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GDP-fucose: β-*N*-acetylglucosamine (Fuc to (Fucα1 → 6GlcNAc)-Asn-peptide) α1 → 3-fucosyltransferase activity in honeybee (*Apis mellifica*) venom glands

The difucosylation of asparagine-bound *N*-acetylglucosamine

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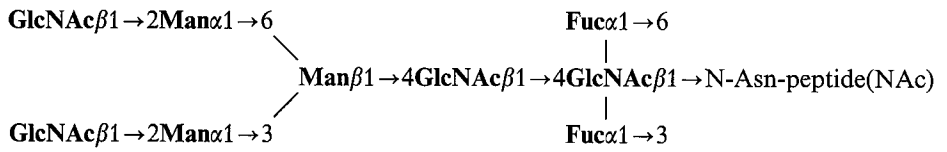
Incubation of honeybee (*Apis mellifica*) venom-gland extracts with GDP-[^{14}C]fucose and GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAcβ1 → N-Asn-peptide(NAc) gave a labeled product in 40% yield. Analysis by 500-MHz ^{1}H-NMR spectroscopy indicated the transferred fucose (Fuc) residue to be α1 → 3-linked to the Asn-bound GlcNAc. Further proof was provided by one-dimensional and two-dimensional ^{1}H-NMR analysis of the incubation mixture, after incubation with β-*N*-acetylhexosaminidase. The established carbohydrate structure



proves the existence of a novel α1 → 3-fucosyltransferase with the ability to effect difucosylation of the Asn-bound GlcNAc in *N*-glycans.

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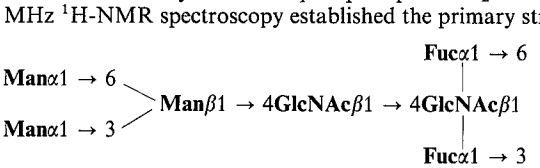
Abbreviations. Fuc, L-fucose; GnGn + F, GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAcβ1 → N-Asn-peptide; MM + F, Manα1 → 6(Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAcβ1 → N-Asn-peptide; GnGn + F(Ac), GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAcβ1 → N-Asn-peptide(NAc); GnGn + 2F(Ac), GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6) (Fucα1 → 3)GlcNAcβ1 → N-Asn-peptide (NAc); MM + F(Ac), Manα1 → 6(Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAcβ1 → N-Asn-peptide(NAc); MM + 2F(Ac), Manα1 → 6(Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6) (Fucα1 → 3)GlcNAcβ1 → N-Asn-peptide (NAc); 2D HOHAHA, two-dimensional homonuclear Hartmann-Hahn.

Enzymes. β-*N*-Acetylhexosaminidase from jack beans (EC 3.2.1.30); Phospholipase A_2 from honey bee venom (EC 3.1.1.4); UDP-*N*-acetylglucosamine: α1 → 3-D-mannoside (GlcNAc to Manα1 → 3) β1 → 2-*N*-acetylglucosaminyltransferase I, GlcNAc-transferase I (EC 2.4.1.101); GDP-fucose: β-*N*-acetylglucosamine (Fuc to Asn-linked GlcNAc) α1 → 6-fucosyltransferase (EC number unknown).

Note. We have subsequently isolated a difucosylated *N*-glycan variant from honeybee-venom phospholipase A_2. Analysis by 500-MHz ^{1}H-NMR spectroscopy established the primary structure



A detailed account on this structure will be published.

Only scattered data exist on the protein glycosylation capacity of insect cells, especially on their ability to carry out elongation reactions such as fucosyl, galactosyl and sialyl additions in the course of *N*-glycan processing [1]. A recent report, however, demonstrates that *Spodoptera frugiperda* cells have the potential to produce complex-type oligosaccharides [2]. This is of importance since insect cells are being used as expression systems for recombinant glycoproteins [3, 4]. The generation of a pool of authentic glycoforms representing a set of structural variants of a given *N*-glycan may be essential for maintaining the whole spectrum of biological activities of a glycoprotein [5].

Fucosylation contributes to the carbohydrate-dependent diversity of glycoproteins. In animal glycoprotein glycans α-Fuc has been found 1 → 6-linked to the Asn-bound GlcNAc, and, as part of several immunodeterminants, 1 → 3-linked to the GlcNAc residue of Galβ1 → 4GlcNAc units, 1 → 4-linked to the GlcNAc residue of Galβ1 → 3GlcNAc units and 1 → 2-linked to peripheral Gal [6–11]. In plants, Fuc occurs either α1 → 3-linked to the Asn-bound GlcNAc [12] or in an α1 → 6-linkage to peripheral GlcNAc [13]. Quite frequently, two or more Fuc residues are found within the same *N*-glycan chain [9, 11, 13]. However, the presence of more than one Fuc residue linked to the same monosaccharide in the carbohydrate chain has not as yet been shown. In the course of our work on phospholipase A_2 from honeybee (*A. mellifica*) venom, an allergenic glycoprotein with a single N-glycosylation site [14, 15], we noticed a significant Fuc content of the *N*-glycan. Preliminary mass spectrometry and methylation analysis of

the phospholipase-A_2 glycopeptide indicated the occurrence of α1 → 6-linked and α1 → 3-linked Fuc residues at the Asn-bound GlcNAc (unpublished results).

Here, we report that honeybee-venom-gland extracts contain a novel α1 → 3-fucosyltransferase with the ability to convert a monofucosylated *N*-glycan acceptor substrate into a difucosylated structure with two Fuc residues at the Asn-bound GlcNAc.

EXPERIMENTAL PROCEDURES

Biochemicals

GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAcβ1 → N-Asn-peptide (GnGn + F) and Manα1 → 6(Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAcβ1 → N-Asn-peptide (MM + F) were prepared as previously described [16]. *N*-Acetylation of GnGn + F (0.3 μmol) was carried out with acetic anhydride (20 μl) in 0.5% (mass/vol.) $NaHCO_3$ (1 ml) for 6 h at room temperature, yielding GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAcβ1 → N-Asn-peptide(NAc) [GnGn + F(Ac)]. Jack bean β-*N*-acetylhexosaminidase was purchased from Sigma, GDP-L-[^{14}C]fucose (specific activity 270 mCi/mmol) was from Amersham and GDP-L-fucose from BioCarb. All other chemicals were of the highest purity available.

Venom-gland extracts

European honeybees (*A. mellifica*) were obtained from the Institut für Bienenkunde, Bad Vöslau, Austria. The bees were anaesthesised with chloroform and the venom glands including the sting were withdrawn, suspended in an ice-cold 0.25-M sucrose solution (100 glands/ml) and extracted using a Potter-Elvehjem homogenizer at 300 rpm for 10 s. The resulting suspension was allowed to settle and the brown, viscous supernatant (protein content approximately 3 mg/ml; Bio-Rad Protein Assay with bovine serum albumin as a standard) was used in the subsequent experiments.

Fucosyltransfer assay

The standard incubation mixture contained, in a total volume of 20 μl, 0.5 mM GnGn + F(Ac), 0.25 mM GDP-[^{14}C]Fuc (4.8 dpm/μmol), 0.1 M Mes, pH 6.8, 10 mM $MnCl_2$, 0.1% (mass/vol.) Triton X-100, 0.2 M GlcNAc (inhibitor of β-*N*-acetylhexosaminidase), 5 mM AMP (inhibitor of pyrophosphatase) and 5 μl venom-gland extract. After incubation for 3 h at 37° C the reaction was terminated by adding 0.5 ml 20 mM ice-cold sodium borate containing 2 mM EDTA. The samples were then applied to Pasteur pipettes filled with 0.5 ml AG 1 × 8 (chloride form, 100–200 mesh, Bio-Rad). Fucose and glycopeptides were eluted with water, mixed with 4 ml Pico Aqua™ scintillation cocktail (Canberra Packard) and the radioactivity counted. Each incubation was run at least in duplicate; appropriate controls, omitting acceptor or enzyme, were included. Radioactivity of the eluates obtained from controls, usually 200–600 dpm due to free [^{14}C]Fuc in the GDP-[^{14}C]Fuc preparation, was subtracted from that of the complete mixture for the calculation of enzyme activity. Less than 5% of the substrate was converted to product in the standard incubation. For the determination of the pH optimum, Mes was replaced by Tris/HCl at pH 8.0 and pH 8.5.

Incubation on a preparative scale

GnGn + F(Ac) (292 nmol) was dissolved in 0.6 ml 0.1 M Mes, pH 6.0, containing 10 mM $MnCl_2$, 0.1% (mass/vol.) Triton X-100, 0.1 M GlcNAc and 0.5 mM GDP-[^{14}C]Fuc (860 dpm/nmol), and 0.2 ml venom-gland extract was added. After incubation for 24 h at 37°C, the mixture was passed through a column (50 × 1 cm) of Bio-Gel P-2 (200–400 mesh; Bio-Rad), using water as the eluent. The radioactive product was collected, lyophilized, investigated by ^{1}H-NMR spectroscopy and then subjected to a second incubation under the same conditions, followed again by ^{1}H-NMR analysis.

β-N-Acetylhexosaminidase treatment

The product of the 2 × 24-h incubation was dissolved in 0.5 ml 0.05 M sodium citrate buffer, pH 5.0, containing 0.02% (mass/vol.) NaN_3 and incubated with 1 unit of β-*N*-acetylhexosaminidase for 48 h at 37°C. The digestion product was desalted on a column (21 × 0.6 cm) of Bio-Gel P-2 (200–400 mesh), and analysed by ^{1}H-NMR spectroscopy.

500-MHz ^{1}H-NMR spectroscopy

Glycopeptide samples were repeatedly exchanged in ^2H_2O (99.96 atom % ^{2}H, MSD Isotopes) with intermediate lyophilization. Resolution-enhanced ^{1}H-NMR spectra were recorded on a Bruker AM-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) operating at 500 MHz with a probe temperature of 27°C. Chemical shifts (δ) are expressed in ppm relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone in ^2H_2O (δ = 2.225 ppm) [17]. Two-dimensional homonuclear Hartmann-Hahn (2D HOHAHA) spectra [18] were obtained with a MLEV-17 mixing time of 100 ms.

RESULTS

Kinetics of Fuc incorporation

Incubation of GnGn + F(Ac) with honeybee-venom-gland extract in the presence of GDP-[^{14}C]Fuc resulted in the formation of a ^{14}C-labeled product. The incorporation was proportional to the time of incubation over a period of 4 h and increased with time for at least 22 h (Fig. 1). Linearity of product formation suggests the absence of significant fucosidase activity. Prolonged incubation of the venom-gland extract with GDP-[^{14}C]Fuc in the absence of acceptor did not

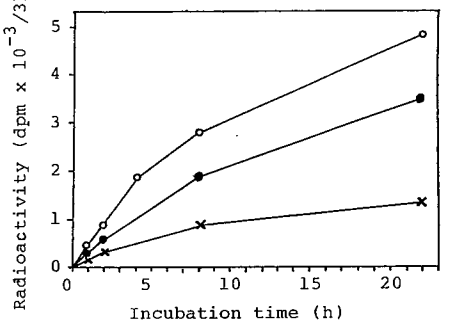


Fig. 1. *Time course of α1 → 3-fucosyl transfer to GnGn + F(Ac).* Assays were carried out under standard conditions for the times indicated. The difference (●) was calculated from assays with (○) and without (×) exogenous acceptor substrate

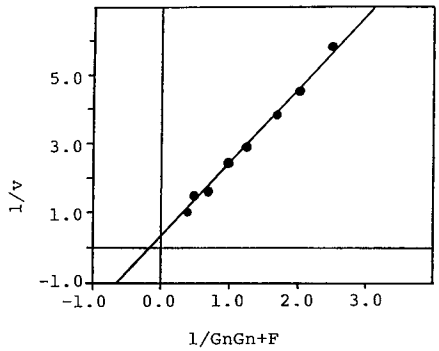


Fig. 2. *Reciprocal plot of α1 → 3-fucosyltransferase activity against the GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAcβ1 → N-Asn-peptide (GnGn + F) concentration.* The incubations were carried out as described in Experimental Procedures. The GDP-Fuc concentration was 0.25 mM. Velocity is calculated as nmol · 3 h^{-1} · 5 μl^{-1} gland extract while the GnGn + F concentration is in mM

cause an increase in the concentration of fucose. The pH optimum was between 6.5 and 7.0. Mn^{2+} was required with optimal activity at 20 mM and the reaction was inhibited at Mn^{2+} concentrations above 40 mM. Triton X-100 had no significant effect on the enzyme at concentrations below 0.5%, but caused a decrease in activity at higher concentrations. Linear $1/v$ versus $1/s$ plots indicated excellent Michaelis-Menten kinetics (Fig. 2). With GDP-[^{14}C]Fuc kept constant at a concentration of 0.25 mM, the $K_{m(app)}$ for GnGn + F(Ac) was 6.7 mM and the apparent v_{max} was 0.21 nmol · h^{-1} · μl aliquot^{-1} extract, corresponding to 69 nmol · h^{-1} · mg protein^{-1}.

Product identification

500-MHz ^{1}H-NMR spectroscopy was carried out on the reaction mixture, containing both substrate and product, at 24 h and 48 h after initiation of the reaction. Comparison of the ^{1}H-NMR spectrum of the 24-h incubation mixture with that of the substrate GnGn + F(Ac) showed the presence of several additional signals which increased in intensity during a further 24-hour incubation. After a total of 48 h of incubation, 40% of the substrate was converted to product. The new NMR signals (Figs. 3 A and 3 B, and Table 1, discussed below) indicate that the venom gland extract has catalyzed the incorporation of a second Fuc residue into GnGn + F(Ac) to form GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6) (Fucα1 → 3)GlcNAcβ1 → N-Asn-peptide(NAc), [GnGn + 2F(Ac)].

NMR spectra of GnGn + F(Ac) (Fig. 3 B) and of the GnGn + F(Ac)/GnGn + 2F(Ac) mixture (Fig. 3 A)

Typical new signals of Fuc at δ = 5.128 ppm (H-1) and δ = 1.282 ppm (CH_3) are present in the spectrum of the incubation mixture GnGn + F(Ac)/GnGn + 2F(Ac). The positions of these Fuc signals are similar to those reported earlier for the α1 → 3-linked Fuc residue in the bromelain glycopeptide Manα1 → 6(Xylβ1 → 2)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 3)GlcNAcβ1 → N-Asn-peptide (H-1, δ = 5.136 ppm; CH_3, δ = 1.285 ppm) [19] and the *Sophora japonica* lectin glycopeptide Manα1 → 6(Manα1 → 3) (Xylβ1 → 2)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 3)GlcNAcβ1 → N-Asn-peptide (H-1, δ = 5.134 ppm; CH_3, δ = 1.290 ppm) [20]. Additional satellite signals in the spectrum of GnGn + F(Ac)/GnGn + 2F(Ac) are observed for Man-4′ H-1 (δ = 4.908 ppm), GlcNAc-**5** and/or GlcNAc-**5**′ H-1 (δ = 4.549 ppm), α1 → 6Fuc H-1 (δ = 4.904 ppm), α1 → 6Fuc H-5 (δ = 4.150 ppm), GlcNAc-**1** NAc (δ = 2.006/1.997 ppm) and GlcNAc-**2** NAc (δ = 2.066 ppm). The values of the NAc singlets of GlcNAc-**1** and GlcNAc-**2** fit those reported for the bromelain glycopeptide (δ = 1.994 ppm and 2.066 ppm) [19]. It should be noted that in general the position of the GlcNAc-**1** NAc signal is influenced by the peptide part of the glycopeptide, explaining the two signals at δ = 2.014 ppm and δ = 2.006 ppm for the GnGn + F(Ac) substrate. From a comparison of the intensity ratio of the GlcNAc-**1** NAc singlets at δ = 2.014 ppm, δ = 2.006 ppm and δ = 1.997 ppm in GnGn + F(Ac)/GnGn + 2F(Ac) (Fig. 3 A), and at δ = 2.014 ppm and δ = 2.006 ppm in GnGn + F(Ac) (Fig. 3 B), and also taking into account the ratio in the spectrum of the 24-h incubation mixture (spectrum not shown), it can be concluded that the GlcNAc-**1** NAc signal at δ = 2.006 ppm in Fig. 3 A stems from both GnGn + F(Ac) and GnGn + 2F(Ac). In summary, the appearance at 24 h of incubation of several characteristic new structural-reporter groups and the increased intensity of these signals after a further 24-h incubation, indicate the incorporation of a Fuc residue at the C-3 position of GlcNAc-**1**.

NMR spectra of the product after digestion with β-N-acetylhexosaminidase

To obtain further evidence for the primary structure of GnGn + 2F(Ac), the 2 × 24-h incubation mixture was treated with *β-N*-acetylhexosaminidase, affording products which can be directly compared with the bromelain glycopeptide [19]. The ^{1}H-NMR spectrum of the digested mixture of GnGn + F(Ac)/GnGn + 2F(Ac) (Fig. 4) indicated the existence of Manα1 → 6(Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 6)GlcNAcβ1 → N-Asn-peptide(NAc) [MM + F(Ac)] and fucosylated MM+F(Ac), denoted as MM + 2F(Ac). Relevant parts of the two-dimensional homonuclear Hartmann-Hahn (2D HOHAHA) NMR spectra of MM + F(Ac)/MM + 2F(Ac) and the reference spectrum of MM + F are presented in Figs 5 and 6, respectively. Pertinent ^{1}H-NMR data of MM + F(Ac)/MM + 2F(Ac) are included in Table 1, together with data of reference compound MM + F [21]. Inspection of the 2D-HOHAHA spectrum (Fig. 5) of MM + F(Ac)/MM + 2F(Ac) shows a series of cross-peaks at δ = 3.72 ppm, δ = 3.97 ppm and δ = 3.81 ppm on the H-1 track of the newly introduced Fuc residue (δ = 5.126 ppm), which correspond to Fuc H-2, H-3 and H-4, respectively. On the CH_3-track of this Fuc residue (δ = 1.282 ppm) a cross-peak at δ = 4.72 ppm, assigned to Fuc H-5, is observed. These assignments are made on the basis of a comparison with the values found for α1 → 3Fuc in the bromelain glycopeptide Manα1 → 6(Xylβ1 → 2)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 3)GlcNAcβ1 → N-Asn-peptide (α1 → 3Fuc at GlcNAc-**1**: H-1, δ = 5.136 ppm; H-2, δ = 3.721 ppm; H-3, δ = 3.981 ppm; H-4, δ = 3.814 ppm; H-5, δ = 4.722 ppm; CH_3, δ = 1.285 ppm) [19]. Furthermore, the satellite NAc signals of GlcNAc-**1** and GlcNAc-**2** in the spectrum of MM + F(Ac)/MM + 2F(Ac) at δ = 2.007/1.997 ppm and δ = 2.064 ppm, respectively, occur at similar positions as found in the bromelain glycopeptide (δ = 1.994 ppm and δ = 2.066 ppm for GlcNAc-**1** and GlcNAc-**2**, respectively). Finally, the attachment of a Fuc residue at C-3 of GlcNAc-**1** is supported by the observed cross-peak patterns on the GlcNAc-**1** and GlcNAc-**2** H-1 tracks of the 2D-HOHAHA spectrum of MM + F(Ac)/MM

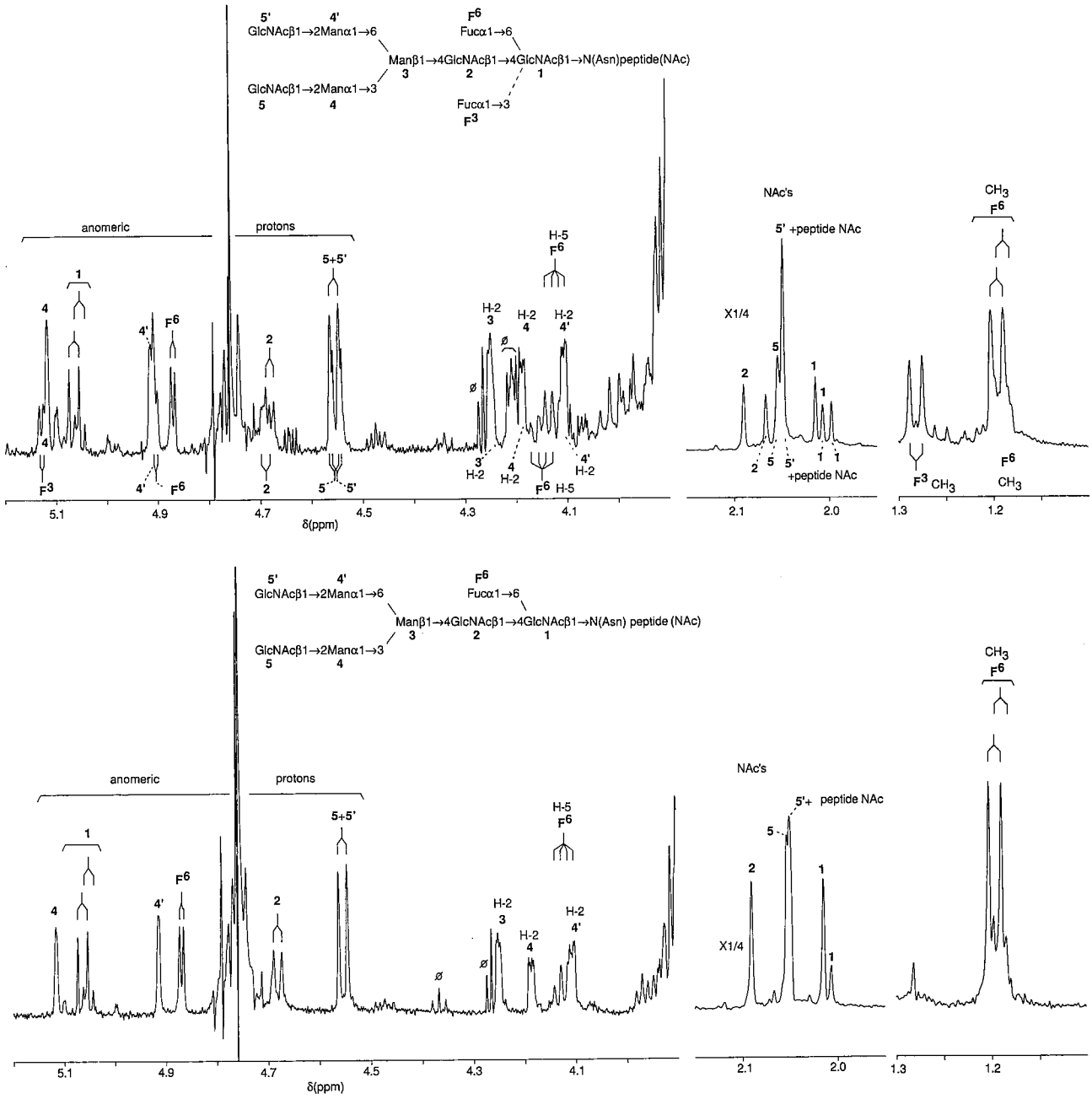


Fig. 3. *Structural-reporter-group regions of the resolution-enhanced 500-MHz ^1H-NMR spectra of (A) the 2 × 24-h incubation mixture GnGn + F(Ac)/GnGn + 2F(Ac) and (B) the substrate GnGn + F(Ac) recorded in ^2H_2O at 27°C.* The numbers in the spectra refer to the corresponding residues in the structures. The relative intensity scale of the *N*-acetyl and Fuc CH_3 regions differs from that of the other parts of the spectrum as indicated. The assignments above the peak pattern in (A) belong to GnGn + F(Ac) and those below the peak pattern to GnGn + 2F(Ac). ø, signals stemming from non-carbohydrate material

+ 2F(Ac) (Fig. 5). On the GlcNAc-**1** H-1 track of the MM + F(Ac) component at δ = 5.063 ppm, cross peaks are observed for GlcNAc-**1** H-2 at δ = 3.85 ppm and H-3 at δ = 3.76 ppm. The assignments of GlcNAc-**1** H-2 and H-3 are based on assignments in the 2D-HOHAHA spectrum of MM + F (Fig. 6; H-2, δ = 3.86 ppm and H-3, δ = 3.77 ppm) and NMR data of a conventional non-fucosylated disialo diantennary glycopeptide [GlcNAc-**1** H-2, δ = 3.85 ppm; H-3, δ = 3.77 ppm; H-4, δ = 3.65 ppm; H-5, δ = 3.58 ppm (De Waard and Vliegenthart, unpublished results)]. The GlcNAc-**1** H-1 track of the MM + 2F(Ac) component at δ = 5.08 ppm shows cross peaks at δ = 4.01 ppm and δ = 3.92 ppm, positions similar to those found for GlcNAc-**1** H-2 and H-3 of the bromelain glycopeptide (δ = 4.033 ppm and δ = 3.924 ppm for H-2 and H-3, respectively) [19]. Concerning the GlcNAc-**2** H-1 tracks of both components, that of the MM + F(Ac) component at δ = 4.681 ppm yields a similar pattern of cross peaks as observed in the 2D-HOHAHA spectra of MM + F (Fig. 6) and of a conventional non-fucosylated disialo diantennary glycopeptide [GlcNAc-**2** H-2, δ = 3.80 ppm; H-3, δ = 3.75 ppm; H-4, δ = 3.73 ppm; H-5, δ = 3.60 ppm; H-6, δ = 3.87 ppm; H-6′, δ = 3.56 ppm (De Waard and Vliegenthart, unpublished results)]. However, the GlcNAc-**2** H-1 track of the MM + 2F(Ac) component at δ = 4.692 ppm

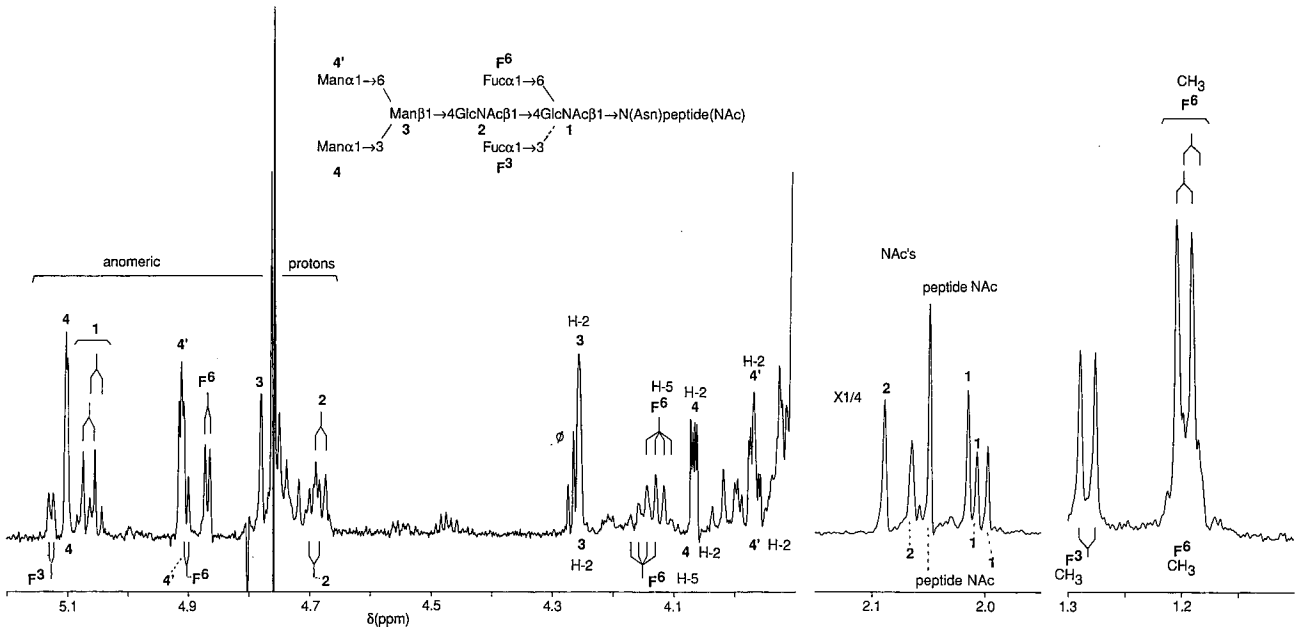


Fig. 4. *Structural-reporter group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of the 2 × 24-h incubation mixture GnGn + F(Ac)/GnGn + 2F(Ac) digested with β-*N*-acetylhexosaminidase, yielding MM + F(Ac)/MM + 2F(Ac), recorded in ^2H_2O at 27°C.* The numbers in the spectrum refer to the corresponding residues in the structures. The relative intensity scale of the *N*-acetyl and Fuc CH_3 regions differs from that of the other parts of the spectrum as indicated. The assignments above the peak pattern belong to MM + F(Ac) and those below the peak pattern to MM + 2F(Ac)

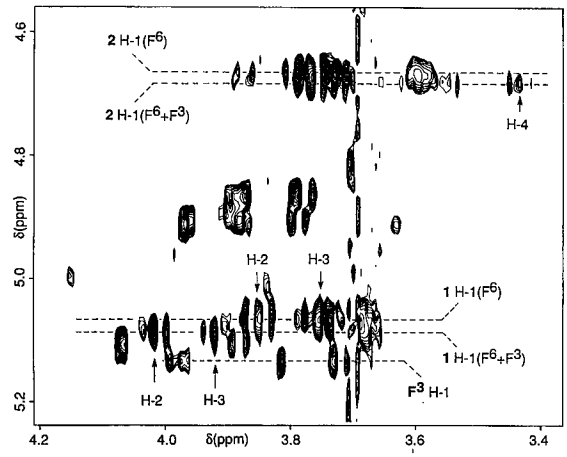


Fig. 5. *Part of the 2D-HOHAHA spectrum of MM + F(Ac)/MM + 2F(Ac) using a MLEV-17 mixing time of 100 ms, recorded in ^2H_2O at 27°C.* **2** H-1 (F^6) denotes H-1 of GlcNAc-**2** in MM + F(Ac); **2** H-1 (F^6 + F^3) denotes H-1 of GlcNAc-**2** in MM + 2F(Ac); **1** H-1 (F^6) denotes H-1 of GlcNAc-**1** in MM + F(Ac); **1** H-1 (F^6 + F^3) denotes H-1 of GlcNAc-**1** in MM + 2F(Ac); F^3 H-1 denotes H-1 of α1-3Fuc in MM + 2F(Ac)

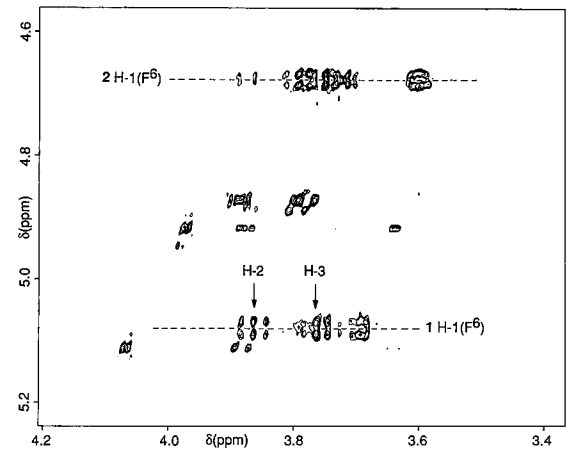
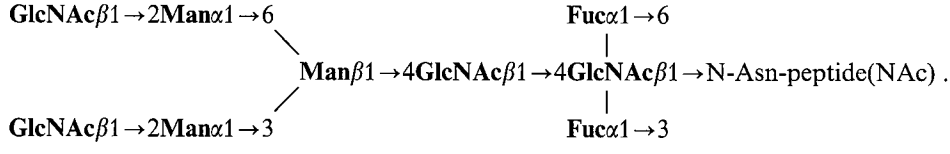


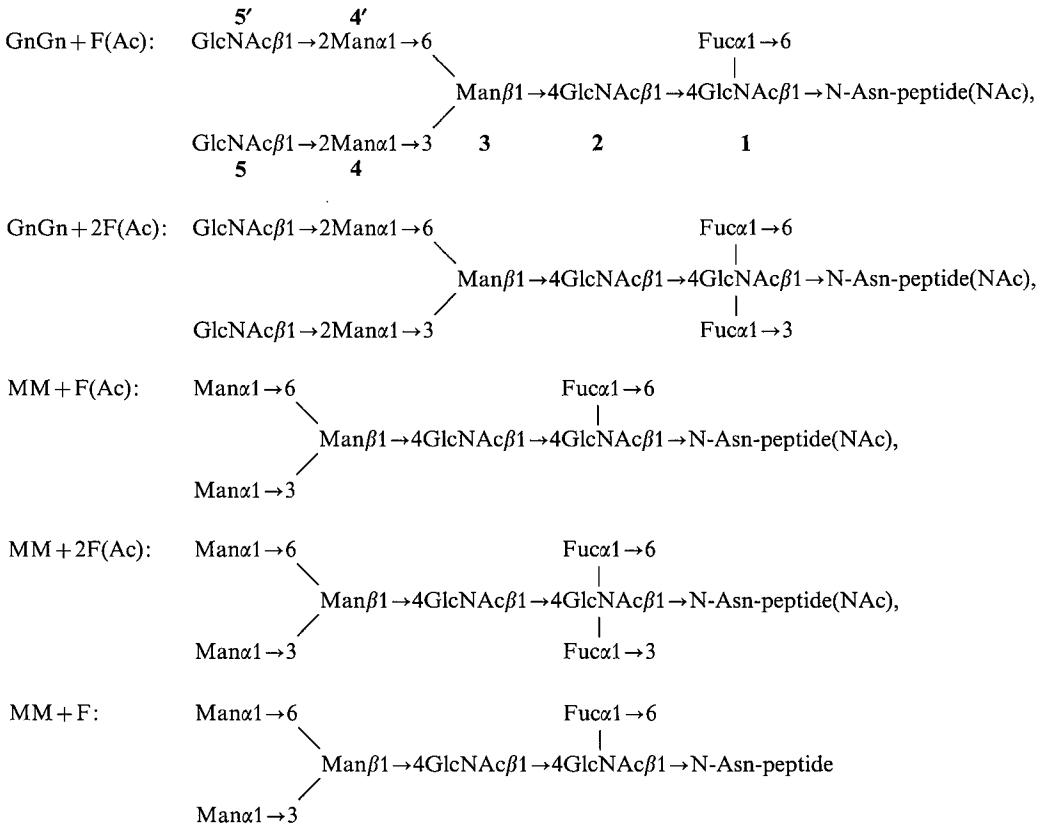
Fig. 6. *Part of the 2D-HOHAHA spectrum of MM + F using a MLEV-17 mixing time of 100 ms, recorded in ^2H_2O at 27°C.* **2** H-1 (F^6) denotes H-1 of GlcNAc-**2** in MM + F; **1** H-1 (F^6) denotes H-1 of GlcNAc-**1** in MM + F

shows a clearly shifted cross peak at δ = 3.45 ppm, a value similar to that found for GlcNAc-**2** H-4 in the bromelain glycopeptide (δ = 3.458 ppm). In conclusion, the NMR data clearly establish that the product GnGn + 2F(Ac) formed by the venom-gland extract has the following structure:



It should be emphasized that the presence of unconverted substrate GnGn + F(Ac) does not interfere with the definitive identification of the GnGn + 2F(Ac) product because of the presence of several characteristic new signal-reporter groups due to product besides the signals due to GnGn + F(Ac).

Table 1. *Relevant ^{1}H-NMR parameters of structural-reporter groups of constituent monosaccharides for GnGn + F(Ac), GnGn + F(Ac)/ − GnGn + 2F(Ac), MM + F(Ac)/MM + 2F(Ac) and reference compound MM + F.* Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate (acetone, δ = 2.225 ppm) in ^2H_2O (27°C). The codes represent the following structures, with the numbering of monosaccharide residues included in GnGn + F(Ac)



Reporter group	Residue	Chemical shift in			
		GnGn + F(Ac)	GnGn + F(Ac)/ GnGn + 2F(Ac)	MM + F(Ac)/ MM + 2F(Ac)	MM + F
		ppm			
H-1	GlcNAc-**1**	5.064; 5.054[a]	*n.d.*/5.063; 5.053[a]	*5.08*[b, c]/5.063; 5.052[a]	5.071
	GlcNAc-**2**	4.681	*4.689*/4.680	*4.692*/4.681	4.683
	Man-**3**	n.d.	n.d.	4.780	4.780
	Man-**4**	5.117	5.116	5.100	5.102
	Man-**4′**	4.916	4.914/*4.908*	4.913/*4.909*	4.914
	GlcNAc-**5**	4.554	4.554	–	–
	GlcNAc-**5′**	4.554	4.554/*4.549*	–	–
	α1 → 6Fuc	4.870	*4.904*/4.869	*4.904*/4.869	4.870
	α1 → 3Fuc	–	*5.128*	*5.126*	–
H-2	Man-**3**	4.252	4.248	4.255	4.256
	Man-**4**	4.187	4.187	4.066	4.066
	Man-**4′**	4.106	4.105	3.970/*3.963*	3.971
H-5	α1 → 6Fuc	4.124	*4.150*/4.124	*4.151*/4.122	4.126
	α1 → 3Fuc	–	*n.d.*	*4.72*[c]	–
CH_3	α1 → 6Fuc	1.196; 1.191[a]	1.196; 1.191[a]	1.197; 1.190[a]	1.200
	α1 → 3Fuc	–	*1.282*	*1.282*	–
NAc	GlcNAc-**1**	2.014; 2.006[a]	2.014; 2.006/*2.006*; *1.997*[a]	2.014; 2.007[a]/*2.007*; *1.997*[a]	2.011
	GlcNAc-**2**	2.090	2.090/*2.066*	2.088/*2.064*	2.089
	GlcNAc-**5**	2.052	2.054	–	–
	GlcNAc-**5′**	2.049	2.048	–	–
	peptide-NAc	2.049	2.048	2.048	–

[a] Two values due to heterogeneity in the peptide part.
[b] Italic values are characteristic for the α1 → 3-fucosylated products.
[c] Values with two decimals are taken from the 2D-HOHAHA spectrum.

Rotamer population

Recently, it has been demonstrated for the bromelain glycopeptide that the presence of an α1 → 3-linked Fuc at GlcNAc-**1** strongly influences the rotamer population about the C-5–C-6 bond of Man-**3**, affording mainly the $P_{\omega = 180}$ (gg) rotamer instead of a mixture of $P_{\omega = 180}$ (gg) and $P_{\omega = -60}$ (gt) [19]. The ^{1}H-NMR data of GnGn + 2F(Ac), as compared to those of GnGn + F(Ac), demonstrate that the introduction of α-Fuc in a 1 → 3-linkage to the Asn-bound GlcNAc influences the resonance positions of several hydrogens, including the anomeric signals of Man-**4′** and in connection with this, GlcNAc-**5′**. The observed small shifts for Man-**4′** H-1 and GlcNAc-**5′** H-1 can be the result of a change in rotamer population, caused by the presence of an α1 → 3Fuc when going from GnGn + F(Ac) to GnGn + 2F(Ac).

DISCUSSION

The presence of a single Fuc, either α1 → 6-linked or α1 → 3-linked, at the Asn-bound GlcNAc of *N*-glycans, has been shown to occur in many glycoproteins of animal and plant origin. Here we demonstrate for the first time, that an insect tissue contains an α1 → 3-fucosyltransferase with the ability to convert a monofucosylated (Fuc α1 → 6) *N*-glycan acceptor substrate into a difucosylated structure with two Fuc residues at the Asn-bound GlcNAc residue. Pig liver [22] can incorporate fucose into GlcNAcβ1 → 2Manα1 → 6(GlcNAcβ1 → 2Manα1 → 3)Manβ1 → 4GlcNAcβ1 → 4GlcNAcβ1 → N-Asn-peptide (GnGn-F) to form GnGn + F; this incorporation requires the prior formation of the GlcNAcβ1 → 2Manα1 → 3Manβ1 → 4 moiety due to the action of GlcNAc-transferase I [22]. Bee venom glands have a similar activity (unpublished results). Our data therefore indicate that bee-venom-gland extract can catalyze the synthesis of the difucosylated structure GnGn + 2F(Ac) by the addition of α1 → 6Fuc to GnGn-F followed by addition of α1 → 3-Fuc to GnGn + F; we have not as yet ruled out the possible existence of the reverse-order pathway (3 before 6). Both Fuc-transferase activities require the prior action of GlcNAc-transferase I since we have found that bee venom-gland extracts are unable to transfer Fuc to either MM −F or MM +F (unpublished results).

In conclusion, we have established the existence of a novel bee venom-gland α1 → 3-fucosyltransferase capable of synthesizing the product GnGn + 2F(Ac) containing the Fucα1 → 6(Fucα1 → 3)GlcNAc-Asn moiety, which has not as yet been described in the literature. The bee venom-gland enzyme is presently being used to synthesize compounds in amounts sufficient for the study of the structural and antigenic properties of this moiety. It is clear that whenever insect cells are used as hosts for the expression of recombinant DNA, the possible existence of this enzyme in the host cell must be considered.

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