

## Primary structures of the N-linked carbohydrate chains from honeybee venom phospholipase A<sub>2</sub>

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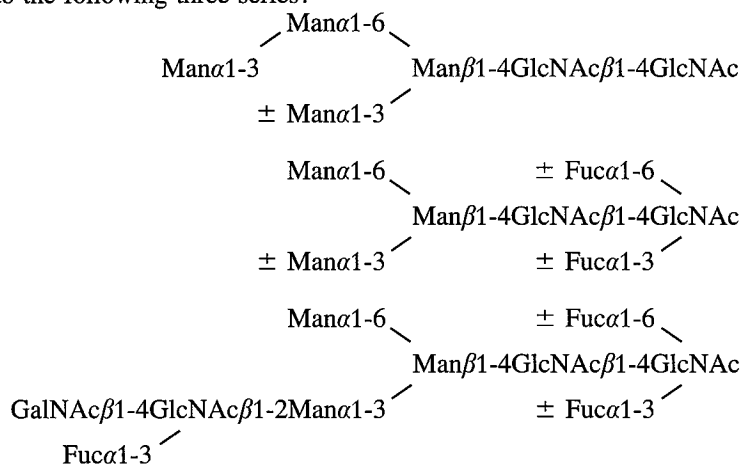
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The N-linked carbohydrate chains of phospholipase A<sub>2</sub> from honeybee (*Apis mellifera*) were released from glycopeptides with peptide-*N*-glycanase A and reductively aminated with 2-aminopyridine. The fluorescent derivatives were separated by size-fractionation and reverse-phase HPLC, yielding 14 fractions. Structural analysis was accomplished by compositional and methylation analyses, by comparison of the HPLC elution patterns with reference oligosaccharides, by stepwise exoglycosidase digestions which were monitored by HPLC, and, where necessary, by 500-MHz <sup>1</sup>H-NMR spectroscopy.

Ten oligosaccharides consisted of mannose, *N*-acetylglucosamine and fucose  $\alpha$ 1-6 and/or  $\alpha$ 1-3 linked to the innermost *N*-acetylglucosamine. Four compounds, which comprised 10% of the oligosaccharide pool from phospholipase A<sub>2</sub>, contained a rarely found terminal element with *N*-acetylgalactosamine. The structures of the 14 N-glycans from honeybee phospholipase A<sub>2</sub> can be arranged into the following three series:



Considerable knowledge on the structure, the biosynthesis and the catabolism of protein-bound carbohydrate has been accumulated, and particular emphasis has been put on the deciphering of the structure/function relation [1]. In con-

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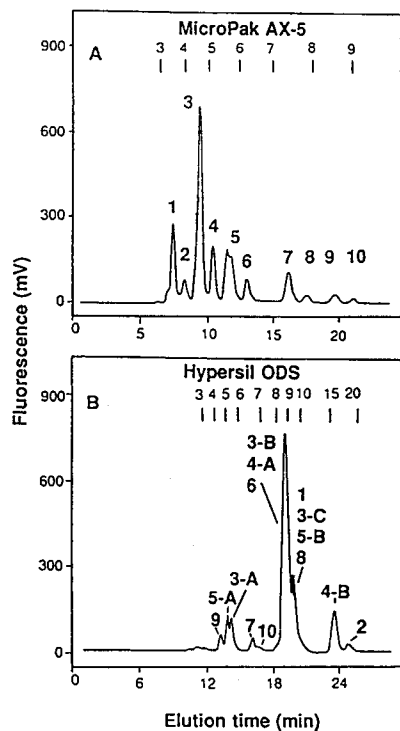
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*Abbreviations.* Fuc, L-fucose; Glc units, number of glucose units of standard oligomer, i.e. *n* in (Glc)<sub>*n*</sub>;  $\beta$ -HexNAcase,  $\beta$ -*N*-acetylhexosaminidase; PLA, phospholipase A<sub>2</sub>.

*Enzymes.*  $\beta$ -*N*-Acetyl-D-hexosaminidase from jack beans (EC 3.2.1.30); bromelain from pineapple stem (EC 3.4.22.4);  $\alpha$ -L-fucosidase from bovine kidney (EC 3.2.1.51);  $\alpha$ -D-mannosidase from jack beans (EC 3.2.1.24); pepsin from porcine mucosa (EC 3.4.23.1); peptide-*N*-glycanase A from sweet almonds (EC 3.5.1.52); phospholipase A<sub>2</sub> from honeybee venom (EC 3.1.1.4);  $\beta$ -D-xylosidase from sweet almonds (EC 3.2.1.37).

trast to the glycoproteins of vertebrates, in particular those from mammalian sources, insect glycoproteins have received little attention. Very recently, the use of lepidopteran cells for the production of recombinant (glyco)proteins has stimulated the interest in the protein-glycosylation potential of insects [2–7].

Glycoproteins produced by insect cells were shown to contain oligomannose structures with 2–9 Man residues [2, 3]. While it was formerly assumed that insect cells are able to assemble oligomannose-type oligosaccharides only, Fuc was found in  $\alpha$ 1-6 linkage to the innermost GlcNAc of Man<sub>2-3</sub>GlcNAc<sub>2</sub> [2, 3, 5]. The expression of glycoproteins in insect cells using the baculovirus vector was reported to be accompanied by the assembly of sialylated *N*-acetylglucosamine type of N-glycans [6, 7]. These results were derived from studies with recombinant glycoproteins or with pools of membrane components from cultured cells. Naturally occur-



**Fig. 1. HPLC profiles of pyridylaminated oligosaccharides from honeybee PLA.** The oligosaccharides released from PLA by peptide-*N*-glycanase A were pyridylaminated and fractionated according to size on MicroPak AX-5 (A). Elution positions of the individual compounds on reverse-phase HPLC can be deduced from the profile of the unfractionated mixture (B). A total of 2 nmol was injected for each chromatogram. Numbers at the top indicate the elution positions of isomaltose standards with the respective degree of polymerization.

**Table 1. Elution positions of individual pyridylamino-oligosaccharides obtained from honeybee venom PLA.** The structures of the reference oligosaccharides are given in Materials and Methods.

Glycan	Elution positions on		Corresponding reference
	MicroPak AX-5	Hypersil ODS	
	Glc units		
1	3.6	9.8	ST-4
2	4.0	18.0	
3-A	4.8	5.7	ST-5
3-B	4.8	8.8	ST-2
3-C	4.8	9.4	
4-A	5.1	8.7	
4-B	5.1	16.4	ST-1
5-A	5.8	5.3	
5-B	5.6	9.0	
6	6.3	8.7	
7	7.5	6.7	
8	7.9	10.7	
9	8.7	4.4	
10	9.1	7.3	

ring insect glycoproteins have not, however, been studied in detail.

Phospholipase A<sub>2</sub> (PLA), the major protein constituent of honeybee venom, is an allergenic, basic glycoprotein with a

**Table 2. Effect of  $\alpha$ -mannosidase treatment on pyridylamino-oligosaccharides.** The higher enzyme dose was applied for the removal of  $\alpha$ 1,6-linked Man residues. Conversion to products was not necessarily quantitative. For an explanation of I and II, see text.

Compound	Enzyme dose	Elution positions of products on		Product coeluting with
		MicroPak AX-5	Hypersil ODS	
	mU	Glc units		
1	25	2.6	7.9	
3-B	5	3.6	9.8	1
3-C	5	3.6	9.6	1, ST-4
	25	2.7	7.9	
4-B	5	4.0	18.2	2
5-A	5	4.8	5.7	3-A, ST-5
5-B	5	I: 4.8	9.6	3-C
		II: 3.6	9.6	1, ST-4
	25	2.6	7.7	
6	5	5.1	8.7	4-A
7	25	6.5	6.5	
8	25	6.9	10.5	
9	25	7.7	4.4	
10	25	8.1	7.2	

**Table 3. Digestions of pyridylamino-oligosaccharides with  $\alpha$ -fucosidase from bovine kidney.** The lower enzyme concentration was found to remove Fuc in  $\alpha$ 1,6-linkage to the innermost GlcNAc, while the higher dose is required for at least partial removal of  $\alpha$ 1,3-linked Fuc residues.

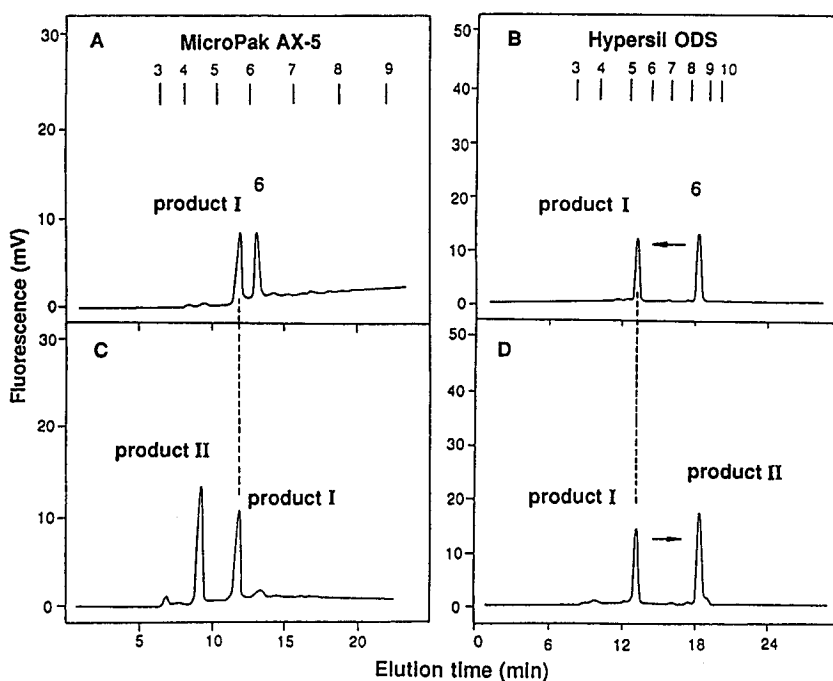
Compound	Enzyme dose	Elution positions of products on		Product coeluting with
		MicroPak AX-5	Hypersil ODS	
	mU	Glc units		
2	0.1	3.6	9.8	1, ST-4
3-A	10	3.6	9.8	1, ST-4
4-A	0.1	4.8	5.7	3-A, ST-5
	10	3.6	9.8	1, ST-4
4-B	0.1	4.8	8.8	3-B, ST-2
5-A	10	4.8	8.8	3-B, ST-2
6	0.1	5.7	5.3	5-A
	10	4.8	8.8	3-B, ST-2
8	0.1	7.5	6.7	7
10	0.1	8.7	4.4	9

molecular mass of 15.7 kDa [8], consisting of 134 amino acids with a single glycosylation site at Asn13 [9]. SDS/PAGE separates PLA into three isoforms which differ in their carbohydrate content [10]. The smallest isoform (PLA-16) is not glycosylated. The main variant (PLA-18, 75%) contains Man, Fuc and GlcNAc only, whereas PLA-20 additionally bears GalNAc [10].

Sera of some bee-venom-allergic individuals were shown to react with the carbohydrate moiety of PLA [11]. This observation stimulated our interest in the structures of these N-glycans. In a preliminary study, a carbohydrate chain with

**Table 4. Methylation analysis of pyridylamino-oligosaccharides from honeybee venom PLA.** The partially methylated alditol acetates are quantitated relative to the respective derivative of the  $\beta$ -mannosyl residue (underlined). The pyridylaminated GlcNAc residue could not be analyzed. The somewhat high value for terminal mannose in **3-B** is unexplained.

Glycan	Partially methylated alditol acetates derived from:								
	Fuc-	Man-	-2Man-	-3Man-	-6Man-	<u>3</u> Man-	GalN-	-4GlcN-	<u>3</u> GlcN-
	mol/mol								
<b>1</b>	—	1.1	—	—	<u>1.0</u>	—	—	0.8	—
<b>2</b>	0.7	0.8	—	—	<u>1.0</u>	—	—	0.5	—
<b>3-A</b>	0.5	1.0	—	—	<u>1.0</u>	—	—	0.4	—
<b>3-B</b>	—	2.6	—	—	—	<u>1.0</u>	—	1.1	—
<b>3-C</b>	—	1.0	—	0.6	<u>1.0</u>	—	—	0.8	—
<b>4-B</b>	0.7	1.8	—	—	—	<u>1.0</u>	—	1.0	—
<b>5-A</b>	0.4	1.5	—	—	—	<u>1.0</u>	—	0.6	—
<b>5-B</b>	—	1.6	—	0.7	—	<u>1.0</u>	—	0.8	—
<b>6</b>	1.1	1.6	—	—	—	<u>1.0</u>	—	1.2	—
<b>7</b>	0.8	1.3	0.7	—	—	<u>1.0</u>	0.6	1.3	0.5



**Fig. 2. Elution pattern of pyridylamino-oligosaccharide 6 obtained by stepwise degradation with  $\alpha$ -fucosidase.** Structure 6 was first digested with a low amount of  $\alpha$ -fucosidase, thereby yielding product I (A, B). This compound was isolated and then treated with a higher concentration of  $\alpha$ -fucosidase to give product II (C and D). Refer also to Table 3.

two fucosyl residues linked to the asparagine-bound *N*-acetylglucosamine was detected [12]. In this paper we report on the structural elucidation of the collection of the *N*-linked oligosaccharides from honeybee venom phospholipase  $A_2$ .

## MATERIALS AND METHODS

### Bee venom

Batches of lyophilized whole bee venom were purchased from Nectarcorp (Sofia, Bulgaria), from Ch. Mraz, Champlain Valley Apiaries (Middlebury, Vermont, USA) and from H. Mack-Nachfolger (Illertissen, Germany).

### Enzymes

Peptide-*N*-glycanase A [almond glycopeptidase, peptide-*N*-(*N*-acetyl- $\beta$ -glucosaminyl)asparagine amidase A] was

purchased from Seikagaku Kogyo (Tokyo, Japan).  $\alpha$ -Fucosidase from bovine kidney was obtained from Boehringer Mannheim. Pepsin,  $\alpha$ -mannosidase and  $\beta$ -*N*-acetylhexosaminidase from jack beans and crude  $\beta$ -galactosidase from *Aspergillus oryzae* were obtained from Sigma.  $\beta$ -Galactosidase was purified by ion-exchange chromatography on S-Sepharose Fast Flow (Pharmacia) using a linear gradient of 0–500 mM NaCl in 10 mM sodium acetate pH 4.6.  $\beta$ -Xylosidase, considered to be a co-activity of a  $\beta$ -glucosidase isoenzyme, was prepared from sweet almonds [13].  $\alpha$ -Fucosidase from almonds was purchased from Genzyme Co. (Cambridge, MA, USA).

### Other materials

2-Aminopyridine, sodium cyanoborohydride and bovine fibrin were purchased from Sigma. Human IgG was a gen-

**Table 5. Determination of the *N*-acetylhexosamine content of fractions 7–10.** The molar contents of the underivatized *N*-acetylhexosamines were calculated by correlation of the peak areas of the derivatized hexosaminotols with that of the unhydrolyzed pyridylamino-glycans, which were chromatographed in parallel. **7 $\beta$ -HexNAcase** denotes the product of digestion of **7** with  $\beta$ -*N*-acetylhexosaminidase. The detector response ratio was assessed from analogous analyses of pyridylamino-oligosaccharides of known composition.

Pyridylamino-glycan	GlcNAc	GalNAc
	mol/mol	
<b>7</b>	2.1	1.1
<b>7<math>\beta</math>-HexNAcase</b>	2.2	0
<b>8</b>	2.2	1.3
<b>9</b>	1.9	1.2
<b>10</b>	2.0	1.0

erous gift of Immuno AG (Vienna, Austria).  $^2\text{H}_2\text{O}$  (99.8% and 99.96%  $^2\text{H}$ ) was obtained from MSD Isotopes (Montreal, Canada).

#### Preparation of pyridylaminated oligosaccharides from phospholipase

Phospholipase  $A_2$  was purified from honeybee venom [10] and then digested with pepsin in 5% (by vol.) formic acid. After desalting of the digest over Sephadex G-25 using 1% (by vol.) acetic acid as the eluent, the oligosaccharides were released with peptide-*N*-glycanase A [14]. The completion of the release was monitored by thin-layer chromatography [12]. To remove peptides, the resulting mixture was passed over Dowex 50W-X2 ( $\text{H}^+$ ) using 0.5% (by vol.) acetic acid. The carbohydrate-positive eluate was lyophilized and reductively aminated with 2-aminopyridine [15]. After desalting of the reaction mixture on Sephadex G-15 using 10 mM ammonium acetate pH 6.0 as the eluent, the pyridylamino-oligosaccharides were pooled and lyophilized. PLA-20 and concanavalin A fractions of PLA were prepared as described [10, 11].

#### Exoglycosidase digestions

Incubations with exoglycosidases were generally performed with 0.1 nmol pyridylamino-oligosaccharide for 20 h

at 37°C. The following buffers and enzyme quantities were employed for selective deglycosylation: 0.1 or 10 mU of  $\alpha$ -fucosidase from bovine kidney in 50 mM sodium citrate, pH 4.5; 5 or 25 mU  $\alpha$ -mannosidase in 50 mM sodium acetate, 0.1 M  $\text{ZnCl}_2$ , pH 4.2; 5 mU  $\beta$ -*N*-acetylhexosaminidase in 0.1 M sodium citrate pH 5.0; 0.01 mU  $\alpha$ -fucosidase from almonds in 50 mM sodium acetate pH 5.0.

#### Reference oligosaccharides

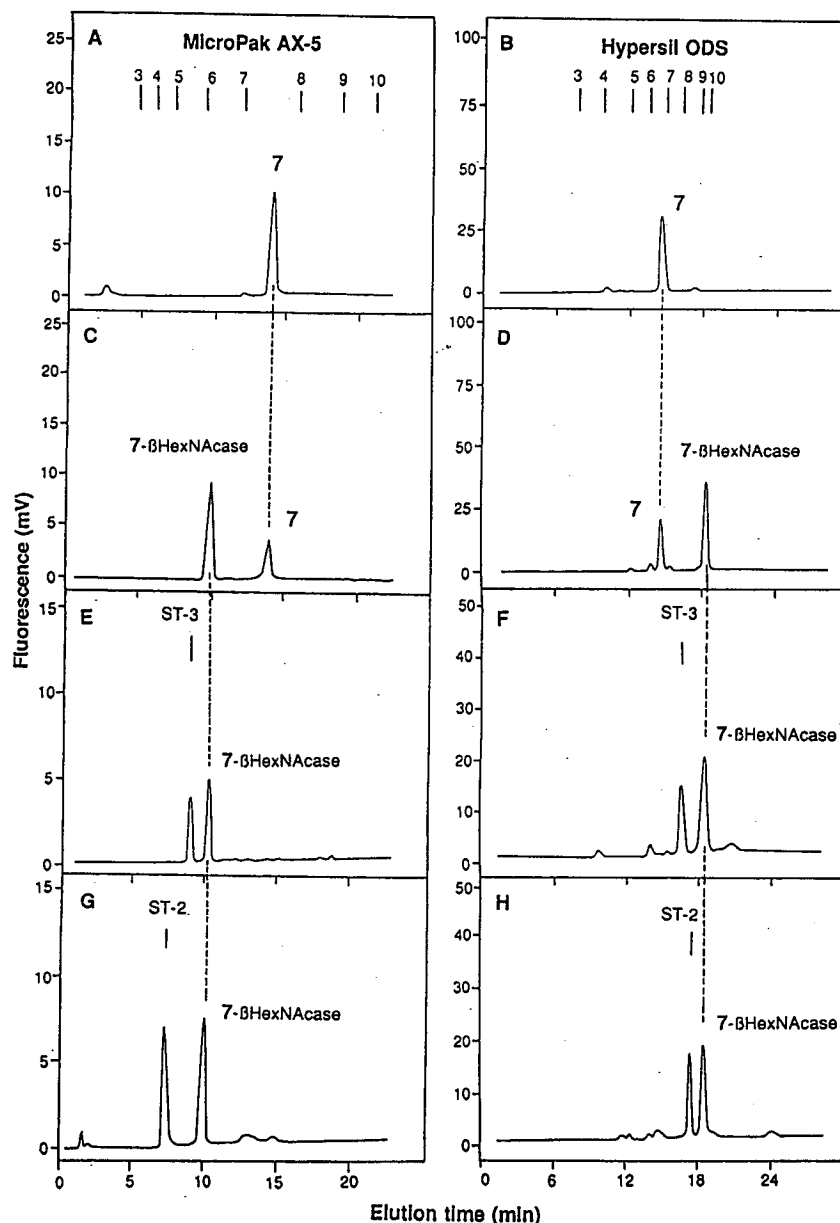
Degradation of the pyridylaminated asialo-oligosaccharides from human IgG [16] with  $\beta$ -galactosidase and  $\beta$ -*N*-acetylhexosaminidase yielded  $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4(\text{Fuc}\alpha 1-6)\text{GlcNAc}$ -pyridylamine (ST-1) as the major product. Likewise,  $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$ -pyridylamine (ST-2) was obtained from bovine fibrin [16]. Limited digestion of the agalacto-pyridylamino-oligosaccharides of fibrin with  $\beta$ -*N*-acetylhexosaminidase (as above but with 5 mU/100 nmol) followed by isolation of the products by HPLC yielded  $\text{Man}\alpha 1-6(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$ -pyridylamine (ST-3) and  $\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$ -pyridylamine (not used as reference compound), which are easily discernible by HPLC [17]. Stepwise degradation of these two structures with  $\alpha$ -mannosidase and  $\beta$ -*N*-acetylhexosaminidase afforded  $\text{Man}\alpha 1-3\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$ -pyridylamine and  $\text{Man}\alpha 1-6\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$ -pyridylamine (ST-4), respectively.  $\text{Man}\alpha 1-6\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4(\text{Fuc}\alpha 1-3)\text{GlcNAc}$ -pyridylamine (ST-5) was obtained from pineapple stem bromelain [18, 19] by treatment with  $\beta$ -xylosidase. The structures of these standard oligosaccharides were confirmed by methylation analysis (data not shown). Pyridylaminated isomaltose oligosaccharides ranging over 1–20 Glc units were prepared from an acid hydrolysate of dextran.

#### Fractionation of pyridylaminated oligosaccharides

Fractionation according to size was accomplished on a MicroPak AX-5 HPLC-column (4 × 300 mm, Varian) exactly as described previously [17, 20]. Individual peaks were collected, rechromatographed if necessary, and lyophilized. Further separation was achieved by reverse-phase HPLC on a 5- $\mu\text{m}$  Shandon-Hypersil ODS column (4 × 250 mm; ÖFZ Seibersdorf, Austria). This column was equilibrated with 0.1 M ammonium acetate pH 4.0 and eluted with a linear gradient of 0–7.5% (by vol.) methanol in 25 min at a flow

**Table 6. Exoglycosidase digestions of fractions 7–10.**

Substrate	Glycosidase dose	Elution positions of products on		Product coeluting with
		MicroPak AX-5	Hypersil ODS	
	mU	Glc units		
<b>7</b>	$\beta$ -HexNAcase 5	6.2	9.7	(= <b>7<math>\beta</math>-HexNAcase</b> )
<b>8</b>		6.6	16.0	
<b>9</b>		7.3	6.3	
<b>10</b>		7.8	8.9	
<b>7</b>	$\alpha$ -fucosidase 5	7.5	6.7	( <b>7</b> )
<b>7</b>	$\beta$ -HexNAcase 5 + $\alpha$ -fucosidase 0.5	4.7	8.7	<b>3-B</b> ST-3
<b>7<math>\beta</math>-HexNAcase</b>		$\alpha$ -fucosidase 0.5	5.7	



**Fig. 3. Elution pattern of pyridylamino-oligosaccharide 7 upon stepwise degradation with exoglycosidases.** The following samples were analyzed: compound 7 (A, B); 7 incubated with  $\beta$ -*N*-acetylhexosaminidase (C, D) to yield a product termed 7- $\beta$ -HexNAcase; 7- $\beta$ -HexNAcase incubated with  $\alpha$ -fucosidase after complete inactivation of  $\beta$ -*N*-acetylhexosaminidase (E, F);  $\alpha$ -fucosidase treatment of 7- $\beta$ -HexNAcase without prior removal of  $\beta$ -*N*-acetylhexosaminidase (G, H).

rate of 1.5 ml/min. In both HPLC systems, pyridylamino-oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 nm and 400 nm, respectively. Glucose oligomers, (Glc)<sub>n</sub> were used for calibration of both columns and as internal standards; retention times are expressed in terms of Glc units *n* [21].

#### Analytical techniques

Monosaccharides were analyzed as their alditol acetates after hydrolysis with 2 M trifluoroacetic acid for 4 h at 100°C [15]. Hexosamines were quantitated by HPLC of the corresponding alditols using precolumn derivatization with *o*-phthalaldehyde according to [22]. *N*-Acetyl- and *N*-glycolyl-neuraminic acids were analyzed after acid hydrolysis

and reaction with 1,2-diamino-4,5-methylene-dioxybenzene [23]. Pyridylamino-oligosaccharides were permethylated by the method of Ciucanu and Kerek [24, 25], and partially methylated alditol acetates were analyzed by GLC/MS [15].

#### <sup>1</sup>H-NMR spectroscopy

Prior to <sup>1</sup>H-NMR analysis, carbohydrate samples were exchanged twice in 99.8% <sup>2</sup>H<sub>2</sub>O. Then samples were dissolved in 450  $\mu$ l 99.96% <sup>2</sup>H<sub>2</sub>O and transferred to NMR tubes. <sup>1</sup>H-NMR spectra at 500 MHz were recorded on a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR spectroscopy, Utrecht University) as described [26]. The probe temperature was 27°C. Chemical shifts are expressed by reference to internal acetone ( $\delta$  = 2.225 ppm) [27].

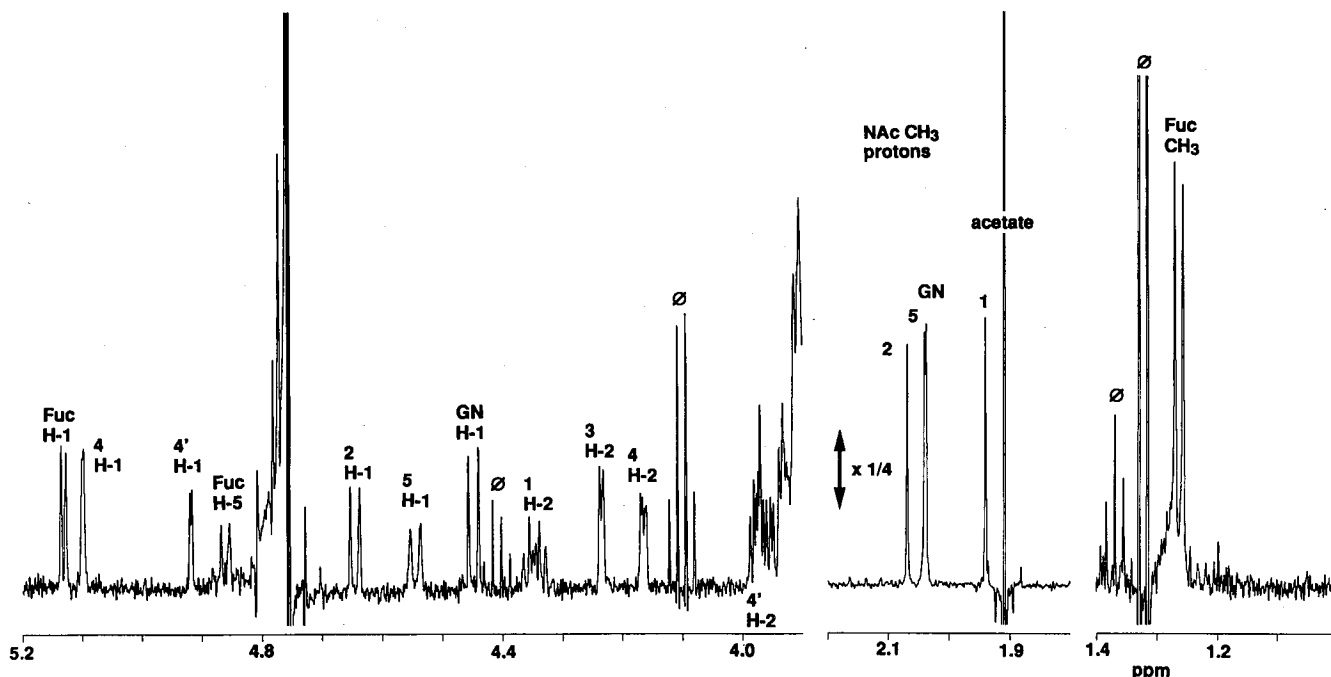


Fig. 4. Structural-reporter-group regions of the resolution-enhanced 500-MHz  $^1\text{H}$ -NMR spectrum of compound **7** recorded in  $^2\text{H}_2\text{O}$  at  $27^\circ\text{C}$ . The numbers in the spectrum refer to the corresponding residues in the structure. The relative intensity scale of the *N*-acetyl and Fuc  $\text{CH}_3$  regions differs from that of the other parts of the spectrum as indicated. Signals close to the HOD line appear with slightly reduced intensity due to the applied water suppression scheme.  $\phi$ , signals stemming from non-carbohydrate material.

## RESULTS

### Separation of pyridylamino-oligosaccharides

The oligosaccharides released from phospholipase  $A_2$  by peptide-*N*-glycanase A were pyridylaminated and size-fractionated on MicroPak AX-5 (Fig. 1A), yielding fractions 1–10. This technique separates primarily according to the number of monosaccharide residues in an oligosaccharide [17, 28], although other factors can exert profound effects on retention (see below). The MicroPak-fractions 3, 4 and 5 could be further fractionated by reverse-phase HPLC (Fig. 1B shows the profile of unfractionated oligosaccharide pool) to yield a total of 14 subfractions (Fig. 1). Their exact elution positions in both HPLC systems were determined and compared with those of defined reference compounds (Table 1). Extensive collections of retention data of pyridylaminated N-glycans exist [17, 21, 29]. However, some differences in the relative elution times, and in a few cases even reversed elution orders of closely eluting peaks, have been observed in the present work. While this may be explained by the different column and organic modifier used, it demonstrates the necessity of verifying conclusions drawn from the two-dimensional mapping either by the use of authentic reference compounds or by additional techniques of structural analysis.

### Structural analysis

The distinctive effects of fucosylation of the reducing-end GlcNAc on the elution behavior of pyridylamino-oligosaccharides [16, 21, 29, 30] were of great help in the structural assignments, especially since in permethylation analyses the derivative of the pyridylaminated GlcNAc can usually not be found. Briefly, in the MicroPak system, an  $\alpha$ 1,3-linked Fuc residue counts as 1.0 Glc unit and an  $\alpha$ 1,6-

linked Fuc as 0.4 Glc unit. On reverse-phase HPLC,  $\alpha$ 1,3-fucosylation causes a significant decrease, and  $\alpha$ 1,6-fucosylation a considerable increase in elution time [30]. Furthermore,  $\alpha$ -L-fucosidase from bovine kidney removes Fuc linked  $\alpha$ 1-6 to the inner GlcNAc residue much faster (about 20 times, data not shown) or at much lower enzyme concentrations than an  $\alpha$ 1,3-linked residue [31]. Likewise, the preferential cleavage of the  $\alpha$ 1,3-linked Man of the pentasaccharide core by  $\alpha$ -mannosidase from jack beans [32] was helpful. To exploit these glycosidase specificities, most digestions were performed with various enzyme concentrations.

Oligosaccharides **1** and **3-B** consisted of Man and GlcNAc only. The product of limited hydrolysis of **3-B** with  $\alpha$ -mannosidase eluted like **1** (Table 2). With a higher dose of enzyme, the apparent size of both glycans decreased to that of Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc-pyridylamine (Table 2) and the products were now sensitive to  $\beta$ -mannosidase (data not shown). Methylation analysis and the coelution of **1** and **3-B** with added standards (ST-4 and ST-1, respectively) supported the structural assignment given in Table 8.

Oligosaccharides **3-C** and **5-B** also consisted of Man and GlcNAc only.  $\alpha$ -Mannosidase converted **5-B** readily into an intermediate product with the elution positions of **3-C** (product I in Table 2), and this compound, just as **3-C** itself, was subsequently degraded to **1** (II in Table 2). Since **1** and its structural isomer, having an  $\alpha$ 1,3-linked instead of an  $\alpha$ 1,6-linked Man, are easily distinguishable on reverse-phase HPLC (data not shown), it was concluded that the  $\beta$ -Man residue in **3-C** was substituted at position 6. Adding the data from methylation analysis, the structure of **3-C** was deduced as shown in Table 8. Methylation analysis indicated that **5-B** differs from **3-C** by the di-substitution of the  $\beta$ -Man residue (Table 8). The results of the  $\alpha$ -mannosidase degradations are thus in agreement with the reported sequence of action of this enzyme [32].

**Table 7.**  $^1\text{H}$  chemical shifts of structural-reporter-group protons of the constituent monosaccharides of the pyridylaminated N-linked oligosaccharides **7** and **9**, derived from honeybee venom PLA, and of the Man $\alpha$ 1-3 branch of reference compound N1.4A. Chemical shifts are given at 27°C and were measured in  $^2\text{H}_2\text{O}$  relative to acetone ( $\delta = 2.225$  ppm [27]). For numbering of the monosaccharide residues, see text. Superscript numbers at the name of a monosaccharide residue indicate the linkage positions of the residue itself and of the adjacent residues.

Reporter group	Residue	Chemical shift in		
		7	9	GalNAc $\beta$ 1-4GlcNAc $\beta$ 1-2Man $\alpha$ 1-3 Fuc $\alpha$ 1-3 from N1.4A [36]
ppm				
H-1	GlcNAc-2	4.643	4.604	
	Man-4	5.096	5.105	5.096
	Man-4'	4.917	4.922	
	GlcNAc-5	4.543	4.547	4.546
	GalNAc-GN	4.447	4.448	4.445
	Fuc <sup>3,2</sup>	5.130	5.132	5.127
	Fuc <sup>3</sup>	—	5.052	
H-2	GlcNAc-1	4.345	4.48 <sup>a</sup>	
	Man-3	4.234	4.246	
	Man-4	4.163	4.167	4.164
	Man-4'	<4.0	3.983	
H-5	Fuc <sup>3,2</sup>	4.860	4.862	4.862
	Fuc <sup>3</sup>	—	4.25 <sup>a</sup>	
NAc	GlcNAc-1	1.939	1.931	
	GlcNAc-2	2.068	2.050 <sup>c</sup>	
	GlcNAc-5	2.039 <sup>b</sup>	2.038 <sup>c</sup>	2.038
	GalNAc-GN	2.037 <sup>b</sup>	2.045 <sup>c</sup>	2.038
CH <sub>3</sub>	Fuc <sup>3,2</sup>	1.262	1.27 <sup>a</sup>	1.260
	Fuc <sup>3</sup>	—	1.20 <sup>a</sup>	

<sup>a</sup> Obtained from a two-dimensional homonuclear Hartmann-Hahn experiment.

<sup>b</sup> Assignments may have to be interchanged.

<sup>c</sup> Assignments may have to be interchanged.

Linkage analysis of oligosaccharides **2** and **4-B** closely resembled that of structures **1** and **3-B**, respectively. The glycans were easily converted into **1** and **3-B**, respectively, by treatment with  $\alpha$ -fucosidase (Table 3). The elution positions of **4-B** coincided with those of the fucosylated core pentasaccharide Man<sub>3</sub>GlcNAc<sub>2</sub>-pyridylamine derived from human IgG (ST-1). Mild  $\alpha$ -mannosidase digestion converted structure **4-B** into **2** (Table 2). Obviously, **2** and **4-B** are the  $\alpha$ 1,6-fucosylated analogues of **1** and **3-B**, respectively. Oligosaccharide **4-B** has recently been isolated from PLA by different methods and identified by  $^1\text{H-NMR}$  spectroscopy [12].

Oligosaccharides **3-A** and **5-A** exhibited monosaccharide compositions and linkage analysis data comparable to those of glycans **2** and **4-B**, respectively (Table 4), but quite different elution positions on both HPLC columns (Table 1). **5-A** was transformed into **3-A** by a low dose of  $\alpha$ -mannosidase (Table 2). When applied at high concentration,  $\alpha$ -fucosidase converted **3-A** and **5-A** into **1** and **3-B**, respectively (Table 3). The coelution of **3-A** with the dextrosylated glycan from pineapple bromelain (ST-5) supported the conclusion that **3-A** and **5-A** are  $\alpha$ 1,3-fucosylated at the inner GlcNAc residue (Table 8).

Oligosaccharide **6** contained a trimannosyl core (Table 4) and was converted into **4-A** with a low dose of  $\alpha$ -mannosidase (Table 2). Upon limited digestion with  $\alpha$ -fucosidase, **4-A** and **6** moved to the elution positions of **3-A** and **5-A**, respectively (Table 3 and Fig. 2). Higher amounts of  $\alpha$ -fucos-

idase led to a further decrease in size and to a substantial increase of retention time on the reverse-phase column (Fig. 2). This behavior indicated the initial removal of  $\alpha$ 1,6-linked Fuc followed by the slow hydrolysis of the  $\alpha$ 1,3-linked fucosyl residue (compare with glycans **3-A** and **5-A**) to yield compounds **1** and **3-B**, respectively. Therefore, the structures for **4-A** and **6** are as presented in Table 8. Compound **6** corresponds to the di-fucosylated glycan previously described [12].

Oligosaccharide **7** was clearly distinguished from the structures described so far, since it contained an additional GlcNAc and a terminal GalNAc residue (hexosamine analysis, Table 5; methylation analysis, Table 4). Incubation of **7** with a high dose of  $\alpha$ -mannosidase caused a shift on MicroPak AX-5 corresponding with the release of one Man residue (Table 2), but  $\alpha$ -fucosidase did not release any Fuc (Table 6). If applied in a rather high concentration, jack bean  $\beta$ -N-acetylhexosaminidase acted on compound **7** (Table 6 and Fig. 3C, D), yielding a product still containing two residues of GlcNAc, but no GalNAc (Table 5). The subsequent addition to this reaction mixture (still containing  $\beta$ -N-acetylhexosaminidase activity) of  $\alpha$ -fucosidase released Fuc, affording **3-B** (Table 6 and Fig. 3G, H). However, addition of  $\alpha$ -fucosidase after removal of the  $\beta$ -N-acetylhexosaminidase resulted in the formation of another product, which displayed the elution behavior of reference ST-3, i.e. the core pentasaccharide Man<sub>3</sub>GlcNAc<sub>2</sub>-pyridylamine with an additional

**Table 8. Structures of N-glycans from honeybee venom phospholipase A<sub>2</sub>.**

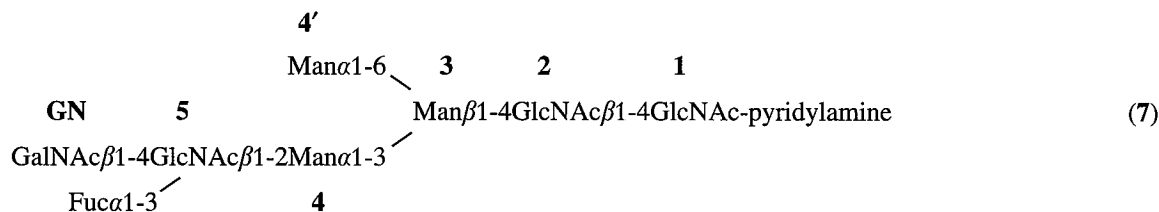
Fraction	Structure	Relative abundance in PLA from		
		Nectarcorp	Mraz	Mack
		%		
1	$\begin{array}{l} \text{Man}\alpha 1-6 \\ \quad \backslash \\ \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \end{array}$	12.0	14.6	13.6
2	$\begin{array}{l} \text{Man}\alpha 1-6 \qquad \text{Fuca}\alpha 1-6 \\ \quad \backslash \qquad \quad \backslash \\ \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \end{array}$	4.4	3.4	3.2
3-A	$\begin{array}{l} \text{Man}\alpha 1-6 \\ \quad \backslash \\ \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \qquad \qquad \qquad \text{Fuca}\alpha 1-3 \end{array}$	6.6	5.9	6.1
3-B	$\begin{array}{l} \text{Man}\alpha 1-6 \\ \quad \backslash \\ \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \text{Man}\alpha 1-3 \end{array}$	31.4	30.6	31.8
3-C	$\begin{array}{l} \text{Man}\alpha 1-6 \\ \text{Man}\alpha 1-3 \quad \backslash \\ \qquad \qquad \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \end{array}$	7.4	4.7	5.0
4-A	$\begin{array}{l} \text{Man}\alpha 1-6 \qquad \text{Fuca}\alpha 1-6 \\ \quad \backslash \qquad \quad \backslash \\ \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \qquad \qquad \qquad \text{Fuca}\alpha 1-3 \end{array}$	1.8	2.1	2.2
4-B	$\begin{array}{l} \text{Man}\alpha 1-6 \qquad \text{Fuca}\alpha 1-6 \\ \quad \backslash \qquad \quad \backslash \\ \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \text{Man}\alpha 1-3 \end{array}$	11.1	8.0	8.6
5-A	$\begin{array}{l} \text{Man}\alpha 1-6 \\ \quad \backslash \\ \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \text{Man}\alpha 1-3 \qquad \qquad \text{Fuca}\alpha 1-3 \end{array}$	3.9	6.6	6.7
5-B	$\begin{array}{l} \text{Man}\alpha 1-6 \\ \text{Man}\alpha 1-3 \quad \backslash \\ \qquad \qquad \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \text{Man}\alpha 1-3 \end{array}$	10.1	8.0	9.0
6	$\begin{array}{l} \text{Man}\alpha 1-6 \qquad \text{Fuca}\alpha 1-6 \\ \quad \backslash \qquad \quad \backslash \\ \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \text{Man}\alpha 1-3 \qquad \qquad \text{Fuca}\alpha 1-3 \end{array}$	3.7	4.2	4.2
7	$\begin{array}{l} \text{Man}\alpha 1-6 \\ \quad \backslash \\ \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \text{GalNAc}\beta 1-4\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3 \\ \text{Fuca}\alpha 1-3 \end{array}$	4.7	7.1	5.6



Table 8. (continued).

Fraction	Structure	Relative abundance in PLA from		
		Nectarcorp	Mraz	Mack
		%		
8	$\begin{array}{c} \text{Man}\alpha 1-6 \quad \text{Fuca}\alpha 1-6 \\ \quad \quad \quad \diagdown \quad \quad \quad \diagdown \\ \quad \quad \quad \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \quad \quad \quad \diagup \\ \text{GalNAc}\beta 1-4\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3 \\ \quad \quad \quad \diagup \\ \text{Fuca}\alpha 1-3 \end{array}$	1.3	1.9	1.3
9	$\begin{array}{c} \text{Man}\alpha 1-6 \\ \quad \quad \quad \diagdown \\ \quad \quad \quad \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \quad \quad \quad \diagup \\ \text{GalNAc}\beta 1-4\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3 \\ \quad \quad \quad \diagup \\ \text{Fuca}\alpha 1-3 \end{array}$	1.2	1.9	1.8
10	$\begin{array}{c} \text{Man}\alpha 1-6 \quad \text{Fuca}\alpha 1-6 \\ \quad \quad \quad \diagdown \quad \quad \quad \diagdown \\ \quad \quad \quad \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \\ \quad \quad \quad \diagup \\ \text{GalNAc}\beta 1-4\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3 \\ \quad \quad \quad \diagup \\ \text{Fuca}\alpha 1-3 \end{array}$	0.4	1.0	0.9

GlcNAc residue linked to the Man $\alpha$ 1-3 arm (Table 6 and Fig. 3E, F). The  $^1\text{H-NMR}$  spectrum of **7** (Fig. 4) indicates the presence of the following novel pyridylaminated octasaccharide:



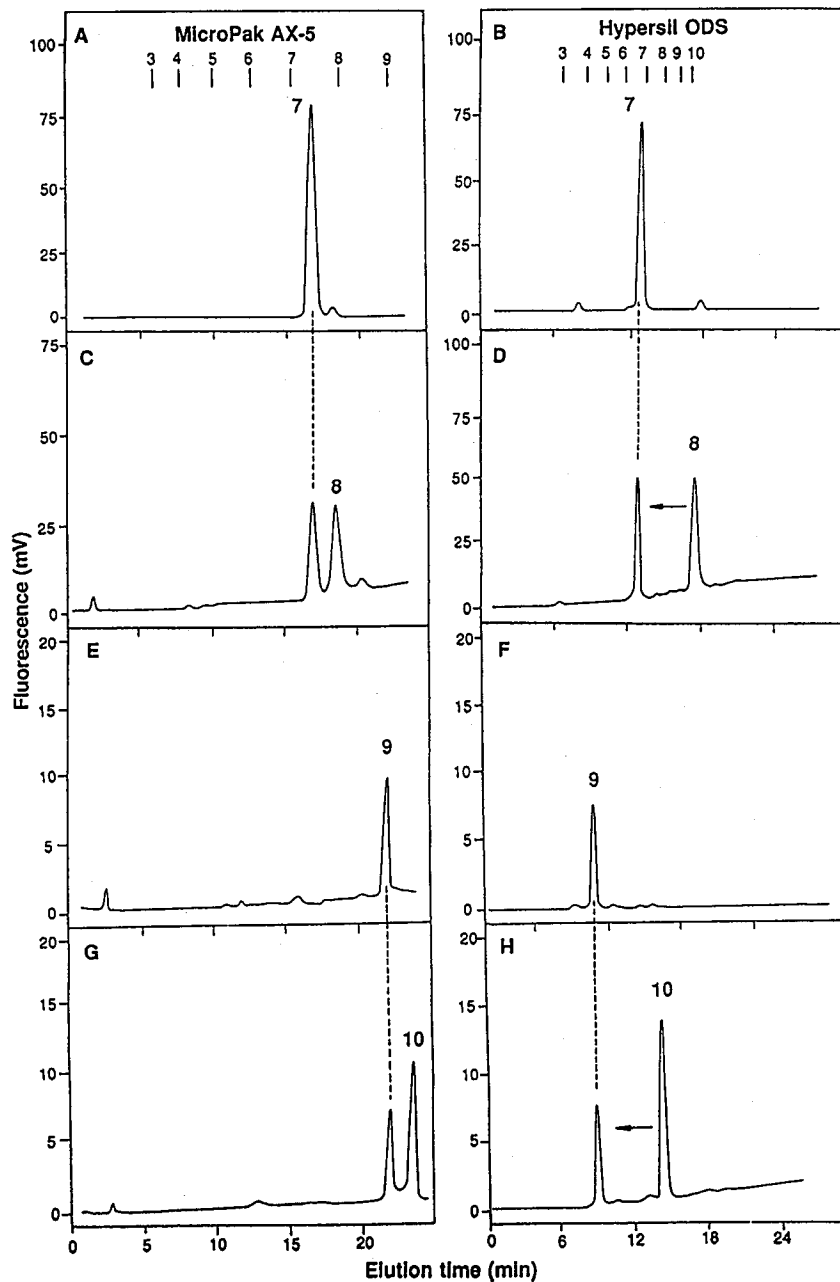
Relevant  $^1\text{H}$  chemical shifts of **7** are compiled in Table 7. The pyridylaminated GlcNAc $\beta$ 1-4GlcNAc element is evident from the H-2 and NAc methyl signals of GlcNAc-1, and the H-1 and NAc methyl signals of GlcNAc-2 [33–35]. The Man H-1/H-2 patterns indicate a trimannosyl structure with a terminal Man-4' [27]. Finally, all structural-reporter-group signals of the GalNAc $\beta$ 1-4(Fuca $\alpha$ 1-3)GlcNAc $\beta$ 1-2Man $\alpha$ 1-3 element match those observed for the same Man $\alpha$ 1-3 branch in an N-linked diantennary oligosaccharide obtained from human urokinase (see N1.4A in [36]).

Oligosaccharide **9** contained one GalNAc and two GlcNAc residues like **7** (Table 5), but appeared to be larger than **7** by approximately one Glc unit (Table 1). On reverse-phase HPLC, **9** eluted 4.6 min before **7**. This retention-time shift is typical for  $\alpha$ 1,3-fucosylation of the innermost GlcNAc as was observed with the structural pairs 1/3-A (4.9 min) and 3-B/5-A (4.1 min). Incubation of **9** with almond  $\alpha$ -fucosidase led to an increase of retention-time of only 0.7 Glc unit on reverse-phase HPLC and to a decrease of 0.9 Glc unit on Micro-Pak, indicating the selective removal of the antennary Fuc residue. The  $^1\text{H-NMR}$  spectrum of **9** (not shown) is very similar to that of **7**, except for a set of signals characteristic for an  $\alpha$ 1,3-linked Fuc to GlcNAc-1 (Table 7) [35, 37, 38].

Incubation of compounds **7** and **9** with  $\alpha$ -fucosidase from almonds decreased their elution time on MicroPak AX-5 by 0.9–1.0 Glc unit. On reverse-phase HPLC, the products eluted later by 1.8 Glc unit (**7**) and 0.8 (**9**) Glc unit. Thus **9** is

not converted into **7** and we conclude that almond fucosidase removes the Fuc residue, which is  $\alpha$ 1,3-linked to the antennary GlcNAc in compounds **7** and **9**. This compares to the reported action of almond fucosidase on glycans containing the Le $^x$  antigen [39].  $\alpha$ -Fucosidase from almonds did not release Fuc residues linked to the innermost GlcNAc from compound **6** and others under the conditions employed.

Oligosaccharides **8** and **10** were not available in amounts sufficient to allow for chemical analyses other than determination of hexosamines (Table 5). The ratio of GlcNAc/GalNAc resembled that in **7** and **9**. The structures were susceptible to  $\beta$ -N-acetylhexosaminidase and  $\alpha$ -mannosidase and both the doses required, as well as the observed retention-time shifts, correlated with the respective results for compounds **7** and **9** (Tables 2 and 6) indicating the release of one GalNAc and one Man residue, respectively. Treatment of **8** and **10** with  $\alpha$ -fucosidase from bovine kidney, at an enzyme concentration which had been shown with **2**, **4-B**, **4-A**, **6** and ST-1 to be just sufficient to remove  $\alpha$ 1,6-linked Fuc from the innermost GlcNAc residue, afforded products eluting at the positions of **7** and **9**, respectively (Table 3 and Fig. 4). The significant retardation of **8** and **10** on reverse-phase HPLC effected by this Fuc residue further indicates that Fuc is  $\alpha$ 1,6-linked to the innermost GlcNAc. A Fuc residue linked  $\alpha$ 1,6



**Fig. 5. Elution patterns of pyridylamino-oligosaccharides 8 and 10 obtained upon treatment with  $\alpha$ -fucosidase.** Glycans 7 and 9 served as reference compounds (A, B and E, F, respectively). The product of a digest of 8 with  $\alpha$ -fucosidase (C, D) appears at the elution positions of 7. Chromatograms G and H demonstrate that the product of the treatment of 10 with  $\alpha$ -fucosidase elutes like 9.

to an antennary GlcNAc, the only other conceivable attachment site in 8 and 10, is reported to affect the elution behavior only marginally [29, 40]. Finally, this means that compounds 7–10 are structural analogues exhibiting the same pattern of fucosylation of the innermost GlcNAc as the other, smaller oligosaccharides.

#### Sialic acids

No *N*-acetyl- and *N*-glycolyl-neuraminic acids could be found at a level of sensitivity that would have guaranteed the detection of 0.2 mmol sialic acid/mol glycoprotein.

#### Oligosaccharide profiles of phospholipase from different venom batches

PLA was isolated from honeybee venom produced in three different geographical regions (Bulgaria, Germany, USA) and their carbohydrate moieties were analyzed by the two-dimensional mapping technique described above. The three batches exhibited the same qualitative and very similar quantitative glycan profiles (Table 8).

#### Analysis of fractions of phospholipase

The glycosylated variants of PLA can be fractionated on immobilized concanavalin A by successive desorption with 15 mM and 500 mM methyl  $\alpha$ -D-mannopyranoside [11].

Analysis of the pyridylaminated oligosaccharides derived from these two fractions revealed that those compounds having a complete trimannosyl core exhibited higher affinity to the lectin than those without an  $\alpha$ 1-3 branch at the  $\beta$ -Man residue. Thus, compounds **1**, **2**, **3-A**, **3-C**, **4-A**, but as the exception to the rule also **5-B**, could be eluted with only 15 mM methyl  $\alpha$ -D-mannopyranoside. Although the GalNAc-containing oligosaccharides were mainly found in the 500 mM methyl  $\alpha$ -D-mannopyranoside fraction, small amounts also appeared in the weakly binding fractions.

The oligosaccharides of the electrophoretically largest isoform of phospholipase, PLA-20, were investigated by the same procedure as applied to the unfractionated phospholipase. The preparation of PLA-20, which was obtained in a purity of at least 80%, was found to contain predominantly the structures **7**, **8**, **9** and **10**. The relatively large size of these GalNAc-containing carbohydrate chains explains the different electrophoretic migration of PLA-20 [10].

## DISCUSSION

Small N-linked oligomannose-type structures and their  $\alpha$ 1,6-fucosylated analogues together account for approximately 70% of the glycans of honeybee venom phospholipase A<sub>2</sub>. Such structures have already been found in several other insect glycoproteins [2, 3, 5, 12].

In PLA, however, Fuc additionally occurs in  $\alpha$ 1,3-linkage to the asparagine-bound GlcNAc, leading to structures with mono- and di-fucosylated cores. In plant glycoproteins, the asparagine-linked GlcNAc is likewise  $\alpha$ 1,3-fucosylated, and there is evidence that this common structural element significantly contributes to the immunological cross-reactivity of plant and insect glycoproteins [19, 41, 42]. Remarkably, anti-(horseradish peroxidase) serum is used for the detection of a neural-specific carbohydrate antigen in *Drosophila* [19, 43, 44].

Although mammalian and probably also plant fucosyltransferases require the foregoing action of *N*-acetylglucosaminyltransferase I [45, 46], the small  $\alpha$ 1,6 and/or  $\alpha$ 1,3-fucosylated structures found in PLA do not display the GlcNAc $\beta$ 1-2Man $\alpha$ 1-3 moiety. However, as can be deduced from the GalNAc-containing structures **7**–**10**, GlcNAc-transferase I is actively expressed in the venom gland cells. This observation constitutes a further analogy to some plant N-glycoproteins in which the peripheral GlcNAc is either substituted or absent even though the innermost GlcNAc residue is fucosylated [31]. It is at the moment not clear whether these fucosylated structures can be explained by a different specificity of the insect fucosyltransferases or by the subsequent removal of the GlcNAc residue. The presence of significant levels of  $\beta$ -*N*-acetylhexosaminidase in bee venom (data not shown) renders the latter pathway feasible.

Recently, the GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-2 moiety has been described as a structural element of the N-linked diantennary carbohydrate chains of human urokinase [36] and *Schistosoma mansoni* glycoproteins [47]. Moreover, this terminal trisaccharide element was found in the allergenically active O-glycans from a sea squirt, where it is  $\beta$ 1,3 and/or  $\beta$ 1,6-linked to GalNAc [48].

The present study is the first complete analysis of the N-linked carbohydrate chains from a pure, naturally occurring, insect glycoprotein; it reveals that insects are capable of performing chain-elongation reactions starting with the transfer of a GlcNAc residue to the Man $\alpha$ 1-3Man $\beta$  arm. However,

with regard to the lack of sialic acids, to the fucosylation pattern at the asparagine-bound GlcNAc and to the type of the antennary structure, the oligosaccharides reported here differ substantially from the usual *N*-acetylglucosamine type carbohydrate chains found in mammalian glycoproteins.

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## REFERENCES

1. Rademacher, T. W., Parekh, R. B. & Dwek, R. A. (1988) *Annu. Rev. Biochem.* **57**, 785–838.
2. Kuroda, K., Geyer, H., Geyer, R., Doerfler, W. & Klenk, H.-D. (1990) *Virology* **174**, 418–429.
3. Williams, P. J., Wormald, M. R., Dwek, R. A., Rademacher, T. W., Parker, G. F. & Roberts, D. R. (1991) *Biochim. Biophys. Acta* **1075**, 146–153.
4. Chen, W., Shen, Q.-X. & Bahl, O. P. (1991) *J. Biol. Chem.* **266**, 4081–4087.
5. Wathen, M. W., Aeed, P. A. & Elhammer, A. P. (1991) *Biochemistry* **30**, 2863–2868.
6. Davidson, D. J. & Castellino, F. J. (1991) *Biochemistry* **30**, 6167–6174.
7. Davidson, D. J. & Castellino, F. J. (1991) *Biochemistry* **30**, 6689–6696.
8. Nordvall, S. L., Uhlin, T., Einarsson, R., Johansson, S. G. O. & Öhman, S. (1984) *Clin. Allergy* **14**, 341–350.
9. Kuchler, K., Gmachl, M., Sippl, M. J. & Kreil, G. (1989) *Eur. J. Biochem.* **184**, 249–254.
10. Altmann, F., Kubelka, V., Staudacher, E., Uhl, K. & März, L. (1991) *Insect Biochem.* **21**, 467–472.
11. Weber, A., Schröder, H., Thalberg, K. & März, L. (1987) *Allergy* **42**, 464–470.
12. Staudacher, E., Altmann, F., März, L., Hård, K., Kamerling, J. P. & Vliegthart, J. F. G. (1992) *Glycoconjugate J.* **9**, 82–85.
13. Kiss, L., Berki, L. K. & Nánási, P. (1981) *Biochem. Biophys. Res. Commun.* **98**, 792–799.
14. Tretter, V., Altmann, F. & März, L. (1991) *Eur. J. Biochem.* **199**, 647–652.
15. Hase, S., Ibuki, T. & Ikenaka, T. (1984) *J. Biochem. (Tokyo)* **95**, 197–203.
16. Staudacher, E., Kubelka, V. & März, L. (1992) *Eur. J. Biochem.* **207**, 987–993.
17. Tomiya, N., Awaya, J., Kurono, M., Endo, S., Arata, Y. & Takahashi, N. (1988) *Anal. Biochem.* **171**, 73–90.
18. Van Kuik, J. A., Hoffmann, R. A., Mutsaers, J. H. G. M., van Halbeek, H., Kamerling, J. P. & Vliegthart, J. F. G. (1986) *Glycoconjugate J.* **3**, 27–34.
19. Kurosaka, A., Yano, A., Itoh, N., Kuroda, Y., Nakagawa, T. & Kawasaki, T. (1991) *J. Biol. Chem.* **266**, 4168–4172.
20. Natsuka, S., Hase, S. & Ikenaka, T. (1987) *Anal. Biochem.* **167**, 154–159.
21. Tomiya, N., Kurono, M., Ishihara, H., Tejima, S., Endo, S., Arata, Y. & Takahashi, N. (1987) *Anal. Biochem.* **163**, 489–499.
22. Altmann, F. (1992) *Anal. Biochem.* **204**, 215–219.
23. Hara, S., Takemori, Y., Yamaguchi, M., Nakamura, M. & Ohkura, Y. (1987) *Anal. Biochem.* **164**, 138–145.
24. Ciucanu, I. & Kerek, F. (1984) *Carbohydr. Res.* **131**, 209–217.
25. Barr, J. R., Anumula, K. R., Vettese, M. B., Taylor, P. B. & Carr, S. A. (1991) *Anal. Biochem.* **192**, 181–192.

26. Hård, K., van Zadelhoff, G., Moonen, P., Kamerling, J. P. & Vliegthart, J. F. G. (1992) *Eur. J. Biochem.* 209, 895–915.
27. Vliegthart, J. F. G., Dorland, L. & Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem.* 41, 209–374.
28. Mellis, S. J. & Baenziger, J. U. (1981) *Anal. Biochem.* 114, 276–280.
29. Hase, S. & Ikenaka, T. (1990) *Anal. Biochem.* 184, 135–138.
30. Hase, S., Koyama, S., Daiyasu, H., Takemoto, H., Hara, S., Kobayashi, Y., Kyogoku, Y. & Ikenaka T. (1986) *J. Biochem. (Tokyo)* 100, 1–10.
31. Takahashi, N., Hitotsuya, H., Hanzawa, H., Arata, Y. & Kurihara, Y. (1990) *J. Biol. Chem.* 265, 7793–7798.
32. Oku, H., Hase, S. & Ikenaka, T. (1990) *Anal. Biochem.* 185, 331–334.
33. Kimura, Y., Hase, S., Kobayashi, Y., Kyogoku, Y., Ikenaka, T. & Funatsu, G. (1988) *J. Biochem. (Tokyo)* 103, 944–949.
34. Yamaguchi, H., Funaoka, H. & Iwamoto, H. (1992) *J. Biochem. (Tokyo)* 111, 388–395.
35. Kamerling, J. P. (1991) *Pure Appl. Chem.* 63, 465–472.
36. Bergwerff, A. A., Thomas-Oates, J. E., van Oostrum, J., Kamerling, J. P. & Vliegthart, J. F. G. (1992) *FEBS Lett.* 314, 389–394.
37. Hase, S., Koyama, S., Daiyasu, H., Takemoto, H., Hara, S., Kobayashi, Y., Kyogoku, Y. & Ikenaka, T. (1986) *J. Biochem. (Tokyo)* 100, 1–10.
38. Takahashi, N., Hitotsuya, H., Hanzawa, H., Arata, Y. & Kurihara, Y. (1990) *J. Biol. Chem.* 265, 7793–7798.
39. Scudder, P., Neville, D. C. A., Butters, T. D., Fleet, G. W. J., Dwek, R. A., Rademacher, T. W. & Jacob, G. S. (1990) *J. Biol. Chem.* 265, 16472–16477.
40. Lee, Y. C., Lee, B. I., Tomiya, N. & Takahashi, N. (1990) *Anal. Biochem.* 188, 259–266.
41. Faye, L. & Chrispeels, M. J. (1988) *Glycoconjugate J.* 5, 245–256.
42. Prenner, C., Mach, L., Glössl, J. & März, L. (1992) *Biochem. J.* 284, 377–380.
43. Snow, P. M., Patel, N. H., Harrelson, A. L. & Goodman, C. S. (1987) *J. Neurosci.* 7, 4137–4144.
44. Katz, F., Moats, W. & Jan, Y. N. (1988) *EMBO J.* 7, 3471–3477.
45. Longmore, G. D. & Schachter, H. (1982) *Carbohydr. Res.* 100, 365–392.
46. Johnson, K. D. & Chrispeels, M. J. (1987) *Plant Physiol.* 84, 1301–1308.
47. Srivatsan, J., Smith, D. F. & Cummings, R. D. (1992) *Glycobiology* 2, 445–452.
48. Ohta, M., Matsuura, F., Kobayashi, Y., Shigeta, S., Ono, K. & Oka, S. (1991) *Arch. Biochem. Biophys.* 290, 474–483.