Carbohydrate Research 345 (2010) 2361–2367

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Glycan structures and antiviral effect of the structural subunit RvH2 of Rapana hemocyanin

Pavlina Dolashka [a,*], Ludmyla Velkova [a], Stoyan Shishkov [b], Kalina Kostova [b], Aleksander Dolashki [a], Ivan Dimitrov [a], Boris Atanasov [a], Bart Devreese [c], Wolfgang Voelter [d], Jozef Van Beeumen [c]

[a] Institute of Organic Chemistry, Bulgarian Academy of Sciences, G. Bonchev 9, Sofia 1113, Bulgaria
[b] The Sofia University, Biological Faculty, Laboratory of Virology, 8 'Dragan Tsankov' St., 1421 Sofia, Bulgaria
[c] Laboratory of Protein Biochemistry and Biomolecular Engineering, Ghent University, KL Ledeganckstraat 35, 9000 Ghent, Belgium
[d] Interfacultary Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Strasse 4, D-72076 Tübingen, Germany

ARTICLE INFO

Article history:
Received 13 May 2010
Received in revised form 22 July 2010
Accepted 12 August 2010
Available online 19 August 2010

Keywords:
Glycosylation
Hemocyanin
MALDI-TOF/TOF
Q-Trap
Rapana venosa
HSV virus

ABSTRACT

Molluscan hemocyanins are very large biological macromolecules and they act as oxygen-transporting glycoproteins. Most of them are glycoproteins with molecular mass around 9000 kDa. The oligosaccharide structures of the structural subunit RvH2 of *Rapana venosa* hemocyanin (RvH) were studied by sequence analysis of glycans using MALDI-TOF-MS and tandem mass spectrometry on a Q-Trap mass spectrometer after enzymatical liberation of the N-glycans from the polypeptides. Our study revealed a highly heterogeneous mixture of glycans of the compositions Hex_{0-9} $HexNAc_{2-4}$ Hex_{0-3} $Pent_{0-3}$ Fuc_{0-3}. A novel type of N-glycan, with an internal fucose residue connecting one GalNAc(β1-2) and one hexuronic acid, was detected, as also occurs in subunit RvH1. A glycan with the same structure but with two deoxyhexose residues was observed as a doubly charged ion. Antiviral effects of the native molecules of RvH and also of *Helix lucorum* hemocyanin (HlH), of their structural subunits, and of the glycosylated functional unit RvH2-e and the non-glycosylated unit RvH2-c on HSV virus type 1 were investigated. Only glycosylated FU RvH2-e exhibits this antiviral activity. The carbohydrate chains of the FU are likely to interact with specific regions of glycoproteins of HSV, through van der Waals interactions in general or with certain amino acid residues in particular. Several clusters of these residues can be identified on the surface of RvH2-e.

1. Introduction

Hemolymph of molluscs and arthropods contains a large amount of bioactive compounds 'hemocyanins' (Hcs).[1–4] There are large differences in the molecular mass, structure, carbohydrate content and monosaccharide composition of Hcs from arthropods and molluscs. The mass values of 4.47×10^6, 8.67×10^6 and 13.40×10^6 Da were obtained for representative decameric, di-decameric, and tri-decameric components of molluscan hemocyanins. Also, the oligosaccharides are markedly different in both types of Hcs.[4–10] The carbohydrate content in arthropodan Hcs is usually between 0.1% and 2%, and only D-Man and D-GlcNAc have been detected.[8] Molluscan Hcs usually have a higher carbohydrate content (2–9%, w/w) and may contain unusual monosaccharides.[11–15] Structural studies of keyhole limpet hemocyanin (KLH), for example revealed that it is very heterogeneously glycosylated carrying mainly high mannose type glycans with 5–7 mannosyl residues, hybrid-type species with a five mannose and one *N*-acetylgalactosamine-containing chain, as well as truncated sugar chains derived thereof.[13,16,17] Carbohydrate structures and glycosylation sites of hemocyanins of *Helix pomatia*, *Sepia officinalis*,[11] and *Arion lusitanicus*[18] were also identified, using several methods.

The carbohydrate moiety of molluscan Hcs has recently received particular interest due to its immunostimulatory properties.[9,16,17,19–23] It was found that the Hcs of *Helix vulgaris* (HvH),[9] (renamed to *Helix lucorum*) the Chilean gastropod *Concholepas concholepas* (CcH),[24] and *Rapana venosa* (RvH)[25–27] have significant antitumor activity. The occurrence of xylose in *H. pomatia* Hc is considered to be highly immunogenic in mammalian species.[11] The gastropod mollusc *Oncomelania hupensis* (Oh) is a unique intermediate host for the human parasite *Schistosoma japonicum*. OhH exhibits o-diphenoloxidase activity after limited proteolysis, and shares carbohydrate epitopes with glycoconjugates of *S. japonicum*.[28]

Hemocyanins have also been found to be active against viruses. Such activity, among others against white spot syndrome virus (WSSV) and Singapore grouper iridovirus (SGIV), is assigned to the protein of the arthropod *Penacus monodon*.[29] Also the Hc of

* Corresponding author. Tel.: +359 29606163; fax: +359 8700225.
E-mail address: pda54@abv.bg (P. Dolashka).

0008-6215/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2010.08.005

the gastropod *R. venosa* has antiviral properties, more in particular against respiratory syncytial virus (RSV). The property seems to be associated with the glycosylation of functional unit RvH-c, one of the functional units of either structural subunit RvH 1 or RvH 2,[10] whereas there is no inhibitory effect on this virus for native RvH and for the non-glycosylated functional unit RvH-b.[10] It was also established that C- and N-terminal hemocyanin fragments have different roles in hemocytes of the shrimp *Penaeus vannamei* during Taura Syndrome Virus (TSV) infection.[30]

Hemocyanin from *R. venosa* (RvH) consists of eight functional units (FUs), each of a molecular mass of 45–50 kDa, named RvH-a to RvH-h, all of them constituting RvH1 as well as RvH2.[31,6] The structure of some oligosaccharide moieties of the native RvH have been determined,[10] as well as that of some moieties bound to glycopeptides of the structural subunit RvH1[32] and, more specifically, of some glycopeptides bound to the FUs RvH1-a[5,7] and RvH1-b.[33] An oligosaccharide structure has also been determined in functional unit RtH2-e of the species *Rapana thomasiana*[12] which, in fact is identical to *R. venosa*.

In this communication, we present data of a more complete structure determination of the oligosaccharide of subunit RvH2, based on mass spectrometric evidence. Subsequently, the potential role is proposed of some of these structures in the antiviral effect of hemocyanins.

2. Results

We analysed the carbohydrate structures of RvH2, but reanalysed those of RvH1 under the same experimental conditions in order to compare both structural subunits. The subunits were subjected to PNGase F digestion and the glycans were separated from the protein.

Some glycans were identified after detection by Q-Trap analysis after digestion of RvH2 with PNGase F (Fig. 1 and Table 1). Some of them appear to be methylated. Methylated sugars were also identified in the structure of other molluscan hemocyanins, such as 3-*O*-methyl-D-mannose and 3-*O*-methyl-D-galactose in *H. pomatia* Hc and *Lymnaea stagnalis* Hc.[14,3] It has been suggested by Gutternig et al. that a high degree of methylation in the gastropod *A. lusitanicus* is an important regulating event in this organism.[18]

Several glycans of the high mannose type were identified in RvH2. The sequence of one of them, represented in Figure 1 for the species at *m/z* 1419.4 is shown in Figure 2. Following the sequence of B and Y ions in the MS/MS spectrum, the structure of the glycan was found to contain six hexoses and two *N* acetyl glucosamine residues.

The most important class of the glycans isolated from RvH2 includes structures with α1,6-fucose linked to an inner or an external HexNAc residue, as previously also identified for RvH1 by Sandra et al.[32] It now appears that RvH2 has the same novel types of N-glycans (at *m/z* 842.3 $(M+2Na)^{2+}$ and at *m/z* 915.6 $(M+2Na)^{2+}$), as they comprise one HexNAc and one hexuronic acid linked to an internal fucosyl residue. The MS/MS spectrum of the first doubly-charged sodium adduct at *m/z* 842.3 is shown in Figure 3. Z-, X-, and A-ions in the spectra can be differentiated from the Y- and B-ions without derivatization because of the asymmetrical nature of the molecule. The sequence can most easily be determined when considering the Y ions (*m/z* 1485.2, 1136.3, 933.2) and combining B and Y ions. Usually, the loss of HexNAc linked to an α1,3-mannose is a very favourable event, but in the present case, the hexosamine is connected to an internal Fuc residue. MS/MS analysis revealed $Y_{6\beta}$ and $Y_{6\alpha}Y_{6\beta}$ ions at *m/z* 1485.2 and 1282.4, indicative for the presence of terminal HexNAc and HexA (176 Da), respectively. Furthermore, the Y_5 ion at 1136.3 represents the glycans after removal of the fragment HexNAc HexA Fuc. The observed difference of 525 mass units between the B (*m/z* 1661.6) and *m/z* 1136.3 fragments accounts for the loss of this fragment. Underivatised sugars frequently produce fragment ions which are derived from multiple cleavages that cannot be attributed to a unique structure, forming cross-linkages. Cross-ring cleavages are effectively present in the spectrum. The $^{4.5}A$, $^{2.5}A$ are the most abundant ones of this type of cleavage, but $^{5.6}X$ and $^{3.5}X$ cleavages can also be seen in the spectrum.

The ion at *m/z* 915.6 in the MS spectrum of glycan 19 (Table 1) corresponds to a structure similar to the one of *m/z* 842.3, but now contains two fucose residues, one internal and one terminal, attached to carbon 6 of GlcNAc (Fig. 4) as deduced from ions $Y_{3\beta}$ (917.2), Y_4 (1079.3) and Y_5 (1282.2). One Fuc is internally located, while the other is positioned at the proximal GlcNAc (see the intense Z_1 ion at *m/z* 372.1 and the $Y_{6\beta}$ ion at *m/z* 1631.2).

Further evidence for the presence of hexuronic acid in the glycans from both structural subunits of *R. venosa* hemocyanin was obtained following amidation of the glycan mixture from RvH2. The method involves the modification of carboxyl groups by

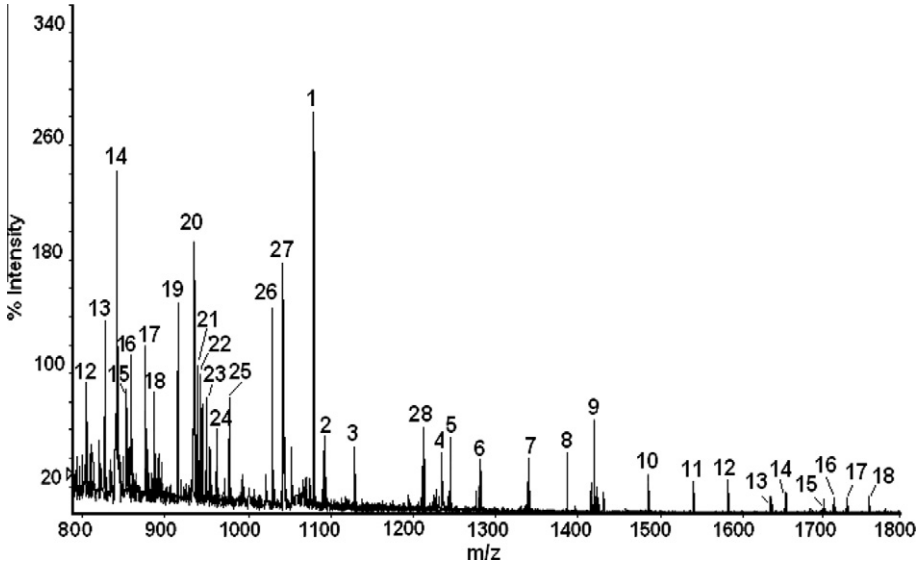

Figure 1. MS spectrum of the released glycans after treatment of RvH_2 with PNGase F. The peaks in the mass range 800–900 nm represent doubly charged ions. All the other peaks are single charged species.

Table 1
Isolated N-glycans from structural subunit RvH2 of *Rapana venosa* hemocyanin

N	Composition	MALDI $[M+Na]^+$	LCMS-Q trap
1	Fuc Man_3 $GlcNAc_2$	1079.3	1079.5 $[M+Na]^+$
2	$HexMan_3$ $GlcNAc_2$	1095.2	1095.5 $[M+Na]^+$
3	HexNAc Man_3 $GlcNAc_2$	1136.5	1136.4 $[M+Na]^+$
4	$HexMan_3$ $GlcNAc_2$	1241.6	1241.4 $[M+Na]^+$
5	Hex_2 Man_3 $GlcNAc_2$	1257.7	1257.5 $[M+Na]^+$
6	Fuc HexNAc Man_3 $GlcNAc_2$	1282.1	1282.2 $[M+Na]^+$
7	$HexNAc_2$ Man_3 $GlcNAc_2$	1339.6	1339.4 $[M+Na]^+$
8	Hex_2 $XylMan_3GlcNAc_2$	1389.8	1389.9 $[M+Na]^+$
9	Hex_3 $Man_3GlcNAc_2$	1419.6	1419.4 $[M+Na]^+$
10	Fuc $HexNAc_2$ Man_3 $GlcNAc_2$	1485.7	1485.3 $[M+Na]^+$
11	$HexNAc_3$ $Man_3GlcNAc_2$	1541.7	1541.9 $[M+Na]^+$
12	Hex_4 $Man_3GlcNAc_2$	1581.9	802.6 $[M+2Na]^{2+}$
13	Fuc_2 $HexNAc_2$ Man_3 $GlcNAc_2$	1631.8	827.5 $[M+2Na]^{2+}$
14	$HexA_1$ $HexNAc_2$ Fuc Man_3 $GlcNAc_2$	1661.7	842.3 $[M+2Na]^{2+}$
15	MeHexNAc $HexNAc_2$ Fuc $Man_3GlcNAc_2$	1702.9	862.7 $[M+2Na]^{2+}$
16	MeHex Hex_2 Fuc Xyl $Man_3GlcNAc_2$	1712.3	867.5 $[M+2Na]^{2+}$
17	Hex_5 Man_3 $GlcNAc_2$	1744.0	883.5 $[M+2Na]^{2+}$
18	Hex_3 HexNAc Fuc $Man_3GlcNAc_2$	1768.4	895.4 $[M+2Na]^{2+}$
19	$HexA_1$ $HexNAc_2$ Fuc_2 Man_3 $GlcNAc_2$	1807.9	915.6 $[M+2Na]^{2+}$
20	Hex_4 Xyl Fuc $Man_3GlcNAc_2$	1861.0	942.2 $[M+2Na]^{2+}$
21	Hex_2 $HexNAc_3$ $Man_3GlcNAc_2$	1866.6	944.8 $[M+2Na]^{2+}$
22	Hex_5 Fuc $Man_3GlcNAc_2$	1890.6	957.0 $[M+2Na]^{2+}$
23	Hex_6 $Man_3GlcNAc_2$	1906.6	964.6 $[M+2Na]^{2+}$
24	Hex_5 HexNAc $Man_3GlcNAc_2$	1947.5	985.4 $[M+2Na]^{2+}$
25	MeHex Hex $HexNAc_3$ Fuc Man_3 $GlcNAc_2$	2028.3	1025.6 $[M+2Na]^{2+}$
26	MeHex Hex_2 $HexNAc_3$ $Man_3GlcNAc_2$	2042.6	1032.6 $[M+2Na]^{2+}$
27	Hex_3 HexNAc Fuc_3 $Man_3GlcNAc_2$	2061.0	1041.6 $[M+2Na]^{2+}$
28	Hex_3 $HexNAc_2$ $XylFuc_3$ $Man_3GlcNAc_2$	2395.6	1209.2 $[M+2Na]^{2+}$

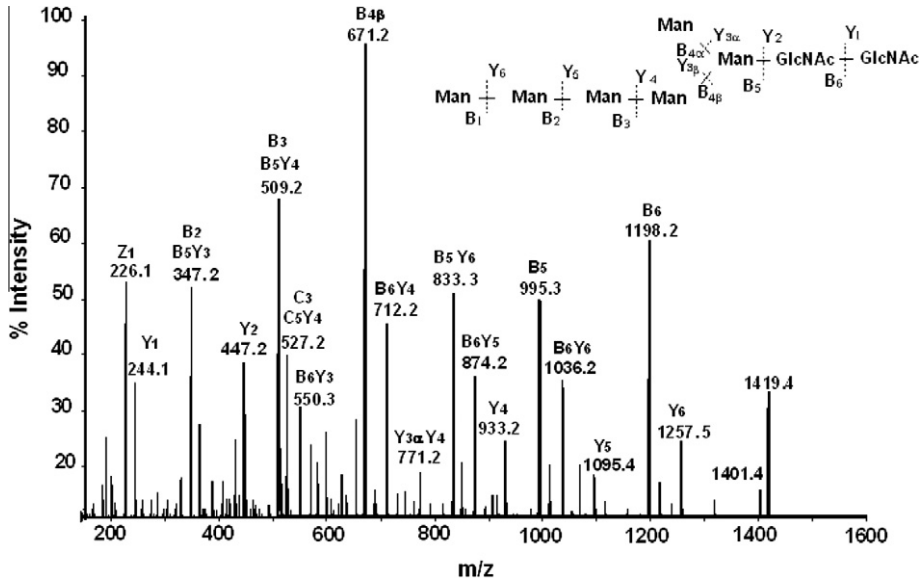

Figure 2. MS/MS spectra and one of more structures with fragmentation nomenclature of the double charged $[M+2Na]^+$ of the glycan at *m/z* 1419, isolated from RvH2.

converting the acid to the amide as described by Sandra et al.[32,34,35] Analysis of the glycans using MALDI-TOF-TOF revealed that the signals at *m/z* 1661.7 and 1807.9 were reduced by 1 Da as presented in Figure 5. These results confirm a branching of HexA to the internal Fuc.[32]

The native molecules of RvH, HlH (*H. lucorum*) and KLH, of structural subunits RvH1 and HlH1, and of glycosylated FU RvH2-e and a non-glycosylated FU of HlH added in non-toxic concentrations of 200, 100, and 50 μg/mL on the Herpes simplex virus type 1, strain Vic, (HSV-1), effectively inhibited the replication of the virus in a dose-dependent way (Fig. 6). FU of RvH2-e was found to be the most effective inhibitor. When applied in a concentration of 200 μg/mL it inhibits the growth of HSV-1 by 62%. In comparison, KLH applied at the same concentration suppresses viral replication by 52%. The respective curves show the significant difference in their ED_{50} values, which are respectively 100 μg/mL and 168 μg/mL. The antiviral effects of other compounds are much smaller.

3. Discussion

It is known that molluscan Hcs are powerful immunogens, probably due to their high carbohydrate content and specific monosaccharide composition.[25] In several cases the incompletely known, oligosaccharide structures of *H. vulgaris*,[7] *R. venosa*[10,33] and *Megathura crenulata* hemocyanins,[13,17,23] appear to have different oligosaccharide structures, which very likely is the reason why

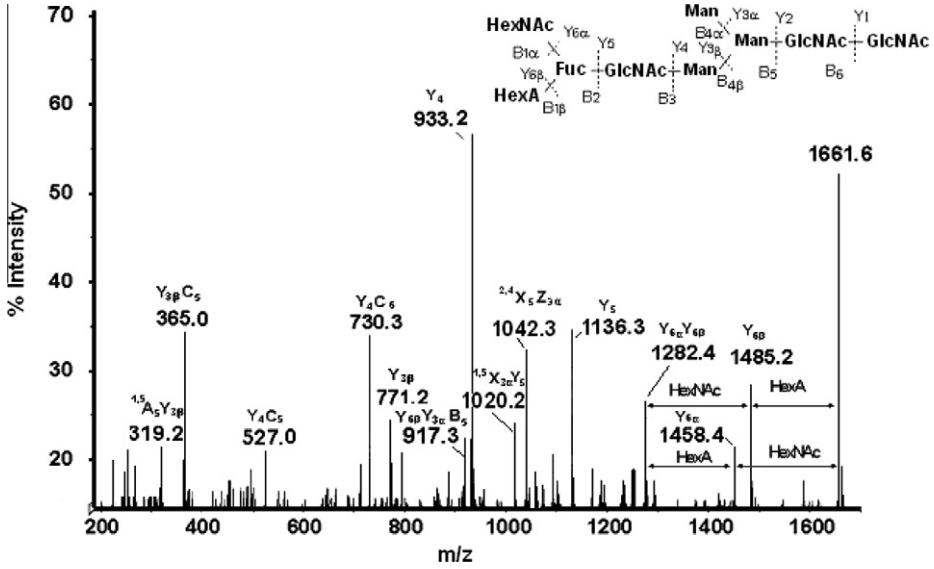

Figure 3. MS/MS spectra and structure with fragmentation nomenclature of the double charged $[M+2Na]^{2+}$ of the glycan at *m/z* 842.3, isolated from RvH2.

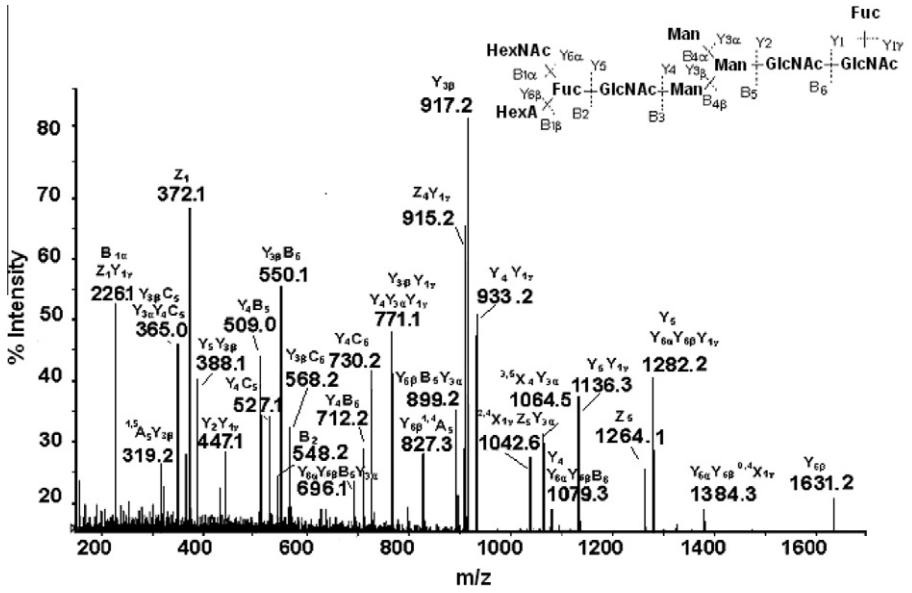

Figure 4. MS/MS spectra and structure with fragmentation nomenclature of the double charged $[M+2Na]^{2+}$ of the glycan at *m/z* 915.6, isolated from RvH2.

the immunostimulatory properties of these hemocyanins[26–28] are different.

Whereas the glycans of subunit RvH1 have previously been determined, we, in the present paper determined the glycosylation structure of the second structural subunit of RvH (RvH2).

Although it is very likely that we have not detected all the glycan structures of RvH2, there are sufficient data now (Table 1) to compare the structures with those found for RvH1,[32] which have probably not all been detected either. It appears that most of the structures are identical in both structural subunits. Fifteen of the 28 structures mentioned in Table 1 for RvH2 have also been detected in RvH1 (numbers 1–7, 9, 10, 12–14, 17, 19 and 23). Most of the structures are of high mannose type as was observed in other molluscan and arthropodan nemocyanins.[36]

Aside from the core structure $Man_3GlcNAc_2$ and except for the structures 5, 7–9, 12, 17 and 23, they all carry at least one fucose residue, mainly bound to the carbohydrate chain outside the core structure. In the cases where 2 fucose were detected, the second residue was found to be linked to the first GlcNAc of the core structure. The latter is also the case for glycan 1. Another glycan in common between RvH2 and RvH1 are 2 structures containing terminal hexuronic acid (numbers 14 and 19 in Table 1), but it remains unclear to which functional subunit they belong. In fact, the presence of hexuronic acid residue, with a neighbouring internal fucose to which also an *N*-acetylhexosamine is linked, stands for a novel N-glycan motif.[32] Moreover, RvH1 contains 3 structures with more than one hexuronic acid, but none of their counterparts have yet been identified in RvH2. We could analyse 3 oligosaccharide structures with a methylated hexose as terminal residue (numbers 16, 25 and 26), but none of them was detected in RvH1. The methylated glycan 25 (Table 1) very closely resembles the structure of the glycan Gplb, described in Ref. 12 to belong to FU of species *R. thomasiana* (RtH), which in fact is identical to *R. venosa*. Oligosaccharide 15, on the other hand, has a composition that is nearly

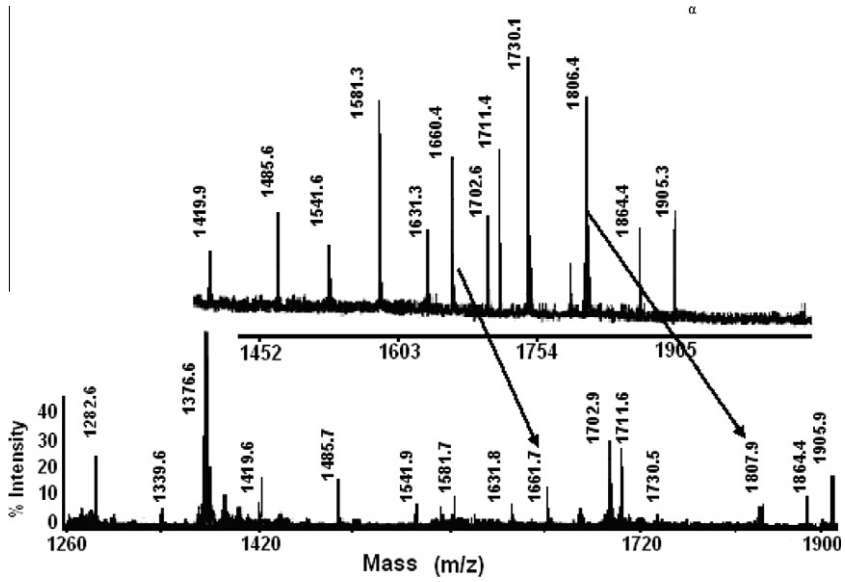

Figure 5. MALDI-TOF-MS spectra of the RvH2 N-glycans before (bottom) and after (top) amidation.

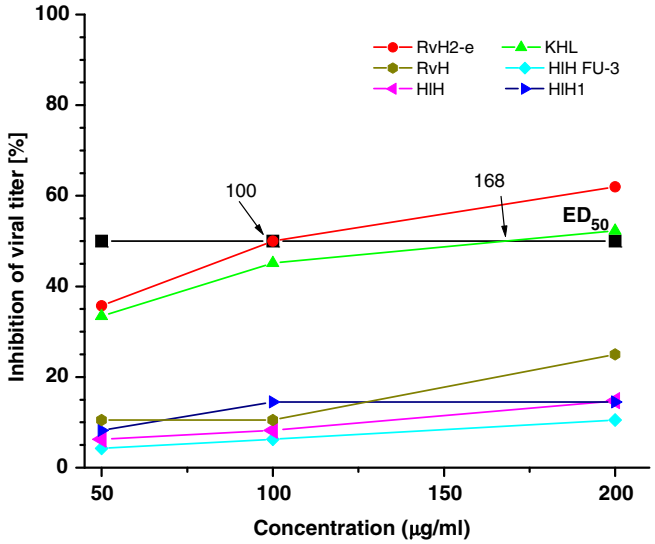

Figure 6. Influence of different hemocyanins and functional units on the replication of HSV type 1, strain Vic, infecting cell line MBDK.

identical to that of glycopeptides Gpla of RtH,[12] the only difference being that the methylated residue is MeHexNAc in RvH2 instead of MeGal in RtH2-e. It is possible, however, that the MeGal-moiety of Gplb has been erroneously identified and in fact is a hexuronic acid, having a nearly identical molecular mass (194 Da).

As a particular feature, we finally detected 4 xylose-containing structures in RvH2 (numbers 8, 16, 20 and 28), but none of these types of glycans has been found in RvH1, so far. Also noteworthy is the fact that we did not detect the core structure $Man_3GlcNAc_2$ in RvH2, although it was detected as a synthetically incomplete structure (of glycopeptides Gplla) in RvH2-e,[12] and it is certainly also present in RvH1 where it is bound as a glycan subfragment to at least 6 glycopeptides containing different glycosylation sites.[32]

The importance of these findings resides in the fact that, in vitro, hemocyanin displays antiviral effects on several types of viruses, for example, on poliovirus type 1, coxsackievirus B4 and respiratory syncytial virus, and that these effects are associated with either one or two glycosylated functional units RvH-c. Native RvH, however, does not show any antiviral activity against the viruses mentioned, and so does also non-glycosylated FU RvH-b. We have previously found that glycosylated FU RvH2-c inhibits the replication of HSV.[10] We now found that glycosylated FU RvH2-e has the same capacity.

It was not the goal of the present paper to set up experiments that can specifically explain the antiviral activity, but on the basis of what has been reported for the interaction between glycoprotein 120 in HIV[37] and specific lectins, we would like to propose a similar scenario. At least nine of the eleven glycoproteins in HSV-1 are known in detail, as well as their role in virus replication. On the other hand, four N-linked glycosylation sites have been found in glycoprotein G-2 (gG-2) of herpes simplex virus type 2.[38] GP120 contains manly high mannose type glycans and this type is known to interact with the GlcNAc-specific lectin of the nettle *Urtia dioica*.[39,40] Three binding models have been suggested between these molecules, one of them via a calcium ion, as found in 'C-type lectin'. A second one consists of the interaction of a single terminal carbohydrate, or of multiple sugar rings, without the need of a metal ion. In the latter case, three aromatic residues of the lectin form ring stackings with the sugar moieties of the individual GlcNAc residues of tri-*N*-acetylchitotriose (NAG3). With respect to *R. venosa* FU2-e, the unit with the highest antiviral activity, it is striking that it contains 6 tryptophan residues in its polypeptide chain, 18 tyrosines and 7 histidines. The position of the 6 tryptophans is shown in Figure 7, which represents a 3D model of the functional unit. It is not unlikely that some of these aromatic residues are also involved in ring stacking with carbohydrate moieties of HSV. We also like to consider the possibility of interaction, through van der Waals forces, of some of the residues shown in Figure 8A with the hydroxyl groups at C-2, C-3 and C-4 of mannose moieties of the glycans of one or several (gB, gD and gHgL) of the 9 viral glycoproteins that have been proposed to be involved in membrane fusion in the process of viral infection.[41] Such an interaction has already been detected between lectin 7 of *Narcissus pseudonarcissus* complexes to Man (1–3) Man.[42]

Finally, we propose that the reason why the complete molecule of HvH does not have antiviral activity is due to the fact that the carbohydrate chains are buried in between the structural subunits of the global proteins and therefore, are unable to interact with viral glycoproteins.

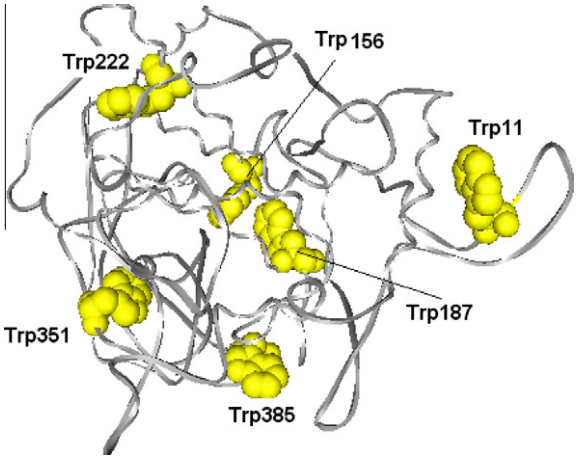

Figure 7. 3D model of functional unit RvH2-e. Eight tryptophan residues are shown in green.

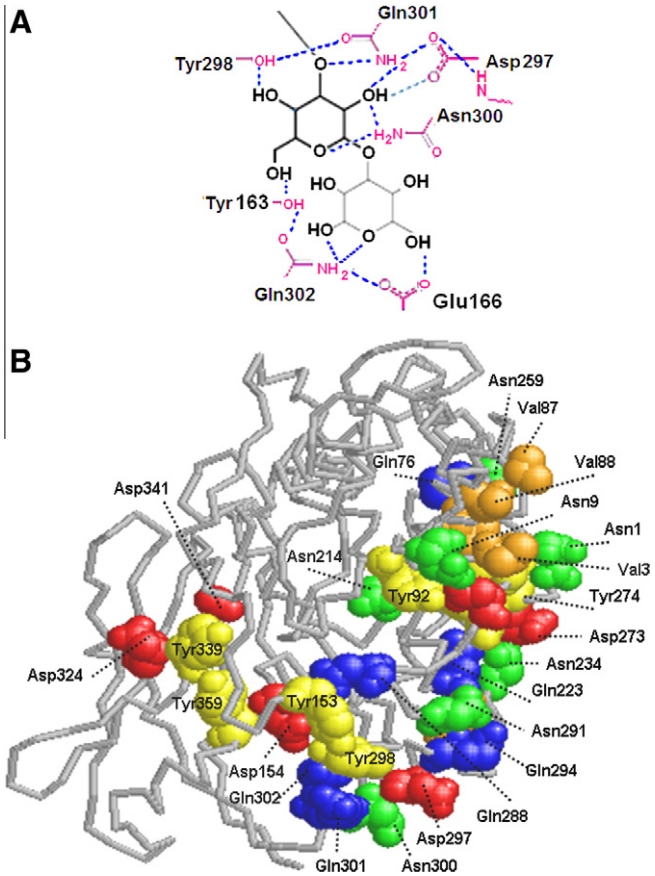

Figure 8. (A) Hydrogen-bonding pattern and van der Waals contacts of the RvH2-e and HSV based on the *Narcissus pseudonarcissus* lectin 7 (NPL7) carbohydrate-binding domain complexed to Manα(1,3)Man66 (PDB ID 1npl) (27). The mannose residue in the main binding pocket is shown in bold lines, with the hydrogen bonds. (B) 3D model of functional unit RvH2-e. Amino acid residues included in the loops are shown in blue and green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Materials and methods

R. venosa Hc was isolated from marine snails of the Black Sea, as described before.[31] The two structural subunits, RvH1 and RvH2, were separated on a Resource Q column using an FPLC system.[6] All (bio)chemicals, unless noted, were purchased from Sigma–Aldrich.

4.1. Isolation and sequencing of glycans from RvH2 by MALDI-TOF/TOF-MS

The sugar stock solution and exoglycosidase digestions 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 dihydroxybenzoic acid, initially dissolved in 50% AcN (10 mg/mL).

A 4700 Proteomics Analyser with TOF/TOF optics (Applied Biosystems, Framingham, MA) was used in this study. A total of 1500 laser shots (355 nm) were acquired in the MS mode. Spectra in the range from *m/z* 900 to 3000 were recorded. Deduced monosaccharide compositions are assigned to $(M+Na)^+$ ions.

4.2. Permethylation and amidation

After permethylation and amidation,[34,35] released glycans (1 μL of the stock solution) were dissolved in 25 μL of 1 M NH_4Cl (ammonium chloride) and then mixed with 15 μL of 1 M 4-(4,6-dimethoxy-1,3,5-triazin-2yl)-4-methylmorpholinium chloride (DMT-MM). After incubation at 50 °C for 24 h, the reaction mixture was desalted by hydrophilic affinity isolation of the oligosaccharides. The sample solution was therefore mixed with 100 μL of a 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, followed by centrifugation, the supernatant was removed. The suspension was washed with the same solvent (3 × 500 μL). Ethanol/H_2O (1:1, v/v) (250 μL) was then added and after incubation for 30 min, the solution phases were recovered and dried. The sample was dissolved in 10 μL of water for MALDI-MS analysis.

4.3. Q-Trap analyses (MS and MS/MS)

Off-line ESI-MS measurements of the glycans were performed on a Q-Trap mass spectrometer equipped with a nanospray ion source (Proxeon, Odense, Denmark) 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. For operation in modes, the resolution of Q1 was set to 'low'. Excitation time was set at 100 ms.

4.4. Bioinformatics

Using programmes RASMOL and 2LNL, the positions of tryptophans and some particular other amino acid residues in the three-dimensional protein structure of RvH2-e were identified.

4.5. Cytotoxicity and antiviral assay

Herpes simplex virus type 1, strain Vic (HSV-1), was supplied by the National centre of infectious and parasitic diseases, Sofia, Bulgaria. The stock viral titer was $10^{5,5}$ $CCID_{50/mL}$. The cell line MDBK (Madin-Darby Bovine Kidney) was grown in medium DMEM (AppliChem GmbH, Germany) containing 10% new born calf serum and antibiotics.

Confluent monolayers were covered with media containing different concentrations of extract, and cultured at 37 °C for 96 h. Cells grown in extract-free medium served as a control. The cytotoxic effect was determined by the MTT test and by microscopy. The maximal concentration which did not alter either the morphology and the viability of the cells was recognized as maximal tolerated concentration (MTC). Experiments were carried out in

multicycle growth conditions. Confluent cell monolayers in 96-well microplates were infected with 320 $CCID_{50/0.1mL}$ of the appropriate virus. After a one hour adsorption at room temperature the investigated compounds (molecules RvH, HlH and KLH, structural subunits RvH1 and HlH1, glycosylated FU RvH2-e and a non-glycosylated FU of HlH), in appropriate dilutions, were added to the monolayer. Every dilution was applied in threefold repetitions. Each experiment was carried out in triplicate. The viral cytopathic effect was determined by a four-cross system, with a full destruction of the cell monolayer as control. The average value from three wells for every dilution was presented as percentage of the viral control. The effective concentration required to inhibit the replication by 50% (ED_{50}) was determined by making a dose–response curve.

Acknowledgements

This work was supported by a research grant by the Bulgarian National Science Fund TK01-496/2009 and UV-L-301, DAAD-17/2007 and DFG-01/2008 (Germany) and the Exchange Agreements VS.011.06N and VS.016.09N between the Fund for Scientific Research-Flanders (FWO-Vlaanderen) and the Bulgaria Academy of Sciences (BAS). We thank the National Snail Breeding Cluster, Sofia (NOC) Ing. H. Stoyanov, Director of 'Delta Industry' AD, Sozopol, for providing the animals.

References

1. Salvato, B.; Beltramini, M. *Life Chem. Rep.* **1990**, *8*, 1–47.
2. Van Holde, K. E.; Miller, K. I. *Adv. Protein Chem.* **1995**, *47*, 1–81.
3. Van Holde, K. E.; Miller, K. I.; Decker, H. *J. Biol. Chem.* **2001**, *276*, 15563–15566.
4. Dolashka-Angelova, P.; Beltramini, M.; Dolashki, A.; Salvato, B.; Voelter, W. *Arch. Biochem. Biophys.* **2001**, *389*, 153–158.
5. Dolashka-Angelova, P.; Beck, A.; Dolashki, A.; Beltramini, M.; Stevanovic, S.; Salvato, B.; Voelter, W. *Biochem. J.* **2003**, *374*, 185–192.
6. Dolashka-Angelova, P.; Schwarz, H.; Dolashki, A.; Stevanovic, S.; Fecker, M.; Saeed, M.; Voelter, W. *Biochim. Biophys. Acta* **2003**, *1646*, 77–85.
7. Dolashka-Angelova, P.; Beck, A.; Dolashki, A.; Stevanovic, S.; Beltramini, M.; Salvato, B.; Hristova, R.; Velkova, L.; Voelter, W. *Micron* **2004**, *35*, 101–104.
8. Dolashka-Angelova, P.; Dolashki, A.; Savvides, S. N.; Hristova, R.; Van Beeumen, J.; Voelter, W.; Devreese, B.; Weser, U.; Di Muro, P.; Salvato, B.; Stevanovic, S. *J. Biochem.* **2005**, *138*, 303–312.
9. Dolashka-Angelova, P.; Stefanova, T.; Livaniou, E.; Velkova, L.; Klimentzou, P.; Stevanovic, S.; Neychev, H.; Schwarz, H.; Voelter, W. *Immunol. Invest.* **2008**, *37*, 822–840.
10. Dolashka-Angelova, P.; Lieb, B.; Velkova, L.; Heilen, N.; Sandra, K.; Nikolaeva-Glomb, L.; Dolashki, A.; Galabov, A.; Van Beeumen, J.; Stevanovic, S.; Voelter, W.; Devreese, B. *Bioconjugate Chem.* **2009**, *20*, 1315–1322.
11. Gielens, C.; De Geest, N.; Compernolle, F.; Préaux, G. *Micron* **2004**, *35*, 99–100.
12. Gielens, C.; Idakieva, K.; Van den Bergh, V.; Siddiqui, N. I.; Parvanova, K.; Compernolle, F. *Biochem. Biophys. Res. Commun.* **2005**, *33*, 562–570.
13. Kurokawa, T.; Wuhrer, M.; Lochnit, G.; Geyer, H.; Markl, J.; Geyer, R. *Eur. J. Biochem.* **2002**, *269*, 5459–5473.
14. Lommerse, J. P. M.; Thomas-Oates, J. E.; Gielens, C.; Preaux, G.; Kamerling, J. P.; Vliegenthart, J. F. G. *Eur. J. Biochem.* **1997**, *249*, 195–222.
15. Van Kuik, J. A.; Sijbesma, R. P.; Kamerling, J. P.; Vliegenthart, J. F. G.; Wood, E. J. *Eur. J. Biochem.* **1986**, *160*, 621–625.
16. Wuhrer, M.; Dennis, R. D.; Doenhoff, M. J.; Geyer, R. *Mol. Biochem. Parasitol.* **2000**, *110*, 237–246.
17. Wuhrer, M.; Robijn, M. L. M.; Koeleman, C. A. M.; Balog, C. I. A.; Geyer, R.; Deelder, A. M.; Hokke, C. H. *Biochem. J.* **2004**, *378*, 625–632.
18. Gutternigg, M.; Ahrer, K.; Grabher-Meier, H.; Burgmayr, S.; Staudacher, E. *Eur. J. Biochem.* **2004**, *271*, 1348–1356.
19. Geyer, H.; Wuhrer, M.; Resemann, A.; Geyer, R. *J. Biol. Chem.* **2005**, *280*, 40731–40748.
20. Harris, J. R.; Markl, J. *Micron* **1999**, *30*, 597–623.
21. Harris, J. R.; Markl, J. *J. Eur. Urol.* **2000**, *37*, 24–33.
22. Kantelhardt, S. R.; Wuhrer, M.; Dennis, R. D.; Doenhoff, M. J.; Bickle, E. Q.; Geyer, R. *Biochem. J.* **2002**, *366*, 217–223.
23. Maras, M.; Callewaert, N.; Piens, K.; Claeyssens, M.; Martinet, W.; Dewaele, S.; Contreras, H.; Dewerte, I.; Penttila, M.; Contreras, R. *J. Biotechnol.* **2000**, *77*, 255–263.
24. Moltedo, B.; Faunes, F.; Haussmann, D.; De Ioannes, P.; De Ioannes, A. E.; Puente, J.; Becker, M. I. *J. Urol.* **2006**, *176*, 2690–2695.
25. Iliev, I.; Toshkova, R.; Dolashka-Angelova, P.; Yossifova, L.; Hristova, R.; Yaneva, J.; Zacharieva, S. *C. R. Acad. Bulg. Sci.* **2008**, *61*, 203–210.
26. Toshkova, R.; Velkova, L.; Voelter, W.; Dolashka-Angelova, P. *C. R. Acad. Bulg. Sci.* **2006**, *59*, 977–982.
27. Toshkova, R.; Ivanova, E.; Nastke, M.-D.; Stevanovic, S.; Velkova, L.; Voelter, W.; Dolashka-Angelova, P. *IDOSY, Global J. Mol. Sci.* **2006**, *1*, 22–32.
28. Guo, D.; Zhang, Y.; Zeng, D.; Wang, H.; Li, X.; Li, Y.; Fan, X. *Exp. Parasitol.* **2009**, *123*, 277–281.
29. Zhang, X.; Huang, C.; Qin, Q. *Antiviral Res.* **2004**, *61*, 93–99.
30. Chongsatja, P.; Bourchookarn, A.; Lo, C. F.; Thongboonkerd, V.; Krittanai, C. *Proteomics* **2007**, *7*, 3592–3601.
31. Dolashka, P.; Genov, N.; Pervanova, K.; Voelter, W.; Geiger, M.; Stoeva, S. *Biochem. J.* **1996**, *315*, 139–144.
32. Sandra, K.; Dolashka-Angelova, P.; Devreese, B.; Van Beeumen, J. *Glycobiology* **2007**, *17*, 141–156.
33. Beck, A.; Hillen, N.; Dolashki, A.; Stevanovic, S.; Salvato, B.; Voelter, W.; Dolashka-Angelova, P. *Biochimie* **2007**, *89*, 938–949.
34. Sandra, K.; Devreese, B.; Stals, I.; Claeyssens, M.; Van Beeumen, J. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 413–423.
35. Sandra, K.; Van Beeumen, J.; Stals, I.; Sandra, P.; Claeyssens, M.; Devreese, B. *Anal. Chem.* **2004**, *76*, 5878–5886.
36. Debeire, P.; Montreuil, J.; Goyffon, M.; van Kuik, A. J.; Van Halbeek, H.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1986**, *151*, 305–310.
37. Balzarini, J. *Nat. Rev. Microbiol.* **2007**, *5*, 583–597.
38. Su, H. K.; Fetherston, J. D.; Smith, M. E.; Courtney, R. J. *J. Virol.* **1993**, *67*, 2954–2959.
39. Gallaher, W. R.; Ball, J. M.; Garry, R. F.; Martin-Amedee, A. M.; Montelaro, R. C. *AIDS Res. Hum. Retroviruses* **1995**, *11*, 191–202.
40. Harata, K.; Muraki, M. *J. Mol. Biol.* **2000**, *297*, 673–681.
41. Turner, A.; Bruun, B.; Minson, T.; Browne, H. *J. Virol.* **1998**, *72*, 873–875.
42. Sauerborn, M. K.; Wright, L. M.; Reynolds, C. D.; Grossmann, J. G.; Rizkallah, P. J. *J. Mol. Biol.* **1999**, *290*, 185–199.