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Glycan structures of the structural subunit (HtH1) of *Haliotis tuberculata* hemocyanin

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Abstract The oligosaccharide structures of the structural subunit HtH1 of *Haliotis tuberculata* hemocyanin (HtH) were studied by mass spectral sequence analysis of the glycans. The proposed structures are based on MALDI-TOF-MS data before and after treatment with the specific exoglycosidases β1-3,4,6-galactosidase and α1-6(>2,3,4) fucosidase followed by sequence analysis via electrospray ionization MS/MS-spectra. In total, 15 glycans were identified as a highly heterogeneous group of structures. As in most molluscan hemocyanins, the glycans of HtH1 contain a terminal MeHex, but more interestingly, a novel structural motif was observed: MeHex[Fuc(α1-3)-]GlcNAc, including thus MeHex and (α1-3)-Fuc residues being linked to an internal GlcNAc residue. While the functional unit (FU) c (HtH1-c) is completely lacking any potential glycosylation site, FU-h possesses a second exposed sugar attachment site between beta-strands 8 and 9 within the beta sandwich domain compared to the other FUs. The glycosylation pattern/sites show a high degree of conservation. In FU-h two prominent potential glycosylation sites can be detected. The finding that HtH1 is not able to form multidecameric structures *in vivo* could be explained by the presence of the exposed glycan on the surface of FU-h.

Keywords Electrospray ionization · *Haliotis tuberculata* · Hemocyanin · Mass spectrometry · Oligosaccharide structures

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Introduction

Hemocyanins (Hcs) are oligomeric blue copper-containing respiratory proteins with a complex quaternary structure that play a role as dioxygen carriers in the hemolymph of different species of molluscs and arthropods [1]. They have recently received particular interest because of their extremely high molecular mass, large differences in oligosaccharide structures [2–8] and immunological properties [9–13].

Structural studies of molluscan hemocyanins of *Helix pomatia* (HpH) [13], *Lymnaea stagnalis,* [14, 15], *Rapana venosa* (RvH) [4–9] and keyhole limpet *Megathura crenulata* (KLH) [16] revealed that they are heterogeneously glycosylated proteins, carrying mainly high mannose-type glycans with 5–7 mannosyl residues, hybrid-type species with a five mannoses and one *N*-acetylgalactosamine-containing chain, as well as truncated sugar chains derived thereof. Unique features for KLH are the presence of a Gal(β1–6)Man determinant, which has not been found in glycoprotein-*N*-glycans so far [12, 16] as well as a novel type of *N*-glycans exhibiting Gal(β1–4)Fuc(α1–6)- or Gal(β1–4)Gal(β1–4)Fuc (α1–6) core modifications [17].

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Studies on the complete N-glycan spectrum of hemocyanin from *Arion lusitanicus* [18] revealed that it contains an enormous potential for generating a large set of structural elements commonly found in eukaryotic *N*-glycosylation [17–20]. It carries either α1-6-linked as well as α1-3-linked fucose, as shown for some hemocyanins from insects, nematodes and trematodes, a β1-2-linked xylose, as found in plants and trematodes, and methylated terminal sugars (mannose and galactose) as found in nematodes [18]. Thus, these glycans combine structural features from mammals, plants, insects, nematodes and trematodes. Recent studies demonstrated the presence of unusual N-glycan structures, with an internal fucose residue connecting one GalNAc (ß1-2) and one hexuronic acid in both the subunits RvH1 and RvH2 of *Rapana venosa* hemocyanin [9, 21, 22]. Such fundamental differences in glycosylation of hemocyanins could well play a biological role, as was observed in the helminth species, including schistosomes and *Haemonchus contortus*, which produce unusual core-modified *N*-glycans implicated in the glycan-induced immune responses that are particularly observed in parasitic helminth infections [23].

The carbohydrate moiety of molluscan Hcs has recently received particular interest because of its immunostimulatory properties [9, 16, 18, 22–28]. Keyhole limpet hemocyanin (KLH1) is even widely used in clinical studies due to these properties [27, 28]. It was found that the Hcs of *Helix vulgaris* (HvH) [31, 32, 34], the Chilean gastropod *Concholepas concholepas* (CcH) [29], and *Rapana venosa* (RvH) [33] also have significant antitumor activities. One isoform (β-HlH) of *Helix lucorum* hemocyanin and RvH activate the immune system in Guerin ascites tumor-bearing animals, and may therefore be a good alternative for the treatment with KLH [31, 34]. Indeed, the latter has been used as an immunotherapeutic agent in the treatment of certain types of cancer (mainly bladder carcinoma) and as a carrier for vaccines [27, 30, 35, 36].

The effects of the native molecules of hemocyanins, of their structural subunits, and of the glycosylated and the non-glycosylated functional units were studied against different viruses. We showed that the glycosylated FUs RvH2-e and RvH-c have antiviral properties against the HSV virus type 1 and respiratory syncytial virus (RSV), respectively, whereas native RvH and the non-glycosylated FUs did not exhibit antiviral activity [9, 22, 26]. The antiviral effects of carbohydrate chains of the FUs are likely to occur by the interaction with specific regions of HSV glycoproteins through van der Waals interactions in general, or with given amino acid residues in particular [9, 26].

Also the Hc from the marine gastropod *Haliotis tuberculata* (Prosobranchia, Archaeogastropoda) is considered to be a possible substitute for KLH as an immunostimulant [35]. However, the oligosaccharide structures of the two-hemocyanin isoforms from *H. tuberculata* are not known. So far, 13 potential *N*-glycosylation sites were identified for the hemocyanin isoform HtH1, sequenced *via* its cDNA [37]. The aim of the present study is therefore to identify and characterize the carbohydrate structures of the hemocyanin from this marine gastropod based on mass spectrometric evidence.

Materials and methods

Isolation of glycans from structural subunits HtH1

Intact HtH1 was obtained from total HtH as described previously by Keller *et al*. [38]. For deglycosylation, approximately 4 mg of HtH1 were dissolved in 50 µl of denaturing solution (1% SDS, 0.5 M mercaptoethanol, 0.1 M EDTA), followed by incubation at room temperature for 30 min. A volume of 300 µl of Na-phosphate buffer (200 mM, pH 8.6) was added and the solution was placed in a boiling water bath for 5 min. After cooling to room temperature, 50 µl of Triton X100 and 5 µl of PNGase F (2 units) (Roche Diagnostics GmbH, Mannheim, Germany) were added. This mixture was incubated during 20 h at 37°C. The liberated *N*-glycans were purified from the reaction mixture by solid phase extraction on a Carbograph column (Alltech, Lokeren, Belgium), and the glycans were eluted with 2 ml of 25% acetonitrile/0.05% TFA. The collected fraction was dried and dissolved in 30 µl H_2O (stock solution) for further analyses.

Mass spectrometric analysis of the glycans by MALDI-TOF/TOF-MS

After treatment of HtH1 with PNGase F, the isolated oligosaccharides were sequenced by specific glycosidases as described by Sandra *et al*. [21]. The exoglycosidases β1-3,4,6-galactosidase (from bovine testes) and α1-6(>2,3,4)-fucosidase (from bovine kidney), both obtained from Calbiochem, were used in sequence, and the reactions were started with the addition of 5μl of these enzymes to the glycans dissolved in either acetate (100 mM) or phosphate buffers (50 mM) at different pHs (pH 4–8). After incubation for 24 h at 37°C, the samples were analysed by MALDI-TOF/TOF-MS.

The undigested sugar stock solution and the exoglycosidase digests were diluted 100- and 10-fold, respectively, and 1 µl of a 1:1 sugar-matrix mixture was applied onto the MALDI target. The matrix was a dihydroxybenzoic acid solution in 50% acetonitrile (10 mg/ml). The analyses were carried on a 4700 Proteomics Analyser with TOF/TOF optics (Applied Biosystems, Framingham, MA). The mass spectrometer has a 200 Hz frequency-tripled Nd-YAG laser operating at a wavelength of 355 nm. A total of

1500 shots was acquired in the MS mode. Spectra from *m/z* 900 to 3000 were recorded. Deduced monosaccharide compositions are assigned to $[M+Na]^+$ ions.

Electrospray mass spectrometry analyses (MS and MS/MS)

Off-line ESI-MS measurements of the glycans were performed on a Q-Trap mass spectrometer (Applied Biosystems), equipped with a nanospray ion source (Proxeon, Odense, Denmark), and using Proxeon medium nanospray needles. Typically, 10 μl of sample in 50% MeOH was introduced. The needle voltage was set at 1000 V. In the product ion-scanning mode, the scan speed was set to 1000 Da/s, with Q-trapping being activated. The trap fill-time was 200 ms in the MS/MS- scan mode. The resolution of Q1 was set to 'low'. Excitation time was set at 100 ms.

HtH1 modelling and glycan structure localization

The models of the tertiary structures of *H. tuberculata* hemocyanins HtH1abcdefg and HtH2abcdefg [37, 39] are based on the X-ray structure of *Octopus dofleini* hemocyanin [(OdH-g) [40]. The models of HtH1-h and HtH2-h are derived from the X-ray structure of KLH1-h [41]. The raw sequences of the single functional units of HtH1 and HtH2 were aligned and fitted onto the ternary structures of KLH and OdH, respectively, using SwissPdb Viewer [42]. Potential N-glycosylation sites were identified by their consensus sequences NXT or NXS. Carbohydrate side chains are examplarily visualized by attaching the known structure of the OdH-g glycan tree [40].

Results and discussion

Isolation and identification of glycans in HtH1 by MALDI-TOF-MS

In total 13 N-linked glycosylation sites were identified in the gene sequence of HtH1, but only some of them appear to be glycosylated. To identify the carbohydrate structure of HtH1, the glycans were removed after digestion with glycosidase PNGase F. The liberated N-glycans were then purified from the reaction mixture by solid phase extraction on a Carbograph column and analysed by mass spectrometry.

Two approaches were applied to analyse the isolated glycans. The first approach included sequencing of the glycans by specific glycosidases and analysis of the fragments via MS before and after treatment with the enzymes giving only preliminary results about the structures of the glycans. Therefore, the second approach, tandem mass spectrometry was applied, and the glycan structure being derived from their MS/MS spectra, obtained on a hybrid quadrupole- linear ion trap mass spectrometer.

The partial compositional information about the isolated glycans was obtained based upon exoglycosidase treatment with two specific glycosidases, β1-3,4,6-galactosidase and α1-6(>2,3,4)-fucosidase. The MALDI-TOF-MS mass spectrum of the underivatised HtH1 N-glycans is presented in Fig. 1a, showing the Na^+-adducts. We could identify all the labeled peaks (Table 1). As shown in Fig. 1b, the spectrum did not significantly change after treatment of the sugar stock solution with β1-3,4,6-galactosidase. This indicates that there are no β1-3,4,6-linked galactose residues in N-glycans of HtH1. However, in general, β1-3,4,6-galactosidase is not capable of cleaving the galactose residues in the Lewis X and Lewis Y structures and those modified with sulfate and/or methyl groups. Therefore, in the methylated glycans, Man or Gal were not differentiated and were represented as MeHex.

Identification of fucose (Fuc) in the glycan chains was performed after treatment with the enzyme α1-6(>2,3,4) fucosidase, which cleaves α1-6-linked fucose residues on trimannosyl cores of N-linked oligosaccharides more efficiently than other α-fucose linkages. After treatment with the enzyme, a series of prominent peaks present in Fig. 1a disappeared at *m/z* 2129.5, 2115.5, 2101.4 and 1079.4 (Fig. 1c), while new peaks appeared at *m/z* 1969.6, 1955.6, 1837.5, 1636.5 and 1298.5, respectively. The latter peaks originated from glycans at *m/z* 2115.1, 2101.4, 1893.4, 1782.4 and 1444.4, respectively, after removal of one α1-6 linked Fuc residue (146 Da) that was terminally attached to the HexNAc core (Fig. 1a and c). Further changes were also observed for the peaks at *m/z* 1983.6, 1590.1, and 1458.2, increasing considerably their intensity, and for the peaks at *m/z* 1782.4, and 1604.5 having reduced intensity upon α1-6(>2,3,4) fucosidase treatment. The strong peak intensities at *m/z* 1983.6 and 1458.2 are probably due to the removal of α1-6 Fuc from glycans at *m/z* 2129.5 and 1604.4 (Fig. 1c and a). The glycan peaks at *m/z* 1257.4, 1271.4, 1285.4, and 1823.4 did not change in intensity in the spectra 1a-1 c, indicating that no α1-6 Fuc residue is present in these ions.

Analyses of the glycans by Q-Trap-MS/MS

Sequencing and determination of the configurations of the 15 *N*-glycans released from HtH1 after PNGase F-treatment were performed by Q-Trap tandem mass spectrometry. Based on these data we propose the structures of these N-glycans isolated from HtH1 as presented in Table 1. For the glycans 1, 5, 8, 10, 12, 13, 14 and 15, a common structural feature was found, one α1-6 linked fucose being attached to the trimannosyl core.

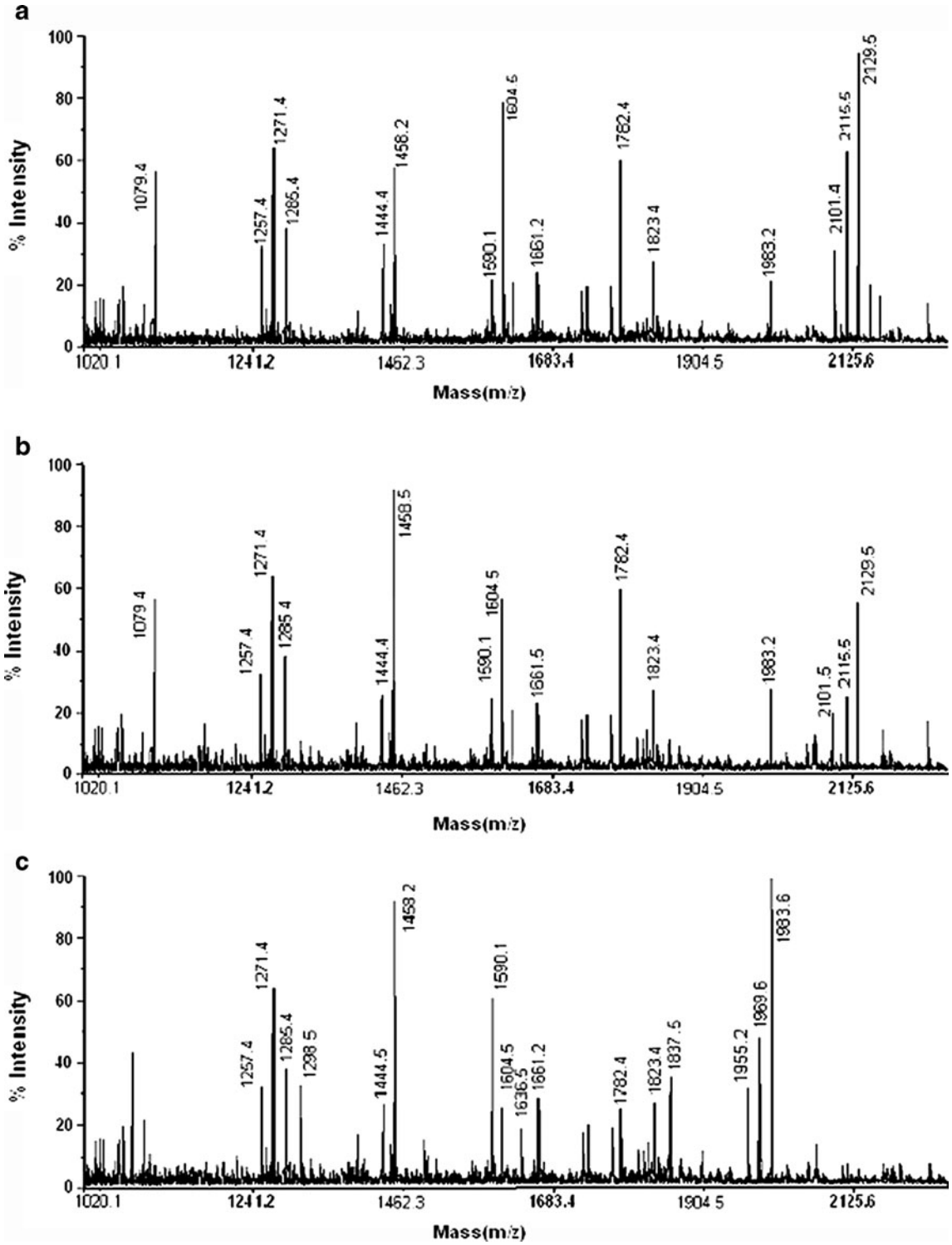
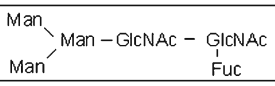
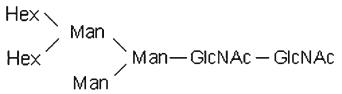
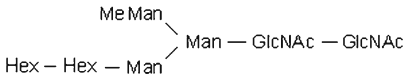
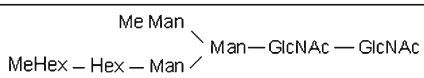
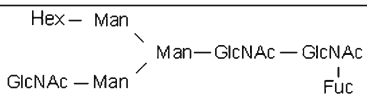
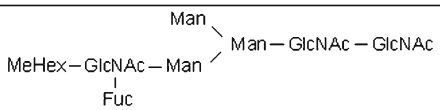
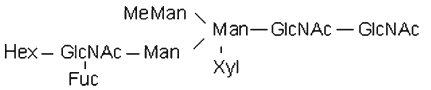
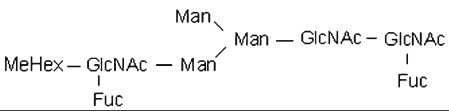
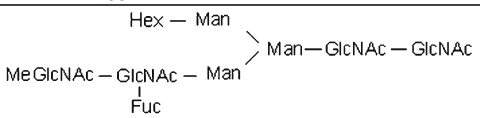
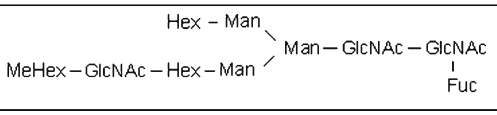
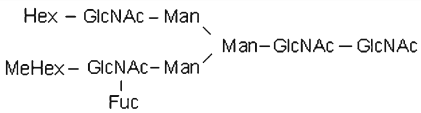
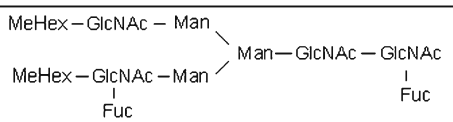
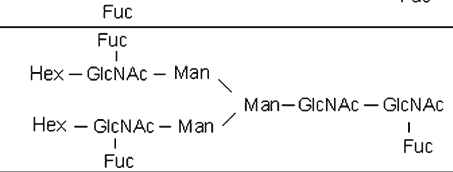
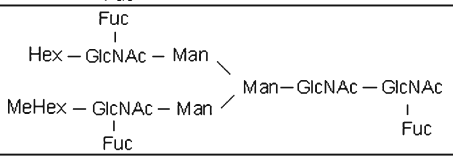
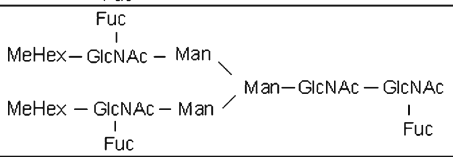


Fig. 1 MALDI-TOF-MS spectra of the N-glycans of HtH1 before (a) and after treatment with the exoglycosidas β1-3-galactosidase (b) and α1-6(>2,3,4) fucosidase (c). The sugar stock solution and the exoglycosidase digests were diluted 100- and 10-fold, respectively, and 1 μl of a 1:1 sugar-matrix mixture was applied onto the MALDI target

To illustrate the structure determination of these sequences, we selected three examples for a more detailed description. Figure 2 shows the sequence of one of these glycans, represented as $[M+H]^+$ at *m/z* 1248.6 (Table 1, glycan № 3) in the electrospray spectrum. Following the sequence of B- and Y- ions, the structure of the glycan was found to contain 4 hexose, one 3-*O*-methyl-D-mannose and two *N*-acetyl glucosamine residues, the core structure thus being

Table 1 Glycans structures from HtH1. The $[M+Na]^+$ ions are shown in the spectrum of Fig. 1

№	Glycan's structures from HtH1	Q-Trap/MS (m/z)	MALDI-TOF/MS (m/z)
1		$[M+H]^+$ 1056.8	$[M+Na]^+$ 1079.4
2		$[M+H]^+$ 1235.0	$[M+Na]^+$ 1257.4
3		$[M+H]^+$ 1248.6	$[M+Na]^+$ 1271.4
4		$[M+Na]^+$ 1285.0	$[M+Na]^+$ 1285.4
5		$[M+H]^+$ 1422.0	$[M+Na]^+$ 1444.4
6		$[M+H]^+$ 1436.0	$[M+Na]^+$ 1458.2
7		$[M+H]^+$ 1568.0	$[M+Na]^+$ 1590.1
8		$[M+H]^+$ 1582.0	$[M+Na]^+$ 1604.5
9		$[M+H]^+$ 1639.0	$[M+Na]^+$ 1661.2
10		$[M+2Na]^{2+}$ 902.8	$[M+Na]^+$ 1782.4
11		$[M+2Na]^{2+}$ 923.2	$[M+Na]^+$ 1823.4
12		$[M+2Na]^{2+}$ 1002.8	$[M+Na]^+$ 1983.2
13		$[M+2Na]^{2+}$ 1062.2	$[M+Na]^+$ 2101.4
14		$[M+2Na]^{2+}$ 1069.2	$[M+Na]^+$ 2115.5
15		$[M+2Na]^{2+}$ 1076.3	$[M+Na]^+$ 2129.5

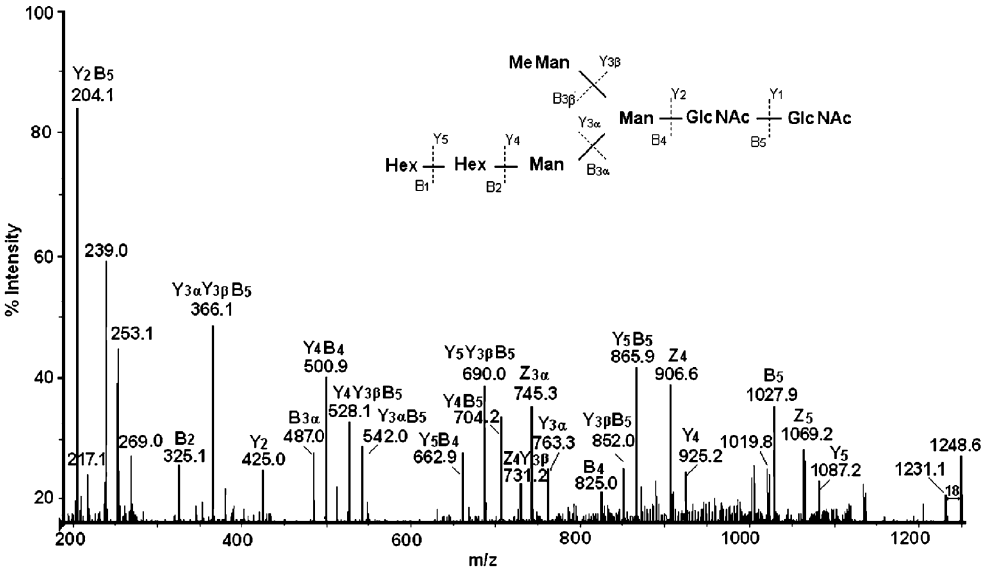


Fig. 2 MS/MS spectra and structures with fragmentation nomenclature of the $[M+H]^+$ of the glycan at *m/z* 1248.6 with high mannose type structure, isolated from HtH1

partially modified by the methyl group. The methylated sugars 3-*O*-methyl-D-mannose and 3-*O*-methyl-D-galactose have also been identified in the structure of other molluscan hemocyanins, such as *Helix pomatia*, *Lymnaea stagnalis* and *Arion lusitanicus* [11, 13–15, 18]. The linkage positions of the hexose and methylhexose residues could be assigned from the MS/MS spectrum (Fig. 2). The Y_4B_4 ion detected at *m/z* 500.9 (Man_2 MeMan) demonstrates that three hexose residues are linked, as well as the $B_{3\alpha}$ ion at *m/z* 487.0 corresponding to the composition Man3. Furthermore, the ion fragment Y_4B_5 at *m/z* 704.2 (Man_2 MeMan GlcNAc) corresponds to one GlcNAc residue and the Z4 ion at *m/z* 906.6 (Man_2MeManGlcNAc$_2$) to two GlcNAc residues. Tracking the typical ions Y_2B_5, $Y_{3\alpha}Y_{3\beta}B_5$, $Y_4Y_{3\beta}B_5$, $Y_5Y_{3\beta}B_5$, $Y_{3\beta}B_5$ at *m/z* 204.1, 366.1, 528.1, 690.0, and 852.0, the fragment Hex_4GlcNAc was determined. The observed B_5 ion at *m/z* 1027.9 corresponds to Hex_4MeHexGlcNAc, resulting from the loss of one GlcNAc from ion $[M+H]^+$ at *m/z* 1231.1. The fragmental ions Y_5B_5 at *m/z* 865.9 ($HexMan_2$ MeManGlcNAc), Y_4B_5 at *m/z* 704.2 (Man_2MeManGlcNAc) and $Y_{3\alpha}B_5$ at *m/z* 542.0 (ManMeManGlcNAc), arise by a sequential loss of one Hex. Further evidence of the proposed partially modified core of the structure by a methyl group are the ions as $Y_{3\alpha}B_5$ at *m/z* 542.0 (ManMeManGlcNAc), $Z_{3\alpha}$ at *m/z* 745.3, Z_4 at *m/z* 906.6, and Z_5 at *m/z* 1069.2. Moreover, the suggested structure was confirmed by the corresponding ions $Y_{3\alpha}$ at *m/z* 763.3 ($ManMeManGlcNAc_2$), Y_4 at *m/z* 925.2 (Man_2MeManGlcNAc$_2$), and Y5 at *m/z* 1087.2 ($HexMan_2$-MeManGlcNAc$_2$), respectively.

It was also found that only one of the glycans carried a xylose residue linked to the inner core (glycan № 7, Table 1). The core-xylosylated structure comprising one xylose residue linked to the β-mannose of the core was detected at *m/z* 1568.0. This position is the usual one for a single xylose attachment and is often found in plant glycans. N-glycans comprising (β1-2)-linked xylose residues were also found in hemocyanins of *Helix pomatia* as major carbohydrate constituents [11, 13].

Based on both the MALDI-TOF-MS analysis after treatment of the glycans with the specific α1-6(>2,3,4) fucosidase (Fig. 1a and c) and the Q-Trap-MS/MS analysis, core- structures containing Fuc(α1-6) GlcNAc, were detected (Table 1, glycans №1, 5, 8, 10, 12–15). Few glycans contained a second or a third fucosyl residue in the Fuc(α1-3)GlcNAc motif (glycans №8, and №12-15, respectively).

The MS/MS spectrum of the *N*-glycan at *m/z* 1582.0 (Fig. 3, Table 1, № 8) illustrates the observation of this peculiar (α1-6) fucosylation. The spectrum clearly demonstrates the presence of a core-linked Fuc(α1-6)GlcNAc, an external α1-3 linked Fuc (corresponding to Fuc(α1-3) GlcNAc), and a terminal MeHex residue, linked to internal GlcNAc. The most dominant Y- and B-ions provide information on the sequence and the branching, but the positions of the monosaccharides are confirmed by the C-, Z-, X- and A-ions. The fragment ion C_1 at *m/z* 195.0 in the MS/MS spectrum obviously corresponds to one MeHex and, being followed by the ion at *m/z* 380.2, which corresponds to MeHex GlcNAc, allows to conclude that one terminal MeHex is linked at the internal GlcNAc residue outside the core of the N-glycan. The cross-ring fragment ions $^{3,5}A_2$ at *m/z* 251.0 and $^{0,3}A_2$ at *m/z* 267.0 are additional evidence for the presence of the linked methylhexose to internal GlcNAc and α1–3 fucose residue to internal GlcNAc, supporting the suggested structure MeHexGlcNAc Fuc(α1-3). Additional evidence for the composition MeHexGlcNAcMan is the ion $Y_{5\gamma}B_{3\alpha}$ at *m/z* 542.0. Moreover, the composition (MeHexGlcNAcFuc) carrying a (α1-3)-fucose terminal linkage at GlcNAc is confirmed by the fragments B_2 at *m/z* 526.1 and $Y_5B_{3\alpha}$ at

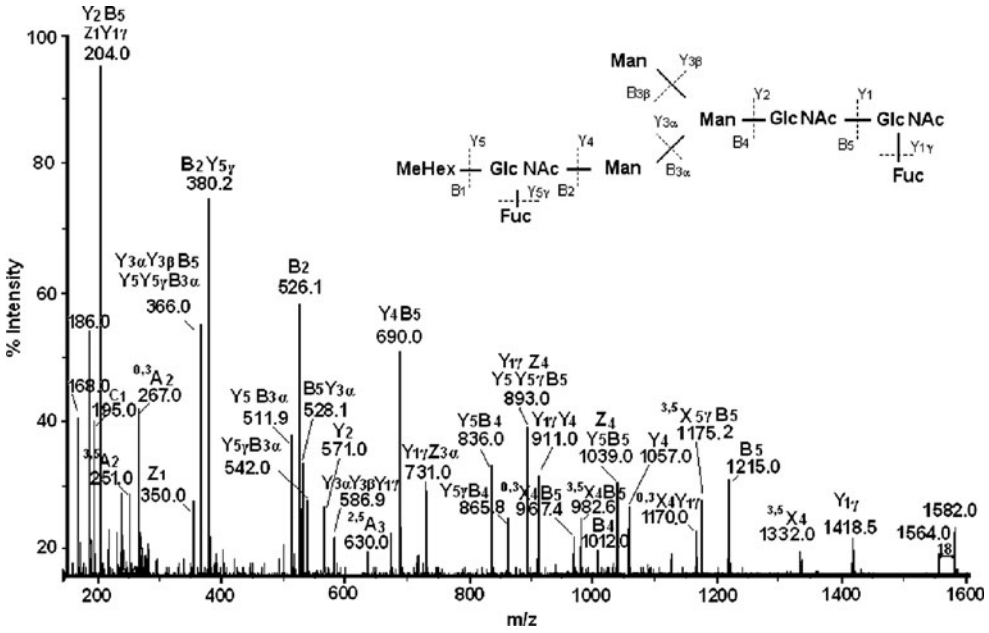


Fig. 3 MS/MS spectra and structures with fragmentation nomenclature of the $[M+H]^+$ of the glycan with complex type structure at *m/z* 1582.0 isolated from HtH1

m/z 511.9, which refer to the composition GlcNAcFucMan. A confirmation of the position of the monosaccharides in glycan 8 are the cross-ring cleavages observed in the spectrum. The cross-ring fragment ion $^{2,5}A_3$ at *m/z* 630.0 shows the presence of a methyl-mannose and a α1–3 linked external fucose residue to internal GlcNAc, supporting the suggested structure MeHex GlcNAc Fuc(α1-3) Man. Branching of Fuc to the core is also confirmed by the Y_2 ion at *m/z* 571.0. The observed mass difference between the registered fragmental ion Z_4 at *m/z* 1039.0 and Y4 at *m/z* 1057.0 and the fragmental ion $Y_{1\gamma}Z_4$ at *m/z* 893.0 and $Y_{1\gamma}Y_4$ at *m/z* 911.0 corresponding to the core is 146 mass units, which is in agreement with a loss of the Fuc unit that is α1-6 linked to the terminal GlcNAc residue of the core. The ions at *m/z* 1039 and *m/z* 893 are also represented as Y_5B_5 and as $Y_5Y_{5\gamma}B_5$, respectively. Additional evidences confirming α1–3 linkage of an external fucose residue to internal GlcNAc are the cross-ring fragment ions $^{0,3}X_4B_5$ at *m/z* 967.4 and $^{3,5}X_4B_5$ at *m/z* 982.6. Moreover, the cross-ring fragment ion $^{0,3}X_4Y_{1\gamma}$ at *m/z* 1170.0 corresponds to the glycan after removal of the fragment ion $^{0,3}A_2$ at *m/z* 267.0 and the terminal core-linked Fuc(α1-6). The ion $^{3,5}X_4$ at *m/z* 1332.0 represents the glycan without the fragment ion $^{3,5}A_2$ (containing MeHex) supporting the suggested structure for the N-glycan at *m/z* 1582.0. Furthermore, the B_5 ion at *m/z* 1215.0 corresponds to the glycan moiety after removal of

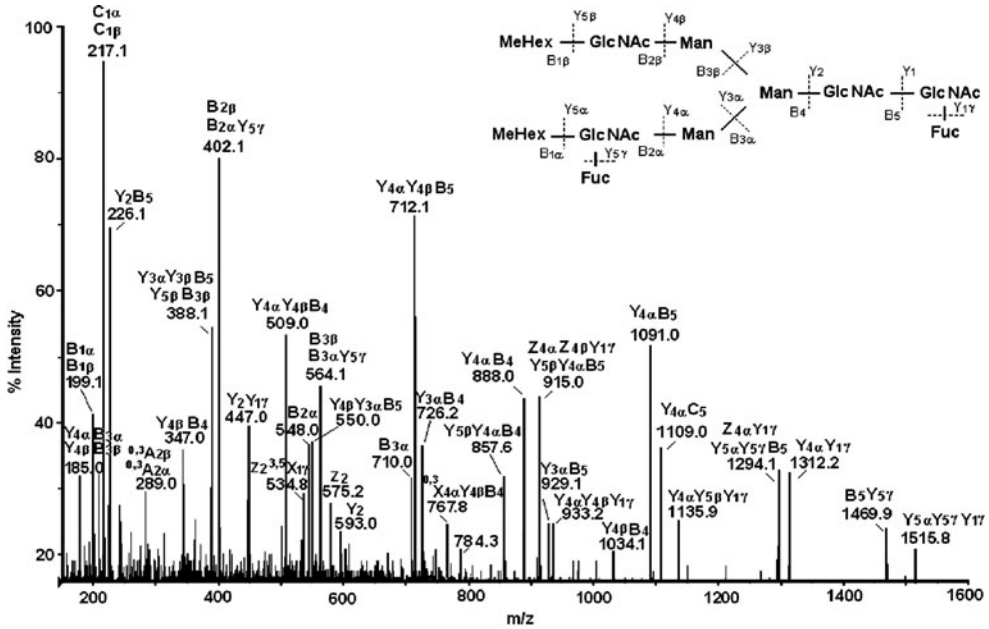


Fig. 4 MS/MS spectra and structures with fragmentation nomenclature of the double charged $[M+2Na]^{2+}$ of the glycan at *m/z* 1002.8 isolated from HtH1

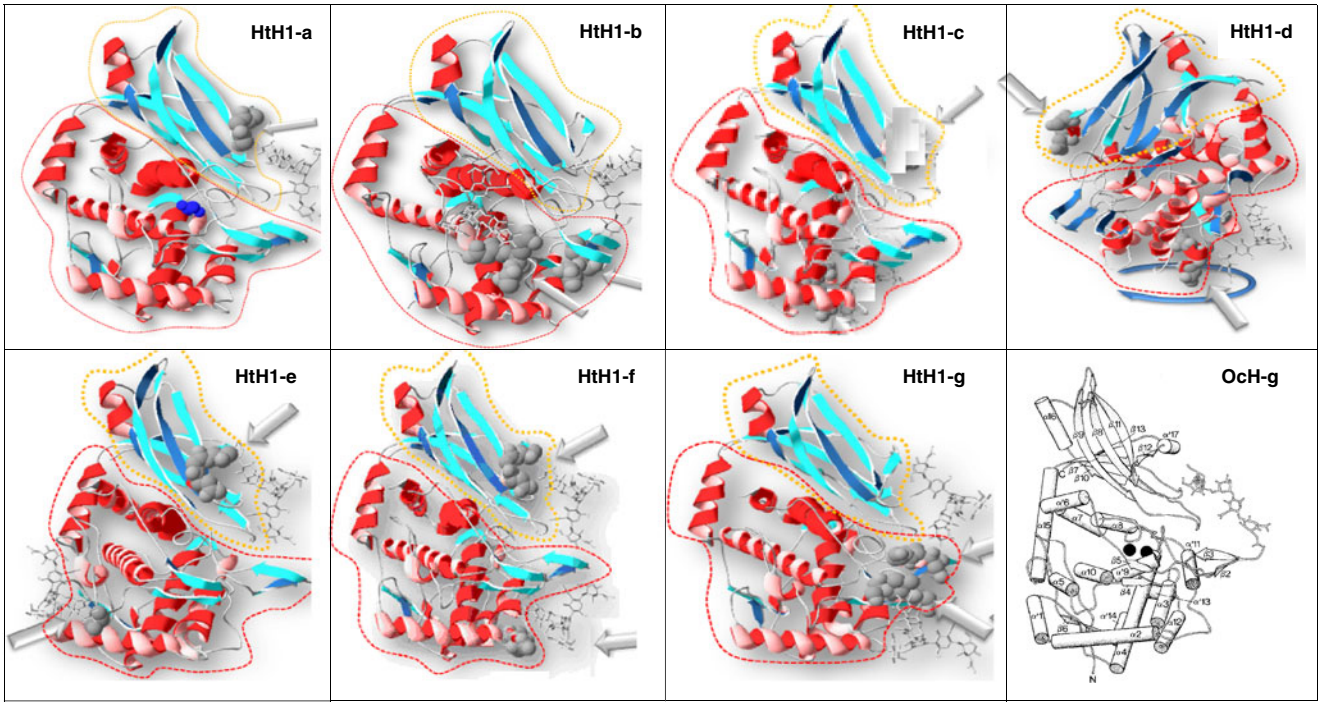


Fig. 5 Modeled of functional units (FU-a to FU-g) with potential N-linked sugar trees (derived from *Octopus dofleini* FU-g), red dotted: alpha core; yellow dotted: beta sandwich; grey balls: N- sugar attachment site; arrow: glycans. HtH1-c is lacking an N-linked sugar. Nomenclature of beta strands and alpha helices is as firstly described by Cuff *et al*. [40]

GlcNAcFuc, which is an additional proof for the presence of the terminal α1-6 Fuc moiety. The observed mass difference between the parent ion at *m/z* 1582.0 and fragment ion $Y_{1\gamma}$ (*m/z* 1418.5) amounts to 146+18 mass units, in agreement with the loss of one Fuc residue and a molecule of water.

Another interesting structure is glycan 12, which was sequenced from the doubly-charged species ($[M+2Na]^{2+}$, *m/z* 1002.8, Fig. 4). As shown in the spectrum, two deoxyhexose and two terminal methyl-Hex residues are linked to the internal GlcNAc of a molecule with the composition $MeHex_2HexMan_3GlcNAc_4Fuc_2$. Two ions, Z_2 $^{3,5}X_{1\gamma}$ at *m/z* 534.8, Z_2 at *m/z* 575.2 and Y_2 at *m/z* 593.0, demonstrate the presence of a core-linked Fuc(α1-6) residue. Confirmation of additional Fuc(α1-3) branching to GlcNAc are the ions $B_{2\alpha}$ at *m/z* 548.0 and $B_{3\alpha}$ at *m/z* 710.0, as well as the ions $B_{3\beta}$ at *m/z* 564.1 and $B_5Y_{5\gamma}$ at *m/z* 1469.9, supporting the branching of two terminal MeHex residues. The observed cross-ring fragment ions $X_{4\beta}Y_{4\alpha}B_4$ at *m/z* 767.8, as well as $^{0,3}A_{2\alpha}$, are additional evidence for the suggested structure Fuc(α1-3)GlcNAc. The ion $Y_{4\beta}B_4$ at *m/z* 1034.1 corresponds to the composition $Man_3GlcNAcFucMeHex$ (carrying a (α1-3)-fucose terminal linkage at GlcNAc). Consequently, cross-ring fragment ion $X_{4\alpha}Y_{4\beta}B_4$ at *m/z* 767.8 is resulting from the ion $Y_{4\beta}B_4$ at *m/z* 1034.1 without $^{0,3}A_{2\alpha}$ fragment, containing the MeHex moiety. The evidence of MeHex being linked to GlcNAc, as observed in most molluscan hemocyanins, is derived from the ions C1α and C1β at *m/z* 217.1, ions $B_{2\beta}$ and $B_{2\alpha}Y_{5\gamma}$ at *m/z* 402.1, as well as from the ions $B_{3\beta}$ and $B_{3\alpha}Y_{5\gamma}$ at *m/z* 564.1. Two alternative interpretations of the signal at *m/z* 1294 as $Y_{5\alpha}Y_5\gamma B_5$ and $Z_{4\alpha}Y_{1\gamma}$ confirm the positions of two Fucoses.

Branching of Fuc to the core was also identified in the glycans 13, 14 and 15 (Table 1). The oligosaccharide structures of these glycans are derivatives of the representative structure of this glycan 12 (Fig. 4).

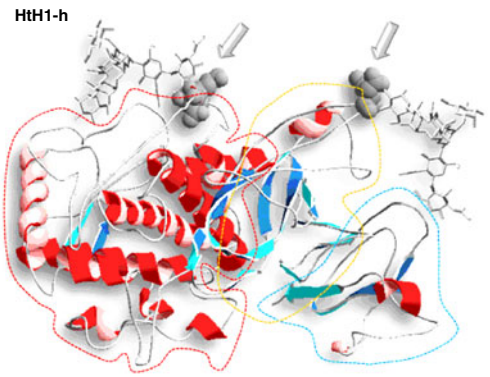


Fig. 6 Modeled HtH1-h with a potential N-linked sugar tree (derived from *Octopus dofleini* FU-g), red dotted: alpha core; yellow dotted: beta sandwich; blue: cupredoxin-like; arrow NKS sugar attachement site

In summary, the structures of 15 glycans liberated from structural subunit HtH1 were identified as a very heterogeneous group of N-glycans. As in most molluscan hemocyanins, the glycans of HtH1 mainly contain a terminal MeHex residue, in some cases even two of these residues occur (glycans 4, 12 and 15). One of the difficulties in assigning the MS/MS data is the existence of mixtures of isomers, such as (α1–3)Man or (α1–6)Man from the core. Some glycans reveal the existence of a novel structural motif MeMan[Fuc(α1-3)-]GlcNAc, including MeHex and (α1-3)-Fuc residues that are linked to an inner (internal) GlcNAc residue, as was found in oligosaccharide structures of glycans 6, 8, 11, 12, 14 and 15 (Table 1). Their common feature can be described as a $Man_3GlcNAc_2$ pentasaccharide core, the 3-linked mannose of which is further substituted at carbon C2 by the MeHex[Fuc(α1–3)]GlcNAc]β1-moiety, which may be further substituted by a methyl-Hex residue, in agreement with the present data (Table 1). Further modifications of the core structure include a substitution of the central Man at carbon C2 by Xyl (glycan 7) and/or the attachment of an additional Fuc residue to C6 of the innermost GlcNAc. However, a *N*-glycan with an internal fucose residue, substituted at two positions with a *N*-acetylhexosamine and one hexuronic acid, as was detected in the oligosaccharide structures of RvH1 and RvH2 [9, 21, 22, 26], was not found in the glycan structures of HtH1.

Multiple sequence alignments of different hemocyanins [43] show a high degree of conserved potential *N*-glycosylation sites. Analysis of the potential glycosylation sites on a 3D-model of the HtH1 FUs (Fig. 5), reveals different locations of these sites within the ternary structure. The majority of the sites are located in the beta-sandwich domain of the functional units, around or between beta strands 11 and 12. One site in HtH1-d is located between beta strands 2 and 3. Some less conserved glycosylation sites can be observed in the alpha core domains of all glycosylated FUs. Attachment of sugar side chains to locations at the interface between the alpha core and the beta sandwich domain may function to stabilize the distinct FUs and also the whole molecule. Other sugar side chains are predominantly located within the alpha core domain, building up the outer or inner surface of the decameric protein [44]. NXT or NXS consensus sites within the beta sandwich domain are exclusively found on one side of this domain. The opposite site is absolutely void of any glycosylation site, probably due to its assumed function in associating and folding the ternary structure of the complete subunit. As mentioned above, the functional unit c (HtH1-c) completely lacks any glycosylation site (Fig. 5), whereas FU-h possesses an additional exposed sugar attachment site located between beta strands 8 and 9 within the beta sandwich domain (Fig. 6). The finding that HtH1 is not able to form multidecameric structures *in vivo* may be explained by the presence of this exposed glycan on the surface of FU-h.

Conclusion

The oligosaccharides found in gastropods reveal a complex *N*-glycan pattern combining typical structural features of different higher organisms (mammals, plants, insects, nematodes, trematodes). Therefore, they are extremely interesting for the further investigation of the structural and functional role of protein glycosylation. Moreover, they are a potential source of novel *N*-glycans that are important for the stimulation of the immune response in humans and/or for the production of antibodies used in diagnosis and therapy. Several carbohydrate chains in the hemocyanins we analyzed are core-fucosylated, and also possess a high degree of methylation. As observed in many glycan structures of gastropod hemocyanins, this suggests that the methylation of *N*-glycans is very important feature in these organisms.

Some of the glycans in HtH1 revealed the presence of the novel structural motif MeHex[Fuc(α1-3)-]GlcNAc, including one or two MeHex and (α1-3)-Fuc residues being linked to the internal GlcNAc residue, as well as an additional Xyl. It is worth reminding that the core (β1-2)-Xyl and (α1-3)-Fuc residues are relatively common in plant and insect glycoproteins [45–47] and that they have a strong contribution in IgE binding to plant glyco-allergens [48].

Based on built models of the FUs, the positions of the linkage sites were shown to likely occur on the surface of the subunits, at least for seven of these FUs. Such a site is missing in FU-c, but there are even two putative sites in FU-h. We here suggest that the native complex of HtH1 is not able to attach further decamers to its didecameric core due to the exposed glycan in FU-h, thus preventing the formation of larger aggregates.

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