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Analysis of a glycopeptide from structural subunit (βc-HlH) of *Helix lucorum* hemocyanin by mass spectrometry

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Introduction

Hemocyanins (Hcs) are copper-containing respiratory glycoproteins with quaternary structure localized in the hemolymph of of several arthropods and molluscs. Molluscan hemocyanins are large glycoproteins, usually have a higher carbohydrate content (1-9 %, w/w) with different structures and quantities of the oligosaccharide moieties [1, 2]. Molluscan Hcs usually have are powerful immunogens, probably due to their high carbohydrate content and specific monosaccharide composition [3, 4]. One of the most investigated and widely used in experimental immunology and clinical practice as adjuvant and molecular bearer is Keyhole limpet hemocyanin (KLH) isolated from hemolymph of the marine snail *Megatura crenulata*.

We have now analyzed the oligosaccharides and the carbohydrate linkage sites of the structural subunit βc-HlH isolated form land snail *Helix lucorum* using tandem mass spectrometry. Mass spectrometry is the method of choice for the sensitive and comprehensive analysis of proteins and glycoproteins. The comprehensive analysis of protein glycosylation is a major requirement for understanding glycoprotein function in biological systems, and is a prerequisite for producing recombinant glycoprotein therapeutics.

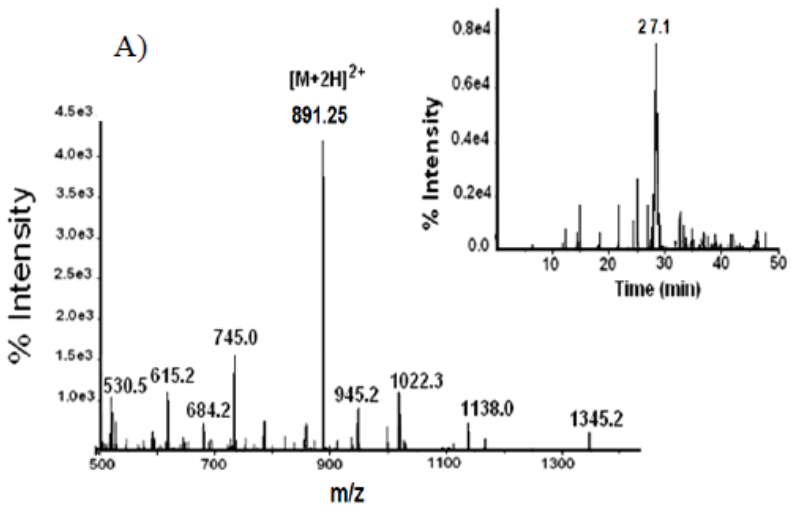
Results and Discussion

In total 10 potential sites for N-glycosylation (Asn-Xaa-Ser/Thr), were identified in the gene sequence of the hemocyanin isoform βc-HlH, but only some of them appear to be glycosylated [5]. However, the oligosaccharide structures of the βc-HlH hemocyanin isoform from *Helix lucorum* are not known yet. The aim of the present study is therefore to identify and characterize the carbohydrate structures of the hemocyanin from this garden snail gastropod based on mass spectrometric analysis.

Glycopeptides were isolated after overnight digestion of the structural subunit βc-HlH with trypsin digestion, and the resulting peptides were subjected to reversed phase separation using a Nucleosil 7 C18 column. Fractions testing positive in the orcinol test were lyophilized and studied by mass spectrometry, using Q-Trap-LC/MS/MS.

A glycopeptide, selectively detected in a proteolytic mixture by the appearance of collisionally induced marker oxonium ions such as *m/z* 163 $[Hex+H]^+$, 204 $[HexNAc+H]^+$, or 366 $[HexHexNAc+H]^+$, was sequenced. The insert of Fig.1A 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 enhanced resolution scan (not shown) showed that the glycopeptide with mass 1781.5 eluting at this moment was doubly charged at *m/z* 891.25 $[M+2H]^{2+}$.

The MS/MS spectrum (Fig. 1B) is dominated by glycan fragmentation series of Y- and B-ions, corresponding to the Domon/Costello nomenclature. However, peptide fragmentation (Roepstorff/Biemann cleavages) became more dominant when the collision energy was increased, allowing one to deduce the peptide sequence DH**NTT**R from the series of y- and b-ions (Fig. 1B). The ion b6 (m/z 725.5) or y6 (m/z 743.4) correspond to the peptide which contains one potential glycosylation site (-NTT-).



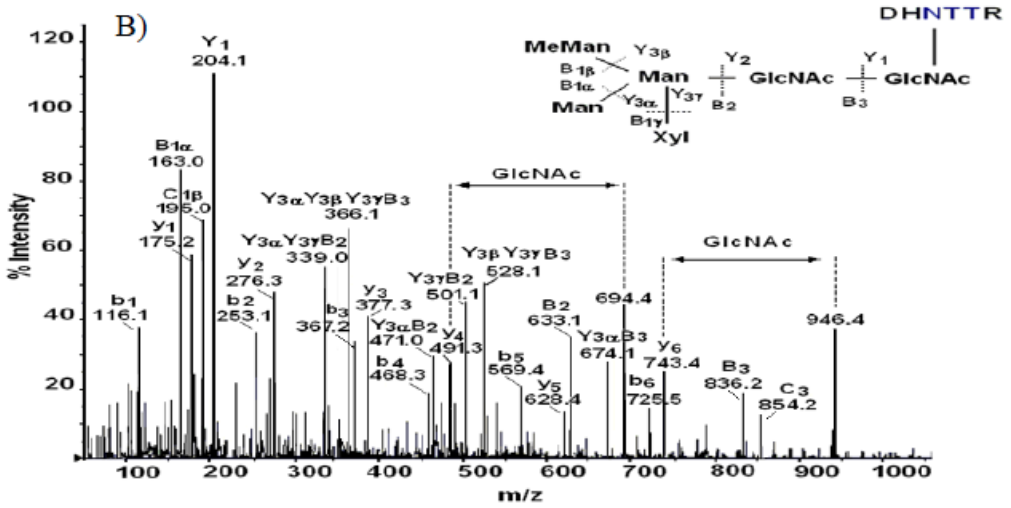


Fig. 1. Precursor scan (A) of the ion at time 27.1 min of the chromatographic separation (insert) and enhanced product ion (EPI) scan of the ion (B) at m/z 891.25 $[M+2H]^{2+}$.

The ion y4 at *m/z* 491.3 correspond to the C-terminal fragment of the peptide **-NTTR**, and the ion at m/z 694.4 represents the same fragment still containing one GlcNAc moiety. This suggests that the linkage site -NTT- is glycosylated. The ion at *m/z* 946.4 corresponds to the intact peptide, represented as ion y6 (*m/z* 743.4), which is N-glycosylated with a single GlcNAc residue. The peptide sequence DH**NTT**R was determined from mass spectrometric fragmenting and fully consistent with the fragment of gene sequence of βc-HlH-g, at Asn 125 [5]. The glycan structure of this peptide could be revealed by this MS/MS spectrum, which displayed the typical ions: B1α at *m/z* 163.0 (Man), C1β at *m/z* 195.0 (MeMan), Y1 *m/z* 204.1 (GlcNAc), Y3αY3βY3γB3 *m/z* 366.2 (Man1GlcNAc1), *m/z* 528.3 (Man2GlcNAc1). The MS/MS-spectrum (Fig.1B) clearly demonstrated the presence of core-linked xylose as well as terminal methyl-hexose linked at the central mannose residue of the trimannosyl core: Y3αY3γB2 at m/z 339.0 (MeMan1Man1), Y3αB2 at m/z 471.0 (D ion: MeMan1Man1Xyl1), Y3γB2 at m/z 501.1 (MeMan1Man2), B2 at *m/z* 633.1 (MeMan1Man2Xyl1), Y3αB3 at m/z 674.1 (MeMan1Man2Xyl1GlcNAc1), B3 as and C3 at m/z 836.2 and 854.2, respectively (Xyl1MeMan1Man2GlcNAc). Combining all data, the carbohydrate structure of 1038.0 Da (Xyl1MeMan1Man2GlcNAc2) could be suggested. The occurrence of xylose residue in βc-HlH from *H. lucorum* is considered to be highly immunogenic in mammalian species [3]. The oligosaccharide constituents of Hcs are of prime significance for its antigenicity and biomedical properties, knowledge on the carbohydrate structures of this glycoprotein are still incomplete. Therefore, they are extremely interesting for the further investigation of the structural and functional role of protein glycosylation. 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 [1-4].

Acknowledgments

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