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# STRUCTURE OF A COMPLEX ASPARAGINE BOUND GLYCOPEPTIDE FROM HORSE PANCREATIC RIBONUCLEASE CONTAINING (2→3) AND (2→6) LINKED SIALIC ACID RESIDUES

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Summary: The primary structure of the main glycopeptide obtained by pronase digestion of horse pancreatic ribonuclease has been investigated by 360 MHz $^1$H-NMR spectroscopy and methylation analysis. The results demonstrate that this glycopeptide has the following structure:

NeuNAcα(2→6)Galβ(1→4)GlcNAcβ(1→2)Manα(1→6)\
Manβ(1→4)GlcNAcβ(1→4)GlcNAcβ1→Asn\
NeuNAcα(2→3)Galβ(1→4)GlcNAcβ(1→2)Manα(1→3)/ ↑ α(1→6)\
Fuc

This is the first time that the presence of both (2→3) and (2→6) linked sialic acid residues in a glycopeptide has been demonstrated.

Introduction: The carbohydrate content of horse and pig pancreatic ribonuclease is high (20-30%) (1,2) in comparison to pancreatic ribonucleases from other animals (3).

It has been proposed (3) that in these highly glycosidated ribonucleases the carbohydrate moieties prevent uptake of the enzyme from the gut, thereby enabling it to reach the large intestine for digestion of the RNA of the extensive microflora.

The amino acid sequence of horse pancreatic ribonuclease has been reported (1). Glycan chains are attached to asparagine at positions 21, 34 and 62; position 21 being only partially glycosidated (1).

To investigate the primary structure of these carbohydrate chains the purified enzyme was extensively digested with pronase and fractionated on Dowex 50W-X2, yielding one main glycopeptide upon elution with water (Eq.GPI)

This paper deals with the structure determination of Eq.GPI by 360 MHz $^1$H-NMR spectroscopy and methylation analysis.

Materials and Methods: Fresh horse pancreatic tissue was obtained from a local slaughter house, cleaned from fat and connective tissue and then stored at -18°C for one night. Ribonuclease was extracted from the homogenized tissue with 0.2 M sodiumacetate pH 5.2 and purified by fractionated ethanol precipitation (25-66% ppt.), chromatography on DEAE-Sephadex (3) and affinity chromatography on Agarose-APUP (Miles) (4). The purified

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enzyme was heterogeneous as could be shown by polyacrylamide gel electrophoresis (5). All protein bands have ribonuclease activity as could be demonstrated by specific staining in duplicate gels (6).

After extensive digestion of the protein with pronase (7) the glycopeptides were purified by gel filtration on Biogel P30 and Biogel P2 and then fractionated on Dowex 50W-X2 (200-400 mesh) (7). Elution with water yielded one main glycopeptide Eq.GPI.

Qualitative and quantitative sugar analyses were carried out after methanolysis (MeOH/HCl 0.5 M, 24 hr., 80°C) by gas-liquid chromatography of the trifluoroacetylated derivatives (8). Permethylation was performed according to Hakomori (9). Partially methylated monosaccharides were identified as described by Fournet et al. (10).

The amino acid composition of the glycopeptide was determined after acid hydrolysis (5.6 N HCl, 24 hr., 100°C under vacuum) on a TSM amino acid analyser

360 MHz $^1$H-NMR spectra of a 25 mM neutral solution were recorded on a Bruker HX-360 spectrometer, operating in the Fourier Transform mode at a probe temperature of 25°C. Chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone in $D_2O$ : $\delta$ = 2.225 ppm).

Results and Discussion: Determination of the sugar composition by gas-liquid chromatography yielded fucose, mannose, galactose, N-acetylglucosamine and N-acetylneuraminic acid in a ratio 0.8/3.0/2.0/3.8/1.8. Amino acid analysis showed the presence of asparagine only.

The partially methylated monosaccharides obtained by methanolysis of the permethylated glycopeptide are given in table 1.

The 360 $^1$H-NMR spectrum of glycopeptide Eq.GPI is given in fig. 1. The chemical shifts of the characteristic well resolved resonances are summarized in table 2.

The assignment of the proton signals was made by using as reference data the 360 $^1$H-NMR spectra of the glycopeptides Man$\alpha$(1→6)Man$\beta$(1→4)GlcNAc$\beta$(1→4)[Fuc$\alpha$(1→6)]GlcNAc$\beta$1→Asn (11), Fuc$\alpha$(1→6)GlcNAc$\beta$1→Asn (12) and the asialo glycopeptides obtained from human serotransferrin (11).

Spectral integration showed that ten anomeric protons were present. The resonance positions of the H-2 protons of Man 3, Man 4 and Man 4' (4.252, 4.196 and 4.113 respectively) are indicative for the presence of a mannotriosido branching core, surrounded by the N-acetyl-glucosamine residues 2, 5 and 5' (c.f. fig. 2) (11,13).

The anomeric signals at 5.067 ppm ($J_{1,2} \sim 7$ Hz), 4.874 ppm ($J_{1,2} \sim 3$ Hz) and 4.683 ppm ($J_{1,2} \sim 8$ Hz) in combination with the N-acetyl signals at 2.094 and 2.014 ppm demonstrate the presence of an asparagine linked fucosyl-di-N-acetyl chitobiose unit, with fucose $\alpha$(1→6) linked to GlcNAc 1 (11,12).

The type of linkage of the N-acetylneuraminic acid residues to galactose can be derived from the chemical shifts of the H-3 protons: the two sets of H-3 ax. and H-3 eq. protons in a 1 : 1 ratio are characteristic for the occurrence of $\alpha$(2→3) besides $\alpha$(2→6) linked N-acetylneuraminic acid residues

Table 1. Partially methylated monosaccharides obtained by methanolysis of permethylated Eq.GPI.

| | |
|---|---|
| methyl-2,3,4-tri-O-methyl-fucoside | 0.7 |
| methyl- 2,4-di -O-methyl-mannoside | 0.7 |
| methyl-3,4,6-tri-O-methyl-mannoside | 2.0 |
| methyl-2,4,6-tri-O-methyl-galactoside | 1.1 |
| methyl-2,3,4-tri-O-methyl-galactoside | 1.0 |
| 3,6-di-O-methyl-N-methyl-glucosamine | 3.0 |
| 3-mono-O-methyl-N-methyl-glucosamine | 1.0 |

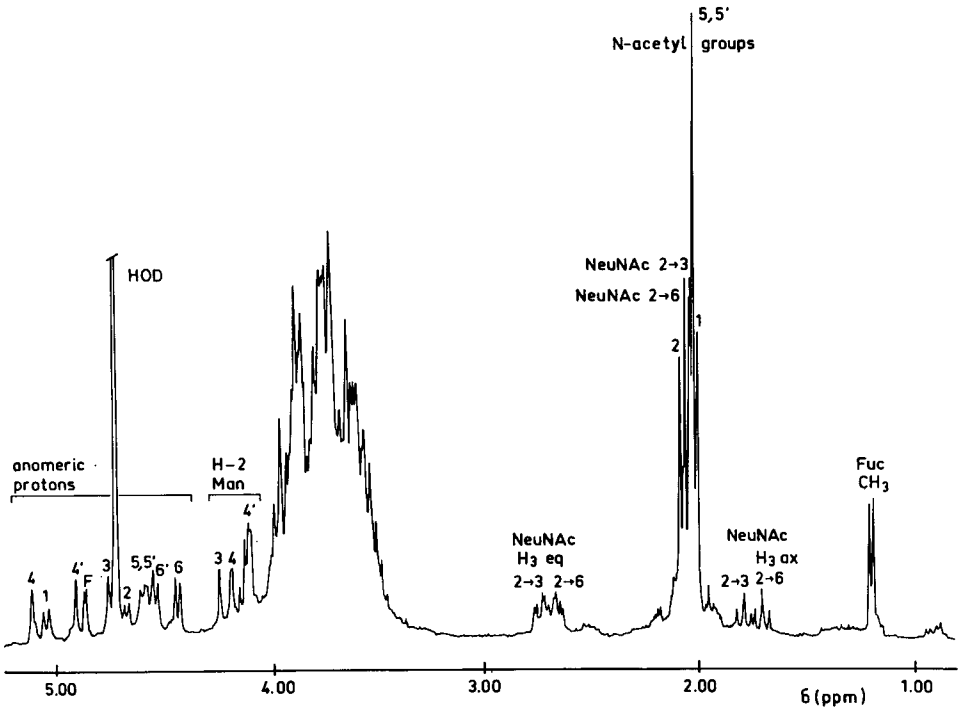


Fig. 1. 360 MHz $^1$H-NMR spectrum of glycopeptide Eq.GPI isolated from horse pancreatic ribonuclease.

(13). From the shift increment of the anomeric proton of mannose 4 with respect to the corresponding resonance position of the asialo glycopeptide from human serotransferrin (11) (5.119→5.135) it was deduced that the (2→6) linked N-acetylneuraminic acid residue is connected to galactose 6 (13). By consequence the (2→3) linked N-acetylneuraminic acid residue is present in the other branch.

On the basis of the data of the carbohydrate composition, permethylation

Table 2. $^1$H-NMR data of anomeric, mannose H-2, N-acetyl, N-acetylneuraminic acid H-3 and fucose $CH_3$ protons for glycopeptide Eq.GPI isolated from horse pancreatic ribonuclease.

| | | |
|---|---|---|
| | anomeric protons | |
| GlcNAc | 1 | 5.067 |
| GlcNAc | 2 | 4.683 |
| Man | 3 | 4.766 |
| Man | 4 | 5.135 |
| Man | 4' | 4.923 |
| GlcNAc | 5 | 4.608 |
| GlcNAc | 5' | 4.579 |
| Gal | 6 | 4.447 |
| Gal | 6' | 4.549 |
| Fuc | | 4.874 |
| | H-2 protons | |
| Man | 3 | 4.252 |
| Man | 4 | 4.196 |
| Man | 4' | 4.11[a] |
| | N-acetyl protons | |
| GlcNAc | 1 | 2.014 |
| GlcNAc | 2 | 2.094 |
| GlcNAc | 5, 5' | 2.033 |
| NeuNAc | (2→3) | 2.046 |
| NeuNAc | (2→6) | 2.070 |
| | H-3 protons | |
| NeuNAc H-3 ax. | (2→3) | 1.800 |
| | (2→6) | 1.719 |
| NeuNAc H-3 eq. | (2→3) | 2.757 |
| | (2→6) | 2.669 |
| Fuc $CH_3$ | | 1.204 |

a) Less accurate due to the presence of the H-3 proton of Gal 6' and the H-5 proton of Fuc at about the same resonance position.

analysis and 360 MHz $^1$H-NMR spectroscopy the primary structure as given in fig. 2 was deduced. The glycopeptide sample is contaminated with a few percent of the asialo compound as is evident from a small amount of methyl--2,3,4,6-tetra-O-methyl-galactoside in the methylation analysis and the small peaks at $\delta$ = 5.119 and $\delta$ = 4.470 ppm in the NMR spectrum (11,13).

The structure as given in fig. 2 is novel since this is the first time that in a glycopeptide the occurrence of both (2→6) as well as (2→3) linked N-acetylneuraminic acid residues has been demonstrated. Sofar only in sialyl--oligosaccharides, isolated from urine of patients with mucolipidosis, the presence of (2→3) and (2→6) linked N-acetylneuraminic acid residues in one structure has been reported (14).

Comparison of the $^1$H-NMR spectrum of the asialo glycopeptides from human serotransferrin (11) with that of Eq.GPI shows that extention of the asialo

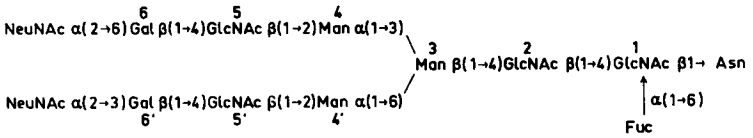


Fig. 2. The primary structure of glycopeptide Eq.GPI from horse pancreatic ribonuclease.

Table 3. Comparison of the chemical shift values of the asialo glycopeptide from human serotransferrin (11) and Eq.GPI.

| proton | asialo glycopeptide serotransferrin | Eq.GPI | |
|---|---|---|---|
| H-1 Man 4 | 5.119 | 5.135 | a |
| H-1 GlcNAc 5 | 4.581 | 4.608 | |
| H-1 Gal 6 | 4.470 | 4.447 | |
| N-Ac GlcNAc 5 | 2.047 | 2.033 | |
| H-1 Gal 6' | 4.470 | 4.549 | b |
| H-3 Gal 6' | < 4.0 | 4.11[d] | |
| N-Ac GlcNAc 5' | 2.047 | 2.033 | |
| H-1 GlcNAc 2 | 4.616 | 4.683 | c |
| N-Ac GlcNAc 2 | 2.076 | 2.094 | |

a) influence of *NeuNAcα(2→6)* Gal 6 -----
b) influence of *NeuNAcα(2→3)* Gal 6'-----
c) influence of *Fucα(1→6)* GlcNAc 1 -----
d) less accurate due to the presence of Fuc H-5 and Man 4' H-2 protons resonating at about the same position.

structure with sialic acid residues linked (2→3) and (2→6) to Gal 6' and Gal 6 respectively and a fucosyl residue to GlcNAc 1 is reflected in the resonance positions of a number of protons as is summarized in table 3.

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