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Carbohydrate structure of molluscan hemocyanins from snails *Helix lucorum* and *Rapana venosa*, determined by mass spectrometry

ABSTRACT

Molluscan hemocyanins (Hcs) have recently particular interest due to their significant immunostimulatory properties. This is mainly related to their high carbohydrate content and specific monosaccharide composition. The oligosaccharide structures of the structural subunits RvH2 from *Rapana venosa* hemocyanin (RvH) and βc-*Helix lucorum* hemocyanin (βc-HlH) were comparative investigated by mass spectrometry. Our study revealed a highly heterogeneous mixture of N-glycans with compositions $Hex_{3-7}HexNAc_{2-5}MeHex_{0-4}Pent_{0-1}Fuc_{0-1}$ and $Hex_{0-9}HexNAc_{2-4}MeHex_{0-1}HexA_{0-1}Pent_{0-1}Fuc_{0-3}$, isolation from βc-HlH and RvH2, respectively. N-glycans from βc-HlH contain mainly a terminal methyl-hexose (MeHex) residue, in some cases even more than one. Several carbohydrate moieties from βc-HlH are core-fucosylated, and also possess on methylation. In contrast with RvH2, N-glycans with an internal fucose residue, substituted at two positions with N-acetylhexosamine and hexuronic acid, as and glycans containing internal fucose were not found in βc-HlH. The glycosylation sites occupancy was subsequently elucidated by precursor ion scanning of the intact glycopeptides from RvH and βc-HlH using a nano-ESI mass spectrometry and Q-trap-LC/MS system. The oligosaccharide moieties found in HlH and RvH reveal a complex N-glycan pattern combining typical structural features of different higher organisms. Moreover, they are a potential source of novel N-glycans that are important for the stimulation of the immune response and/or for the production of antibodies used in diagnosis and therapy.

Key words: hemocyanin, *Rapana venosa*, *Helix lucorum*, oligosaccharide structures, glycans, glycosylation, mass spectrometry.

Introduction

Hemocyanins act as oxygen-transporting glycoproteins in many arthropod and mollusc species (Van Holde et al., 1995). There are large differences in the molecular masses, structure, carbohydrate content and composition of Hcs from the two species (Van Holde et al., 1995; 2001; Salvato et al., 1990; Decker et al., 2007). Molluscan hemocyanins are glycoproteins with high molecular masses and complicated quaternary and oligosaccharide structures. Native RvH is organized by two structural subunits RvH1 and RvH2 with molecular masses of 420 and 400 kDa, respectively (Dolashka et al., 1996; Dolashka-Angelova et al., 2003a; Dolashka-Angelova et al., 2007). In contrast, the hemocyanin of the garden snail *H. lucorum* is organized by three different isopolypeptides which were isolated from the hemolymph and named as βc-HlH, α_D-HlH, and α_N-HlH structural subunits. Each structural subunit contains 8 functional units (FU) with masses of about 50–60 kDa (Velkova et al., 2010). Both hemocyanins (RvH and HlH), differ not only in their quaternary structure, but also in their oligosaccharide structures.

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An important feature of hemocyanin structures are their carbohydrate structures, which play fundamental roles in their organization and immunological efficacy and antitumor activity (Riggs et al., 2005; Jurincic et al., 1988; Jurincic-Winkler et al., 2000; Liedtke et al., 2001; Moltedo et al., 2006Iliev et al., 2008; Dolashka-Angelova et al., 2010; Dolashka et al., 2011;). The relevance of glycosylation on the molluscan hemocyanin as a factor for their immunostimulatory properties has already been raised in studies on the hemocyanin isoforms KLH1 and KLH2 from *M. crenulata* (Dickleret al., 1999; Wuhrer et al., 2004; Hulíková et al., 2010), which are ones of the most used in experimental immunology and clinical practice, as an immunotherapeutic agent in the treatment of bladder carcinoma and other cancer types (Dickleret al., 1999; Harris & Markl, 2000; Lamm et al., 2000; Hulíková et al., 2010; Kanteleet al., 2011). The clinical success of intravesical administration of KLH to patients with bladder carcinoma based to assumed expression of the disaccharide epitope Gal β(1-3) GalNAc determinants that are cross reactive with an equivalent epitope on the bladder tumor cells surface (Wirguinet al., 1995; Kurokawa et al., 2002). The proposed mechanism of action of the best studied hemocyanin-keyhole limpet lemocyanin (KLH) involves immune activation due to the presence of cross-reacting epitopes, such as the Thomson–Friederich antigen and N-linked oligosaccharides carbohydrates motifs, as well as T helper type-1 immunity enhancement (Kurokawa et al., 2002; Liedtke, 2001; Geyer et al., 2005; Moltedo et al., 2006).

Apart from KLH, other hemocyanins such as *Rapana venosa* (RvH), *Helix lucorum* (HlH) (previously called *Helix vulgaris* (HvH), *Concholepas concholepas* (CcH) hemocyanins have been shown to be immunogenic and have significant antitumor activities (Dolashka-Angelova et al., 2008; Dolashka-Angelova et al., 2009; Dolashka et al., 2011; Iliev et al., 2008; Moltedo et al., 2006; Slovin et al., 2005; Del Campo et al., 2011).

The βc-HlH and RvH have immunological and antitumor potential (Dolashka-Angelova 2008; Toshkova et al., 2009; Iliev et al., 2008). They are active against Guerin ascites tumor as well as progressing myeloid Graffi tumors (Toshkova et al., 2007; Dolashka, et al., 2011). The native molecule of HlH and the structural subunits from RvH, are alternative candidates for the treatment of human superficial bladder cancer (Boyanova et al., 2013; Antonova et al., 2014).

Nevertheless, knowledge on their carbohydrate structure is still incomplete. Carbohydrate structures of some functional units and first structural subunit of *Rapana venosa* hemocyanin have been studied by different methods and techniques (Dolashka-Angelova et al., 2003b; Dolashka-Angelovaet al., 2004; Idakieva et al., 2004; Beck et al., 2007). The attachment of carbohydrate structure Man3GlcNAc2 to Asn 127 was shown by Gielens et al. (Gielens et al., 2005) and besides the core structure for N-glycosylation, they also reported on more complex glycans with internal fucose residues substituted with 3-O-methyl-Gal and GalNAc. However, the presence of hexuronic acid and an internal fucose were proposed for these structures by Sandra et al. (Sandra et al., 2007).

Detailed knowledge of protein glycosylation at the proteome level is becoming an important aspect of post-genomic research. By determining how glycopeptides are structured and in order to better understand how they work. Therefore, the development and application of different analysis techniques will increase the knowledge of their structure and function. Mass spectrometric (MS) techniques play a key role in glycoprotein and glycan analysis, to study protein glycosylation at the glycopeptide level.

In the present paper, we present comparative research about carbohydrate structures of both gastropod hemocyanins (*Rapana venosa* and *Helix lucorum* hemocyanins), based on mass spectrometric evidence.

Materials and Methods

Materials

Rapana venosa Hc was isolated from the hemolymph of marine snails living in the Black sea as described earlier (Dolashka-Angelova et al., 2003a). Two structural subunits RvH1 and RvH2 were separated using a Resource Q column using an FPLC system (Dolashka-Angelova et al., 2003a, 2004, 2007) and purified by HPLC.

Helix lucorum hemocyanin was isolated from the hemolymph of the garden snails as described in (Velkova et al., 2010).

All other chemicals, unless noted, were purchased from Sigma-Aldrich.

Mass spectrometric analysis of glycopeptides by MS/MS.

About 4 mg of RvH1, RvH2 and bc-HlH were digested with trypsin, and the glycopeptides were isolated as described by (Dolashka-Angelova et al., 2004; 2007). HPLC

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fractions, detected at a wavelength of λ=210 nm, were collected, lyophilized and analyzed for carbohydrates with orcinol/H_2SO_4 on silica gel plates (Dolashka-Angelova et al., 2007). Glycopeptides containing fractions were analyzed by nano-electrospray ionization (ESI) mass spectrometry (MS) on a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF; Micromass, Manchester, United Kingdom) as described (Sandra et al., 2007). Alternatively, glycopeptides were sequenced on a Q-Trap LC/MS/MS system (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with a nanospray ion source (Protana, Odense, Denmark) (Sandra et al., 2007).

Isolation of N-glycans from structural subunits RvH1, RvH2, and βc-HlH

For deglycosylation, approximately 4 mg of each structural subunit was dissolved in 50 µL of denaturing solution (1% SDS, 0.5 M mercaptoethanol, 0.1 M EDTA). Following incubation at room temperature during 30 min, 300 µl of Na-phosphate buffer (200 mM at 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 N-glycosidase F (PNGase F) (2 enzyme 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). 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).

Amidation

Amidation was carried out as described recently (Sekiya et al., 2005). Released glycans from RvH1 and RvH2 (1 µL of the stock solution) were dissolved in 25 µL of 1M NH4Cl and then mixed with 15 µL of 1M 4-(4,6-dimethoxy-1,3,5-triazin-2yl)-4-methylmorpholinium chloride. After incubation at 50°C for 24 h, the reaction mixture was desalted by hydrophilic affinity isolation of the oligosaccharides. The sample solutions were mixed with 100 µL Sepharose CL-4B slurry (Amersham Biosciences, Uppsala, Sweden) in 1 mL of 1-butanol/ethanol/H_2O (4:1:1, v/v). After gentle shaking for 45 min, the supernatants were removed by centrifugation. The gels were subsequently washed with the same solution (3 x 500 µL). Ethanol/H2O (1:1, v/v) (250 µL) was added to the gels and after incubation for 30 min., the solution phases were recovered and dried. The two samples were then dissolved in 10 µL of water prior to MALDI-MS analysis.

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

The isolated oligosaccharides were analysed by MALDI-TOF-MS. For glycan analysis, the matrix solution was prepared as a 10 mg/mL dihydroxybenzoic acid solution in 50% acetonitrile. The analyses were carried on a 4700 Proteomics Analyser with TOF/TOF optics (Applied Biosystems, Framingham, MA). The mass spectrometer had a 200 Hz frequency-tripled Nd-YAG laser operating at a wavelength of 355 nm. A total of 1500 shots were acquired in the MS mode. Spectra from m/z 900 to 3000 were recorded. Deduced monosaccharide compositions are assigned to $[M + Na]^+$ ions.

Q-Trap analyses (MS and MS/MS)of glycans

Off-line ESI-MS measurements of the glycans on a Q-Trap massspectrometer, equipped with a nanospray ion source (Proxeon, Odense, Denmark) and using Proxeon medium nanospray needles were performed. 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 Q0-trapping being activated. The trap fill-time was 200 ms in the MS/MS- scan mode. For operation the reflecting mode, the resolution of Q1 was set to 'low'. Excitation time was set at 100 ms (Sandra et al., 2004).

Results and Discussion

Glycosylation is one of the most important post-translational modifications found in nature. Identifying and characterizing glycans is an important step in correlating glycosylation structure to the glycan's function. Glycans on a protein can be characterized by a variety of methods. The methods to study glycoproteins, glycopeptides or glycans in MS-based analyzes vary according to the specific research question. We extended this study implementing mass spectrometric techniques to analyze the binding sites and the carbohydrate heterogeneity of the structural subunits RvH1, RvH2 and βc –HlH.

The strategy that was applied for the characterisation of oligosaccharide structure of structural subunit from RvH and βc-*Helix lucorum* hemocyanin is presented in Figure 1. Two approaches are applied mainly.

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The first approach included the isolation of glycopeptides from structural subunits, and analysis of their amino acid sequences and structures of the linked oligosaccharides by mass spectrometry. This analytical approach is useful in characterizing glycan heterogeneity and correlating glycan compositions to their attachment sites on the protein. Tryptically digested structural subunits (RvH1 and RvH2 as and β-HlH) were separated and fractionated by reversed phase chromatography on a Nucleosil 7 C18 column, on HPLC. Subsequently all fractions were tested for carbohydrate content using the orcinol/H_2SO_4 staining method on silica-gel plates. The positive fractions tested for carbohydrates were further analyzed by different mass spectrometry techniques and methods.The information obtained from this approach can serve as the foundation for understanding how glycan compositions affect protein function, in both normal and aberrant glycoproteins.

The second way includes isolation and characterization of glycans from RvH1, RvH and 2b-HlH. The intact structural subunits were subjected to PNGase F digestion and the glycans were separated from the protein. Preliminary analysis of N-glycans could be perform by MALDI-TOF-TOF before and after treatment with specific exoglycosidases as α-1,2,3-mannosidase, β-galactosidase, β1-2,3,4,6-*N*-acetylglucosaminidase and α-1,2,3,4,6-fucosidase. The following step, was to sequencing and determination of the configurations of N-glycans released from structural subunits after PNGase F treatment performed by Q-Trap tandem mass spectrometry.

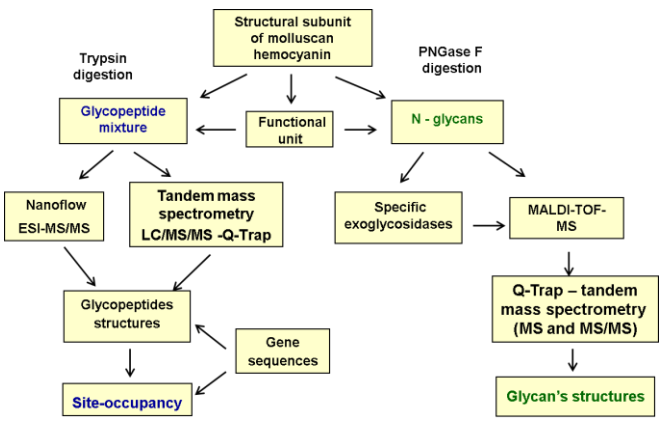

Figure 1. *The strategy, following for characterization of oligosaccharide structure of structural subunits RvH2 and βc-HlH.*

Characterization of glycopeptides using ESI-MS and Q-Trap-LC/MS/MS

Since the protein sequence of RvH is currently unknown, de novo MS sequencing had to be performed on the glycopeptides. Proteomic techniques, such as HPLC coupled to tandem mass spectrometry (LC-MS/MS), have proven to be useful for the identification of specific glycosylation sites of glycoproteins. Two methods to analyse the glycosylation sites in *Rapana venosa* hemocyanin have used. The first included - characterization of glycopeptides using HPLC fractions analyzed by flow injection analysis on a Q-TOF ESI-MS and the second - characterization of glycopeptides by Q-Trap LC/MS/MS system. Six glycopeptides were characterized in detail by analyzing the corresponding HPLC fraction by ESI-MS/MS and seven were identified by Q-Trap-LC/MS/MS (Dolashka-Angelova et al., 2009).

Through the gene sequence of βc-HlH is already known (De Smet et al., 2011) the identification of specific glycosylation sites of a glycoprotein was actually easier. The characterization of glycopeptides has performed using Q-Trap LC/MS/MS system.

The Q-Trap system with its capabilities to perform typical triple quadrupole scans allows for selectively detected of glycopeptides. They, selectively detected in a proteolytic mixture by the appearance of collisionally induced marker oxoniumions such as m/z 163 $[Hex]^+$, 204 $[HexNAc]^+$, or 366 $[HexHexNAc]^+$, were sequenced. The insert of Figure 2a shows the LC/MS/MS total ion current(TIC) chromatogram of the precursor ion scan (monitoring *m/z* 204) of the HPLC fraction at time 27.1 min. The enhancedresolution scan (not shown) showed that the glycopeptide withmass 1781.5 eluting at this moment was doubly charged at *m/z* 891.25 $[M+2H]^{2+}$.The precursor ion scan at time 31.24 min is presented in Figure 2a.

The MS/MS spectrum (Figure 2b) is dominated by glycan fragmentation series of Y- and B-ions, corresponding to the Domon/Costello nomenclature (Domon and Costello, 1988). However, peptide fragmentation (Roepstorff/Biemann cleavages) became more dominant when the collision energy was increased, allowing one to deduce the peptide sequence DHNTTR from the series of y- and b-ions (Figure 2b). The ion b_6 (m/z 725.5) and y_6 (m/z 743.4) correspond to a intact peptide contains one potential glycosylation site (-NTT-). The ion y_4 at *m/z*491.3 correspond to the C-terminal fragment of a peptide fragment –NTTR. The observed mass difference between ions at m/z 694.4 and y4 (m/z 491.3) amounts to one

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GlcNAc moietystill containing to Asn of a fragmental ion y4. The ion at *m/z* 946.4 corresponds to the intact peptide, represented as ion y_6 (*m/z*743.4), which is N-glycosylated with a single GlcNAc residue. The peptide sequence DHNTTR has determined from mass spectrometric fragmenting and fully consistent with the fragment of gene sequence at Asn 125 of βc-HlH-g (De Smet et al., 2011). This is evidence that the site -NTT- at Asn-125 of βc-HlH-g is actually glycosylated.

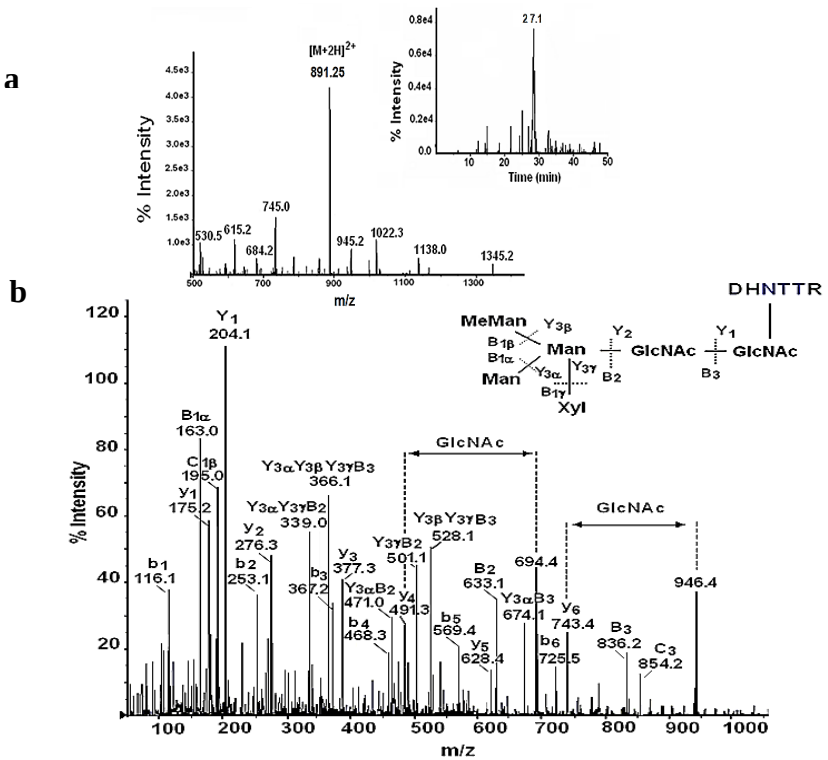

Figure 2. *Fragment spectra of the glycopeptide isolated from βc-HlH. Precursor scan (A) of the ion at time 27.1 min of the chromatographic separation (insert) and enhanced product ion (EPI) scan of the ion (B) at m/z 891.25 [M+2H]$^{2+}$.*

The glycan structure of this peptide could be revealed by thisMS/MS spectrum, which displayed the typical ions (representedas Y, B and C): $B_{1\alpha}$at *m/z* 163.0 (Man), $C_{1\beta}$at *m/z* 195.0 (MeMan), Y_1 at *m/z* 204.1 (GlcNAc), $Y_{3\alpha}Y_{3\beta}Y_{3\gamma}B_3$ at *m/z* 366.2 (Man1GlcNAc1),at*m/z* 528.3 (Man2GlcNAc1). The MS/MS-spectrum (Figure 2b) clearly demonstrated the presence of core-linked xylose as well as terminal methyl-hexose linked at the central mannose residue of thetrimannosyl core: $Y_{3\alpha}Y_{3\gamma}$B2 at m/z 339.0 (MeMan1Man1), $Y_{3\alpha}B_2$ at m/z 471.0 (MeMan1Man1Xyl1), $Y_{3\gamma}B_2$ at m/z 501.1 (MeMan1Man2), B_2 at *m/z* 633.1 (MeMan1Man2Xyl1), $Y_{3\alpha}B_3$ at m/z 674.1 (MeMan1Man2Xyl1GlcNAc1), B_3 as and C_3 at m/z 836.2 and 854.2, respectively (Xyl1MeMan1Man2GlcNAc). Combining all data, the carbohydratestructure with composition Xyl1MeMan1Man2 GlcNAc2 has suggested. The occurrence of xylose residue in βc-HlH from *H. lucorum* is considered to be highly immunogenic (Dolashka et al., 2011).

Structural characterization of N-glycans

Analyse of oligosaccharide structures of the structural subunits.

The oligosaccharide structures of the structural subunit of *Rapana venosa* hemocyanin and βc-HlH were studied by sequence analysis using MALDI-TOF-MS and tandem mass spectrometry on a Q-Trap mass spectrometer.

After enzymatical liberation of the N-glycans from the polypeptides with specific exoglycosidasePNGase F, and purified from the reaction mixture by solid phase extraction on a Carbograph column, the isolated glycans have analysed by mass spectrometry.

Two ways were applied to analyse the isolated glycans.

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The first, 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. MALDI-TOF-MS in combination with exoglycosidase sequencing was used to reveal compositional information and partial structural information on the enzymatically liberated N-glycans. This approach provided only preliminary results about the structures of the glycans.

Therefore, in the second approach, tandem mass spectrometry was applied. The glycan structure was derived from the MS/MS spectra, obtained on a hybrid quadrupole-linear ion trap mass spectrometer. Analysis of the purified N-glycans by Q-Trap is a very sensitive and useful method and several glycans were identified from MS/MS spectrum.

Q-Trap analyses (MS and MS/MS) of glycans from Rapana venosa Hc and βc-Helix lucorum hemocyanin

Monosaccharide sequencing and determination of the configurations of N-glycans, released from structural subunits RvH2 and βc-*Helix lucorum* hemocyanin (βc-HlH) after PNGaseF-treatment have performed by Q-Trap tandem mass spectrometry. We analysed the carbohydrate structures of RvH2, but reanalyzed those of RvH1 under the same experimental conditions in order to compare both structural subunits. The glycan structure was derived from the MS/MS spectra, obtained on a hybrid quadrupole-linear ion trap mass spectrometer. In most cases, the dominant ions are Y and B that arise from glycosidic cleavages, but C and Z ions are also observed. They can be differentiated from the Y- and B-ions without derivatisation, because of the asymmetrical nature of the molecule.

The glycans were identified after detection by Q-Trap analysis. On Figure 3 is shown MS- spectrum of the released glycans after treatment of RvH2 with PNGase F.The peaks are represented as doubly and single charged sodium adduct. Some of them appear to be methylated. We have analyzed three oligosaccharide structures with a methylated hexose as terminal residue $[M+2Na]^{2+}$ at m/z 867.5 with composition MeHex1Hex2Fuc1Xyl1 Man3GlcNAc2; $[M+2Na]^{2+}$ at m/z 1025.3 (MeHex1 Hex1 HexNAc3 Fuc1 Man3GlcNAc2), $[M+2Na]^{2+}$ at m/z 1032.3 (MeHex1 Hex2 HexNAc3 Man3GlcNAc2) from RvH2, but none of them was detected in RvH1 (Dolashka-Angelova et al., 2010).

Similar structures containing one or more methyl-hexose and Xyl and/or Fuc residue, were detected in βc-*Helix lucorums* hemocyanin. As is shown in the spectrum of doubly-charged species $[M+2Na]^{2+}$ at m/z 1077.1 (Figure 4), one deoxyhexose, one pentose, and two terminal methyl-Hex residues are linked to the internal GlcNAc of a molecule with the composition MeHex2 Hex1 Man3 GlcNAc4 Fuc1 Xyl1.The fragment ion C_1 (as and $C_{2\beta}$) at m/z 217.1 in the MS/MS spectrum obviously corresponds to one terminal MeHex and being followed by the ions B2α at m/z 361.1 and C2α at m/z 379.1, which corresponds to MeHex-Hex, as and the ion B3Y6α at m/z 402.1 allows to conclude that second terminal MeHex is linked at the internal GlcNAc residue outside the core of the N-glycan. The deduced structure with outer two methylhexose residues was further confirmed by the ion B_3 at m/z 740.1 corresponds of fragment MeHex-Hex-[(MeHex-)]-GlcNAc, followed by B_4 at m/z 943.4, $B_{5\alpha}$ at m/z 1105.2 and B_6 at m/z 1561.2. Additional evidences for Xylresedue included in composition Xyl(β1→2)Man(β1→4)GlcNAcβ1-4GlcNAc is D-ion Y3αB6 at m/z 479.2, as and fragmental ions Y_4B_6 at m/z 641.2 and Y_4C_6 at m/z 658.9, Y_4B_7 and Y_5B_6 at m/z 844.3 (respective of Xyl1 Man3 GlcNAc1), Y_5B_7 and $Z_4Y_{1\gamma}$ at m/z 1047.5 (Xyl1 Man3 GlcNAc2).

The presence of the core Fuc residue was confirmed by the Z_4 at m/z 1193.4 and Y_4 at m/z 1211.3, and also Z_5-ion at m/z 1396.3 and Y_5-ion at m/z 1414.4, as well as the fragmental ion $Y_{6\alpha}Y_{6\beta}$ at m/z 1617.3. Confirmations of the position of the monosaccharides in glycan of the Figure 4 are the cross-ring cleavages observed in the spectrum. The cross-ring fragment ion $^{2,4}X_0$ at m/z 330.1 and the $^{1,5}X_1$ at m/z 418.1 show the presence of Fuc α(1–6) - linked to internal GlcNAc to the core. The cross-ring fragment ion $^{0,2}X_2$ at m/z 767.2 reaffirmed too presence of Fuc α(1–6) and core-linked β(1-2)-xyloseresidue. The α1-6 fucosylation of the Asn-bound GlcNAc in *H. lucorum* hemocyanin fits the usual finding in higher-animal glycoproteins.

However, Sandra et al. (2007) were detected the most important class of the glycans isolated from RvH1 containing internal fucose branching to hexuronic acid and HexNAc. Based on the obtained results, after Q-trap MS/MS analyses of glycans from RvH2 it was found same types N-glycans (at m/z 842.3 $[M+2Na]^{2+}$ and at m/z 915.6 $[M+2Na]^{2+}$), as they comprise one internal fucose residue, substituted at two positions with a N-acetylhexosamine and a hexuronic acid

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(Dolashka-Angelova et al., 2010).

On the other hand, the mass difference between a methyl-Hex residue and a HexA is only 0.036 Da, hence, MS and MS/MS cannot differentiate between these two residues. Further proof for the presence of the HexA in the glycans from RvH2 was obtained by amidation, the resulting effect being analyzed by MALDI-MS. The method involves the modification of carboxyl groups (present in HexA) by converting the carboxylic acid to the amide in the presence of both an amine constituent (NH_4Cl) and a condensing agent [4-(4,6-dimethoxy-1,3,5-triazin-2yl)-4-methylmorpholinium chloride (DMT-MM)], and as results in a mass decrease of ≈ 1 Da (0.9840 Da) per carboxylic acid. Further analysis of the glycans from RvH2 using MALDI-TOF-MS revealed that the signals at m/z 1661.6 and 1807.6 were reduced to m/z 1660.4 and 1806.4. As expected, other glycans were unreactive towards amidation and changes in their molecular masses were not observed (including methylated glycans). These results confirm a branching of HexA to the internal Fuc in RvH2 as was suggested for (Sandra et al., 2007), in contrast to methylated Hex as proposed by Gielens et al. (2005). Though, hexuronic acid moieties, which are common constituents of proteoglycans, occur rather rarely in glycoproteins. Nevertheless, they have been observed on mammalian N-glycans as constituent of the HNK-1 carbohydrate epitope (Geyer et al. 2001; Liedtke et al. 2001).

Our results about glycans from βc-HlH revealed that sugar chains were mostly modifications by a xylose β-(1-2) linked at the central mannose residue of the trimannosyl core, an α-fucosylation (mainly α1,6) of the innermost GlcNAc residue (the Asn-bound GlcNAc) as and a high degree methylated Hex residues (Table 1). A xylose residue (β1-2)-linked to a β-mannose unit of the core of an N-linked carbohydrate chain is considered to be a highly immunogenic epitope for mammalian species (Gutternigg et al., 2004; 2007). Methylated sugar residues were also identified in *Helix pomatia* hemocyanin and *Lymnaea stagnalis* Hc. (Gielens et al., 2004;Van Kuik et al., 1987; Lommerse et al., 1997).

The high degree of methylation and its occurrence on so many different structures in gastropod *Arion lusitanicus* was published by Gutternigg et al. (2004), suggesting that the methylation of N-glycans is an important regulating event in this organism.

Table 1. *Oligosaccharide structures with methyl hexose residues (MeHex) detected on RvH2.*

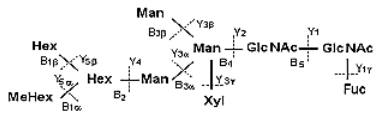
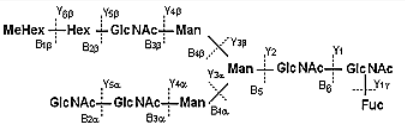
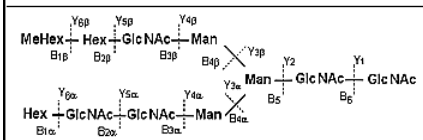
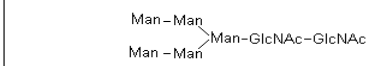
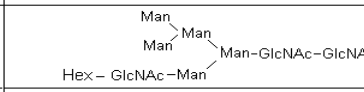
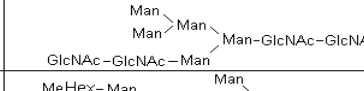
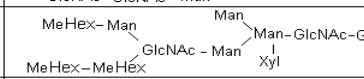
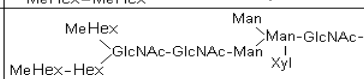
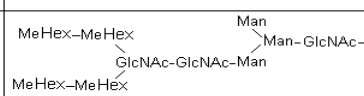
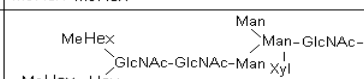
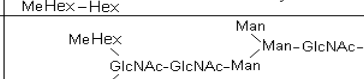
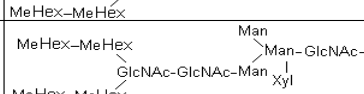
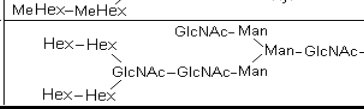
Composition	MALDI-TOF/MS (m/z)	Q- Trap/ MS (m/z)
	$[M+Na]^+$ 1711.6	$[M+2Na]^{2+}$ 867.5
	$[M+Na]^+$ 2028.3	$[M+2Na]^{2+}$ 1025.4
	$[M+Na]^+$ 2042.6	$[M+2Na]^{2+}$ 1032.4

Table 2. *Some oligosaccharide structures of N-glycans detected on βc-Helix lucorum hemocyanin (βc-HlH).*

№	Glycan's structures from βc-HlH	Q-Trap/MS (m/z)	MALDI-TOF/MS (m/z)
1		$[M+H]^+$ 1235,4	$[M+Na]^+$ 1257,5
2		$[M+Na]^+$ 1622,2	$[M+Na]^+$ 1622,5
3		$[M+Na]^+$ 1663,2	$[M+Na]^+$ 1663,4
4		$[M+2Na]^{2+}$ 990,5	$[M+Na]^+$ 1958,2
5		$[M+2Na]^{2+}$ 1004,1	$[M+Na]^+$ 1985,4
6		$[M+2Na]^{2+}$ 1033,1	$[M+Na]^+$ 2043,5
7		$[M+2Na]^{2+}$ 1077,1	$[M+Na]^+$ 2131,4
8		$[M+2Na]^{2+}$ 1018,1	$[M+Na]^+$ 2013,4
9		$[M+2Na]^{2+}$ 1099,1	$[M+Na]^+$ 2175,4
10		$[M+2Na]^{2+}$ 1106,5	$[M+Na]^+$ 2190,3

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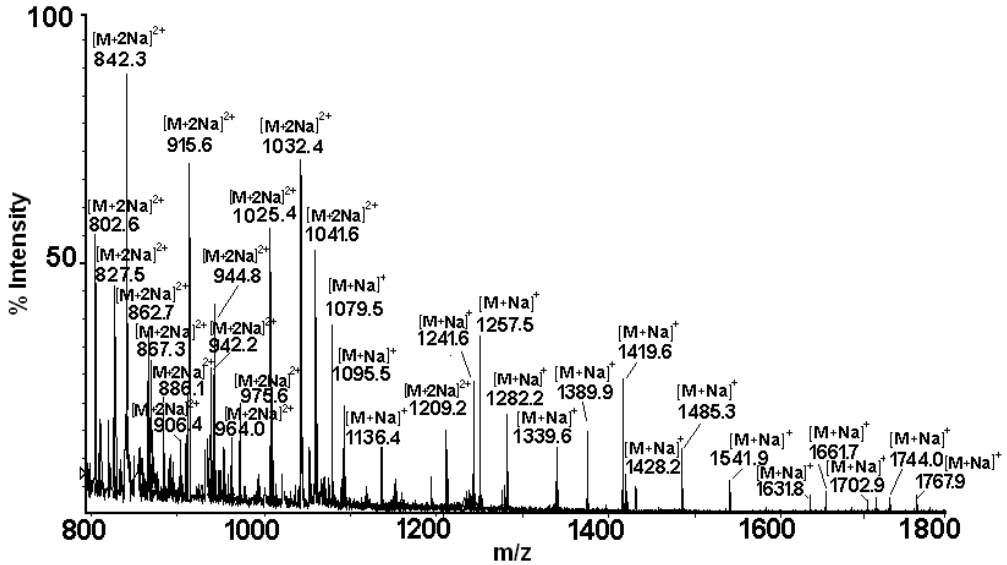

Figure 3. *MS spectrum of the released glycans after treatment of RvH2 with PNGase F. The peaks are represented as doubly and single charged sodium adduct.*

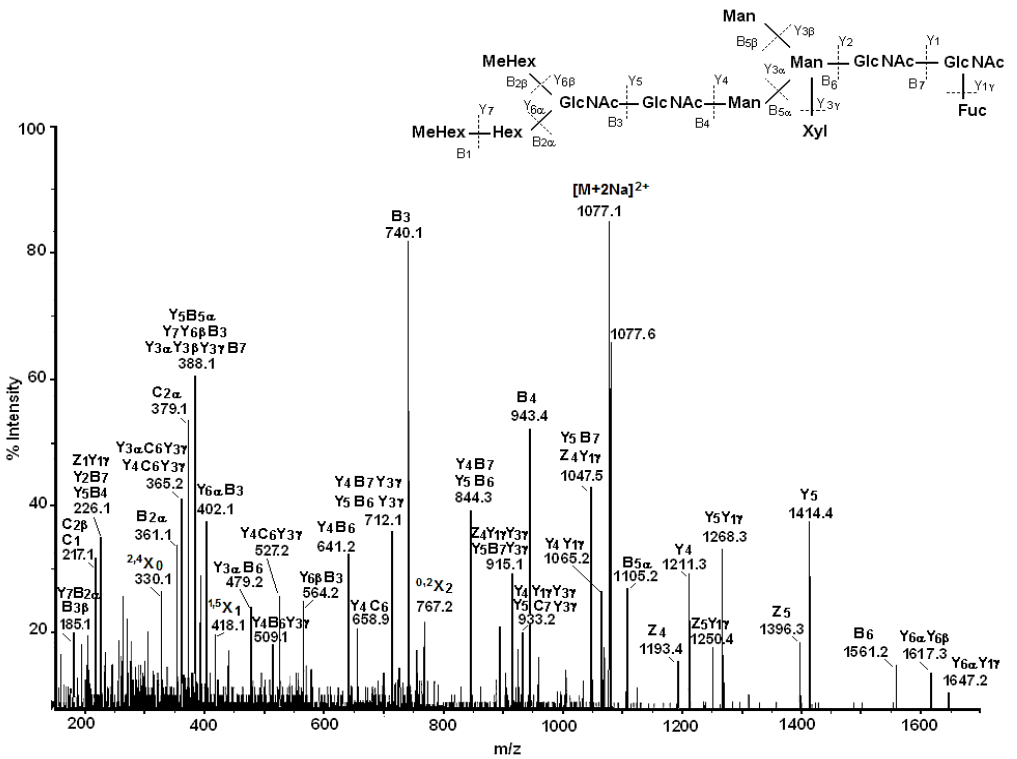

Figure 4. *MS/MS spectra and structure with fragmentation nomenclature of the double charged $[M+2Na]^{2+}$ of the glycan at m/z 1077.1, isolated from βc-Hl.*

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The carbohydrate structures of βc-HlH contained various larger N-glycans with a number of methylated sugars, which could be identified as 3-O-methyl-mannose and 3-O-methylgalactose, in addition Fuc and/or Xyl residues were found. Several carbohydrate chains in the βc-HlH are core-fucosylated, and also possess a high degree of methylation (Table 1). In total many glycans, were identified as a highly heterogeneous mixture with compositions Hex_{3-7} $HexNAc_{2-5}$ $MeHex_{0-4}$ $Pent_{0-1}Fuc_{0-1}$. In contrast with RvH, N-glycans with an internal fucose residue, substituted at two positions with N-acetylhexosamine and one hexuronic acid as well as and glycans containing internal fucose were not found in βc-HlH.

Conclusion

Glycan moieties have a very diverse number of roles in biological systems, making them relevant for biotherapeutics. The carbohydrate chains of some Hcs are involved in their antiviral and antitumor effect, as well in the organization of the quaternary structure of the molecules.

In the present study, the isolated N-glycans of structural subunits of *Rapana venosa* (RvH) and βc-*Helix lucorum* hemocyanin, were comparative investigated by mass spectrometry. The following strategy comprising on two mainly approaches is considered conducive for analysis Hcs with known and currently unknown protein sequence.

Our study revealed a highly heterogeneous mixture of N-glycans with compositions $Hex_{3-7}HexNAc_{2-5}MeHex_{0-4}Pent_{0-1}Fuc_{0-1}$ and $Hex_{0-9}HexNAc_{2-4}MeHex_{0-1}HexA_{0-1}Pent_{0-1}Fuc_{0-3}$, for isoform βc-HlH and subunits of RvH, respectively. Identified glycans of βc-HlH have predominantly complex- and hybrid-type structure. The glycans of βc-HlH contain mainly a terminal MeHex residue, in some cases even two, three and four. Identified glycans in RvH have mainly complex- and high mannose-type structures. A novel type of N-glycan, with an internal fucose residue connecting one GalNAc(β1-2) and one hexuronic acid, was detected in the RvH. The glycosylation sites occupancies were subsequently elucidated by precursor ion scanning of the intact glycopeptides from RvH and βc-HlH using nano-ESI mass spectrometry and Q-Trap-LC/MS/MS system.

The oligosaccharides found in RvH and HlH reveal a complex N-glycan pattern combining typical structural features of different higher organisms. 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.

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