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Oligosaccharide structure of a functional unit RvH_1-b of *Rapana venosa* hemocyanin using HPLC/electrospray ionization mass spectrometry

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Abstract

In the present study the structures of two glycopeptides (G1 and G1′), isolated from FU RvH_1-b and two glycopeptides (G2 and G3), isolated from the structural subunit RvH_1 of *Rapana venosa* hemocyanin, were determined. To structurally characterize the site-specific carbohydrate heterogeneity and binding site of the N-linked glycopeptide(s), a combination of capillary reversed-phase chromatography and ion trap mass spectrometry was used.

The amino acid sequences of glycopeptides G1 and G1′ determined by Edman degradation and MS/MS sequencing demonstrated that the oligosaccharides are linked to N-glycosylation sites. Two peptides (a glycosylated (G1) and non-glycosylated one) were identified in this fraction and no linkage sites were observed in the latter one. Based on the sequencing of the glycosylated fractions G1, G1′, G2 and G3, the carbohydrate structure Man(α1−6)Man(α1−3)Man(β1−4)GlcNAc(β1−4)[Fuc(α1−6)]GlcNAc-R could be identified for glycopeptides G1 and G3, and only the typical core structure Man(α1−6)Man(α1−3)Man(β1−4)GlcNAc(β1−4)GlcNAc-R was found for G1′ and G2. The Fuc residue found in glycopeptides G1 and G3 is attached to *N*-acetyl-glucosamine of the carbohydrate core, as often found in other glycoproteins.

Keywords: Glycopeptides; HPLC/electrospray ionization mass spectrometry; Oligosaccharides; *Rapana venosa* hemocyanin

1. Introduction

Hemocyanins (Hcs) are high-molecular (4.5×10^2 to 9×10^3 kDa) copper-containing oxygen-transporting proteins, freely dissolved in the hemolymph of several arthropodan and molluscan species [1,2]. Most Hcs are glycoproteins and there are large differences in their carbohydrate contents and their monosaccharide composition, and both, O-linked and N-linked oligosaccharides were identified [3,4]. Earlier we reported on *O*-glycosylation and *N*-glycosylation of hexameric *Carcinus aestuarii* Hc (1.6% carbohydrate content) which were found by sequencing of the glycopeptides isolated after tryptic digestion of the subunit [4].

So far, *N*-glycan structures of the hemocyanins of the snails *Helix pomatia* [5], *Lymnaea stagnalis* [6], *Rapana venosa* [7–10] and keyhole limpet *Megathura crenulata* [11–14] were

Abbreviations: API-CID, atmospheric pressure ionization–collision-induced dissociation; ESI-MS, electrospray ionization mass spectrometry; Hc, hemocyanin; FU, functional unit; Fuc, fucose; GlcNAc, *N*-acetyl-D-glucosamine; μLC/ESI-MS/MS, microcapillary liquid chromatography/electrospray ionization mass spectrometry/mass spectrometry; Man, D-mannose; MS/MS, tandem mass spectrometry; nanoES-MS, nanoelectrospray mass spectrometry.

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published. The carbohydrate moiety of molluscan Hcs has recently received particular interest for their immunostimulatory properties [15]. In addition to the already known monosaccharides fucose, xylose, mannose, galactose, glucose, *N*-acetylgalactosamine and *N*-acetylglucosamine, the carbohydrate part of the hemocyanin from *H. pomatia* (Roman snail) contains 3-*O*-methylgalactose, and that from *L. stagnalis* (a freshwater snail) 3-*O*-methylgalactose and 3-*O*-methylmannose [16].

KLH *M. crenulata* hemocyanin is carrying high mannose type glycans and truncated sugar chains derived thereof [11]. As a characteristic feature, a number of the studied *N*-glycans contained a Gal(β1–6)Man unit which has not been found in glycoprotein-*N*-glycans so far. The neutral *N*-glycan structures of *Arion lusitanicus* (gastropod) skin, viscera and egg glycoproteins were identified [17].

Not only oligosaccharide structure but also glycosylation sites were localized in FUs of the cephalopod *Sepia officinalis* (cuttlefish) [18] and of the gastropods *H. pomatia* [18] and *Rapana thomasiana* (marine snail) [7–9]. All of them are N-type and, with the exception of a conserved site near the C-terminus they are dispersed over the FU sequences. The *N*-glycosylation site of FU g of *Octopus dofleini* Hc has been identified by X-ray crystallography [19].

The aim of the present study is to identify and characterize the glycosylation sites and the branching carbohydrate chains of functional unit (FU) RvH_1-b, isolated from *R. venosa* Hc (RvH), previously termed as *R. thomasiana*. The properties of this Hc were studied well [20–22]. In a previous study on the glycosylation of *R. venosa* Hc only the ratios of the oligosaccharides and partial information about the sequence of the monomers and their linkage sites were reported [7–9]. *Rapana* Hc is a glycoprotein, and carbohydrate contents of 8.9% were determined for the native molecule and 12.8% and 4.4% for the structural subunits RvH_1 and RvH_2, respectively [7]. The oligosaccharide content is more abundant in the N-terminal FU RvH_1-a (7%) of the structural subunit RvH_1 than in N-terminal FU RvH_2-a (5.1%) of the structural subunit RvH_2. Two *N*-glycosylation sites with carbohydrate chains of the complex type were identified in FU RvH_1-a [8]. In contrast, in the FUs RvH_2-b and RvH_2-d, only one *N*-glycosylation site carrying glycans of the high mannose type was found whereas the FU RvH_2-c is devoid of any sugars [9].

2. Materials and methods

2.1. Isolation of the FU RvH_1-b from the structural subunit RvH_1 of R. venosa hemocyanin

Native Hc was purified from the hemolymph of *R. venosa* Hc as described previously [20–22]. Subunit RvH_1, previously referred to as RHSS1, was eluted as the first peak from an ion exchange chromatography column in 50 mM Tris/HCl buffer, pH 8.2, with a 0–0.5 M NaCl gradient. Then, 50 mg of the subunit were treated with trypsin (trypsin: Hc ratio 1:400) for 1 h at room temperature in 20 mM NH_4HCO_3 buffer, pH 8.2. The tryptic hydrolysate was loaded on a Resource 6 ml column (FPLC), equilibrated with 50 mM Tris/HCl buffer, pH 8.2, and the Fu RvH_1-b was eluted with a linear gradient (0–0.5 M NaCl in 60 min) at a flow rate of 1 ml min^{-1}.

2.2. Carbohydrate determination and isolation of glycopeptides

RvH_1-b (4 mg) was dissolved in 1 ml of 0.4 M Tris/HCl buffer, pH 8.6, containing 6 M guanidine-HCl and 0.2 M EDTA. To cleave the disulphide bonds, 20 µl of a β-mercaptoethanol solution was added with stirring. After heating at 50 °C for 4 h, 3 µl of 4-vinylpyridine was added and the reaction mixture allowed to stand for 3 h at room temperature. The reaction was terminated by the addition of 50 µl of 2.0 M acetic acid. The sample was dissolved in 200 µl of 0.1 M ammonium bicarbonate buffer, pH 9.0, and 50 µl of the trypsin solution (bovine pancreas, Hc-trypsin ratio 50:1) was added and the reaction mixture incubated at room temperature for 3 h. The glycopeptide mixture was separated on a Superdex 300 gel filtration column (2 × 30 cm), and the fractions were eluted with water at a flow rate of 1 ml min^{-1}. Each chromatographic peak fraction was checked for carbohydrates using the orcinol/H_2SO_4 test [8,23]. The only peak fraction giving a positive reaction was further fractionated by reverse phase HPLC using a Nucleosil 7C 18 column (250 × 10 mm; Macherey–Nagel, Düren, Germany). For elution, a linear gradient of 5% A (0.1% TFA in water) to 100% B (0.085% TFA in acetonitrile) within 70 min at a flow rate of 1 ml min^{-1} was used. The HPLC fractions, detected at a wavelength of $\lambda = 206$ nm, were collected, lyophilized and analyzed for carbohydrates with orcinol/H_2SO_4 on silica gel plates.

2.3. Glycoprotein/peptide-staining on silica gel plates

The lyophilized peptides were dissolved in water, 2–4 µl of these solutions transferred to the plate, taking care to restrict the size of the spot to 2–3 mm in diameter, and air-dried. The plate was sprayed with orcinol/H_2SO_4 and heated for 20 min at 100 °C [23].

2.4. Amino acid sequence analysis

Amino acid sequence analysis was performed from the peptides that were positive in the orcinol test. The fractions were dried and after dissolving in 40% methanol 1% formic acid, subjected to automated Edman N-terminal sequencing (Procise 494A Pulsed Liquid Protein Sequencer, Applied Biosystems GmbH, Weiterstadt, Germany).

2.5. Sample analysis by µLC/ESI-MS/MS and µLC/ESI-API-CID-MS

LC was performed on a 0.5 × 150 mm Zorbax Extend SB-C18 (5 µm) capillary column (Agilent Technologies, Waldbronn, Germany) attached to an HP1100 capillary HPLC system (Agilent Technologies). Mobile phase A was 0.025% TFA in

water, and mobile phase B 0.023% TFA, 80% acetonitrile in water. An aliquot (~10 pmol) of the collected fraction (t_R 36.7 min) from the first dimension LC separation was diluted with mobile phase A and directly injected (7 µl) onto the column. Samples were separated by a gradient program: 0–5 min 5% of mobile phase B, and 5–60 min 5–80% mobile phase B at a flow rate of 15 µl min^{-1}. The outlet of the column was directly interfaced with the electrospray ionization source of an Esquire3000$^+$ ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany), UV spectra (214 nm) were recorded on-line.

The mass spectrometer was operated in the positive ion mode. Dry gas (10 l/min) temperature was set to 185 °C, the nebulizer to 22.0 psi, and the electrospray voltage to −4000 V. Maximal accumulation time was set to 200 msec. Loading of the trap was controlled by the instrument (ICC 50.000).

2.5.1. µLC/ESI-MS/MS experiment

The acquisition method involves one MS precursor scan from *m/z* 200–1750 followed by three data-dependent MS/MS scans (isolation width 6 amu, 1.00 V fragmentation amplitude) on the top three most abundant ions in the MS survey scan. Peptide ions for which sequence information had been obtained were dynamically excluded from reanalysis for 2 min. Capillary exit voltage was set to +136 V (low potential, no in-source fragmentation).

2.5.2. µLC/ESI-API-CID-MS experiment

For the detection of the glycopeptide-specific *m/z* 204 and *m/z* 366 oxonium marker ions (HexNAc and Hex-HexNAc), the capillary exit voltage was increased to +251 V (high potential, in-source fragmentation), scanning from *m/z* 140–400 in the positive ion mode.

2.6. Nanoflow ESI mass spectrometry of glycopeptides

Glycopeptides were analyzed by nanoelectrospray (ES) mass spectrometry (MS) on a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF; Micromass, Manchester, United Kingdom) as described by Schirle et al. [24]. The ions were produced in a nanoflow electrospray ionization source. A potential of 1.2 kV was applied to the gold-coated glass capillary nanoflow needles (Proxeon, type Medium NanoES spray capillaries for the Micromass Q-TOF, Odense, Denmark), resulting in sample flow rates of 20–50 nl min^{-1}. The cone voltage was 35 V for MS and tandem MS experiments and 60–100 V for in-source and collision-induced fragmentation experiments. A quadrupole analyzer was used to select precursor ions for fragmentation in a hexapole collision cell. The collision gas was argon, used at optimized collision energies of 15–70 eV depending on mass and charge state of the precursor ion.

3. Results

Several methods and techniques were applied to study glycopeptides G1 and G1′ (isolated from functional unit RvH_1-b) and glycopeptides G2 and G3, isolated from structural subunit RvH_1. Scheme of characterization of glycopeptides is shown in Fig. 1.

3.1. Isolation of functional units from the structural subunit RvH_1

RvH_1 was first digested with trypsin and then subjected to ion exchange separation as described by Stoeva et al. [20]. Then, to confirm the glycosylation the collected fractions (FUs) were screened for glycopeptides using the orcinol method [23].

3.2. Isolation of glycopeptides G1 and G10 from FU RvH_1-b and Edman degradation

Functional unit RvH_1-b was initially digested with trypsin overnight, and the resulting peptides were subjected to reversed-phase separation using a Nucleosil 7 C18 column (Fig. 2). Fractions were collected manually, lyophilized and tested for their carbohydrate content using the orcinol/H_2SO_4 test on silica gel plates (Fig. 2). As shown in Fig. 2, the fraction eluting after 36.7 min gave a positive test for carbohydrates and was further studied using Edman degradation and LC/ESI-MS.

For the determination of the peptide sequence an aliquot was used for automated Edman degradation (Table 1). Two major peptide sequences could be identified. The sequence obtained for the first peptide was EMGLDHHMPFDIHY with a theoretical average mass of 1741.9 Da. As in this sequence neither serine, threonine nor asparagine residues were identified, classical attachment of carbohydrate chains can be excluded. Based on Edman degradation, the sequence of the second

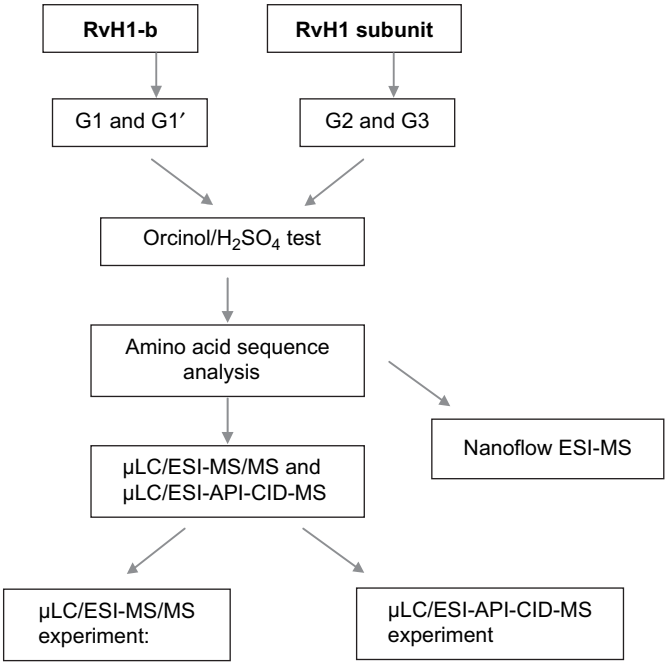

Fig. 1. Schematic representation of the "experimental strategy".

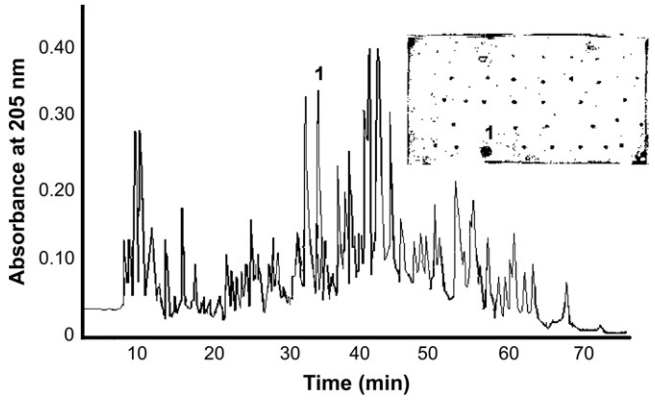

Fig. 2. HPLC profile of a trypsin digest of functional unit RvH_1-b. Chromatographic conditions: Column: 100 × 2.1 mm, Nucleosil RP C18, linear gradient elution: solvent A (0.1% TFA in water) and solvent B (0.085% TFA in acetonitrile) in 70 min at a flow rate of 1 ml min^{-1}; detection: UV, at a wavelength of 206 nm. Insert, orcinol/H_2SO_4 test for (glyco)peptides, eluted by HPLC and applied on a silica gel plate, where glycopeptide G1 was eluted as fraction 1.

peptide could be established as SV*N*GTLLGSQILGKPY with a theoretical average mass of 1646.9 Da and a potential N-linked glycosylation site at position 3 fitting to the general glycosylation motif NXS/T (X = any amino acid except proline) [25–27]. Because of a chymotryptic contamination of the trypsin (cleaves C-terminal to R and K) used for the digestion of the functional unit RvH_1-b, the C-terminal tyrosine residues of both peptides must be due to a chymotryptic cleavage.

3.3. Structural identification of glycopeptide fragments of R. venosa hemocyanin

To detect the attachment site and determine the structure of the N-linked oligosaccharide and the glycopeptide(s), we used a combination of capillary reversed-phase chromatography and electrospray ionization mass spectrometry on an ion trap mass spectrometer.

The first step involved the formation and detection of modification-specific marker ions during the course of an LC/ESI-MS experiment (with in-source fragmentation) [28]. Carbohydrate-specific detection was performed in the positive ion mode leading to $[HexNAc]^+$ (*m/z* 204) and $[Hex\text{-}HexNAc]^+$ (*m/z* 366) oxonium ion fragments which can be derived from terminal sugars as well as by two-bond cleavages from internal sugars. Fig. 3A shows the carbohydrate-specific LC/ESI-MS analysis from an aliquot of the carbohydrate-containing fraction, eluting after 36.7 min during the first RP-HPLC separation (Fig. 2). At a retention time of 25.8 min an intense peak in the extracted ion chromatograms (EICs) of the carbohydrate-specific *m/z* 204 and *m/z* 366 ions (Fig. 3B, upper and lower trace) could be observed, clearly indicating the elution of at least one glycopeptide.

In a second experiment, a second aliquot of the 36.7 min fraction was analyzed using a positive ion LC/ESI-MS/MS experiment (no in-source fragmentation) under identical RP-HPLC conditions (Fig. 3A). As shown in Fig. 3A, two major peaks (t_R 23.2 min and 25.8 min) could be observed in the UV (recorded at 214 nm wavelength, upper panel) as well as in the base peak chromatogram (BPC, lower panel), whereas the second eluting peak was identified as a glycopeptide-containing fraction as described above (Fig. 3B).

Fig. 4A shows the averaged positive ion electrospray (ES) mass spectrum of the first eluting peak (t_R 23.2 min, Fig. 3A). Only one major component with an average mass of 1741.9 was detected, which was calculated based on the $[M + 2H]^{2+}$(*m/z* 871.56), $[M + 3H]^{3+}$ (*m/z* 581.62) and $[M + 4H]^{4+}$ (*m/z* 436.52) molecular ions. This mass fits exactly to the theoretical mass calculated from the amino acid sequence of the peptide EMGLDHHMPFDIHY which was determined by Edman degradation (Table 1). Fig. 4B shows the averaged positive ion electrospray mass spectrum of the glycopeptide-containing peak (t_R 25.8 min) showing the elution of two components. For the first component, the molecular mass could be calculated as 2686.60 Da ($[M + 2H]^{2+}$ at *m/z*

Table 1
Sequence alignment of isolated glycopeptides and peptides from *Rapana venosa* hemocyanin (RvH) with other molluscan hemocyanins: HtH_1 (*Haliotis tuberculata*) CAC20588, Ac (*Aplysia californica*) CAG47100, Od (*Octopus dofleini*) AAK28276

Glycopeptide	Position	Hemocyanin	Carbohydrate composition	Amino acid sequences
G1		RvH_1-b	$Man_3GlcNAc_2Fuc$	SV*N*GTLLGSQILGKPY
G1′		RvH_1-b	$Man_3GlcNAc_2$	SV*N*GTLLGSQILGK
	399	HtH_1		SV*N*GTALSPDLLPQP
	406	Ac		SV*N*GTELPS NL IPSP
	412	Od		A I*N*GTLLPDGTI PRP
	2479	HtH		AV*N*GTVIPSSHLHQP
		RvH		EMGLDHHMPFDIHY
	2011	Od		MTREHSVPFDV
	814	Od		ELGVDMHAEYSIN
G2		RvH	$Man_3GlcNAc_2$	FSWVDGH*N*TSR
	2565	HtH		LCPMPDGH*N*YSC
G3		RvH	$Man_3GlcNAc_2Fuc$	AE*N*ITTTR
	2628	HtH		YL*N*VSTTR
	264	RvH_2-a		PYNL*N*PTT
	2691	HtH		HSL*N*VSTTR

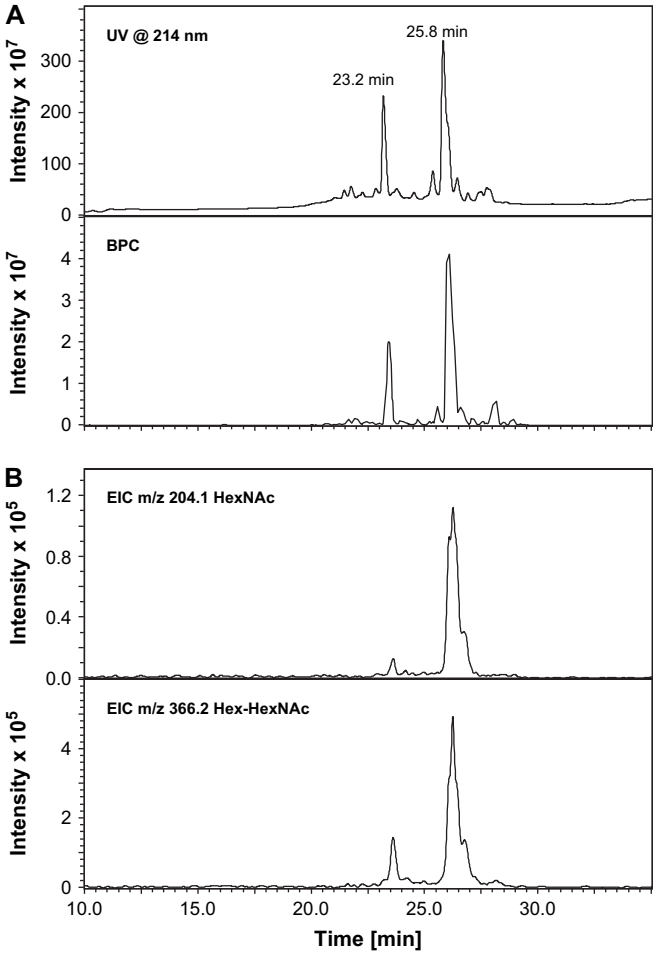

Fig. 3. LC/ESI-MS analysis of the fraction eluted after 36.7 min in the first dimension RP-HPLC separation of a tryptic digest of RvH_1-b (Fig. 2). (A) Positive ion mode LC/ESI-MS/MS experiment. The upper panel shows the chromatogram acquired at 214 nm UV absorption; the lower panel shows the base peak chromatogram (BPC). (B) Carbohydrate-specific positive ion mode LC/ESI-MS experiment with in-source fragmentation. Extracted ion chromatograms (EICs) of the diagnostic $[HexNAc + H]^+$ oxonium ion (*m/z* 204.1, upper panel) and $[Hex\text{-}HexNAc + H]^+$ oxonium ion (*m/z* 366.2, lower panel).

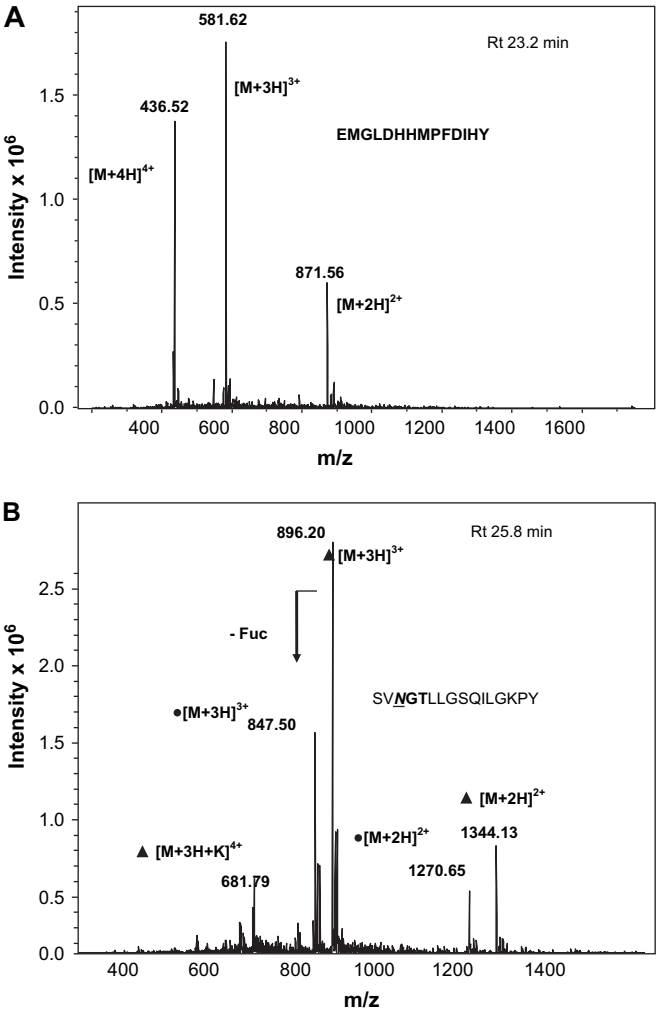

Fig. 4. Averaged positive ion mode electrospray mass spectra of peak 1 (23.2 min) and peak 2 (25.8 min) acquired during LC-MS analysis of the 36.7 min fraction (Fig. 2). (A) Electrospray mass spectrum of the compound eluting at 23.2 min representing a non-glycosylated peptide (1741.83 Da av.). The observed mass corresponds to the amino acid sequence EMGLDHHMPFDIHY (determined by Edman degradation). (B) Electrospray mass spectrum of the compounds eluting at 25.8 min representing two glycopeptides (▲ 2686.56 Da av., ● 2539.49 Da av.). The mass difference of the glycopeptides is 147.1 Da (which accounts for one fucose residue). The amino acid sequence (determined by Edman degradation) of both glycopeptides could be determined as SV*NGT*LLGSQILGKPY (1646.90 Da av.).

1344.13 and $[M + 3H]^{3+}$ at *m/z* 896.20), and 2540.5 Da for the second component ($[M + 2H]^{2+}$ at *m/z* 1270.65 and $[M + 3H]^{3+}$ at *m/z* 847.50) differing from one another by the mass of 146 Da which fits to a deoxyhexose sugar. The extracted mass chromatograms of the $[M + 3H]^{3+}$ ions shows that the components which give rise to these ions do not coelute, suggesting the presence of a pair of related components (glycopeptides) differing in their deoxyhexose content (data not shown). Regarding the facts that (1) two peptide sequences have been observed by Edman degradation (EMGLDHHMPFDIHY and SV*N*GTLLGSQILGKPY), (2) the first peptide (EMGLDHHMPFDIHY) was identified as the first eluting peak (t_R 23.2 min), (3) only the second peak (t_R 25.8 min) was identified as a glycopeptide (formation of carbohydrate-specific marker ions), and (4) the sequence of the second peptide (SV*N*GTLLGSQILGKPY) contains a theoretical site for N-linked glycosylation, we conclude that both components, which were detected in the t_R 25.8 min peak fraction, are related glycopeptides with the identical peptide sequence SV*N*GTLLGSQILGKPY. Based on the molecular mass of this peptide (1646.9 Da) the carbohydrate mass for the first glycopeptide G1 (2686.60 Da) could be calculated as 1039.7 and 893.6 for the second glycopeptide G1′ (2539.49 Da).

The carbohydrate composition of the glycopeptides was analyzed using the fragment ion data (Figs. 5 and 6), acquired during the LC/ESI-MS/MS experiment. The low-energy CID spectrum of the triply-charged precursor ion ($[M + 3H]^{3+}$ at *m/z* 896.2) of the first glycopeptide with a molecular weight of 2686.60 Da (Fig. 4B) is shown in Fig. 5. The major fragment ions observed in the MS^2 spectrum arise from Y_{az}- and Y_{nb}- type cleavages corresponding to the loss of one or

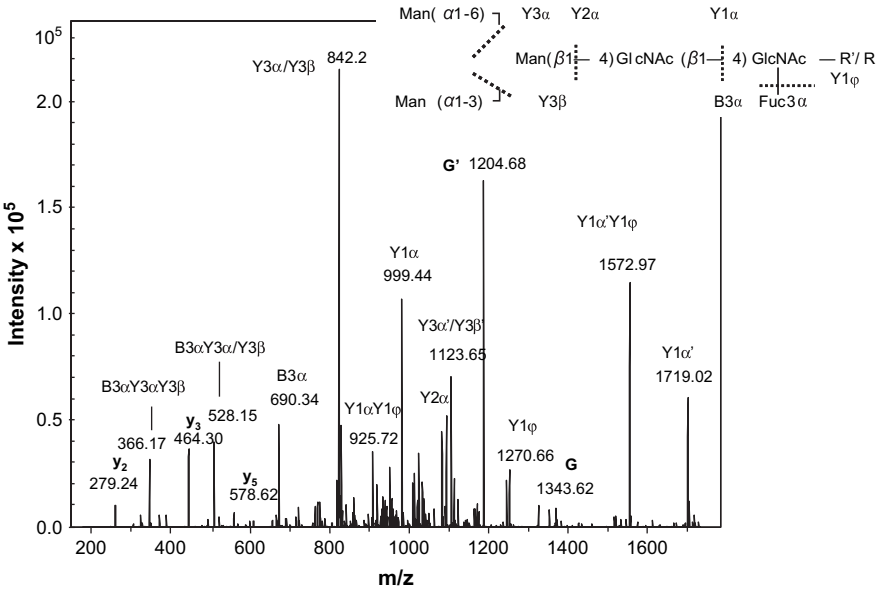

Fig. 5. Positive ion mode electrospray MS/MS spectrum acquired from the triply-charged molecular ion (*m/z* 896.20, $[M + 3H]^{3+}$) of the first eluting glycopeptide G1 (▲ 2686.56 Da, Fig. 3B), with interpretation of the resulting fragment ions (Table 2). G, intact glycopeptide; G′, partially cleaved glycopeptide missing the last two amino acids (PY).

more carbohydrate residues from the glycopeptide as well as B-type ions from the cleavage of the glycosidic bond with the charge retained at the non-reducing terminus (data are summarized in Table 2). From the theoretical carbohydrate content of 1039.7, the composition of the carbohydrate chain was predicted as $HexNAc_2dHexHex_3$.

In the low *m/z* region of the MS^2 spectrum B type oxonium ions could be observed at *m/z* 366.17 ($[HexNAc_2 + H]^+$), *m/z* 528.15 ($[HexNAc_2Hex + H]^+$) and *m/z* 690.34 ($[HexNAc_2Hex_2 + H]^+$) which were accompanied by a series of Y-type ions (cleavage of the peptide backbone at the C-terminus), y_2 at *m/z* 279.24 (PY), y_3 at *m/z* 464.30 (KPY), and y_5 at *m/z* 578.62 (LGKPY), respectively. The high *m/z* region of the MS^2 spectrum was dominated by Y-type fragment ions, which were formed via glycosidic bond cleavages with charge retention at the reducing end. In the tandem mass spectrum two major fragment ion series could be observed (Fig. 5 and Table 2). The first series (R) derived from the intact glycopeptide and the second series (R′) from a partially cleaved glycopeptide missing the last two amino acids (PY) at the C-terminal end. As shown in Fig. 5, the most intense

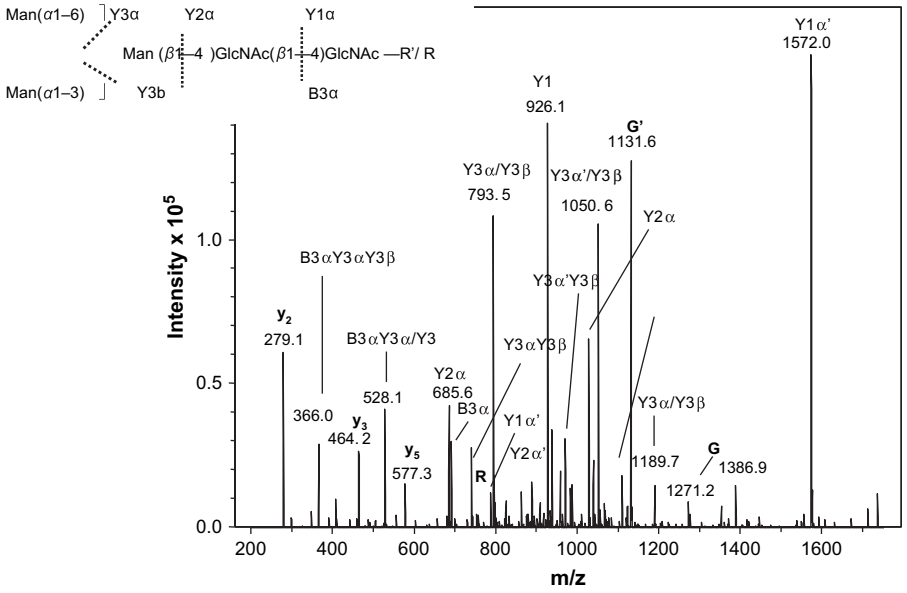

Fig. 6. Positive ion mode electrospray MS/MS spectrum acquired from the triply-charged molecular ion (*m/z* 847.50, $[M + 3H]^{3+}$) of the second eluting glycopeptide G1′ (● 2539.49 Da, Fig. 3B), with interpretation of the resulting fragment ions (Table 3).

fragment ion at *m/z* 842.2 ($[M+3H]^{3+}$) arises from a Y3α- or Y3β-glycosidic cleavage of a hexose residue (162 Da) from the intact triply-charged glycopeptide ion (*m/z* 896.2). Based on the doubly-charged fragment ions of the R series, *m/z* 925.70 (R-HexNAc, Y1α- and Y1φ-glycosidic cleavage), *m/z* 999.14 (R-HexNAcdHex, Y1α cleavage), *m/z* 1101.14 (R-$HexNAc_2$d-Hex, Y2α cleavage), *m/z* 1270.66 (R-$HexNAc_2Hex_3$, Y1φ cleavage), and *m/z* 1343.62 (R-$HexNAc_2dHexHex_3$, no cleavage), the carbohydrate composition could be further elucidated. The R′ series started with the intact $[M+2H]^{2+}$ glycopeptide ion (PY peptide backbone cleavage) at *m/z* 1204.18 (R′-$HexNAc_2dHex$-Hex_3), followed by a sequential loss of hexose residues resulting in the formation of the $[M+2H]^{2+}$ fragment ions at *m/z* 1123.65 (R′-$HexNAc_2Hex_2dHex$, Y3α′ or Y3β′ cleavage) and *m/z* 1042.63 (R′-$HexNAc_2$HexdHex, Y3α′ and Y3β′ cleavage), respectively. The singly-charged signals at *m/z* 1572.97 and *m/z* 1719.02 could be interpreted as the R′-HexNAc and R′-HexNAcd-Hex fragment ions arising from Y1α′ and Y1α′Y1φ′ glycosidic cleavages (Table 2, Fig. 5).

In summary the fragment ion spectra confirmed the carbohydrate structure ($HexNAc_2dHexHex_3$) predicted for the first glycopeptide G1, isolated from FU RvH_1-b.

The second glycopeptide G1′ (2539.49 Da, Fig. 4B) was analyzed identically by the fragmentation of the $[M+3H]^{3+}$ glycopeptide ion at *m/z* 847.5 (Fig. 6 and Table 3). Based on the observed fragment ions, the carbohydrate structure of this glycopeptide could be established as $HexNAc_2Hex_3$ which is indeed identical to the first glycopeptide, with the exception of the missing deoxyhexose residue which is linked to the first HexNAc residue.

With the monosaccharide components identified as mannose (Man), *N*-acetyl-D-glucosamine (GlcNAc) and fucose (Fuc) and based on structural data of other snail Hcs [5,18], the composition of the 2686.56 Da glycopeptide is proposed as Man(α1−6)Man(α1−3)Man(β1−4)GlcNAc(β1−4)[Fuc(α1−6)] GlcNAc (Table 2), and Man(α1−6)Man (α1−3)Man(β1−4) GlcNAc(β1−4)GlcNAc for the 2539.49 Da glycopeptide (Table 3), which is, with exception of the fucose residue, a common type pentasaccharide core structure for N-linked carbohydrates.

3.4. Isolation of glycopeptides G2 and G3 from structural subunit RvH_1

Several fractions of the tryptic digest of the whole RvH_1 were also identified as glycopeptides on silica gel plates and two of them, G2 and G3, were analyzed by nanoES mass spectrometry and compared to glycopeptides G1 and G1′, isolated from FU RvH_1-b.

3.5. MS/MS analysis using nanoES-MS of glycopeptide G2, isolated from structural subunit RvH_1

The oligosaccharide sequences of the glycopeptides were determined by tandem MS. The oligosaccharide structure of the glycopeptide G2 of 2197.0 Da was analyzed by MS/MS of the doubly-charged ion $[M+2H]^{2+}$ observed at *m/z* 1099.5 and the obtained composition of selected MS/MS fragment ions is shown in Table 4 and Fig. 7A. In the MS/MS spectra, two series of both, singly- and doubly-charged fragment ions are observed, in which either the N- or C-terminus is retained. A series of singly-charged ions starts from the C-terminus with the typical signals for sequencing of sugars at *m/z* 204.10 (HexNAc) and 366.16 (HexHexNAc), continued by *m/z* values of 528.24 (HexHexNAc), 690.28 (Man_3GlcNAc), and extending to the doubly-charged ion at *m/z* 1099.54 (2198.08 Da) which represents the complete structure of carbohydrate chain plus peptide. The oligosaccharide structure was determined to be a typical core for N-linked structures.

Further structural details about this fraction could be derived from pseudo-MS/MS/MS scans from in-source and collision-induced fragmentation experiments given in Fig. 7B.

Table 2
Interpretation of the MS/MS spectrum (Fig. 5) acquired from the triply - charged molecular ion (*m/z* 896.20, $[M+3H]^{3+}$) of the first eluting glycopeptide G1, isolated from Fu RvH_1-b (▲ 2686.56 Da, Fig. 3B). G, intact glycopeptide; G′, partially cleaved glycopeptide missing the last two amino acids (PY)

N	*m/z*	Molecular ion	Molecular mass (Da)	Cleavage	Structure
1	279.24	$[M+1H]^{1+}$	278.24	Y_2	PY
2	366.17	$[M+1H]^{1+}$	365.17	B3αY3αY3β	(HexNAc)Hex
3	464.30	$[M+1H]^{1+}$	463.30	Y_3	KPY
4	528.15	$[M+1H]^{1+}$	527.15	B3αY3α/Y3β	$(HexNAc)Hex_2$
5	578.62	$[M+1H]^{1+}$	577.62	Y_5	LGKPY
6	690.34	$[M+1H]^{1+}$	689.34	B3α	$(HexNAc)Hex_3$
7	925.70	$[M+2H]^{2+}$	1849.40	Y1αY1φ	R-(HexNAc)
8	999.44	$[M+2H]^{2+}$	1996.88	Y1α	R-(HexNAc)Fuc
9	1101.14	$[M+2H]^{2+}$	2200.28	Y2α	$R-(HexNAc)_2Fuc$
10	842.24	$[M+3H]^{3+}$	2523.72	Y3α/Y3β	$R-(HexNAc)_2Hex_2Fuc$
11	1270.66	$[M+2H]^{2+}$	2539.32	Y1φ	$R-(HexNAc)_2Hex_3$
12	1343.62	$[M+2H]^{2+}$	2685.24	G	$R-(HexNAc)_2Hex_3Fuc$
13	1204.18	$[M+2H]^{2+}$	2406.36	G′	$R′-(HexNAc)_2Hex_3Fuc$
14	1123.65	$[M+2H]^{2+}$	2245.30	Y3α′/Y3β′	$R′-(HexNAc)_2Hex_2Fuc$
15	1042.63	$[M+2H]^{2+}$	2083.26	Y3α′Y3β′	$R′-(HexNAc)_2HexFuc$
16	1719.02	$[M+1H]^{1+}$	1718.02	Y1α′	R′-(HexNAc)Fuc
17	1572.97	$[M+1H]^{1+}$	1571.97	Y1α′Y1φ′	R′-(HexNAc)

R, SV*N*GTLLGSQILGKPY; R′, SV*N*GTLLGSQILGK.

Table 3
Interpretation of the MS/MS spectrum (Fig. 6) acquired from the triply-charged molecular ion (*m/z* 847.50, $[M + 3H]^{3+}$) of the second eluting glycopeptide G1′, isolated from Fu RvH_1-b (● 2539.49 Da, Fig. 3B)

N	*m/z*	Molecular ion	Molecular mass (Da)	Cleavage	Structure
1	279.19	$[M + 1H]^{1+}$	278.19	Y_2	PY
2	366.17	$[M + 1H]^{1+}$	365.17	B3αY3αY3β	(HexNAc)Hex
3	464.23	$[M + 1H]^{1+}$	463.23	Y_3	KPY
4	528.15	$[M + 1H]^{1+}$	527.15	B3αY3α/Y3β	$(HexNAc)Hex_2$
5	577.30	$[M + 1H]^{1+}$	576.30	Y_5	LGKPY
6	690.34	$[M + 1H]^{1+}$	689.34	B3α	$(HexNAc)Hex_3$
7	685.66	$[M + 3H]^{3+}$	2053.98	Y2α	$R\text{-}(HexNAc)_2$
8	739.43	$[M + 3H]^{3+}$	2215.29	Y3αY3β	$R\text{-}(HexNAc)_2Hex$
9	793.53	$[M + 3H]^{3+}$	2377.59	Y3α/Y3β	$R\text{-}(HexNAc)_2Hex_2$
10	787.10	$[M + 2H]^{2+}$	1572.20	Y1α′	R′-(HexNAc)
11	888.40	$[M + 2H]^{2+}$	1774.80	Y2α′	$R'\text{-}(HexNAc)_2$
12	969.00	$[M + 2H]^{2+}$	1936.00	Y3α′Y3β′	$R'\text{-}(HexNAc)_2Hex$
13	1050.64	$[M + 2H]^{2+}$	2099.28	Y3α′/Y3β′	$R'\text{-}(HexNAc)_2Hex_2$
14	1572.04	$[M + 1H]^{1+}$	1571.04	Y1α′	R′-(HexNAc)
15	1131.62	$[M + 2H]^{2+}$	2261.24	G′	$R'\text{-}(HexNAc)_2Hex_3$
16	824.30	$[M + 2H]^{2+}$	1646.60		R
17	926.12	$[M + 2H]^{2+}$	1850.24	Y1α	R-(HexNAc)
18	1027.60	$[M + 2H]^{2+}$	2053.20	Y2α	$R\text{-}(HexNAc)_2$
19	1108.60	$[M + 2H]^{2+}$	2215.20	Y3αY3β	$R\text{-}(HexNAc)_2Hex$
20	1189.70	$[M + 2H]^{2+}$	2377.40	Y3α/Y3β	$R\text{-}(HexNAc)_2Hex_2$
21	1271.23	$[M + 2H]^{2+}$	2541.40	G	$R\text{-}(HexNAc)_2Hex_3$

R, SV*N*GTLLGSQILGKPY; R′, SV*N*GTLLGSQILGK.

The amino acid sequence of the peptide chain was analyzed by the same method, but using a cone voltage of 95 V and a collision energy of 80 V. Based on the singly-charged ions one peptide ion carrying a GlcNAc at the N-linkage site was determined to be FSWVDGH*N*T*S*R (1508.83 Da) (Table 1). Identity of the N-terminus as well as the assignment of N- or C-terminal fragments were confirmed by modification of the glycopeptides with nicotinic acid [29] followed by mass spectrometry (data not shown). The sequence is homologous to putative glycosylation sites in other molluscan Hcs given in Table 1. The difference between two single ions at *m/z* 1305.81 and *m/z* 1508.83 is 203, corresponding to one HexNAc connected to the peptide, which also demonstrates that this peptide of 1305.81 Da is glycosylated.

3.6. MS/MS analysis of glycopeptide G3 isolated from RvH_1 using nanoflow ESI-MS

Fig. 8A shows a third glycopeptide isolated from RvH_1 with the same carbohydrate chain as glycopeptide G1. It is represented by a doubly-charged ion at *m/z* 972.5 (1944.0 Da). The oligosaccharide structure was determined from the MS/MS data shown in Fig. 8A. Based on the previous structures and following the differences between singly- and doubly-charged ions the structure was determined to be Man(α1−6)Man(α1−3)Man(β1−4)GlcNAc(β1−4)[Fuc(α1−6)] GlcNAc-R, linked to an Asn residue of a peptide with a molecular mass of 905.5 Da. Based on the singly-charged ions in the pseudo-MS/MS/MS scans (Fig. 8B) one peptide ion carrying the N-linkage site was determined to be AE*N*ITTTR

Table 4
MS/MS analysis of glycopeptide G2, isolated from structural subunit RvH and detected at *m/z* 1099.54 with a molecular mass of 2197.08 Da (Fig. 7A)

N	*m/z*	Molecular ion	Molecular mass (Da)	Cleavage	Structure
1	163.07	$[M + 1H]^{+1}$	162.07	B1α/B1β	Hex
2	204.10	$[M + 1H]^{+1}$	203.10	B3αY2α	(HexNAc)
3	366.16	$[M + 1H]^{+1}$	365.16	B3αY3αY3β	(HexNAc)Hex
4	528.24	$[M + 1H]^{+1}$	527.24	B3αY3α/Y3β	$(HexNAc)Hex_2$
5	653.30	$[M + 2H]^{+2}$	1304.60		R-
6	690.28	$[M + 1H]^{+1}$	689.28	B3α	$(HexNAc)Hex_3$
7	754.86	$[M + 2H]^{+2}$	1507.72	Y1α	R-(HexNAc)
8	856.43	$[M + 2H]^{+2}$	1710.86	Y2α	$R\text{-}(HexNAc)_2$
9	937.46	$[M + 2H]^{+2}$	1872.92	Y3αY3β	$R\text{-}(HexNAc)_2Hex$
10	1018.51	$[M + 2H]^{+2}$	2035.02	Y3α/Y3b	$R\text{-}(HexNAc)_2Hex_2$
11	1099.54	$[M + 2H]^{+2}$	2197.08		$R\text{-}(HexNAc)_2Hex_3$
12	1305.61	$[M + 1H]^{+1}$	1304.61		R-
13	1508.68	$[M + 1H]^{+1}$	1507.68	Y1α	R-(HexNAc)
14	1711.46	$[M + 1H]^{+1}$	1710.46	Y2α	$R\text{-}(HexNAc)_2$

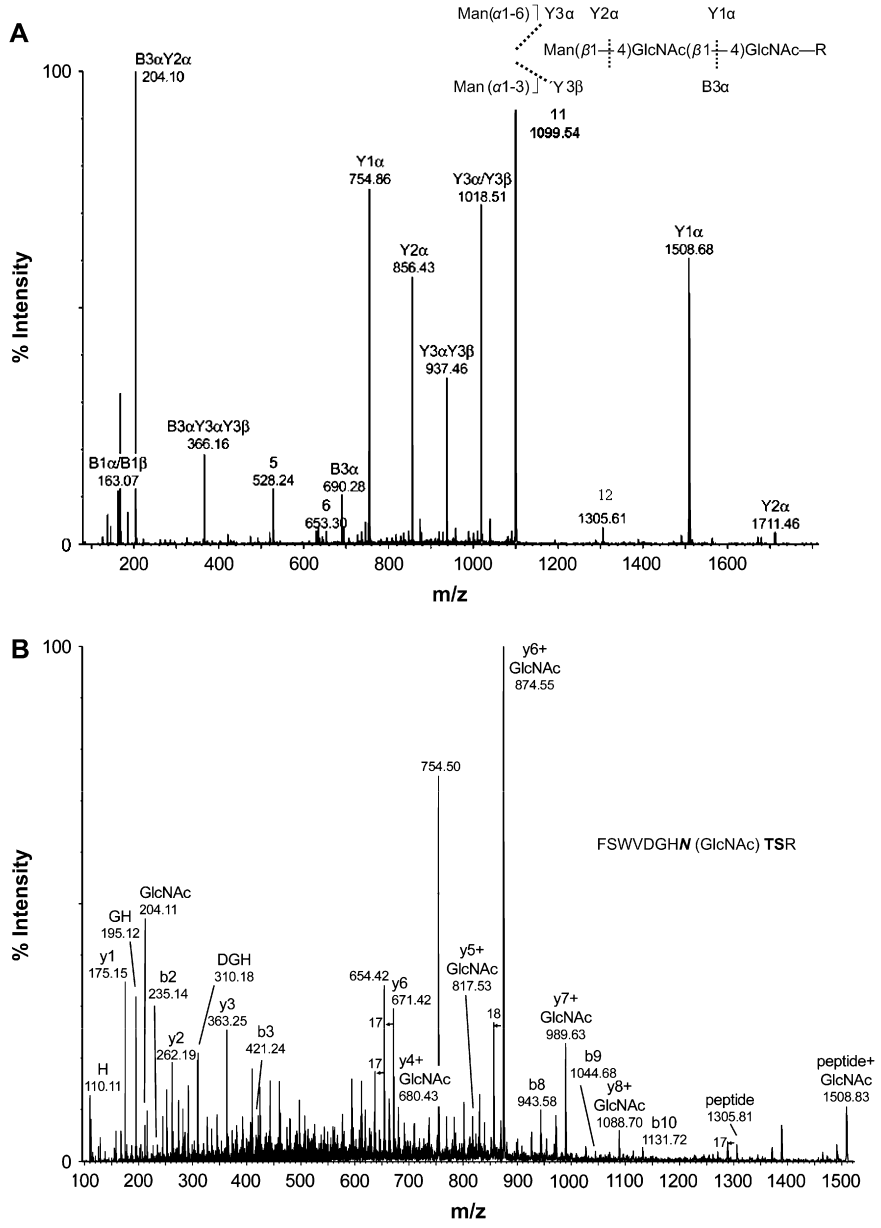

Fig. 7. Fragment spectra of the glycopeptide G2 with determined *m/z* 1099.54 $[M + 2H]^{2+}$. (A) MS/MS analysis of the sugar moiety, using a collision energy of 37 eV. Annotation of sugar fragments, see Table 4. The insert shows the complete carbohydrate structure and the breakpoints leading to the respective fragments. (B) Pseudo-MS/MS/MS experiment (in-source and collision-induced fragmentation) of the peptide moiety still carrying one HexNAc (*m/z* 1508.68), using a cone voltage of 95 V and a collision energy of 80 eV.

(905.51 Da) and *m/z* 1108.67 is the same peptide with one GlcNAc connected to linkage site −NIT− (Table 1). Identity of the N-terminus as well as the assignment of N- or C-terminal fragments were also confirmed by modification of the glycopeptides with nicotinic acid [29] followed by mass spectrometry (data not shown). Also this sequence is homologous to putative glycosylation sites in other molluscan Hcs (Table 1).

4. Discussion

The carbohydrate structure of FU RvH_1-b in comparison with other two glycopeptides, isolated from structural subunit RvH_1, is presented in this study. Following the schema shown in Fig. 1 and using different methods and techniques such as Edman degradation, ESI-MS/MS or nanoES-MS identification of the glycan structure and peptide structure was accomplished.

Based on the sequencing of two glycosylated fractions G1 and G1′ (isolated from FU RvH_1-b) and two glycosylated fractions G2 and G3 (isolated from structural subunit RvH_1), a common trimannosyl-*N*,*N*-diacetylchitobiose core Man(α1−6)[Man(α1−3)]Man(β1−4)GlcNAc(β1−4)GlcNAc-ol was observed in all isolated glycans of RvH_1 but different antennae are attached to the α-Man residues. The carbohydrate structure Man(α1−6)Man(α1−3)Man(β1−4)GlcNAc(β1−4)[Fuc(α1−6)]GlcNAc-R, was identified in fractions G1 and G3 while the

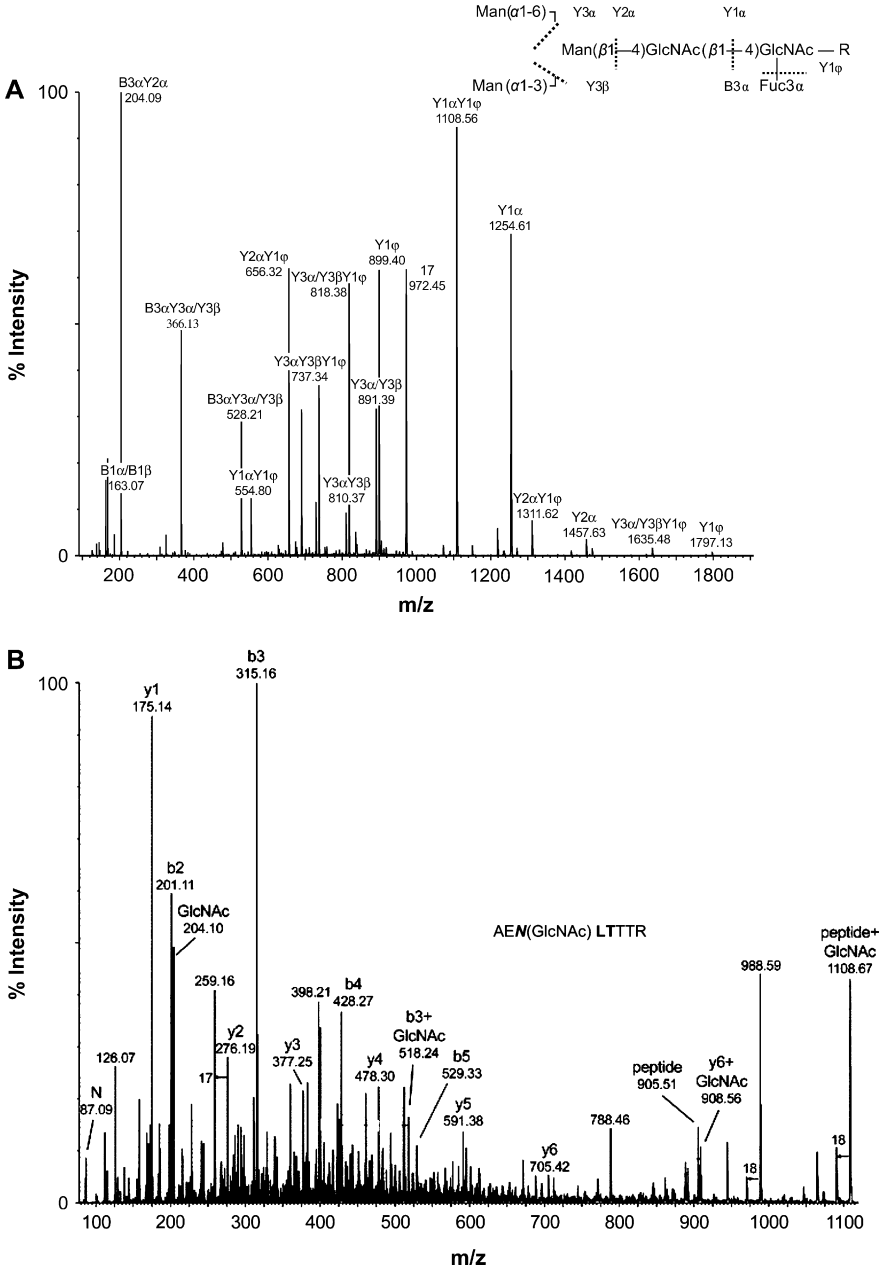

Fig. 8. Fragment spectra of the glycopeptide G3 with determined *m/z* 972.45 $[M + 2H]^{2+}$. (A) MS/MS analysis of the sugar moiety, using a collision energy of 28 eV. Annotation of sugar fragments, see Table 5. The insert shows the complete carbohydrate structure and the breakpoints leading to the respective fragments. (B) Pseudo-MS/MS/MS experiment (in-source and collision-induced fragmentation) of the peptide moiety still carrying one HexNAc (*m/z* 1108.56), using a cone voltage of 75 V and a collision energy of 62 eV.

typical core oligosaccharide Man(α1−6)Man(α1−3)Man(β1−4)GlcNAc(β1−4)GlcNAc-R was identified in fraction G1′ and G2. The Fuc residue found in glycopeptide G1 and G3 is attached to the *N*-acetyl-glucosamine of the core, as found generally in glycoproteins.

Amino acid sequences of glycopeptide G1 isolated from FU RvH_1-b were determined by Edman degradation and via MS/MS sequencing, demonstrating that the oligosaccharides are linked to asparagines residues. Two fractions were sequenced in parallel: a glycosylated one with a linkage site (EMGLDHHMPFDIHY) and a second one with His residues in the sequence (SV*N*GTLLGSQILGKPY). Apart from one His residue no classical carbohydrate linkage sites was observed in the non-glycosylated peptide which confirms our suggestion that the distant site of oligosaccharide might be connected non-covalently to the second peptide. The amino acid sequences of the peptide chains of G2 (FSWVDGH*N*T*S*R) and G3 (AE*N*ITTTR) were analyzed by nanoES-MS, but using a cone voltage of 95 V and a collision energy of 80 V. N-linkage sites of the glycopeptides were determined.

In comparison to the isolated glycans from FU RvH_1-a and 21 monoantennary and diantennary *N*-glycans of *H. pomatia*

Table 5
MS/MS analysis of glycopeptide G3, isolated from structural subunit RvH and detected at *m/z* 972.45 with a molecular mass of 1942.90 Da (Fig. 8A)

N	*m/z*	Molecular ion	Molecular mass (Da)	Cleavage	Structure
1	163.07	$[M+1H]^{+1}$	162.07	B1α/B1β	Hex
2	204.09	$[M+1H]^{+1}$	203.09	B3αY2α	(HexNAc)
3	325.11	$[M+1H]^{+1}$	324.11	B2αY3α/Y3β	Hex_2
4	366.13	$[M+1H]^{+1}$	365.13	B3αY3αY3β	(HexNAc)Hex
5	453.25	$[M+2H]^{+2}$	904.50		R-
6	528.21	$[M+1H]^{+1}$	527.21	B3αY3α/Y3β	(HexNAc)Hex_2
7	554.80	$[M+2H]^{+2}$	1107.60	Y1αY1φ	R-(HexNAc)
8	627.82	$[M+2H]^{+2}$	1253.64	Y1α	R-(HexNAc)Fuc
9	656.32	$[M+2H]^{+2}$	1310.64	Y2αY1φ	R-$(HexNAc)_2$
10	690.25	$[M+1H]^{+1}$	689.25	B3α	(HexNAc)Hex_3
11	729.36	$[M+2H]^{+2}$	1456.72	Y2α	R-$(HexNAc)_2$Fuc
12	737.34	$[M+2H]^{+2}$	1472.68	Y3αY3βY1φ	R-$(HexNAc)_2$Hex
13	810.37	$[M+2H]^{+2}$	1618.74	Y3αY3β	R-$(HexNAc)_2$HexFuc
14	818.38	$[M+2H]^{+2}$	1634.76	Y3α/Y3βY1φ	R-$(HexNAc)_2Hex_2$
15	891.39	$[M+2H]^{+2}$	1780.78	Y3α/Y3β	R-$(HexNAc)_2Hex_2$Fuc
16	899.40	$[M+2H]^{+2}$	1796.80	Y1φ	R-$(HexNAc)_2Hex_3$
17	972.45	$[M+2H]^{+2}$	1942.90		R-$(HexNAc)_2Hex_3$Fuc
18	1108.56	$[M+1H]^{+1}$	1107.56	Y1αY1φ	R-(HexNAc)
19	1254.61	$[M+1H]^{+1}$	1253.61	Y1α	R-(HexNAc)Fuc
20	1311.62	$[M+1H]^{+1}$	1310.62	Y2αY1φ	R-$(HexNAc)_2$
21	1457.63	$[M+1H]^{+1}$	1456.63	Y2α	R-$(HexNAc)_2$Fuc
22	1635.48	$[M+1H]^{+1}$	1634.48	Y3α/Y3βY1φ	R-$(HexNAc)_2Hex_2$
23	1797.13	$[M+1H]^{+1}$	1796.13	Y1φ	R-$(HexNAc)_2Hex_3$

Hc the identified carbohydrate chains of RvH_1-b were found to be shorter [5]. The primary structures of two biantennary *N*-glycans of the N-terminal functional unit RvH_1-a were also determined, applying several techniques, including capillary electrophoresis, matrix-assisted laser desorption ionization-MS and electrospray ionization-MS in combination with glycosidase digestion [7]. Two oligosaccharide chains with molecular mass 1609 and 1653, containing $(SO_4)MeGalGlcNAc_4Man_3$ and $MeGal_2GlcNAc_4Man_3$ were found to be connected to the peptides Glp1 and Glp2, respectively.

Acknowledgements

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