RAPANA VENOSA HEMOCYANIN WITH ANTIVIRAL ACTIVITY

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ABSTRACT

Molluscan hemocyanins (Hcs) have recently particular interest due to their significant immunostimulatory properties. This is mainly related to their high carbohydrate content and highly specific monosaccharide composition. Our study revealed a highly heterogeneous mixture of different glycans isolation from structural subunit RvH2 of Rapana venosa hemocyanin at least 28 different compositions of Hex_{0-9} $HexNAc_{2-4}$ Hex_{0-3} $Pent_{0-3}$ Fuc_{0-3} and deoxyhexose and pentose residues. A novel type of N-glycan, with an internal Fuc connecting one GalNAc(ß1-2) and one hexuronic acid, was detected in RvH2 as was previously found in subunit RvH1.

We compared investigation on antiviral effects of several molluscas hemocyanins (keyhole limpet hemocyanin, Rapana venosa hemocyanin and Helix vulgaris hemocyanin) and the arthropod Carcinus aestuarii hemocanin. For the first time, we demonstrate here the inhibitory effect of one glycosylated functional unit of molluscan hemocyanin against viruses.The FU RvH-1 of Rapana venosa hemocyanin is the most effective inhibitor on the replication of Herpes simplex virus type 1, strain Vic, (HSV-1).

Keyword: Rapana venosa hemocyanin, functional unit, glycosylatin, antiviral effect, mass spectrometry

Introduction

Hemocyanins (Hcs) are high-molecular-mass oxygentransporting glycoproteins, freely dissolved in the hemolymph of several arthropods and molluscs (3,18,20). There are large differences in the molecular mass, structure, carbohydrate content and monosaccharide composition of Hcs from arthropods and molluscs. Molluscan hemocyanins are large glycoproteins, usually have a higher carbohydrate content (2-9 %, w/w) with different structures and quantities of the oligosaccharide moieties (2,5,6,7,8,14). Molluscan Hcs usually have are powerful immunogens, probably due to their high carbohydrate content and specific monosaccharide composition. Although it is generally accepted that the oligosaccharide constituents of Hcs are of prime significance for its antigenicity and biomedical properties, knowledge on the carbohydrate structures of these glycoprotein are still incomplet.

The carbohydrate moiety of molluscan Hcs has recently received particular interest: keyhole limpet hemocyanin (KLH1) is widely used in clinical studies due to its immunostimulatory properties. Structural studies of KLH revealed that it is very heterogeneously glycosylated, carrying mainly high mannose-type glycans with 5-7 mannosyl residues, hybrid-type species with five mannoses and one *N*-acetylgalactosamine chain, as well as truncated sugar chains derived thereof. Keyhole limpet hemocyanin (KLH1) is widely used in research and clinical studies due to its immunostimulatory properties. Present fields of application based on the assumed expression of Gal(β1-3)GalNAc-determinants as cross-reacting epitopes are immunotherapy of bladder cancer (15), and its use as a carrier of polysialic acid or low molecular weight haptens, such as synthetic oligosaccharides, gangliosides or (glyco)peptides, designed for potential application in anticancer therapy (11,12, 16,21). Carbohydrate structures and some glycosylation sites of hemocyanins from *Helix pomatia* and *Sepia officinalis* have been identified using different methods (17 ,9). The occurrence of xylose in *H. pomatia* Hc is considered to be highly immunogenic in mammalian species (9). At present the oligosaccharide structures of several functional units isolated from the Hc of the marine snail

Rapana venosa (RvH), previously referred to as *Rapana thomasiana,* have been studied, but a complete structure is not known yet (1, 5, 4, 10, 19).

We therefore comparison investigation on antiviral effects of several molluscas hemocyanins (keyhole limpet hemocyanin, *Rapana venosa* hemocyanin and *Helix vulgaris* hemocyanin) and the arthropod *Carcinus aestuarii* hemocanin. This is the first report of the fact that also glycosylated functional units of molluscan hemocyanin have antiviral activity.

Materials and methods

Isolation of Rapana venosa hemocyanin

Rapana venosa hemocyanin (RvH) was isolated from the hemolymph of marine snails living in the Black sea. Native Hc was purified from the hemolymph of *Rapana venosa* Hc as described previously Dolashka-Angelova et al. 2003 (8).
After treatment of the isolated structural subunits RvH1 and RvH2 with trypsin in a ratio of 400:1 and incubation at 37ºC, for 4 hours, the tryptic hydrolysate was separated on of an ion exchange Resource 6 ml (Pharmacia) column using an FPLC system and eluted with 50 mM Tris/HCl buffer, pH 8.2. with a 0,0-0.5 M NaCl gradient as described by Dolashka-Angelova et al .2003 (4).

Isolation of glycans from structural subunits RvH1 and RvH2

For deglycosylation, approximately 4 mg of RvH1 and RvH2 each were dissolved separately in 50 µl of denaturing solution. 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 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). The glycans were eluted with 2 ml of 25% acetonitrile /0.05% TFA.

Q-Trap analyses (MS and MS/MS) of glycans from Rapana venosa Hc

Off-line ESI-MS measurements of the glycans were performed on the 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 Q0-trapping being activated. The trap fill-time was 200 ms in the MS/MS- scan modes. For operation in the MS/MS modes, the resolution of Q1 was set to 'low'. Excitation time was set at 100 ms.

Virus

Herpes simplex virus type 1, strain Vic, (HSV-1) was supplied by National center of infectious and parasitic diseases, Sofia, Bulgaria. Stock viral titer was $10^{5,5}$ $CCID_{50/ml}$.

Cell culture

Cell line MDBK (Madin-Darby Bovine Kidney), grown in medium DMEM (AppliChem GmbH, Germany) with 10% new born calf serum and antibiotics.

Cytotoxicity assay

Confluent monolayer were covered with media contain different concentrations of extract and cultured at 37°C for 96h. Cells grown in extract-free medium served as a control. The maximal concentration, which did not alter neither the morphology nor viability of the cells, was recognized as maximal tolerate concentration (MTC).

Results and Discussion

Sequencing of glycans by Q-Trap

Sandra K. et al., 2007, were detect the most important class of the glycans isolated from RvH1 containing internal fucose branching to hexuronic acid and HexNAc (19). Based on the results obtained, it is now found that RvH2 has the same novel types of N-glycans at m/z 842.2 $[M+2Na]^{2+}$ (1661.6) and 915.6 $[M+2Na]^{2+}$ (1807.6), as they comprise one HexNAc and one hexuronic acid linked to fucosyl residue glycans. These structures were identified after analyses of the isolated glycans from structural subunit RvH2 by Q-trap system. The MS/MS spectrum of the first doubly-charged sodium-adduct on glycans isolated from RvH2 is shown in **Figure 1**

The sequence can most easily be determined when considering the Y ions (*m/z* 1485.2, *m/z* 1136.0, *m/z* 933.2) and the combination of B and Y ions. Usually, the loss of HexNAc is a very favourable, event, so the usual HexNAc residue linked to an α1,3-mannose is missing, 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, respectively, indicative for the presence of terminal HexNAc and HexA (176 Da). Furthermore, the Y5 ion at *m/z* 1136.0 represents the glycan after removal of the frag-

ment HexNAc HexA Fuc. The observed difference of 527.4 mass units between fragments at m/z 1661.5 and m/z 1136.0 accounts for the loss of this fragment. Additional evidence for the composition of this fragment is the ion B_2 at m/z 566.6. It can be speculated that the snail enzymes need this HexNAc residue for their action.

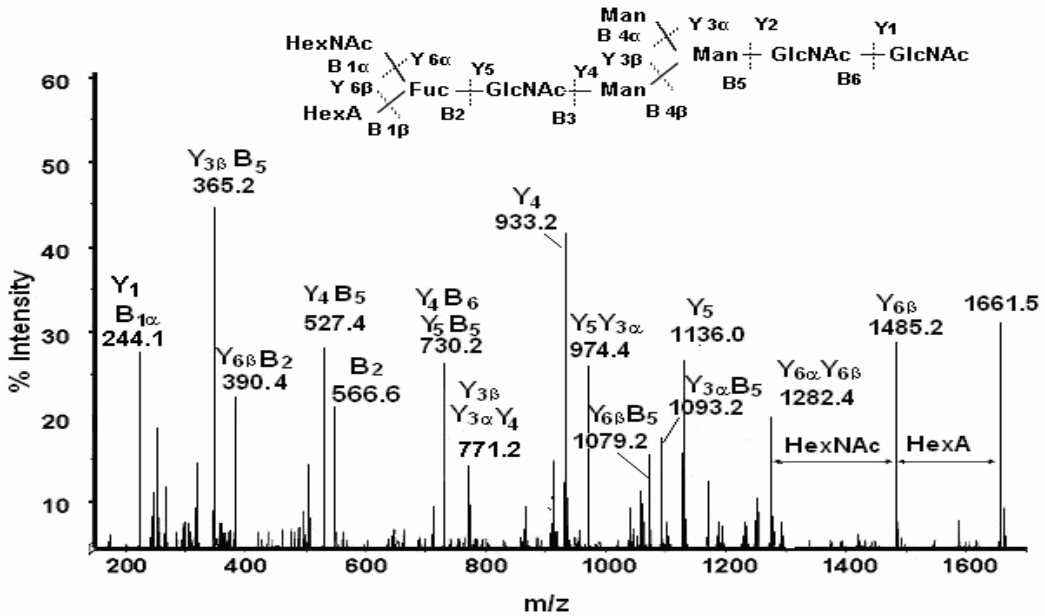

Fig. 1. MS/MS spectra and structures with fragmentation nomenclature of the double charged $[M+2Na]^{2+}$ of the glycan at m/z 842.2, isolated from RvH2.

Further evidence for the presence of hexuronic acid in the structure of glycans isolated from both structural subunits of *Rapana venosa* hemocyanin was obtained following amidation of the glycan mixture from RvH2. The method involves the modification of carboxyl groups by converting the acid to the amide as described by Sandra et al. (19). Further analysis of the glycans using MALDI-TOF-TOF revealed that the signals at m/z 1661.6 and 1807.6 were reduced to m/z 1660.4 and 1806.4 (**Figure2**).

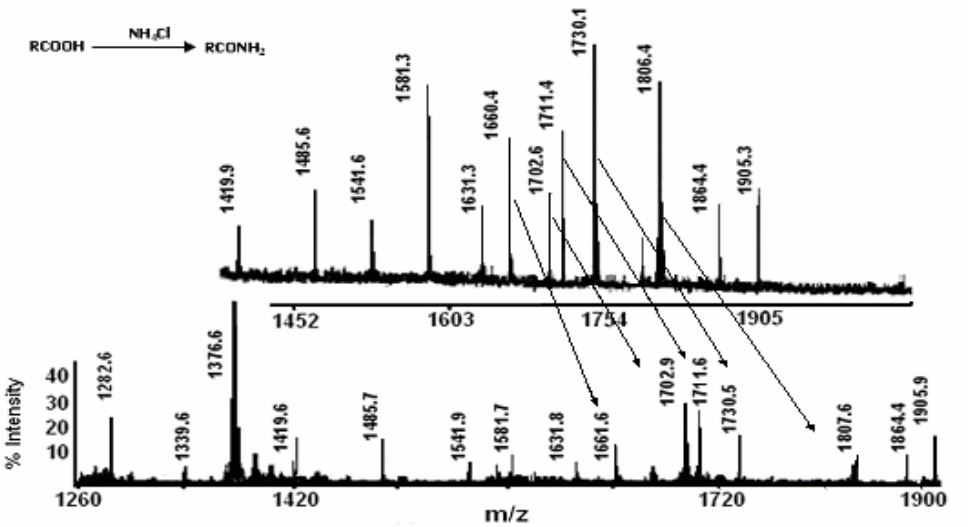

Fig.2. The evidence for the presence of hexuronic acid in the structure of glycans isolated from RvH2.The MALDI-TOF-MS spectra of the RvH2 N-glycans before (botton) and after (top) amidation.

These results confirm a branching of Hex acid to the internal Fuc in RvH2 as was suggested by Sandra et al. (19) for RvH1, in contrast to methylated Hex as proposed by Gielens et al. (10).

Two glycans with one internal fucose residue, substituted at two positions with a N-acetylhexosamine and a hexuronic acid were identified in the carbohydrate structure of RvH2. Though hexuronic acid moieties, which are common constituents of proteoglycans, occur rather rarely in glycoproteins. Nevertheless, hexauronic acid and fucose residues have been observed on mammalian N-glycans (21).

Antiviral assa

Experiments were done in multicycle growth conditions.

Confluent cell monolayers in 96-well microplates were infected with 320 $CCID_{50/0.1ml}$ of the appropriate virus. After one-hour adsorbtion the investigated compounds, in respective dilutions, were added. Every dilution was applied in three-fold repetitions. The viral cytopathic effect was determined by four-cross system when there was a full destruction of the cell monolayer in the viral control. The average value from three wells for every dilution was taken and was presented as percentage of the viral control. Effective concentration required to inhibit the replication by 50% (ED_{50}) was determined by dose-response curve. Each experiment was done in triplicate.

Cytotoxicity assay

The data sowed no affect the morphology of the cell cultures with compounds. Therefore the values of the maximal tolerate concentration for test substances no determined.

Antiviral ass

The examined compounds were added in concentrations 200, 100, and 50μg/ml. They shown antiviral activity and inhibit the viral replication (**Fig.3**). The experimental data suggested dose-depend effects.

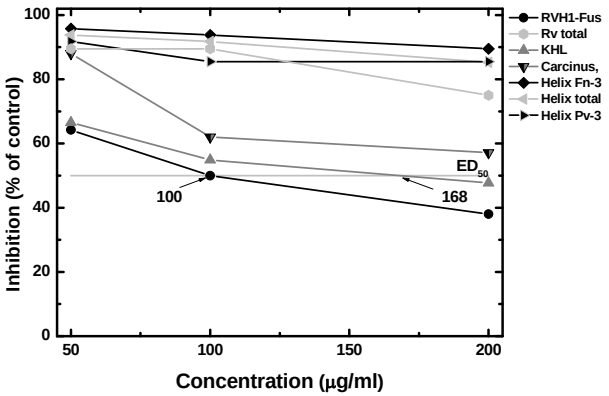

Fig. 3. Antiviral activity on the replication of HSV tape 1, strain Vic.

We founded that RVH-1 Fus was most effective inhibitor. The compound applied in concentration 200 μg/ml inhibited the growth of HSV-1 by 62%. KHL applied in same concentration suppressed viral replication 52%.

The data has been demonstrated by the respective curves and the insignificant difference in the values of ED_{50}, which are respectively 100 μg/ml and 168 μg/ml. Whereas the antiviral effects of other compounds were slightly (Tab. 1).

Thus, for the first time, we demonstrate here the inhibitory effect of one glycosylated functional unit of molluscan hemocyanin HvH against viruses. No inhibitory effect on this viruse could be observed for the native molecule RvH, HvH and structural subunit RvH1. The antiviral effect of glycosylated FU of HvH against the replication of HSV is established, but its antiviral mechanism is still unknown. Therefore, the further investigation of the inhibitory properties of HvH against HSV is under way.

TABLE 3

Anti-HSV effect *in vitro*

Compound (200 μg/ml)	RvH1-Fu	Keyhole limpet	Carcinus aestuarii	Helix Fn-3	Helix total	RvH1 Su	Rv-total
Inhibition (%)	62	52	43	21	21	14	25

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