

CHARACTERISTICS OF THE ASPARAGINE-LINKED OLIGOSACCHARIDE FROM HONEY-BEE VENOM PHOSPHOLIPASE A₂

EVIDENCE FOR THE PRESENCE OF TERMINAL N-ACETYLGLUCOSAMINE AND FUCOSE IN AN INSECT GLYCOPROTEIN

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Abstract—1. Eighty-eight % of phospholipase A₂ from honey-bee (*Apis mellifica*) venom is glycosylated.
2. Its single oligosaccharide exists in several structural variants, which represent consecutive stages of the "N-glycan processing pathway".
3. The carbohydrate carries terminal fucosyl and N-acetyl-glucosaminyl residues. This is in contrast to earlier reports which suggest the lack of respective glycosyl-transferases in insects.

INTRODUCTION

Phospholipase A₂ (EC 3.1.1.4) accounts for 12–14% of the dry weight of honey-bee (*Apis mellifica*) venom. This enzyme has been shown to be a glycoprotein with an oligosaccharide linked to asparagine-13 of the polypeptide (Shipolini *et al.*, 1974). Details of the carbohydrate structure have not yet been reported. Recent evidence suggests a divergence in the processing of asparagine-linked oligosaccharides ("N-glycans") between vertebrates and insects, based on the observation that mosquitoes lack sialyl, galactosyl, fucosyl and N-acetylglucosaminyl transferases (Butters and Hughes, 1981; Butters *et al.*, 1981). Action of these enzymes leads, in higher organisms, to the formation of, sometimes fucosylated, "complex" or of "hybrid" type N-glycans (Montreuil, 1980). In this paper we report evidence for the presence of terminal fucosyl and N-acetylglucosaminyl residues in the carbohydrate part of bee venom phospholipase A₂, which suggests that the transfer of these monosaccharides may not be restricted to vertebrates.

MATERIALS AND METHODS

Chemicals

Pooled lyophilized honey-bee venom (batches 00380 and 110092) were from H. Mack (Illertissen, GFR). Egg yolk lecithin (Type IX-E), phosphatase substrate (Sigma 104^R), hyaluronic acid (Grade III-P), Pronase (Type XXI), and α -L-fucosidase (EC 3.2.1.51; from bovine kidney) were purchased from Sigma, Munich, GFR. α -D-Mannosidase (EC 3.2.1.24, from *Canavalia ensiformis*) and β -N-acetylglucosaminidase (EC 3.2.1.30, from bovine kidney) were supplied by Boehringer-Mannheim, GFR. Endoglycosidase H (*Streptomyces griseus*) was from Miles, USA, N-succinimidyl-[³H]propionate (1 mCi per ml of toluene) from Amersham, UK. Sepharose 4B-Concanavalin A is a product of Pharmacia, Sweden, and Biogels were

obtained from Bio-Rad, Vienna, Austria. α -D-Methylmannoside was purchased from Fluka, Switzerland. All other chemicals were of the highest available purity.

Analytical procedures

Phospholipase A₂ (PA₂) activity was measured by titration of fatty acid liberated from egg yolk lecithin (Grossmann *et al.*, 1974), hyaluronidase (EC 3.2.1.35) was assayed turbidimetrically (Tolksdorf *et al.*, 1949), and acid phosphatase (EC 3.1.3.2) by measuring the cleavage of p-nitrophenyl phosphate (Linhart and Walter, 1962). Monosaccharides were analyzed by gas-liquid chromatography (GLC) according to Lehnhardt and Winzler (1968). The permethylation procedure and GLC analysis of the partially methylated and acetylated products are described elsewhere (Jansson *et al.*, 1976; März *et al.*, 1982).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was done according to Laemmli (1970). Gels were stained with Coomassie brilliant blue R-250 for protein and with the periodic acid-Schiff reagent (PAS) for carbohydrate (Fairbanks, 1971).

Preparations

Phospholipase A₂: 100 mg of crude venom were fractionated over Biogel P-60, 100–200 mesh, in 0.05 M ammonium acetate buffer, pH 4.75. Column size: 1.5 × 100 cm; fraction volume: 1.4 ml. PA₂-containing fractions were pooled, desalted and lyophilized. Amounts of this preparation equivalent to 2 mg of crude venom per ml of gel were applied to a column (88 ml) of Sepharose 4B-Concanavalin A equilibrated with 0.02 M Tris-HCl buffer, 0.5 M in NaCl, 1 mM in Ca²⁺ and Mn²⁺, pH 7.4. The column was eluted with 0.15 and 500 mM solutions of α -methyl mannose in the same buffer (Finne and Krusius, 1982). The PA₂-fractions thus obtained were finally chromatographed on a column (1.5 × 100 cm) of Biogel P-10, 200–400 mesh, in 0.05 M ammonium acetate buffer, pH 4.75.

Glycopeptides: a 2% solution of PA₂ in 0.15 M Tris-HCl, 1 mM in Ca²⁺, pH 7.8, was digested with repeated additions of Pronase (final amount: 5% of sample weight) at 37°C for 72 hr. The digest was desalted over Sephadex G-25 and chromatographed on Biogel P-6, 200–400 mesh, in 1%

acetic acid (column: 1.5×100 cm; fractions: 1.4 ml). Aliquots (100–300 nM) of the glycopeptide pool (GP) were tritium labeled by sonication with 50 μ l of *N*-succinimidyl- ^{3} H]propionate solution at pH 6.0, evaporated and desalted. Exoglycosidases: aliquots of glycopeptide solutions were treated with α -D-mannosidase, α -L-fucosidase, and *N*-acetyl- β -D-glucosaminidase, either sequentially or in combination, in 0.05 M sodium acetate buffer, pH 4.8, at 37°C, for 24 hr per enzyme added.

Endoglycosidase H (Endo H): 100 μ g of denatured phospholipase A₂ or 360 μ g of glycopeptide were treated with 4 μ u of Endo H in 0.15 M citrate-phosphate buffer, pH 5.3, for 24 hr at 37°C (Henner *et al.*, 1983).

RESULTS

Chromatography of bee-venom phospholipase A₂ (PA₂) on Sepharose 4B-Concanavalin A yields three fractions (Fig. 1), which account for 12 (PA₂-O), 33 (PA₂-15), and 55% (PA₂-500) of the total activity recovered. They are free of hyaluronidase, acid phosphatase, and melittin (Fig. 2). PA₂-O does not react with PAS-reagent; direct monosaccharide analysis is negative. Furthermore, its faster moving electrophoretic band can be generated by treatment of PA₂-15 with EndoH, an enzyme known to act upon high-mannosyl-type oligosaccharides by cleavage of the chitobiosyl core (Kobata, 1979). Obviously, PA₂-O lacks carbohydrate and thus represents the unglycosylated enzyme polypeptide.

Unfractionated "glycopeptide pool" (GP) contains mannose, fucose and glucosamine (*N*-acetylated, as shown by enzyme treatment). Sialic acid, galactose and *N*-acetylglucosamine, a monosaccharide known to occur "O-glycosidically" linked to protein (Montreuil, 1980), are absent (Table 1). Hydrazinolysis of glycopeptide, followed by re-*N*-acetylation, reduction with NaB ^{3} H₄, hydrolysis, again re-*N*-acetylation, and high voltage-paper-electrophoresis in 0.06 M sodium borate buffer, pH 9.5 (Takasaki

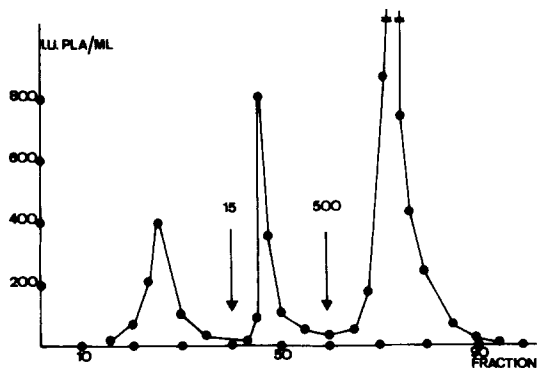


Fig. 1. Elution pattern of phospholipase A₂ on Sepharose 4B-Concanavalin A. The diagram reflects the amount obtained from 150 mg of crude bee venom after chromatography on Biogel P-60. For experimental details see the text. The arrows indicate the changes to 15 and 500 mM α -methylmannoside concentration, respectively. ●—●, International units of enzyme activity per ml of eluant.

*Abbreviations—Tris, tris(hydroxymethyl)-aminomethane; GlcNAc, *N*-acetyl-glucosamine; Man, mannose; Fuc, fucose.

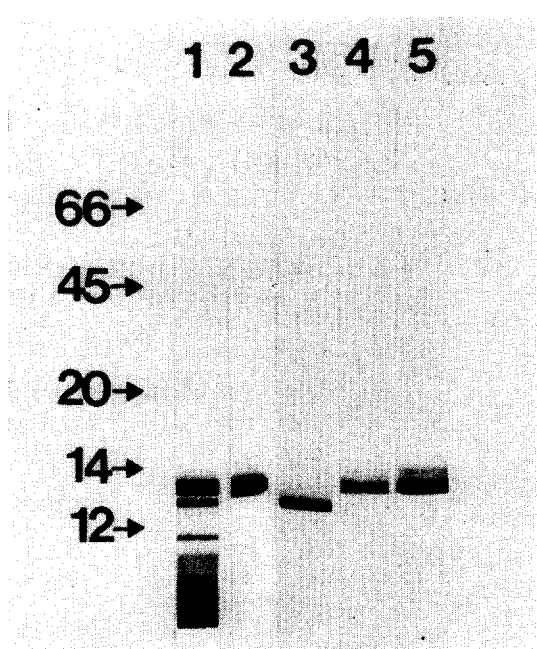


Fig. 2. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of dialyzed honey-bee venom (lanes 1 and 2) and phospholipase A₂ fractions: lane 3, PA₂-O; lane 4, PA₂-15, and lane 5, PA₂-500. Lane 2 is stained for carbohydrate, the others for protein. The numbers (representing kDa) and arrows to the left mark the positions of mol. wt standards.

and Kobata, 1978), yields *N*-acetylglucosaminitol as the sole labeled product to identify GlcNAc* as the monosaccharide linked to the protein. Amino acid analysis of GP (not shown) corroborates asparagine to be the linkage counterpart.

The non-reducing terminal position of fucose and of one fifth of the total GlcNAc content, their anomeric configuration and that of the peripheral mannosyl residues were ascertained by the use of exoglycosidases (Table 2).

The elution pattern of GP on Sepharose 4B-Concanavalin A reflects that of the parent preparation of PA₂ in that it yields two fractions, GP-15 and GP-500, according to their affinities toward the lectin (Fig. 3). The relative proportions of monosaccharides in GP, GP-15, and GP-500 are approximately equal. Methylation data (Table 3) provide clues for their absolute amounts. The methylation pattern of GP-500 is consistent with a "complete"

Table 1. Monosaccharide analysis of honey-bee venom glycopeptide preparations (relative proportions)

	Man	Fuc	GlcNAc ^a
GP	3.70	0.62	2.00
GP-15	3.85	0.42	2.00
GP-500	3.85	0.62	2.00

^aArbitrary calculation basis. Absolute numbers can be inferred from Table 3.

Table 2. Action of glycosidases on honey-bee venom glycopeptide pool (GP)

Enzyme	percentage of total sugar removal		
	GlcNAc	Man	Fuc
α -Mannosidase	0	76,4	0
α -Fucosidase	0	0	62,5
β -N-Acetylglucosaminidase	21,0	0	0

*These numbers represent the highest possible removal rates obtained by combination of the enzymes and are results of a typical experiment.

Man₅-GlcNAc₂-structure (Fig. 4a) most of which is fucosylated and also carries terminal GlcNAc (approx. 60%). The data for GP-15 reflect the lack of either terminal 3-linked or 6-linked mannose in what is a mixture of two isomeric Man₄-GlcNAc₂-variants (Fig. 4b, c). About half of this fraction is fucosylated; very little, if any, terminal GlcNAc is present.

Endo H acts upon roughly 50% of PA₂-15 and GP-15 to liberate a fucose-free oligosaccharide. PA₂-500 and GP-500 remain unaffected by this enzyme.

DISCUSSION

The existence of glycosylated and carbohydrate-free forms of bee venom phospholipase A₂ explains the electrophoretic heterogeneity of this enzyme (Shipolini *et al.*, 1974; Fig. 2). In the glycosylated fraction, several structural variants of a "high-mannosyl" type oligosaccharide (Fig. 4) are found,

which possess different affinities toward Concanavalin A and give rise to further, very subtle, heterogeneities. The oligosaccharides carry terminal substitutions by GlcNAc and fucose, although to different extents. The positions of these residues are demonstrated by their release upon exoglycosidase treatment and by methylation analyses. The linkage of fucose to the proximal GlcNAc of the chitobiosyl core is supported by the liberation of a fucose-free glycan by Endo H from PA₂-15 or GP-15 (40% fucosylated), but not from PA₂-500 or GP-500

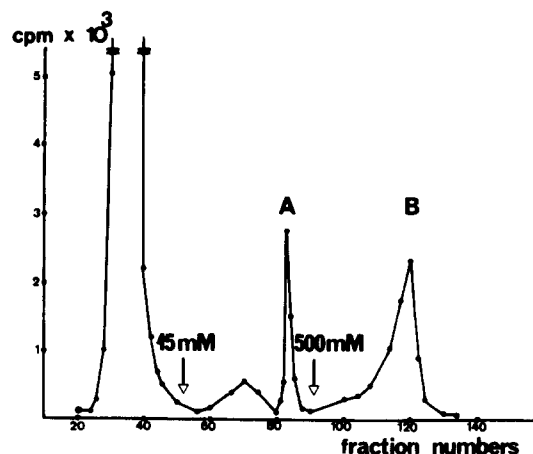


Fig. 3. Chromatography of [³H]-labeled "glycopeptide pool" (GP) from bee-venom phospholipase A₂ on Sepharose 4B-Concanavalin A under the same conditions as in Fig. 1. Only peaks A and B contain carbohydrate; the other peaks represent labeling agent and carbohydrate-free peptides.

Table 3. Methylation analyses of bee-venom glycopeptide fractions

Sugar	moles/mole of oligosaccharide	
	GP-15	GP-500
Methylation Pattern ¹⁾		
Mannose:		
2,3,4,6-Tetra-O-methyl (terminal)	1.9	2.4
3,4,6-Tri-O-methyl (2-O-substituted)	0.1	0.6
2,4 -Di-O-methyl (3-O and 6-O substituted)	1.0	2.0
2,4,6-Tri-O-methyl (3-O-substituted)	0.6	0.0
2,3,4-Tri-O-methyl (6-O-substituted)	0.4	0.0
Fucose:		
2,3,4 -Tri-O-methyl (terminal)	0.4	0.8
GlcNAc:		
3,4,6-Tri-O-methyl (terminal)	0.1	0.6
3,6-Di-O-methyl (4-O-substituted)	1.6	1.2

¹⁾The substitution situation of each residue, inferred from its methylation pattern, is given in parentheses.

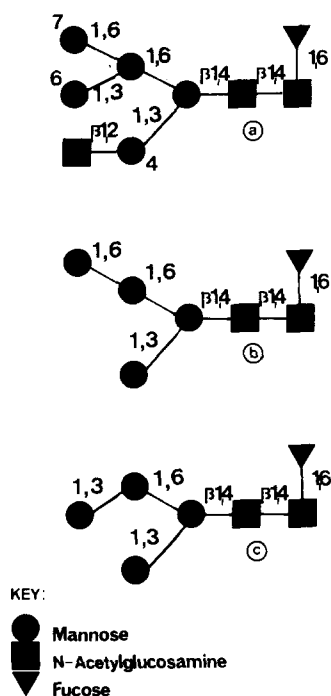


Fig. 4. Postulated structures of the asparagine-linked oligosaccharides found in bee-venom phospholipase A₂. a, GP-500 oligosaccharide; b and c: GP-15 oligosaccharides. The residues numbered 4, 6 and 7 in structure a are the ones referred to in the text (Results and Discussion). The linkages not specified in the structures are α .

(almost completely fucosylated). Endo H has been described not to act upon oligosaccharides with a fucosyl residue in this position (Kobata, 1979).

It has been shown in vertebrate cells, that the transfer of GlcNAc to Man $\alpha(1-3)$ (numbered 4 in Fig. 4) by the so-called " $\beta(1-2)$ GlcNAc-transferase I" is followed by the removal of residues 6 and 7 by α -mannosidase (Schachter *et al.*, 1983). It is not known, which residue is cleaved preferentially. Indeed, GP-15 appears to be a mixture of two isomeric structures lacking either Man-6 or Man-7. Very similar data have been reported on oligosaccharide B from bovine rhodopsin (Liang *et al.*, 1979). GP-500 and GP-15 can thus be considered to represent consecutive stages of the "*N*-glycan processing pathway", whose course in vertebrate cells is well established (Schachter *et al.*, 1983).

Our evidence contradicts in part earlier reports which suggest that insect cells lack the enzymes which transfer fucosyl, sialyl, galactosyl and *N*-acetylglucosaminyl residues to glycoprotein *N*-glycans during their maturation (Butters and Hughes, 1978, 1981; Butters *et al.*, 1981; Hsieh and Robbins, 1984) and are thus not capable of synthesizing "complex" or "hybrid" type structures. The divergence in the oligosaccharide-processing pathway between vertebrates and insects seems therefore less pronounced and general as postulated.

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