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The asparagine-linked carbohydrate of honeybee venom hyaluronidase

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Hyaluronidase from the venom of the honeybee (*Apis mellifera*) has been purified by gelpermeation and cation exchange chromatography. Its asparagine-linked carbohydrate chains were released from tryptic glycopeptides with N-glycosidase A and reductively aminated with 2-aminopyridine. Separation of the fluorescent derivatives by size-fractionation and reversed-phase HPLC afforded eighteen fractions which were analysed by two-dimensional HPLC mapping combined with exoglycosidase digestions. The bulk of the N-linked glycans of hyaluronidase consisted of small oligosaccharides ($Man_{1\text{-}3}GlcNAc_2$), most of which were either α1,3-monofucosylated or α1,3-(α1,6-)difucosylated at the innermost GlcNAc residue. High-mannose type structures constituted the minor fractions, together making up about 5% of the oligosaccharide pool from hyaluronidase. Four fractions, making up 8% of the N-linked glycans, contained the terminal trisaccharide GalNAcβ1-4[Fucα1-3]GlcNAcβ1- in β1,2-linkage to the core α1,3-mannosyl residue. No evidence for the presence of O-glycans or sialic acids could be found.

Keywords: hyaluronidase, *Apis mellifera*, bee venom, N-linked carbohydrate chains, α1,3-fucosylation

Abbreviations: GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; PA, pyridylamino; PLA, phospholipase A_2; 2D-HPLC, two-dimensional HPLC.

Introduction

Hyaluronidases catalyse the hydrolysis of hyaluronic acid, a polymer present in the interstitial ground substance of connective tissues. These enzymes are widely distributed in nature. As constituents of various venoms they serve in breaking down the intercellular matrix, thus facilitating diffusion of other venom components. The enzyme from honeybee venom, originally described as 'spreading factor' [1], belongs to the group of hyaluronoglucosaminidases. It is a basic glycoprotein (pI = 9.0) making up approximately 2% of the venom's dry matter [2]. The sequence of its 349 amino acids, which has recently been determined [3], accounts for a mass of 40.7 kDa and contains three potential sites for N-glycosylation at Asn-82, Asn-190, and Asn-230. Beside phospholipase A_2 (PLA_2), the main protein constituent of honeybee venom, hyaluronidase is the next important allergen of bee venom [4]. The structures of the N-glycans from PLA_2 have recently been elucidated [5]. Some of these glycans contain fucose α1,3-linked to the innermost GlcNAc residue of the core-pentasaccharide. This part-structure has been shown to bind to IgE from bee venom allergic individuals and to contribute to cross-reactivity towards other glycoproteins such as pineapple stem bromelain [6]. An immunogenic and cross-reactive determinant occurring both in plant and insect glycoproteins bears considerable significance, and it is of importance to determine its presence e.g. on allergens.

In this study we have therefore analysed the structures of N-glycans from the allergenic glycoprotein honeybee venom hyaluronidase. In addition we have examined the presence of O-linked glycans and sialic acids in hyaluronidase.

Materials and methods

Purification of hyaluronidase from honeybee venom

Bee venom (Nectarcorp, Sophia, Bulgaria) was fractionated on BioGel P30 (100–200 mesh, Bio-Rad; 2.5 × 120 cm) using ammonium acetate (50 mM, pH 5.0) as the eluent. Fractions containing hyaluronidase activity were pooled and lyophilized. Further purification of hyaluronidase was achieved by cation-exchange chromatography on Mono S HR 5/5 (Pharmacia) using a linear gradient of 0 – 600 mM sodium chloride in 25 mM sodium acetate (pH 4.2). Fractions containing hyaluronidase activity were pooled, dialysed against distilled water and lyophilized.

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Enzymes and related materials N-glycosidase A and α-fucosidase from bovine kidney were obtained from Boehringer Mannheim. Trypsin (TPCK-treated), α-mannosidase, β-*N*-acetylhexosaminidase from jack beans, crude β-galactosidase as well as hyaluronic acid were obtained from Sigma. β-Galactosidase was purified as described [5]. α-Fucosidase from almonds was purchased from Genzyme Co. (Cambridge, MA, USA). Human immunoglobulin G was a gift from Immuno AG (Vienna, Austria). α-Amylase from *Aspergillus oryzae* was purified from the crude enzyme (Fungamyl L800, Novo Nordisk, Denmark) as described [7].

Reference oligosaccharides Pyridylaminated isomalto-oligosaccharides ranging from 1 to 20 glucose units were prepared from a partial acid hydrolysate of dextran. Manβ1-4GlcNAcβ1-4GlcNAc-PA (ST-1) was prepared by sequential exhaustive digestion of pyridylaminated asialo-oligosaccharides from bovine fibrin with β-galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase. Analogous treatment of asialo-PA-oligosaccharides from human immunoglobulin G led to Manβ1-4GlcNAcβ1-4[Fucα1-6] GlcNAc-PA (ST-2). Manβ1-4GlcNAcβ1-4[Fucα1-3]GlcNAc-PA (ST-3) and Manβ1-4GlcNAcβ1-4[Fucα1-3] [Fucα1-6] GlcNAc-PA (ST-4) were prepared from α1,3- and difucosylated PA-oligosaccharides isolated from PLA_2 [5] by digestion with α-mannosidase. Pyridylamination of the oligosaccharides from α-amylase of A. *oryzae* yielded $Man_5GlcNAc_2$-PA (ST-5; M5.1 using the nomenclature of Tomiya *et al.* [8]) and $Man_6GlcNAc_2$-PA (ST-6; M6.1), respectively. In addition, the PA-oligosaccharides obtained from honeybee venom phospholipase A_2 [5] were used as reference compounds.

Analytical methods Hyaluronidase activity was determined turbidimetrically according to the method of Tolksdorf *et al.* [9]. Hyaluronidase activity is expressed in U.S.P. units (1 μg hyaluronic acid hydrolysed in 3 min). *N*-Acetyl- and *N*-glycolylneuraminic acids were analysed by reversed-phase HPLC after acid hydrolysis and reaction with 1,2-diamino-4,5-methylene-dioxybenzene [10]. Hexosamines were quantified by reversed-phase HPLC after reduction with sodium borohydride and precolumn derivatization with o-phthalaldehyde according to [11]. SDS-PAGE was performed in a Mini-Protean II cell (Bio-Rad) with gels containing 12% acrylamide. Protein bands were visualized with Coomassie blue G250. HPLC analysis of hyaluronidase was performed on a Hi-Pore RP-304 column (4.6 × 250 mm, Bio-Rad) using a gradient from 0 to 60 % (v/v) acetonitrile in 0.1 % trifluoroacetic acid containing 10 % isopropanol.

Preparation and HPLC-fractionation of pyridylaminated oligosaccharides Twenty-five mg purified hyaluronidase was S-carboxymethylated [12], dialysed against distilled water and lyophilized. A 2% (w/v) suspension of the carboxymethylated protein in 0.2 M NH_4HCO_3 (pH 8.5) was digested with trypsin (TPCK-treated) at a ratio of enzyme to substrate of 1:50 (w/w) for 24 h at 37°C. After desalting of the digest over Sephadex G-25 using 1% (v/v) acetic acid as the eluent, N-linked oligosaccharides were released with N-glycosidase A [13]. A small portion (5%) of the resulting mixture was fractionated on a C18 cartridge to monitor the release of carbohydrate (see below). The rest was passed over Dowex 50 W-X2 (H^+) using 0.5% (v/v) acetic acid as the eluent to remove the peptides. Carbohydrate-positive fractions were pooled, lyophilized and reductively aminated with 2-aminopyridine [14]. Excess reagent was removed by gel filtration over Sephadex G-15 with 10 mM sodium acetate (pH 6.0) as the eluent and the PA-oligosaccharides were lyophilized.

PA-glycans were separated and analysed by 2D-HPLC. Size fractionation on MicroPak AX-5 (4 × 300 mm, Varian) was used as the first and reversed-phase chromatography on Hypersil ODS (4 × 250mm, ÖFZ Seibersdorf, Austria) as the second dimension exactly as published [5].

Exoglycosidase digestions Digestions with exoglycosidases were generally performed with 50 pmol of PA-oligosaccharide for 20 h at 37°C in a total volume of 20 μl. The following buffers and enzyme quantities were employed: 50 mU of α-mannosidase in sodium acetate buffer (50 mM, containing 0.1 mM $ZnCl_2$, pH 4.2) to achieve complete removal of α-linked mannose residues; 0.1 mU of α-mannosidase to selectively remove α1,3-linked mannose residues; 0.1 mU of α-fucosidase from bovine kidney in sodium citrate buffer (50 mM, pH 4.5) to split off α1,6-linked fucose residues; 0.01 mU of fucosidase from sweet almonds to remove fucose linked α1,3 to peripheral GlcNAc residues; 5 mU of β-*N*-acetylhexosaminidase in a sodium citrate buffer (0.1 mM, pH 5.0). Additionally, 1 nmol of PA-oligosaccharides from hyaluronidase was exhaustively digested with 2 U of jack bean α-mannosidase.

Determination of O-glycans Liberation of O-glycans from hyaluronidase was attempted with 1 M $NaBH_4$ in 0.1 M NaOH at 37°C for 20 h. Bovine fetuin served as a positive control. The samples were desalted by passage over AG 50 × 8 and evaporated to dryness. Finally, the samples were hydrolysed and analysed for the presence of galactosaminitol by HPLC with o-phthalaldehyde derivatization as described [11].

In another approach, we attempted to recover O-glycopeptides after N-glycosidase digestion of the glycopeptide pool. For this purpose, a small portion of the N-glycosidase digest was lyophilized, redissolved in 5% (w/v) acetic acid and loaded on to a Sep-Pak C18 cartridge (Waters) previously conditioned with methanol, 1-propanol and 5% (w/v) acetic acid [15]. Free oligosaccharides were eluted with 5% (w/v) acetic acid followed by 20 and 40% (v/v) 1-propanol in 5% (v/v) acetic acid to desorb (glyco-)peptides. Fractions were lyophilized and subjected to aminosugar analysis.

Results

Hyaluronidase was purified from honeybee venom by gelpermeation chromatography followed by cation exchange on

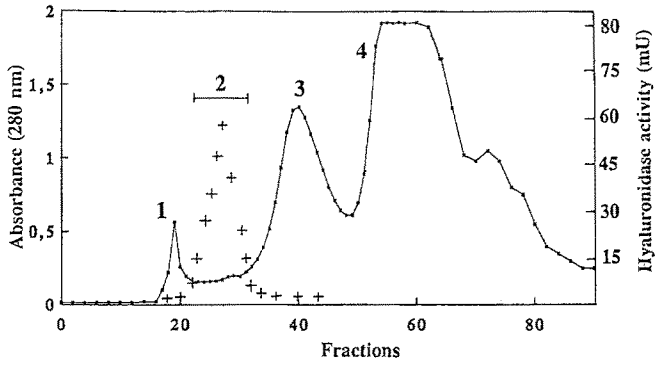
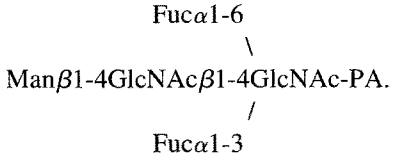


Figure 1. Chromatography of honeybee venom on Biogel P30. Graphs show total protein (———) and hyaluronidase activity (+ +), which is expressed in U.S.P.-units. Peak 1 contained a β-hexosaminidase and some unidentified compounds. Peak 2 contained hyaluronidase, which was further purified (see Fig. 2). Peaks 3 and 4 consisted of phospholipase A_2 and melittin, respectively. Acid phosphatase was found in all fractions.

MonoS™ (Figs 1 and 2). The peak fraction was devoid of acid phosphatase and *N*-acetylhexosaminidase. The product gave a single band when analysed by SDS-PAGE (Fig. 3) or reversed-phase HPLC (not shown), although a trace (< 1%) of phospholipase A_2 may have been present as judged from SDS-PAGE with sample overload.

Purified hyaluronidase was S-alkylated and digested with trypsin. The N-linked oligosaccharides were released with N-glycosidase A and analysed by two-dimensional HPLC as their pyridylaminated derivatives. To get a quick idea of the structural types as well as the fucosylation pattern, a small portion of the PA-oligosaccharide pool was exhaustively digested with α-mannosidase. The major part of the pool was separated into individual fractions, which were further analysed by treatment with exoglycosidases in combination with 2D-HPLC-mapping.

Analysis of the α-mannosidase-treated oligosaccharides In order to acquire information on the ratio of non-, α1,3-, α1,6- and α1,3-(α1,6-)- difucosylated glycans, the PA-oligosaccharide-pool from hyaluronidase was exhaustively digested with α-mannosidase to convert all 'high-mannose' type structures (including the truncated glycans) into the four possible fucosylation variants of the core-trisaccharide:



As described in detail elsewhere [7], these four oligosaccharides can easily be separated by reversed-phase HPLC. Indeed, the mannosidase-digest contained four major peaks (Fig. 4). Since glycans with terminal substitutions other than mannose would lead to the occurrence of additional peaks, it is obvious from Fig. 4 that such structures do not constitute major species. The same products, if in different proportions, were obtained from PLA_2 by an analogous treatment (Fig. 4).

Separation of pyridylaminated oligosaccharides The PA-oligosaccharides were size-fractionated on MicroPak AX-5 (Fig. 5) and rechromatographed if necessary. The twelve fractions obtained from the sizing column were then subjected to reversed-phase HPLC (Fig. 5B shows the profile of the unfractionated oligosaccharide pool). In this system, compounds **4**, **5** and **9** could be further separated to yield a total of eighteen fractions. The elution positions of these compounds are listed in Table 1.

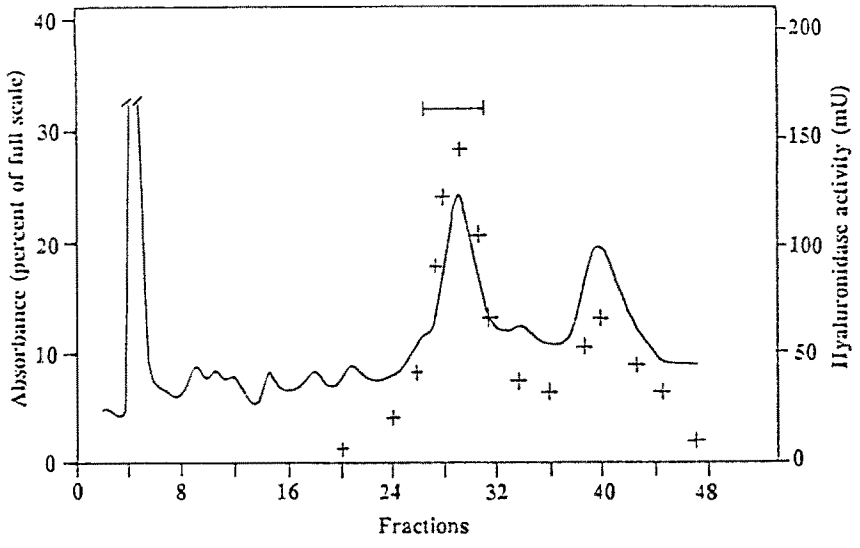


Figure 2. Purification of hyaluronidase by FPLC on MonoS™. The hyaluronidase-enriched pool from Biogel P30 was separated by a linear gradient from 0 to 0.6 M NaCl at pH 4.2.

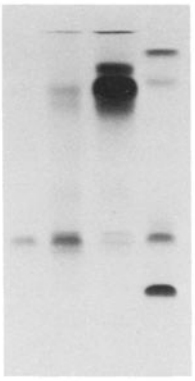


Figure 3. SDS-PAGE of honeybee venom and hyaluronidase. Lane 1: molecular mass markers; lane 2: crude honeybee venom; lane 3: hyaluronidase-rich pool from Biogel P30; lane 4: hyaluronidase after chromatography on MonoS™.

Table 1. Elution positions of PA-oligosaccharides obtained from honeybee venom hyaluronidase. Structures of reference oligosaccharides are given in Materials and methods. The assignments of equivalent structures from honeybee venom phospholipase A_2 (PLA) are based on enzymatic degradation in addition to HPLC-mapping. The fraction codes given for the PLA glycans refer to reference [5]. Elution volumes are expressed as glucose units.

Fraction	*Elution positions on*		*Corresponding reference*	*Equivalent in PLA_2*
	MicroPak AX-5	*Hypersil ODS*		
1	2.6	7.0	**ST-1**	
2	3.6	9.0		**1**
3	4.4	4.5	**ST-3**	
4-A	4.7	5.7		**3-A**
4-B	4.7	7.3	**ST-4**	
4-C	4.7	8.2		**3-B**
4-D	4.7	8.6		**3-C**
5-A	5.2	8.6		**4-A**
5-B	5.2	16		**4-B**
6	5.8	5.3		**5-A**
7	6.3	8.7		**6**
8	6.6	7.7	**ST-5**	
9-A	7.5[a]	6.3[b]		**7**
9-B	7.7[a]	6.5[b]	**ST-6**	
9-C	7.9[a]	10.7		**8**
10	8.8	4.4		**9**
11	9.2	7.3		**10**
12	10.6	5.0		

[a] Originally eluting as one peak with maximum at 7.7 glucose units.
[b] Not resolved, but distinguished by sensitivity to α-mannosidase (see text).

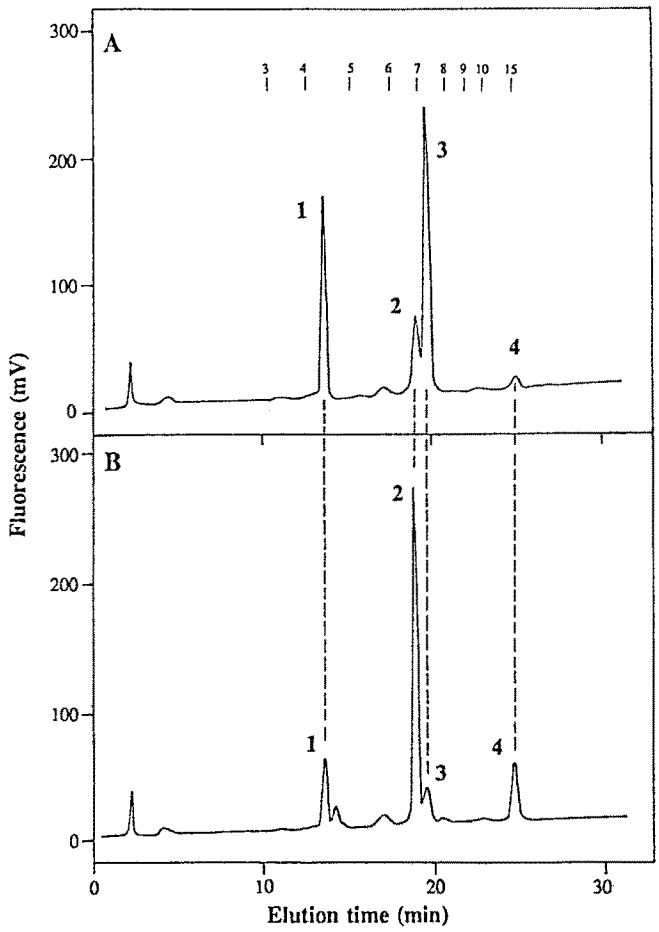


Figure 4. Reversed phase HPLC of the PA-oligosaccharides from honeybee venom hyaluronidase (A) and phospholipase A_2 (B) after extensive digestion with α-mannosidase. In both chromatograms, peak 1 is Manβ1-4GlcNAcβ1-4[Fucα1-3]GlcNAc-PA, peak 2 is Manβ1-4GlcNAcβ1-4GlcNAc-PA, peak 3 is Manβ1-4GlcNAcβ1-4[Fucα1-3][Fucα1-6]GlcNAc-PA, and peak 4 is Manβ1-4GlcNAcβ1-4[Fucα1-6]GlcNAc-PA. Numbers at the top indicate the elution positions of isomaltose standards of the respective degree of polymerization.

Structural analysis In many cases, comparison of the HPLC elution patterns of single compounds with glycans from PLA_2 gave the first clues to the structural assignment. Additionally, valuable conclusions could be drawn from the effects of fucosylation of the reducing-end GlcNAc on the elution behaviour of PA-oligosaccharides on reversed-phase HPLC [5, 7]. Tentative assignments were then tested by exoglycosidase treatment.

Twelve of the eighteen fractions corresponded with structures already found in PLA_2 (Table 2), as deduced from their elution positions and from the effects of digestion with α-mannosidase and bovine kidney α-fucosidase [5]. Since the strategies, data and reasonings for most of these glycans are so similar to previously published data [5, 7], structural identification is only described for the larger glycans **9-C**, **10**, and **11** (for **9-A** see below), which were suspected from their

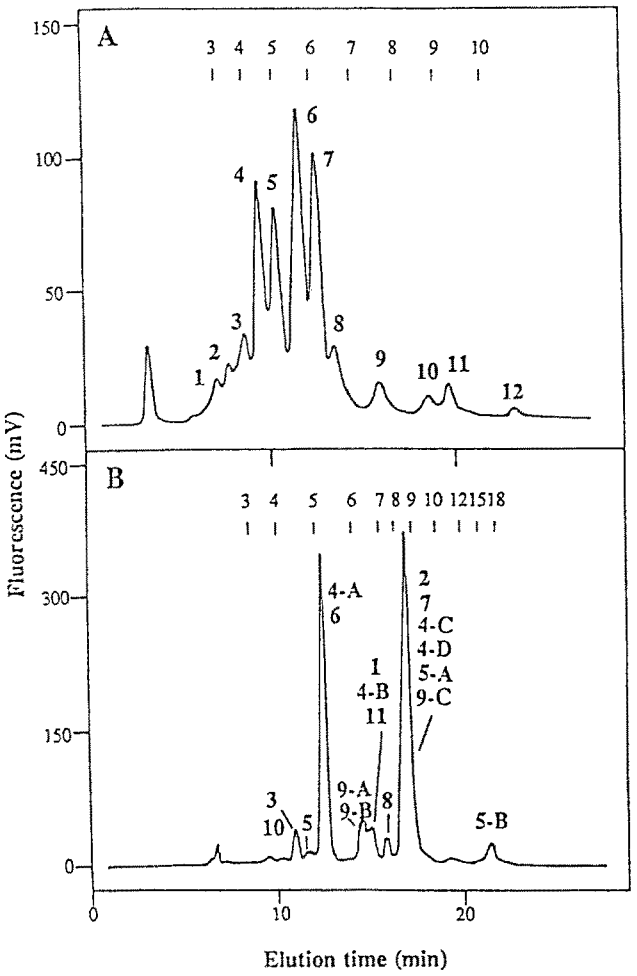


Figure 5. Profiles of pyridylaminated oligosaccharides from hyaluronidase obtained by HPLC using MicroPak AX-5 for size fractionation (A) and reversed-phase HPLC (B). Exact elution positions and structures of individual fractions are given in Tables 1 and 2, respectively.

elution positions to correspond to the GalNAcβ1-4[Fucα1-3]GlcNAc-containing glycans of PLA_2. Digestion of these glycans with a high dose of α-mannosidase resulted in the removal of only one mannose residue — as could be deduced from analyses on the sizing column. The glycans were susceptible to jack bean β-*N*-acetylhexosaminidase if applied in high doses. Together with the elution time shifts on both columns this indicated the presence of a terminal GalNAc-residue. Likewise, the glycans were susceptible to almond α-fucosidase, which had previously been shown to selectively remove α1,3-linked antennary fucose residues [5, 16]. α-Fucosidase from bovine kidney acted upon structure **11** turning it into **10**. These results are convincing evidence for the structural assignments of fractions **9-C**, **10**, and **11** (Table 2). Fraction **9** gave two peaks on the reversed-phase column, the first of which was not homogeneous as discovered during experiments with α-mannosidase. While compound **9-B** (see below) was highly sensitive to moderate amounts of this enzyme, **9-A** could only be degraded with a high dose of α-mannosidase. **9-A** was also digested by β-*N*-acetylhexosaminidase and almond α-fucosidase, thereby exhibiting the same elution time shifts as the GalNAc-containing structure **7** from honeybee venom phospholipase A_2 [5]. The surprising co-fractionation of **9-A** and **9-C** on MicroPak AX-5 is explained by the intermediate elution position of compound **9-B**.

The small glycans **1**, **3**, and **4-B** coeluted with reference oligosaccharides ST-1, ST-3, and ST-4, respectively. Their susceptibility to β-mannosidase firmly identified them as heavily truncated (Table 2).

Exhaustive digestion with α-mannosidase of fractions **8**, **9-B**, and **12** yielded the trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc-PA. The size and, with a very small note of caution, also the structures of these high-mannose glycans were deduced to be as shown in Table 2 from their 2D-HPLC mapping position [7].

O-Glycans The presence of GalNAc raises the possibility that hyaluronidase may also contain O-glycans. These would remain as glycopeptides after enzymatic release of N-glycans. Therefore, a small portion of the N-glycosidase digest of hyaluronidase was fractionated over a Sep-Pak C18 cartridge in order to separate free oligosaccharides from (glyco-)peptides. The 'oligosaccharide fraction' eluted by 5% acetic acid contained GlcNAc and GalNAc in a ratio of 20:1, which is in good agreement with the finding that about 10% of the N-linked glycans contained the trisaccharide GalNAcβ1-4[Fucα1-3]GlcNAcβ1- (see Table 2). Small amounts of aminosugars, again mainly GlcNAc, were found in the fraction eluted with 20% 1-propanol. However, repeated digestion with N-glycosidase A identified these glycopeptides as N- rather than O-glycopeptides.

Moreover, β-elimination under reducing conditions, followed by hydrolysis failed to produce galactosaminitol.

Sialic acids No *N*-acetyl- or *N*-glycolyl-neuraminic acid could be found at a level of sensitivity ensuring the detection of 0.2 mmol sialic acid per mol glycoprotein.

Discussion

Electrophoretically pure hyaluronidase from honeybee venom could be obtained with only two chromatographic steps. All the enzyme's carbohydrate could be released by N-glycosidase A indicating that hyaluronidase contains only N-linked oligosaccharides. Essentially the same N-glycan structures could be identified as previously found on honeybee venom PLA_2 [3]. Truncated ('paucimannose') glycans and their fucosylated analogues together accounted for approximately 85% of the glycans from hyaluronidase. Considering the recently developed hypothesis on N-glycan processing in insect cells [7, 17], these structures should no longer be regarded as high-mannose but rather as modified type glycans, since they prob-

Table 2. Structures of N-glycans from honeybee venom hyaluronidase.

Fraction	*Structure*	*Abundance (%)*
1	Manβ1-4GlcNAcβ1-4GlcNAc	1.5
2	Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc	2.6
3	Manβ1-4GlcNacβ1-4GlcNAc Fucα1-3	3.6
4-A	Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc Fucα1-3	8.1
4-B	Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc Fucα1-3	2.2
4-C	Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3	6.3
4-D	Manα1-6 / Manα1-3 / Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3 / Fucα1-6	0.8
5-A	Manβ1-4GlcNAcβ1-4GlcNAc Fucα1-3	12.1
5-B	Manα1-6 / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3	1.8
6	Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3 / Fucα1-3	20.6
7	Manα1-6 / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3 / Fucα1-3	27.6
8	Manα1-6 / Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3 / Manα1-3	1.2
9-A	Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc GalNAcβ1-4GlcNAcβ1-2Manα1-3 / Fucα1-3	1.9
9-B	Manα1-6 / Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc Manβ1-3 / Manα1-2Manα1-3	2.0
9-C	Manα1-6 / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc GalNAcβ1-4GlcNAcβ1-2Manα1-3 / Fucα1-3	1.4
10	Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc GalNAcβ1-4GlcNAcβ1-2Manα1-3 / Fucα1-3 Fucα1-3	3.2
11	Manα1-6 / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc GalNAcβ1-4GlcNAcβ1-2Manα1-3 / Fucα1-3 Fucα1-3	2.7
12	Manα1-2Manα1-6 / Manα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc Manα1-2Manα1-3 / Manα1-2Manα1-2Manα1-3	0.3

ably have undergone a series of modifications including the action of GlcNAc-transferase I and mannosidase II. Another 7.6% of the glycans exhibited the terminal trisaccharide GalNAcβ1-4[Fucα1-3]GlcNAcβ1- already known from PLA_2 and some mammalian glycoproteins [18, 19]. The remarkable difference from PLA_2 and any other insect glycoprotein so far analysed lies in the high degree of α1,3-fucosylation of the innermost GlcNAc. Not less than 80% of the glycans contain this immunogenic determinant and about 45% are additionally α1,6-fucosylated at the same GlcNAc residue. Thus, α1,3- and difucosylation of the core can constitute major structural features of an insect glycoprotein. The difference in the amounts of α1,3- and α1,6-monofucosylated species is remarkable as it points to restraints effected either by different location or, more probably, by the specificity of the involved fucosyltransferases.

Acknowledgements

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