α1-6(α1-3)-Difucosylation of the asparagine-bound N-acetylglucosamine in honeybee venom phospholipase A2

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Chymotryptic glycopeptides were prepared from a honeybee (Apis mellifica) venom phospholipase A2 (E.C. 3.1.1.4) fraction, with high affinity towards lentil (Lens culinaris) lectin. Treatment of the glycopeptide mixture with peptide-β-N4-(N-acetyl-β-glucosaminyl)asparagine amidase A, followed by HPLC fractionation, yielded two oligosaccharides, which were analysed by 500 MHz 1H-NMR spectroscopy to give the following structures:

\[
\text{Manz1} \quad \text{Fucz1} \quad \text{Manz1} \quad \text{Fucz1}
\]
\[
\text{Manz1-4GlcNAcβ1-4 GlcNAc} \quad \text{Manz1-4GlcNAcβ1-4 GlcNAc}
\]
\[
\text{Manz1} \quad \text{Manz1} \quad \text{Fucz1}
\]

This is the first report on a naturally occurring glycoprotein N-glycan with two fucose residues linked to the asparagine-bound N-acetylglucosamine.

Keywords: Insect glycoprotein, N-glycan, fucosylation.

Abbreviations: Fuc, fucose; PLA2, phospholipase A2; PNGase A, peptide-β-N4-(N-acetyl-β-glucosaminyl)asparagine amidase A; PNGase F, peptide-β-N4-(N-acetyl-β-glucosaminyl)asparagine amidase F; TLCK, Nα-p-tosyl-L-lysine-chloromethylketone; 2D HOHAHA, 2-dimensional homonuclear Hartmann-Hahn.

Frequently, glycoprotein N-glycans of plant or animal origin are found to contain a Fuc residue, α1-3- or α1-6-linked, respectively, to the Asn-bound GlcNAc [1, 2]. Recently, we demonstrated the ability of honeybee (Apis mellifica) venom gland extracts to convert, in the presence of GDP-Fuc, α1-6-fucosylated N-glycan acceptor into a difucosylated product with the additional Fuc residue in α(1-3)-linkage to the Asn-bound GlcNAc [3]. Here, we report the structural characterization of an N-linked carbohydrate chain, containing a Fucz1-6(Fucz1-3)GlcNAc moiety, which was isolated from an insect glycoprotein, honeybee venom phospholipase A2 (PLA2; E.C. 3.1.1.4).

Materials and methods

Materials
Lyophilized honeybee venom was obtained from Nectar-corp, Sofia, Bulgaria. Peptide-β-N4-(N-acetyl-β-glucosaminyl)-asparagine amidase A (PNGase A) was purchased from Seikagaku Kogyo, Tokyo. Bovine pancreas β-chymotrypsin (E.C. 3.4.21.1) type VII, TLCK treated, was obtained from Sigma. Lentil lectin was prepared as described [4] and coupled to CNBr-activated Sepharose 4B (Pharmacia) as recommended by the supplier.

Lentil lectin chromatography and chymotryptic digestion of PLA2
PLA2 was purified from honeybee venom as described [5].
Pure PLAz (185 mg) was dissolved in 10 ml 10 mM Tris-buffer, pH 7.1, containing 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, and 0.1% (w/v) NaN3, and applied to a column (2.5 cm x 30 cm) of lentil lectin-Sepharose 4B. After washing with 180 ml Tris-buffer, bound material was eluted with 0.1 M methyl α-D-mannopyranoside in the same buffer. The elution pattern was monitored at 280 nm, and the protein fractions were pooled, desalted, and lyophilized. The yields were 150 mg (unretarded fraction) and 30 mg (retarded fraction). The lentil lectin-binding PLA2 fraction was reduced with dithioerythritol, S-carboxymethylated, and digested with α-chymotrypsin [6]. After desalting, the digest was fractionated by FPLC on a PepRPC HR 5/5 column (Pharmacia) using 0.1% (by vol) aqueous trifluoroacetic acid (eluent A) and acetonitrile-water, 1:1 by vol (eluent B) at a flow rate of 0.5 ml min⁻¹. The gradient program was: 0–2 min, 0–25% eluent B; 2–10 min, 25–50% eluent B; 10–19 min, 50–75% eluent B; 19–20 min, 75–100% eluent B. The peptide content of the eluate was monitored at 230 nm and the carbohydrate content by an orcinol-H2SO4 spot test. Fractions containing glycopeptide material were pooled and lyophilized.

PNGase A digestion

The glycopeptide fraction of lentil-lectin binding PLA2 (approximately 4.8 mg) was dissolved in 300 μl 0.1 M sodium acetate buffer, pH 5.0, containing 0.01 mM leupeptin and 10 mM phenylmethanesulfonyl fluoride, and digested with 2 mU PNGase A for 48 h at 37 °C, added in two equal portions at 0 and t = 24 h. The release of oligosaccharides was monitored by thin layer chromatography on silica gel 60 plates, developed with butanol–ethanol–pyridine–acetic acid–water, 10:10:10:3:30 by vol, and visualized using an orcinol–H2SO4 spray reagent. Fractions containing glycopeptide material were pooled and lyophilized.

Fractionation of oligosaccharides

HPLC separation of the desalted oligosaccharide fraction (about 290 μg) was carried out on a 5 μm Shandon-Hypersil APS2-column (4 mm x 250 mm; ÖFZ Seibersdorf), using acetonitrile–water, 70:30 by vol, as eluent at a flow rate of 1.0 ml min⁻¹; 1 ml fractions were collected. The fractionation was monitored at 204 nm and carbohydrate-containing fractions were traced by the orcinol–H2SO4 spot test. Appropriate fractions were pooled and lyophilized.

Sugar composition analysis

Samples were hydrolyzed in 4 M trifluoroacetic acid for 4 h at 100 °C, and monosaccharides were analyzed as their corresponding alditol acetates by capillary GLC on DB-1701 (0.25 mm x 30 m, J&W Scientific) using a Finnigan ion trap detector (electron impact mode) [7].

Methylation analysis

Glycopeptides were methylated using lithium dimethylsulfanyl carbanion [8], and hydrolyzed with 4 M trifluoroacetic acid for 4 h at 100 °C. The partially methylated monosaccharides were reduced with NaBH4, acetylated with acetic anhydride, and separated by capillary GLC on a DB1 column (0.25 mm × 60 m, J&W Scientific) using helium at a pressure of 2 bar [9]. The oven temperature was programmed from 100 °C to 140 °C at 10 °C min⁻¹ and then to 280 °C at 3 °C min⁻¹. Detection was carried out with a Finnigan ion trap detector operating in the electron impact mode.

500 MHz 1H-NMR spectroscopy

Oligosaccharide samples were repeatedly dissolved in 2H2O at room temperature, with intermediate lyophilization, finally using 99.96% 2H2O (MSD Isotopes, Canada). 500 MHz 1H-NMR spectra were recorded using a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University), at a probe temperature of 27 °C, unless indicated otherwise. Chemical shifts are given relative to internal acetone (δ 2.225) [10].

Results

Pure honeybee venom phospholipase A2 was separated by immobilized lentil lectin into two fractions, denoted I and II (Fig. 1). Monosaccharide analysis of the unretarded fraction I and the retarded fraction II showed significant differences in Fuc content (Table 1). The affinity of lentil lectin towards N-glycopeptides is known to increase, if an α1-6-fucosyl residue is linked to the Asn-bound GlcNAc [11]. Methylation analysis of the chymotryptic glycopeptide preparation derived from fraction II showed the presence of 4,6-di- and 3,4,6-trisubstituted GlcNAc and that derived from fraction I of 3,4-disubstituted GlcNAc, suggesting that fraction II, but not fraction I, contained Fuc α1-6-linked to GlcNAc (data not shown). Incubation with PNGase F of the chymotryptic glycopeptide preparation derived from fraction II afforded only partial N-deglycosylation (data not shown), whereas treatment with PNGase A led to a complete release of the carbohydrate (not shown). Recently, it has been demonstrated that the action of PNGase F to release acetic acid for 4 h at 100 °C. The partially methylated monosaccharides were reduced with NaBH4, acetylated with acetic anhydride, and separated by capillary GLC on a DB1 column (0.25 mm × 60 m, J&W Scientific) using helium at a pressure of 2 bar [9]. The oven temperature was programmed from 100 °C to 140 °C at 10 °C min⁻¹ and then to 280 °C at 3 °C min⁻¹. Detection was carried out with a Finnigan ion trap detector operating in the electron impact mode.

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Figure 1. Affinity chromatography at 280 nm of pure phospholipase A2 on lentil lectin Sepharose 4B. For experimental details, see the Materials and methods section.