation of the size in the last pattern the isomaltose standard, 3-11 glucose units is shown. Regions I-IV are indicated (I: oligomannosidic structures; II: methylated oligomannisidic structures; III: α1,6-fucosylated structures; IV: large, galactose containing structures). It has to be noticed that none of the reverse-phase peaks is a pure structure

Gutternigg *et al*. [21]; for abbreviations of glycan structures see Fig. 3. An overview on the different types of structures in the gastropod preparations can be found in Table 1. An overview on the elution times on HPLC and on the masses of the structures found in significant amounts in this study is given in Table 2.

In reversed phase area I, most of the typical oligomannosidic structures, which were confirmed by their sensitivity to α-mannosidase and endoglycosidase H, and small paucimannosidic structures, containing up to four mannose residues and additional xylose and/or α1-3-linked fucose residues linked to the inner core were found.

In the next area (area IIa and IIb) only some oligomannosidic structures were found: those which are hydrophobic due to a special conformation of the mannose residues (see above). Characteristic for this second area is the presence of 3-*O*-methylation on terminal mannose residues; the corresponding non-methylated structures are present in area I. This methylation can be complete (all terminal mannoses methylated) or incomplete (only some of the terminal mannoses methylated). In this study the same kind of methylation as in *Arion* was found [21]. No hints on methylation of internal sugar residues could be detected. Furthermore a few mammalian-like biantennary structures with terminal GlcNAc or galactose residues were found in this area in the patterns of some of the species.

Area III was mainly characterized by structures with an α1-6-fucose linked to the inner GlcNAc (Fig. 2), which is sensitive to α-fucosidase from bovine kidney. The shift of −146.1 mass units on MALDI-TOF and especially the characteristic shift to earlier elution times on reversed phase chromatography confirmed the loss of an α1-6-linked fucose to the inner core. While α1-6-linked fucose to the inner GlcNAc residue increases elution time drastically on reverse-phase column, glycans with α1-3-linked fucose to

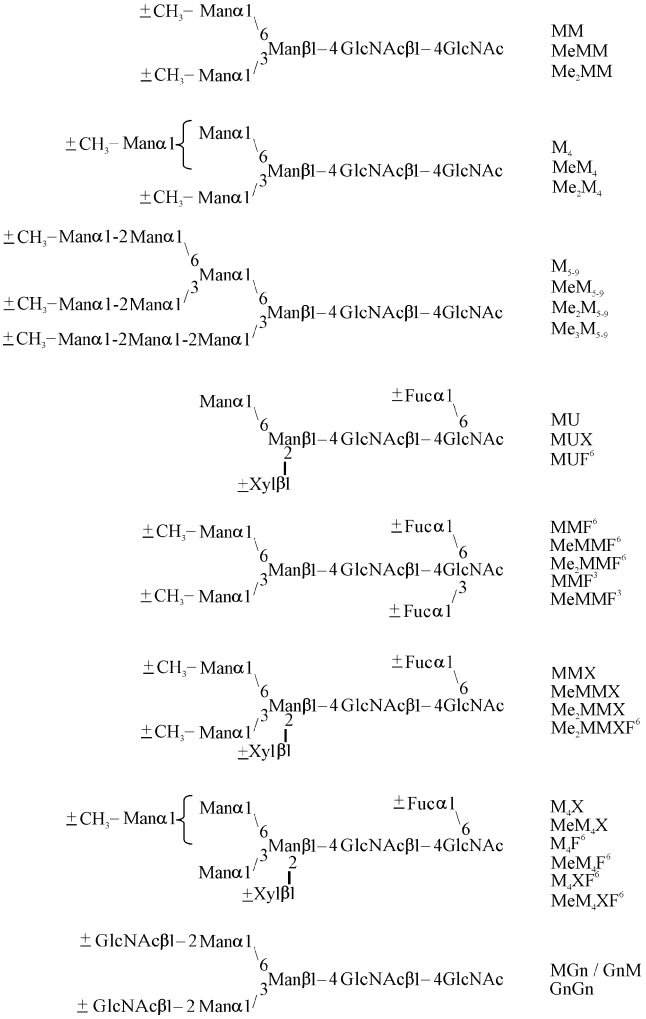

Fig. 3 Structures and nomenclature of N-glycans mentioned in this paper. The abbreviation system applied herein (according to [40]). In the case of the core fucose, which occurs in more than one type of linkage, the linkage is depicted as a superscript

Table 1 Distribution of the different types of neutral N-glycans of *Planorbarius corneus*, *Limax maximus*, *Cepaea hortensis*, *Arianta arbustorum*, *Achatina fulica* and *Arion lusitanicus*

		Mannosidic structures *e.g.* MU, MM, $M_{4–9}$	Methylated mannosidic structures *e.g.* MeMU, $Me_{1–2}MM$, $Me_{1–2}M_4$, $Me_{1–3}M_{5–9}$	α1-6-fucosylated structures *e.g.* MUF^6, MMF^6, $GnGnF^6$, M_4F^6	Methylated α1, 6-fucosylated structures *e.g.* $MeMUF^6$, Me_2MMF^6,	Other pauci-mannosidic structures *e.g.* MMX, M_4X, $MMXF^3$	Other methylated pauci-mannosidic structures *e.g.* MeMMX, MeM_4X, Me_2MMX	Complex type structures *e.g.* GnGn, GalGalX,	Complex type structures with methylated galactose
Planorbarius corneus	Skin	+++ (M_7)	+++ (MeM_4, Me_3M_5)	+	+	+	+	(+)	0
	Viscera	+ (M_5)	+++ (Me_2MM, Me_3M_5, MeMU)	+ (MUF^6)	+	+	(+)	(+)	(+)
Limax maximus	Skin	++ (M_7)	+++ (Me_2M_4, Me_3M_5)	++ (MUF^6)	+	+	+	++	0
	Viscera	+	++ (Me_3M_5)	+++ (MMF^6, M_4F^6)	(+)	++ (MUX)	+	+	(+)
Cepea hortensis	Skin	+++ (M_9)	++	+	+	++ (M_4X)	+	(+)	0
	Viscera	++ (M_9)	+++ (Me_2MM,Me_2M_4)	++ (MUF^6, MMF^6)	+	+	+	(+)	(+)
Arianta arbustorum	Skin	+++	+++ (MeMU, Me_2MM)	(+)	+ (Me_2MMF^6)	+	+	+	(+)
	Viscera	+	++ (Me_2MM)	+ (MUF^6)	+ (Me_2MMF^6)	++ (M_5X)	+	(+)	(+)
Achatina fulica	Muscle	++	+	+	++ (Me_2MMF^6)	+++ (MMX)	+ (MeMMX)	(+)	+
Arion lusitanicus (taken from [21])	Skin	+++ (M_5, M_6, M_9)	+++ (Me_2MM, Me_3M_5, Me_3M_6)	0	+ (Me_2MMF^6)	(+)	+	0	(+)
	Viscera	+	+++ (Me_2MM, Me_3M_5, Me_3M_6)	0	+	(+)	+	0	(+)

Structures are given in parentheses if one single structure is more than 5% of the total amount of neutral N-glycans of the whole tissue
+++ More than 25% of the total neutral N-glycans, ++ 15–25% of the total neutral N-glycans, + 4–15% of the total neutral N-glycans, *0* not detected

Table 2 HPLC retention times [given in glucose units] and molar masses of the main glycans which have been found in the investigated snail tissues

	Retention time on HPLC; Reverse-phase C18 (glucose units)	Retention time on HPLC; Palpak type N (glucose units)	Mass $[M + Na]^+$	Enzymes used successfully for structural confirmation
MU	8.3	3.5	848.9	α-mannosidase
MeMU	13.0	3.5	862.9	
MM	7.8	4.3	1011.8	α-mannosidase
MeMM	8.7	4.3	1025.8	α-mannosidase
Me_2MM	>14	4.3	1039.8	
M_4	9.0	5.2	1174.0	α-mannosidase, Endo H
MeM_4	11.8	5.2	1188.0	α-mannosidase, Endo H
Me_2M_4	>14	5.2	1202.0	Endo H
M_5	7.6	6.4	1336.2	α-mannosidase, Endo H
MeM_5	8.4	6.4	1350.2	α-mannosidase, Endo H
Me_2M_5	10.8	6.4	1364.2	α-mannosidase, Endo H
Me_3M_5	>14	6.4	1378.2	Endo H
M_6	6.2	7.5	1498.4	α-mannosidase, Endo H
Me_2M_6	12.2	7.5	1526.4	α-mannosidase, Endo H
Me_3M_6	>14	7.5	1540.4	Endo H
M_7	5.1 Me_7D1 7.5 Me_7D2 5.7 Me_7D3	8.1	1660.6	α-mannosidase, Endo H
Me_3M_7	>14	8.1	1702.6	Endo H
M_8	4.8 Me_8D13	9.4	1822.8	α-mannosidase, Endo H
Me_3M_8	>14	9.4	1864.8	Endo H
M_9	5.0	10.1	1985.0	α-mannosidase, Endo H
Me_2M_9	12.0	10.1	2013.0	Endo H
Me_3M_9	>14	10.1	2027.0	Endo H
MUF^6	>14	4.0	995.8	α-mannosidase α1,6-fucosidase
$MeMUF^6$	>14	4.0	1009.8	α1,6-fucosidase
MMF^6	>14	4.6	1157.9	α-mannosidase α1,6-fucosidase
$MeMMF^6$	>14	4.6	1171.9	α-mannosidase α1,6-fucosidase
Me_2MMF^6	>14	4.6	1185.9	α1,6-fucosidase
MMF^3	5.3	4.6	1157.9	α-mannosidase HF-treatment
$MeMMF^3$	8.2	4.6	1171.9	α-mannosidase HF-treatment
MUX	4.9	4.2	981.8	α-mannosidase β-xylosidase
MeMUX	7.3	4.2	995.8	
MMX	6.1	5.1	1143.9	α-mannosidase β-xylosidase
MeMMX	9.0	5.1	1157.9	α-mannosidase
Me_2MMX	>14	5.1	1171.9	
$MUXF^6$	12.0	5.8	1127.9	α-mannosidase α1,6-fucosidase β-xylosidase
$MMXF^6$	12.0	6.2	1290.0	α-mannosidase α1,6-fucosidase β-xylosidase
Me_2MMXF^6	>14	6.3	1318.0	α1,6-fucosidase
M_4X	8.2	6.3	1306.1	α-mannosidase β-xylosidase
MeM_4X	9.0	6.3	1320.1	α-mannosidase
M_4F^6	>14	6.5	1320.1	α-mannosidase α1,6-fucosidase

Table 2 (continued)

	Retention time on HPLC; Reverse-phase C18 (glucose units)	Retention time on HPLC; Palpak type N (glucose units)	Mass $[M + Na]^+$	Enzymes used successfully for structural confirmation
MeM_4F^6	>14	6.5	1334.1	α-mannosidase α1,6-fucosidase
M_4XF^6	13.0	6.7	1452.2	α-mannosidase α1,6-fucosidase β-xylosidase
MeM_4XF^6	>14	6.7	1466.2	α-mannosidase α1,6-fucosidase
M_5X	9.1	6.7	1468.3	α-mannosidase β-xylosidase
MGn	8.2	5.6	1215.1	β-hexosaminidase α-mannosidase
GnM	12.2	5.6	1215.1	β-hexosaminidase α-mannosidase
GnGn	11.3	6.6	1418.2	β-hexosaminidase
$Me_3Hex_5HexNAc_4Fuc$	>14	9.8	1930.9	α1,6-fucosidase

Furthermore the enzymes are given, which have been successfully used to confirm the estimated structures. On reverse-phase HPLC a reliable assignment to glucose units can be carried out only from 3 to 14 glucose units, therefore every structure eluting later than 14 glucose units is given as >14

the same GlcNAc residue elute earlier compared to the corresponding nonfucosylated glycans (Table 2) [39].

In some species in region IV of the reverse phase pattern eluted larger N-glycans with a number of galactose residues terminated with methyl groups. Again the linkage of the methyl groups was identified by gas chromatography/mass spectrometry to be a 3-*O*-methylation (data not shown). Due to the high heterogeneity and the low amount of these glycans not all of them could be elucidated completely.

Structures of *Planorbarius corneus*

Planorbarius corneus, the only water snail analysed, showed some small differences between the skin and the viscera patterns (Fig. 2). In viscera we found there mainly M_5, M_8, MMX and M_4X, whereas in skin M_7–M_9 were the dominant structures. In area IIa the corresponding partly methylated and in area IIb the corresponding fully methylated structures were found.

In this species the intermediate structure for the production of complex glycans in the usual biosynthetic pathway, a M_5 already substituted by a GlcNAc-transferase I (GnM_5), was determined (Fig. 4). Staining with anti-HRP antibody (specific for xylosylated and α1-3-fucosylated structures, Fig. 1) and anti-bee venom antibody (specific for α1,3-fucosylated structures) and the monosaccharide analysis supported the finding of a remarkable amount of xylosylated and α1-3-fucosylated structures. While the presence of

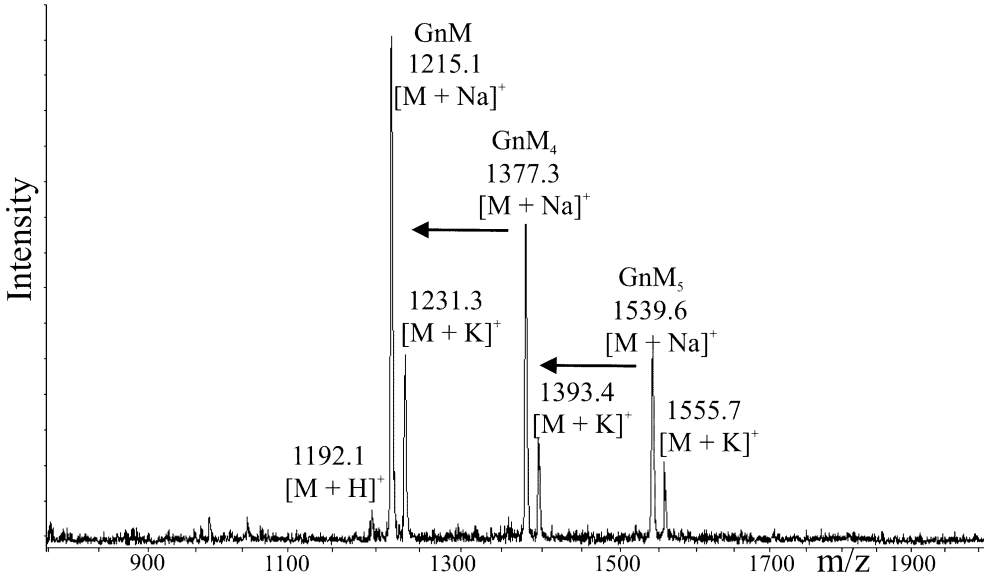

Fig. 4 MALDI-TOF-MS spectrum of pyridylaminated GnM_5 *Planorbarius corneus* viscera after a moderate digest with α-mannosidase. The intermediate product GnM_4 can be seen

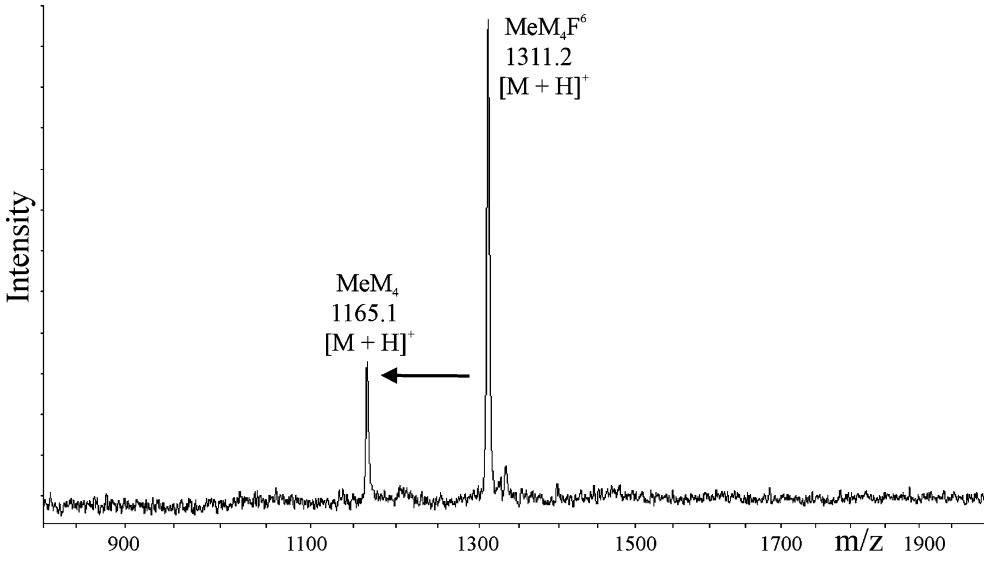

Fig. 5 MALDI-TOF-MS spectrum of pyridylaminated MeM_4F^6 *Planorbarius corneus* viscera after digest with α-fucosidase from bovine kidney

fucose linked to the inner core is easy to determine by digestion with bovine kidney α-fucosidase (for α1,6-linked fucose; Fig. 5) or chemical release with hydrofluoric acid (for α1,3-linked fucose), the verification of the presence of xylose was complicated by the fact that so far all xylosidases, those commercially available and also our own preparation from potatoes [46], which release β1,2-linked xylose from N-glycans, depend on the prior release of the whole α1,3-antenna (including the mannose) linked to the β-mannose. In *Planorbarius corneus* we found paucimannosidic xylosylated and/or fucosylated structures with 2–5 mannose residues and, to a lesser extend, xylosylated and/or fucosylated biantennary glycans terminating with galactose or GlcNAc residues. The α1,6-fucosylated structures were located mainly in area III of the reverse phase pattern, with Me_2MMF^6 being the main structure there.

In contrast to all the other species investigated, a significant amount of highly hydrophobic structures was present and eluted in area IV on reverse phase chromatography (Fig. 2). Surprisingly, some of these structures—especially in skin—were found to be sensitive to endoglycosidase H digest. It turned out that these glycans were methylated oligomannosidic glycans with "degenerated" α1,3-arms which changed their hydrophobicity and made them therefore elute very late on reverse phase. Glycans usually show an earlier retention time on reverse-phase HPLC if one or more sugars of the α1,3-antenna are missing and later retention times if one or more sugars of the α1,6-antenna are missing [49].

Those structures, which were not sensitive to endoglycosidase H, mostly contained two or more terminal galactoses, in the viscera preparation some of them were 3-*O*-methylated, and very often with a fucose linked α1,6 to the inner GlcNAc residue. One of these structures could be identified in detail by sequential exoglycosidase digests. It proved to be a biantennary N-glycan with two β1-4-linked terminal galactoses, one of them 3-*O*-methylated. The glycan contained also two fucoses, one α1-6-linked to the inner GlcNAc-residue and the other one α1-2-linked to the non-methylated terminal galactose. All the other structures in this area where present only in very low amounts.

Structures of *Limax maximus*

The glycans of the local slug *Limax maximus* differed quite strongly from those from the slug *Arion lusitanicus*, which has infiltrated Central Europe during the past decade and which was analysed in detail in our previous paper [21].

Limax maximus skin and viscera preparations showed remarkable differences. In skin most of the glycans eluted in area II. In the areas III and IV only minor peaks were found. Oligomannosidic structures, unmethylated as well as methylated, were mainly found in skin (approximately 50% of total glycans in skin, 25% in viscera), xylosylated structures were mainly in viscera (8% of total glycans in skin, 30% in viscera). The typical structure for the skin was Me_2M_4; with about 10% of total glycans for this structure. Typical for the viscera preparation were Me_3M_5, MUX, M_4F^6 and MMF^6. Also a number of small structures containing xylose as well as α1-6-linked fucose were determined (Fig. 6). Area I contained in the skin as well as in the viscera preparation just one dominant peak which was identified to be a mixture from MMX and M_4X. Furthermore some oligomannosidic structures, mainly M_4, could be detected there. The high amount of xylosylated structures was to be expected due to previous anti-HRP-blots (Fig. 1) and monosaccharide composition analysis. Overall the glycans were small. Large glycans with a

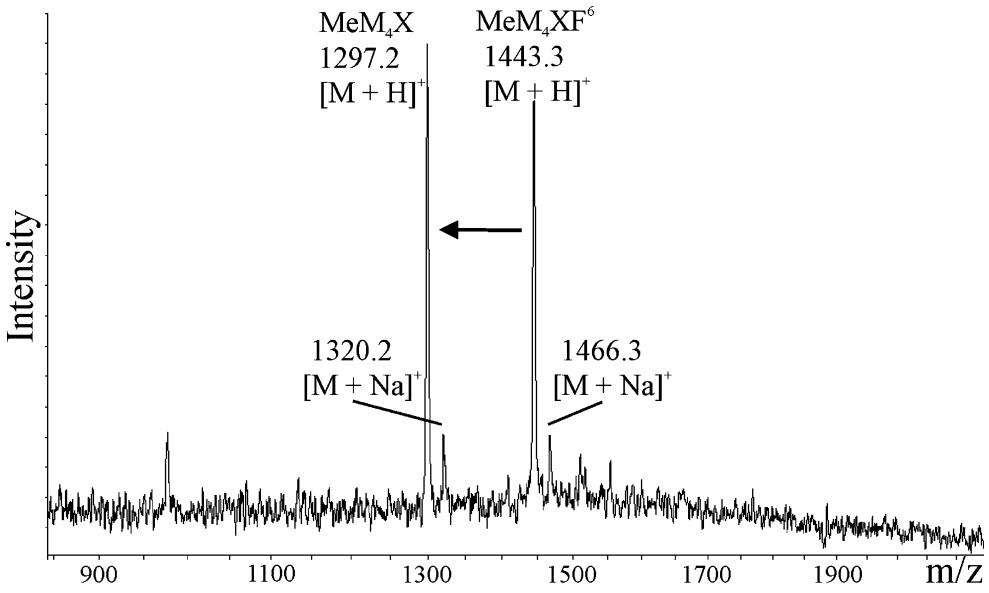

Fig. 6 MALDI-TOF-MS spectrum of pyridylaminated MeM_4XF^6 from *Limax maximus* viscera after digest with α-fucosidase from bovine kidney

number of galactose residues, as seen before in *Planorbarius corneus*, occurred only in minor traces. The methylated oligomannosidic structures contained five or less mannose residues while only a few larger unmethylated structures were found (M_7 and M_8). In skin α1-6-fucosylated structures were present mostly in their unmethylated version, with Me_2MMF^6 as the only exception. The "usual" bi-antennary complex glycans which can be found in nearly all mammalian tissues and also in plants are rarely present in snails. In *Arion lusitanicus* none of these structures have been detected [21]. In the present study only *Limax maximus* showed some of these structures in remarkable amounts. It were in skin 17% of the total glycans and in viscera 10%. In both cases two thirds of the structures terminated with one or two *N*-acetylglucosamine residues (MGn, GnGn, GnM_5) and one third with one or two galactose residues (GalGn or GalGal). In all other snails far less than 3% of total glycans were "mammalian-like" binantennary. An α1-3-fucose, confirmed by retention time on reverse-phase and sensitivity to HF-treatment, linked to the inner GlcNAc residue, which is typical for plant tissues, was found here only in traces linked to very small glycans with two or three mannoses. Analogous to *Planorbarius corneus* skin preparation *Limax maximus* viscera fraction showed some degraded oligomannosidic structures in area IV.

Structures of *Cepea hortensis*

The glycan preparations of *Cepea hortensis* displayed in skin and viscera quite similar patterns. In both preparations more than 70% of the glycans eluted in area II. Compared to the previously described species in area III less glycans and in area IV nearly no glycans could be detected (Fig. 2). Therefore it was not surprising, that some of the total glycans were sensitive to endoglycosidase H, which cleaves oligomannosidic glycans with at least four mannose residues. After an endoglycosidase H digest the remaining glycans were identified to be mainly xylosylated, most of them appearing in the methylated as well as in the unmethylated version (MeMUX, MeMMX, Me_2MMX, MUX, MMX, M_4X). Furthermore some small α1-6-fucosylated glycans (MUF^6, MMF^6, $MeMMF^6$, Me_2MMF^6), a few oligomannosidic structures which were too small to be sensitive to endoglycosidase H (MeMM, Me_2MM) and both xylosylated as well as α1,6-fucosylated glycans ($MUXF^6$, $MMXF^6$) were found.

Structures of *Arianta arbustorum*

Similar to *Cepea hortensis*, most of the glycans of *Arianta arbustorum* eluted in area II and only traces were found in area IV. In area III a significant amount of glycans was detected, in area I only minor amounts were found (Fig. 2). Analysing the structures in detail, differences between the skin and the viscera preparation could be determined. Whereas in skin oligomannosidic structures dominated the pattern, in viscera a significant amount of paucimannosidic structures, again many of them xylosylated, could be identified. M_5X alone made up to 12% of total neutral N-glycan content of *Arianta arbustorum* viscera preparation. M_4F^6 was identified in the skin preparation. Both structures, containing only mannoses on the antennae and xylose or fucose linked to the inner core should not be possible according to the usual biosynthesis pathway and will be discussed later. In both tissues Me_2MMF^6 was the main α1-6-fucosylated (14% in skin, 10% in viscera) glycan.

Structures of *Achatina fulica*

Achatina fulica preparation was not separated into a skin and a viscera fraction but the edable part was taken, the muscle. Therefore, only one sample of this species was analysed.

Most of the structures appeared on reverse-phase chromatography in the first two areas (area I—37% and area IIa and IIb—54% of total glycans). One dominant peak in area III and another one in IV completed the pattern (Fig. 2). Preliminary anti-HRP-blotting experiments (Fig. 1) strongly indicated the existence of large amounts of xylosylated and/or α1,3-fucosylated structures. However, whereas the xylosylation was confirmed, no α1,3-fucosylated structures could be detected in our preparations.

In area I the dominating structures were xylosylated paucimannosidic structures with 2 to 4 mannose residues and only a few larger oligomannosidic glycans were found. In area IIa and IIb the corresponding structures with partly or fully methylated terminal mannoses were detected. A very large amount of structures, carrying xylose as well as α1-6-linked fucose to the innermost GlcNAc-residue were found here. Again, microheterogeneity was due to the absence of one mannose and/or varying numbers of methyl groups linked to terminal mannoses.

In area III Me_2MMF^6, well known from the other species, was the dominating structure which was accompanied by minor amounts of the same glycan missing the α1-6-linked mannose including the methyl group. Of the total glycans, 3.6% eluted in area IV. These structures were resistant to exo- or endoglycosidase digests except the digest with bovine kidney α-fucosidase. Analysis by ESI-LC-MS showed that the main structure consisted of four *N*-acetylhexosamine residues, five hexoses, one fucose and three methyl groups. This glycan was subjected to tandem MS analysis (Fig. 7). The localisation of the fucose was possible due to the fragment with the mass 446.24. It fits to the reducing end *N*-acetylglucosamine of the core carrying a fluorescence pyridylamino label as well as a fucose residue. The fucose was proven to be α1,6-linked to the inner core, which corresponded to the hydrophobicity of the glycan and its sensitivity to bovine kidney fucosidase. The inner core of the N-glycan (three mannoses and two *N*-acetylglucosamines) was terminated on one antenna with a mannose carrying a methyl group. The other antenna was elongated by two additional *N*-acetylhexosamine residues and two methylated hexose residues. Due to the high hydrophobicity of the glycan this elongation was most likely on the α1,3-antenna of the structure.

The two *N*-acetylhexosamines which were not from the core were proven to be located linked in series on the antenna, because of the occurrence of the fragment 407.19 (2 HexNAc without label). The fragment 556.25 fitted to an *N*-acetylhexosamine with two methylated hexoses. This structural element seemed to be the non-reducing terminus on the elongated arm. Monosaccharide analysis indicated that these terminal hexoses were galactose residues. Gas chromatography/mass spectrometry indicated that the methyl-group was 3-*O*-linked similar to the mannose-residues (data not shown). A confirming exoglycosidase digest of the whole glycan was not possible because if methyl groups

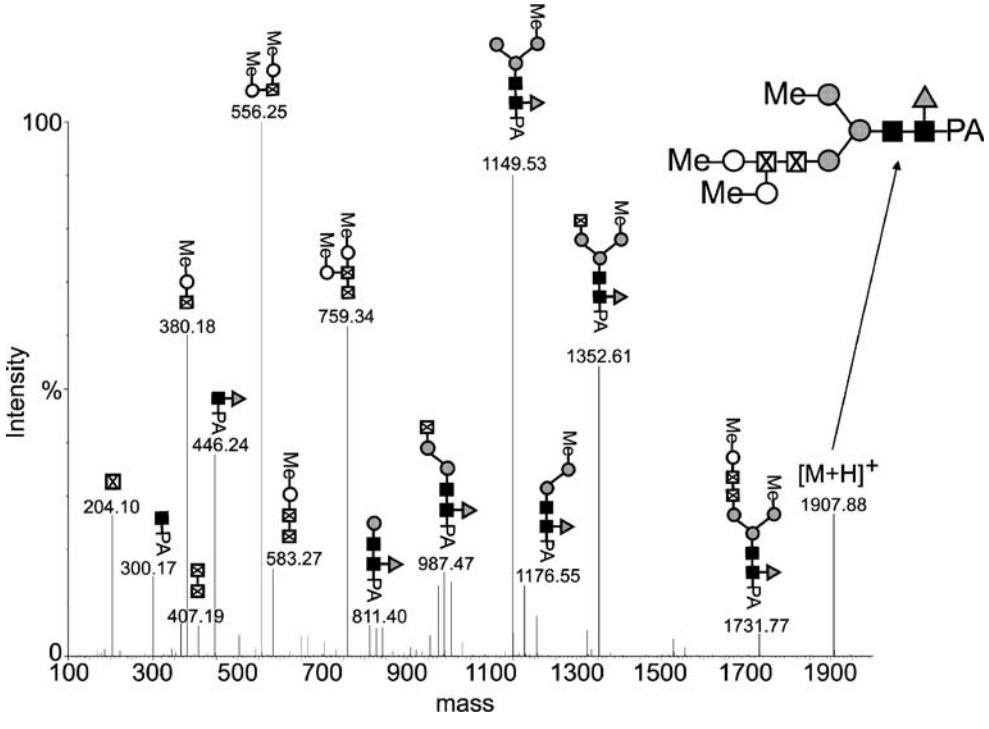

Fig. 7 LC-ESI-MS/MS spectrum of the glycans of a subfraction of area IV of *Achatina fulica*. Mayor peaks are annotated with the corresponding glycan structure in symbols. *Black square*, GlcNAc; *square divided diagonally*, hexosamine; *grey circle*, mannose; *open circle*, galactose; *grey triangle*, fucose; Me, methylgroup; PA, pyridylamino; The nomenclature was established by the CFG. Complete annotation of the spectra can be found at the CFG web site http://www.functionalgenomics.org/glycomics/publicdata/glycoprofiling.jsp)

terminate an antenna, they prevent terminal hexoses from being cleaved by mannosidase and galactosidase, respectively. A very minor compound in the area IV showed the same number of monosaccharide compounds as above, but with a second fucose. It is definitely not linked to the inner GlcNAc to form a difucosylated core-GlcNAc. It is located on one of the antennae and is not sensitive to any of the used fucosidases or treatment by hydrofluoric acid. Also a glycan with similar structure but an additional xylose linked to the core was identified as a very minor compound of area.

Discussion

Not much is known about the glycosylation potential of gastropods. Only scattered data exist on some species in which mostly the hemocyanin has been investigated. This lack of data is surprising as already one successful molluscan continuous cell line exists, the Bge line (American Type Culture Collection) from the freshwater snail *Biomphalaria glabrata*. This snail serves as an intermediate host for the flatworm *Schistosoma mansoni*, which is under constant investigation due to its pathogenic potential.

Other snail species can also be hosts for pathogenic organisms, or at least may cause significant damage to vegetable cultures. On the other hand, these not so well investigated organisms may still hide a powerful spectrum of glycosylation capacity which could be used for closer investigation of glycan biosynthesis or the analysis of carbohydrate functions. In the interplay of parasite and host carbohydrates may play a role somewhere in the very species specific attraction, recognition, adhesion and invasion processes [1, 50].

Closer determination of the neutral N-glycans of five different gastropods, one water snail (*Planorbarius corneus*), one slug (*Limax maximus*) two European (*Cepaea hortensis*, *Arianta arbustorum*) and one African (*Achatina fulica*) shell-carrying land species, should give an overview on the glycosylation potential of gastropods.

SDS-PAGE of the proteins followed by blots with specific lectin or antibody detection gave a first impression of the glycan patterns to expect. Then N-glycans were prepared from the glycoproteins by proteolytic digest, release of the glycans from the peptides and fluorescent labelling. The structures of each tissue were fractionated by reverse phase HPLC into four different areas (I–IV) due to their hydrophobicity. A further fractionation of the obtained peaks by size gave more pure fractions but only in a few cases completely purified structures. The structures were analysed for their monosaccharide composition and for their mass by MALDI-TOF mass spectrometry. To obtain information on the position of the sugars in the glycan, 2-dimensional HPLC (size and hydrophobicity) and MALDI-TOF mass spectrometry before and after digestion with specific exoglycosidases was carried out. In some cases also ESI-LC-MS analysis was carried out. Further information on linkages, especially the linkage of the methylation group to the hexoses, was gained by methylation analysis followed by gas-chromatography/mass spectrometry. This methodical approach gave a fast overview on the complex glycan patterns of the different species. As soon as the main structures of the four areas on reverse phase HPLC were identified, a strategic glycosidase digest (e.g. endoglycosidase H for cleaving all oligomannosidic structures with more than four mannose residues) made it possible to focus on minor structures which had not been seen clearly before. Glycan pools which could not be separated into single structures still could be analysed by the use of moderate amounts of exoglycosidases. A concentration of the enzymes was chosen, where the source structure, the final product and all of the intermediate steps of the digestion could be observed.

Analysis showed, that most of the structural elements occurred in all investigated species independent of their origin in the skin as well as in the viscera fraction. However the relative amounts varied from species to species and also sometimes a significant difference between skin and viscera could be observed. So far it is up to speculation if these differences are due to other needs of function in the different natural environments.

The most obvious structural element of the snail glycans is the 3-*O*-methylation of terminal hexoses (mannoses and galactoses). This kind of modification has been described since the early seventies in polysaccharides of prokaryotes, lower eukaryotes, algae and fungi, in gastropod hemocyanin 3-*O*-methylated mannose and 3-*O*-methylated galactose were found by Hall *et al*. [51]. Mammals, insects or plants do not show this element as far as we know today. May be it is some kind of adaptation in the relation between host and parasite. Nothing is known so far about function of this elongation nor about the enzyme(s) which is/are responsible for this elongation. However, the product turns out to be very hydrophobic and insensitive to all commercially available exoglycosidases. Therefore one function of the glycans could be a shielding of sensitive proteins and preventing them from digestion.

Fucosylation occurs mainly and quite frequently as an α1-6-fucose linked to the innermost GlcNAc residue of the N-glycan core. This kind of fucosylation is usual over a wide range of animals. Low amounts of α1,3-fucosylation to the same GlcNAc have been found in *Arianta* and *Cepaea*, but it has to be noticed that no difucosylation of this GlcNAc, as can be seen in insects [48], was observed. Traces of a further fucose residue linked to the antennae were detected in large hydrophobic glycans. In *Planorbarius* structures the terminal fucose could be removed by α1,2-

fucosidase, for *Achatina* structures this was not possible. However, no indications were found for a branching on fucose, which has been claimed recently for *Rapana thomasiana* hemocyanin [52].

A further common modification of the glycans was one xylose residue linked to the β-mannose of the core. This position is the usual one for xylose often seen in plant glycans. Enzymatic analysis of this sugar is inconvenient as so far all available xylosidases, including our own one [46], which may remove this xylose, work exclusively on glycans where the α1,3-antennae has been already completely removed.

For several organisms it has been shown that a non-reducing terminal GlcNAc residue which is linked to the 3-arm of the antennae is a prerequisite for the action of further modifying enzymes such as other GlcNAc-transferases, core-fucosyltransferases or the xylosyltransferase [39, 53, 54]. Here, in the snail tissues, both decorations of the inner core (fucosylation and xylosylation) are often seen on very small glycans which lack any terminal elongation of the antennae. Overall less than 3% of the structures carry a terminal GlcNAc with or without further elongation. Two explanations for this fact are possible so far. In insects and *Caenorhabditis elegans* a highly active Golgi-located hexosaminidase has been described, which removes the GlcNAc immediately after the transfer by the other enzymes took place [55–57]. This may also be the case in gastropods. Or, analogous to the core α1,3-fucosyltransferase (FUT-1) from *Caenorhabditis elegans*, the enzyme may not need or want any terminal GlcNAc to recognize an N-glycan as a substrate [58]. Even in mammalian tissue a GlcNAc I independent fucosylation pathway has been described recently [59]. A detailed investigation of the related gastropod enzymes is necessary to clarify how the biochemical pathway works in these organisms.

The pool of large hydrophobic structures was small and very heterogenic. Only in *Planorbarius* and *Achatina* some analysis was possible. The structures had usually one long and one short antenna. We concluded from the elution behaviour on HPLC, that the 3-arm can be extended, whereas the 6-arm carries only one or two mannoses substituted by a methyl group. The long arm consists of two *N*-acetylhexosamine residues linked together and is usually terminated by a galactose which is also decorated with a methyl group. These structures correlate in principle to some extend with structures which have been described in *Helix pomatia* and *Biomphalaria glabrata* [15, 26].

Most of these glycans carried one fucose α1-6-linked to the inner core. A second fucose, which was detected on some glycans, could be designed in *Planorbarius* to be α1-2-linked to one antenna by its sensitivity to α1,2-fucosidase treatment, whereas in *Achatina* no exoglycosidase digest with this enzyme was positive. Many of these big structures carried also xylose linked to the inner core.

Comparing the glycosylation patterns of snails so far obtained, keyhole limpet hemocyanin [11, 12], *Lymnea stagnalis* hemocyanin [16, 17], *Helix pomatia* hemocyanin [15], *Arion lusitanicus* [21], *Biomphalaria glabrata* [26] and the 5 species analysed here, no obvious difference can be seen between water and land living animals or between species carrying a shell or not. Also the differences between skin and viscera fraction were not dramatic. The percentages of the structures vary slightly with the area (due to nutritional conditions) where the snails had been collected, which was seen for *Limax,* the age (size) of the individuals and their physiological status (carrying eggs or not), both seen for *Planorbarius* and *Achatina*. However, skin and viscera preparations contained the same spectrum of N-glycans. Therefore it can be ruled out that unusual structures are due to food or environmental contaminants.

The gastropod structures seen in our analysis seem to be a potentially valuable source for a large number of novel N-glycans. Their complex N-glycan pattern combines typical structural features of all kinds of organisms (mammals, plants, insects, nematodes, trematodes) investigated before. This makes them a valuable model for the investigation of the regulation of glycosylation. Some of these structures may be a safe way to stimulate directly the immune response of humans or cattle to recognize and fight against pathogenic nematodes and trematodes, or to induce the production of antibodies in other organisms which then can be used in diagnosis and therapy.

Acknowledgements This project was partly financed by the Austrian Fonds zur wissenschaftlichen Forschung Project number P13928-BIO. We want to thank Dr. Manfred Pintar (Department for Integrative Biology, Institute for Zoology, University of Natural Resources and Applied Life Sciences, Vienna,) for identification and classification of the snails and Dr. Iain Wilson for reading the manuscript. The technical help of Thomas Dalik, and Denise Kerner is highly appreciated.

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