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α1-6(α1-3)-Difucosylation of the asparagine-bound *N*-acetylglucosamine in honeybee venom phospholipase A_2

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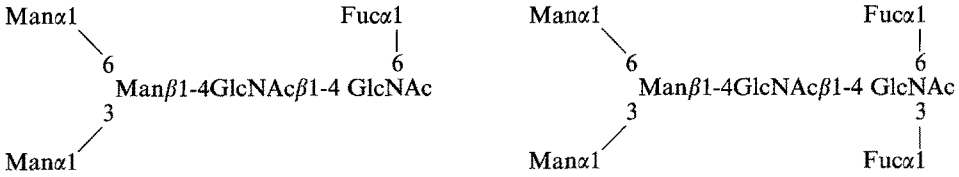
Chymotryptic glycopeptides were prepared from a honeybee (*Apis mellifica*) venom phospholipase A_2 (E.C. 3.1.1.4) fraction, with high affinity towards lentil (*Lens culinaris*) lectin. Treatment of the glycopeptide mixture with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase A, followed by HPLC fractionation, yielded two oligosaccharides, which were analysed by 500 MHz ^{1}H-NMR spectroscopy to give the following structures



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; PLA_2, phospholipase A_2; PNGase A, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase A; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; TLCK, *N*α-*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, an 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 Fucα1-6(Fucα1-3)GlcNAc moiety, which was isolated from an insect glycoprotein, honeybee venom phospholipase A_2 (PLA_2; E.C. 3.1.1.4).

Materials and methods

Materials

Lyophilized honeybee venom was obtained from Nectarcorp, Sofia, Bulgaria. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase A (PNGase A) was purchased from Seikagaku Kogyo, Tokyo. Bovine pancreas α-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 PLA_2

PLA_2 was purified from honeybee venom as described [5].

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Pure PLA_2 (185 mg) was dissolved in 10 ml 10 mM Tris-buffer, pH 7.1, containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 0.1% (w/v) NaN_3, and applied to a column (2.5 cm × 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 PLA_2 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–H_2SO_4 spot test. Fractions containing glycopeptide material were pooled and lyophilized.

PNGase A digestion

The glycopeptide fraction of lentil-lectin binding PLA_2 (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 $t = 0$ 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, 10:100:10:3:30 by vol, and visualized using an orcinol–H_2SO_4 spray reagent.

Fractionation of oligosaccharides

HPLC separation of the desalted oligosaccharide fraction (about 290 µg) was carried out on a 5 µ Shandon-Hypersil APS2-column (4 mm × 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–H_2SO_4 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 × 30 m, J&W Scientific) using a Finnigan ion trap detector (electron impact mode) [7].

Methylation analysis

Glycopeptides were methylated using lithium dimethylsulfinyl carbanion [8], and hydrolyzed with 4 M trifluoroacetic acid for 4 h at 100 °C. The partially methylated monosaccharides were reduced with $NaBH_4$, 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 ^{1}H-NMR spectroscopy

Oligosaccharide samples were repeatedly dissolved in ^2H_2O at room temperature, with intermediate lyophilization, finally using 99.96% ^2H_2O (MSD Isotopes, Canada). 500 MHz ^{1}H-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 A_2 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

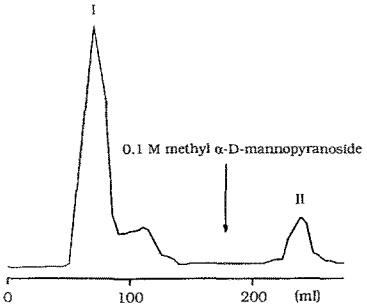


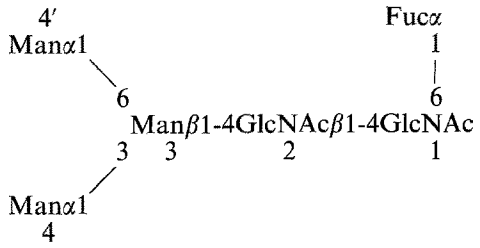
Figure 1. Affinity chromatography at 280 nm of pure phospholipase A_2 on lentil lectin Sepharose 4B. For experimental details, see the Materials and methods section.

Table 1. Sugar component analysis of PLA_2-fractions. Values are based on the assumption of three mannose residues per molecule. Traces of *N*-acetylgalactosamine in the highly purified PLA_2 were not quantified.

Honeybee venom PLA_2	*Fucose*[a]	*Mannose*[a]	*GlcNAc*[a]
Native	0.5	3.0	1.8
Fraction I	0.3	3.0	1.9
Fraction II	1.4	3.0	1.9

[a] Values expressed as mol per mol.

N-linked carbohydrates is inhibited by Fuc α1-3-linked to the Asn-bound GlcNAc [12]. In view of the high Fuc content, the affinity for *Lens culinaris* lectin, and the detection of 3,4,6-trisubstituted GlcNAc in the chymotryptic glycopeptide preparation derived from fraction II, it was reasonable to assume that part of fraction II would contain a Fucα1-6(Fucα1-3)GlcNAc-Asn moiety. HPLC fractionation of the N-linked chains, released by PNGase A from the chymotryptic glycopeptide preparation derived from fraction II (Fig. 2), afforded two fractions, denoted II-A and II-B. Both fractions were subjected to 500 MHz ^{1}H-NMR spectroscopy, and relevant ^{1}H-NMR data are presented in Table 2. The ^{1}H-NMR spectrum of fraction II-A showed the presence of α1-6-fucosylated trimannosyl-*N*,*N*′-diacetylchitobiose. The structural reporter group signals fit exactly those of reference Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc [13].



II-A

The ^{1}H-NMR spectrum of fraction II-B demonstrated the occurrence of an extended trimannosyl-*N*,*N*′-diacetylchitobiose element. As compared to the structural reporter group signals of II-A, typical shift effects were observed for GlcNAc-**1** H-1α (Δδ −0.113), GlcNAc-**1** NAc (1α, Δδ −0.011; 1β, Δδ −0.014), GlcNAc-**2** NAc (Δδ −0.029), Man-**3** H-1 (Δδ −0.04), Man-**4**′ H-1 (Δδ −0.006). Two sets of Fuc structural reporter groups were detected, namely, (i) Fuc H-1 at δ 4.926, H-5 at δ 4.1, CH_3 at δ 1.211 (GlcNAc-**1**α)/1.220 (GlcNAc-**1**β); and (ii) Fuc H-1 at δ 5.121, H-5 at δ 4.71, CH_3 at δ 1.282. On guidance of the earlier reported ^{1}H-NMR data for the α1-6-fucosylated and α1-6(α1-3)-difucosylated trimannosyl-*N*,*N*′-diacetylchitobiosyl glycopeptides [3], compound II-B turned out to be the α1-3-fucosyl extension of II-A.

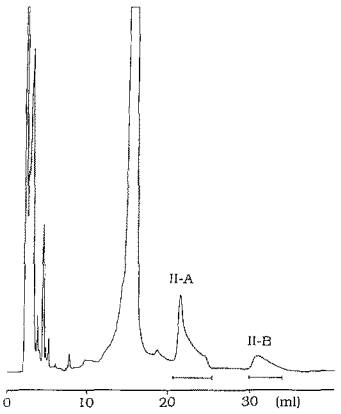
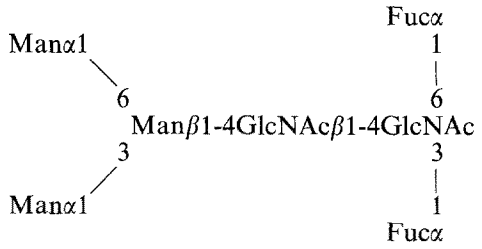


Figure 2. HPLC fractionation pattern at 204 nm of oligosaccharides released by PNGase A from the chymotryptic glycopeptide preparation of lectin-bound phospholipase A_2. For experimental details, see the Materials and methods section.

Table 2. Relevant ^{1}H-NMR parameters of structural reporter groups of constituent monosaccharides for oligosaccharides II-A and II-B, obtained by PNGase A treatment of the chymotryptic glycopeptide preparation of the lentil lectin-bound fraction II of honeybee venom phospholipase A_2.

		Chemical shift[a] *(ppm) in*	
Reporter	*Residue*	*II-A*	*II-B*
H-1	GlcNAc-**1**α	5.180	5.067
	GlcNAc-**1**β	4.693	4.69
	GlcNAc-**2**	4.664(α)/4.669(β)[b]	4.667(α)/4.676(β)
	Man-**3**	4.790	4.748[c]
	Man-**4**	5.100	5.099
	Man-**4**′	4.915	4.909
	Fucα1-6	4.886(α)/4.893(β)	4.926
	Fucα1-3	–	5.121
H-2	Man-**3**	4.255	4.257
	Man-**4**	4.066	4.064
	Man-**4**′	3.971	3.97[d]
H-5	Fucα1-6	4.098(α)/4.133(β)	4.1[d]
	Fucα1-3	–	4.71[d]
CH_3	Fucα1-6	1.209(α)/1.219(β)	1.211(α)/1.220(β)
	Fucα1-3	–	1.282
NAc	GlcNAc-**1**α	2.039	2.028
	GlcNAc-**1**β	2.038	2.024
	GlcNAc-**2**	2.093(α)/2.089(β)	2.064(α)/2.060(β)

[a] Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (acetone, δ 2.225) in ^2H_2O (27 °C).
[b] α and β correspond to GlcNAc-**1**α and GlcNAc-**1**β, respectively.
[c] Recorded at 42 °C.
[d] Obtained from a 2D HOHAHA experiment at 42 °C.



II-B

Comparison of the shift effects when going from II-A to II-B for Fucα1-6 H-1, Man-**4**′ H-1, GlcNAc-**2** NAc and GlcNAc-**1** NAc, shows that they are similar to those observed when going from Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ1-*N*-Asn-peptide(NAc) to Manα1 - 3(Manα1 - 6)Manβ1 - 4GlcNAcβ1 - 4(Fucα1 - 6) - (Fucα1-3)GlcNAcβ1-*N*-Asn-peptide(NAc) [3].

Discussion

The α1-6-fucosylation of GlcNAc-**1** is a common modification of animal glycoprotein N-glycans [1], whereas the Fucα1-3-linkage to this residue is typical for plant glycoproteins [2]. We have recently shown that insect tissue, i.e., honeybee venom glands, contains an α1-3-fucosyltransferase, which converts an N-glycopeptide acceptor, with a single α1-6-linked Fuc residue at the Asn-bound GlcNAc, into a difucosylated product with the second Fuc α1-3-linked to the same residue. The natural occurrence of the Fucα1-6(Fucα1-3)GlcNAc-Asn moiety has hitherto not been documented.

Oligosaccharide II-B, which represents the difucosyl extension of the trimannosyl-*N*,*N*′-diacetylchitobiose core structure, is in several ways remarkable. First, it has been generally accepted that α1-6-fucosylation of the innermost GlcNAc-residue requires the prior action of GlcNAc-transferase I forming the GlcNAcβ1-2Manα1-3Manβ1-4-moiety [14]; the metabolic route leading to the formation of oligosaccharide II-A, having no GlcNAc-**5** residue, also traced in other glycoproteins [15, 16], is not yet understood. A similar question arises now for the biosynthetic formation of oligosaccharide II-B. Second, whereas two or more Fuc residues within the same N-glycan chain have frequently been encountered [17–19], this report represents the first demonstration of more than one Fuc residue being linked to the same monosaccharide. Finally, recent evidence [20] and data from our own experiments (unpublished) strongly suggest that α1-3-fucosylation of the Asn-bound GlcNAc may profoundly contribute to the generation of an antigenic epitope. Our findings may therefore bear important implications for the use of insect cells as expression systems for recombinant glycoproteins.

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