Carbohydrate Research, 247 (1993) 291–297
Elsevier Science Publishers B.V., Amsterdam

Characterization of the oligosaccharide structures on bee venom phospholipase A_2

Tameira Hollander, Paul A. Aeed and Åke P. Elhammer *

The Upjohn Company, 7240-209-622, 301 Henrietta St., Kalamazoo, MI 49001 (USA)

(Received January 18th, 1993; accepted in revised form March 17th, 1993)

ABSTRACT

The *N*-linked oligosaccharide structures on bee venom phospholipase A_2 were investigated. The oligosaccharides on purified phospholipase A_2 were released by hydrazinolysis and labeled in vitro by reduction with NaB^3H_4. Following purification, the labeled oligosaccharides were characterized by size exclusion chromatography in combination with digestion with specific glycosidases. Linkage positions were determined by methylation analysis. Four types of structures were identified on the molecule, all of which were of truncated high-mannose type and none of which contained any α-(1 → 2)-linked mannose residues. The majority of the structures were Man_3 oligosaccharides with (43%) or without (38%) a fucose residue linked α-(1 → 6) to the reducing *N*-acetylglucosamine. The remaining 19% of the oligosaccharides on the molecule were identified as a Man_5 oligosaccharide without core fucose (9.6%) and a core-fucosylated Man_4 structure (9.2%).

INTRODUCTION

Phospholipase A_2 (EC 3.1.1.4.) is the most abundant protein in honey bee (*Apis mellifera*) venom, accounting for 12–14% of the dry weight[1]. The protein consists of a single polypeptide chain of 128 amino acids and is cross-linked by four disulfide bridges[2–4]. The primary structure contains one site for *N*-linked glycosylation, Asn 13, and previous work has demonstrated that this site is glycosylated on the majority of the molecules[3,5].

Bee venom phospholipase A_2 has a heterogenous appearance on SDS-polyacrylamide electrophoresis which has been attributed to heterogeneity in the oligosaccharide structures. In a previous report three different structures on the molecule were tentatively identified by methylation analysis[5].

This report is a characterization of *N*-linked oligosaccharides isolated from bee venom phospholipase A_2. Four different structures were identified, all of which are representative of the truncated high-mannose type oligosaccharides frequently found on insect glycoproteins[6–8].

* Corresponding author.

EXPERIMENTAL

Materials and methods.—Sodium boro[^{3}H]hydride (500 Ci/mmol) was obtained from Dupont. Sodium borohydride, Dowex 50WX8, Amberlite MB-3, Chelating resin, hydrazine, jack bean α-mannosidase, snail β-mannosidase, jack bean *N*-acetyl-β-D-glucosaminidase, and bovine epididymis α-fucosidase were purchased from Sigma. *Aspergillus phoenincis* α-mannosidase was obtained from Oxford GlycoSystems. Bee venom phospholipase A_2 was purchased from Boehringer. Bio-Gel P-4 was from BioRad. The radiolabeled standard Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc-ol was isolated from an in vivo labeled chimeric protein called FG synthesized by the insect cell line Sf9[8]. The radiolabeled standards Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-ol, Man(β1-4)GlcNAc(β1-4)GlcNAc-ol, and GlcNAc(β1-4)GlcNAc-ol were prepared from a standard biantennary oligosaccharide purchased from Oxford GlycoSystems by reduction with sodium boro[^{3}H]hydride followed by sequential exoglycosidase digestion.

Phospholipase A_2 (2.7 mg) was dried extensively and the oligosaccharide structures were released, purified and labeled by reduction with sodium boro[^{3}H]hydride as described by Takasaki et al.[9] with minor modifications. The in vitro labeled oligosaccharides were separated from radioactive contaminants on descending paper chromatography in pyridine–ethyl acetate–glacial acetic acid–water (5:5:1:3). Analysis of the purified radioactive oligosaccharides was carried out on a calibrated Bio-Gel P-4 column (1.6 × 100 cm) essentially as described by Yamashita et al.[10]; since all oligosaccharide structures on the molecule are relatively small, a 100-cm column provides sufficient resolution and was used instead of a 200-cm column. The sequential degradation of the oligosaccharides involved the use of the following exoglycosidases and conditions: jack bean *N*-acetyl-β-D-glucosaminidase (5 U/mL) in 100 mM citrate-phosphate, pH 4.6 for 18 h; *Aspergillus phoenicis* α-(1 → 2)-mannosidase (0.2 mU/mL) in 100 mM sodium acetate, pH 5 for 20 h; jack bean α-mannosidase (14 U/mL) in 100 mM sodium acetate, pH 4.5 for 48 h; snail β-mannosidase (1.5 U/mL) in 100 mM glycine–HCl, pH 3.5 for 24 h; bovine epididymis α-fucosidase (0.15 U/mL) in 100 mM citrate, pH 4.5 for 24 h followed by addition of fresh enzyme and an additional 24 h incubation. All enzyme digestions were done at 37°C under toluene atmosphere. Methylation analysis was done essentially as described[11].

RESULTS AND DISCUSSION

Separation of the in vitro labeled oligosaccharides on Bio-Gel P-4 yielded three radioactive peaks, eluting at the positions of oligosaccharides composed of approximately 8.8, 7.6, and 6.6 glucose units (GU), respectively. None of the radioactivity in these fractions was linked to charged structures, as tested by binding to QAE-Sephadex (data not shown). Fig. 1, panel A shows the earliest eluting, 8.8

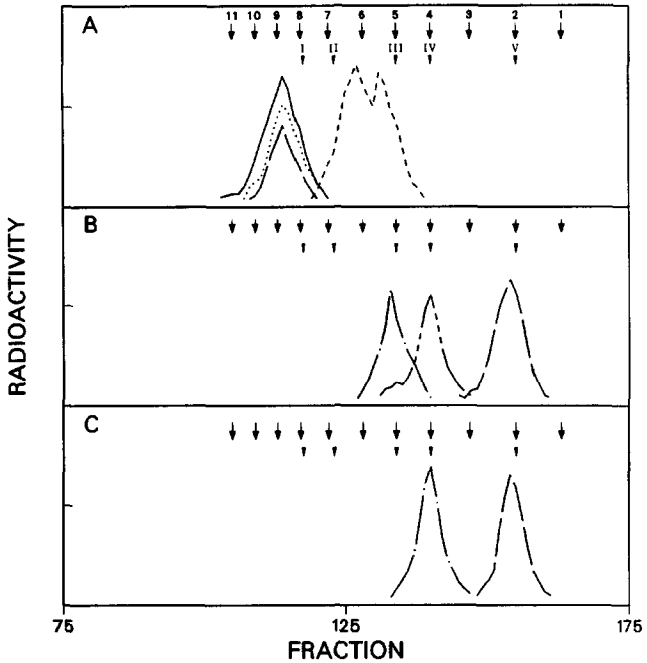

Fig. 1. Sequential exoglycosidase digestion of oligosaccharides co-eluting with dextran oligomers composed of 8.8 glucose units. Oligosaccharides were isolated from purified phospholipase A_2 and labeled in vitro as outlined in the Experimental section. Following size-exclusion chromatography on Bio-Gel P-4, the oligosaccharides eluting at 8.8 glucose units (Panel A, ———) were re-chromatographed on the column after sequential digestion with jack bean *N*-acetyl-β-D-glucosaminidase (Panel A, ——— ———), *Aspergillus phoenicis* α-1,2-mannosidase (Panel A, · · · · · ·), jack bean α-mannosidase (Panel A, — — —), snail β-mannosidase (Panels B and C, – · – · –), bovine epidiymis α-fucosidase (Panel B, – – – – –), and jack bean *N*-acetyl-β-D-glucosaminidase (Panels B and C, ——— ——— ———). Numbers 1 through 10 indicate the elution volumes of dextran oligomers composed of 1–10 glucose units. The elution positions of oligosaccharide standards are indicated by roman numerals: I, Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAcβ(1-4)[Fuc(α1,6)]GlcNAc-ol; II, Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-ol; III, Man(β1-4)GlcNAc(β1-4)GlcNAc-ol; IV, GlcNAcβ(1-4)GlcNAc-ol; V, GlcNAc.

GU, radioactive peak. Digestion of the material in this peak with jack bean *N*-acetyl-β-D-glucosaminidase did not result in a shift in elution volume, indicating the absence of terminal GlcNAc residues. Digestion of the same material with α-(1 → 2)-mannosidase again did not result in a shift, suggesting that the structure(s) in this peak also lacked outer α-(1 → 2)-linked mannose residues. However, further digestion with jack bean α-mannosidase resulted in an ~ 2.6 GU shift in mobility for 45% of the radioactivity in the peak while the remaining 55% shifted ~ 3.5 GU, indicating that the initial peak contained structures from which the treatment removed 3 and 4 α-linked mannoses, respectively (Fig. 1A). Digestion of the two peaks (6.2 and 5.3 GU) in Fig. 1A with snail β-mannosidase, resulted in both cases in an ~ 1 GU shift in elution position, (yielding peaks at 5.3 and 4.1 GU, respectively) consistent with the removal of one β-linked mannose residue

TABLE I

Permethylated alditol acetates identified in oligosaccharides isolated from bee venom phospholipase A_2

1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methylmannitol
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol
1,4,5,6-Tetra-*O*-acetyl-3-*O*-methyl-2-*N*-acetylglucosaminitol
1,4,5-Tri-*O*-acetyl-3,6-di-*O*-methyl-2-*N*-acetylglucosaminitol

(Fig. 1B and C). The 5.3 GU peak was further digested with bovine epididymis α-fucosidase which resulted in a 1 GU shift, consistent with the removal of one terminal α-linked fucose residue (Fig. 1B). The material in the 4.1 GU peak was resistant to this treatment (data not shown). Finally digestion of the material in the 4.1 GU peaks with jack bean *N*-acetyl-β-D-glucosaminidase in both cases resulted in an ~ 2 GU shift. Taken together, these results suggest that the 8.8 GU peak contains two oligosaccharides: a Man_5 structure and a core-fucosylated Man_4 structure, both without α-(1 → 2)-linked mannose residues; the recovery of only 2,3,4-methylated mannose (and the absence of the 2,4,6-tri-*O*-methylated species) in the methylation analysis of the preparation, strongly suggests that one of the outer mannoses on the Man_4 structure is linked to the 6 position of the α-(1 → 6)-linked mannose (Table I). The experimental data also suggest that the fucose residue on the structure is linked to one of the core *N*-acetylglucosamines and control experiments involving digestion of the intact oligosaccharide with endoglucosidase D resulted in a labeled 3 GU fragment which could be further degraded to a 2 GU structure by α-fucosidase digestion, suggesting that the fucose is linked to the innermost (Asn-linked) GlcNAc (Data not shown). Further support for this interpretation comes from the methylation analysis. Two methylated alditol acetates of *N*-acetylglucosamine were observed: 3,6-di-*O*-methyl- and 3-*O*-methyl-GlcNAc. The latter species is predicted from oligosaccharides containing a fucose residue linked to the 6-position of *N*-acetylglucosamine. Previous reports have established that insect cells are capable of synthesizing core-fucosylated oligosaccharides[5,7,8].

The second peak, eluting at ~ 7.6 GU, (Fig. 2) was first digested with bovine epididymis α-fucosidase; this resulted in 1 GU shift in elution volume, indicating the removal of one terminal fucose residue. Digestion of this (the product from α-fucosidase digestion) material with jack bean α-mannosidase resulted in an additional 2 GU shift in elution volume, consistent with the removal of two terminal mannose residues. Further digestion with snail β-mannosidase and jack bean *N*-acetyl-β-D-glucosaminidase resulted in shifts in elution volume of 1 and 2 GU, respectively. Digestion of the intact oligosaccharide with endoglycosidase D confirmed that the fucose residue was linked to the reducing *N*-acetylglucosamine (compare above). Thus the 7.6 GU peak appears to contain only one type of oligosaccharide, a core-fucosylated trimannosyl structure.

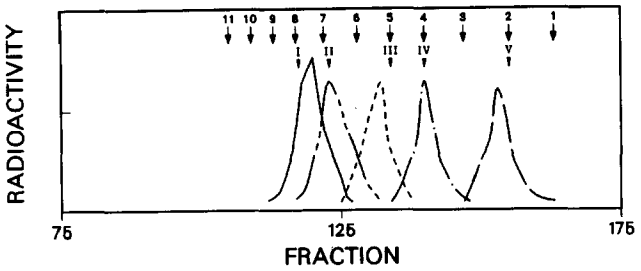

Fig. 2. Sequential exoglycosidase digestion of oligosaccharides co-eluting with dextran oligomers composed of 7.6 glucose units. In vitro labeled phospholipase A_2 oligosaccharides were isolated and separated by Bio-Gel P-4 chromatography as outlined in the legend to Fig. 1. The oligosaccharides eluting at 7.6 glucose units (———) were re-chromatographed on the column after digestion with bovine epidiymis α-fucosidase (-----), jack bean α-mannosidase (— — —), snail β-mannosidase (-·-·-) and jack bean *N*-acetyl-β-D-glucosaminidase (——— ——— ———). Numbers 1 through 10 indicate the elution volumes of dextran oligomers composed of 1–10 glucose units. The elution positions of oligosaccharide standards are indicated by roman numerals: I, Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAcβ(1-4)[Fuc(α1-6)]GlcNAc-ol; II, Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-ol; III, Man(β1-4)GlcNAc(β1-4)GlcNAc-ol; IV, GlcNAc(β1-4)GlcNAc-ol; V, GlcNAc.

The third peak, eluting at ~ 6.6 GU (Fig. 3), was sequentially digested with jack bean α-mannosidase, snail β-mannosidase and jack bean *N*-acetyl-β-D-glucosaminidase. This resulted in the predicted shifts of ~ 2, 1, and 2 GU, for a non core-fucosylated trimannosyl structure.

Methylation analysis of the oligosaccharides isolated from bee venom phospholipase A_2 produced the methylated alditol acetates shown in Table I. The recovered methylated species are consistent with the oligosaccharide structures. Linkage positions are all in agreement with previously published data on glycosylation in insect cells[6–8] and with the common structures of *N*-linked oligosac-

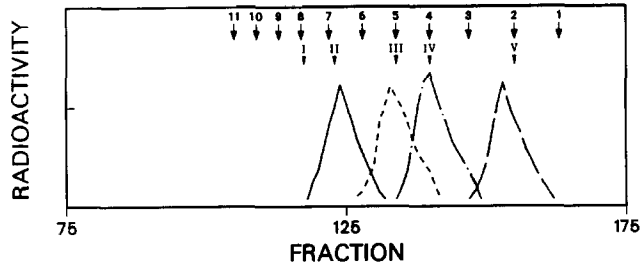

Fig. 3. Sequential exoglycosidase digestion of oligosaccharides co-eluting with dextran oligomers composed of 6.6 glucose units. In vitro labeled phospholipase A_2 oligosaccharides were isolated and separated by Biogel P-4 chromatography as outlined in the legend to Fig. 1. The oligosaccharides eluting at 6.6 glucose units (———) were re-chromatographed on the column after digestion with jack bean α-mannosidase (— — —), snail β-mannosidase (-·-·-) and jack bean *N*-acetyl-β-D-glucosaminidase (——— ——— ———). Numbers 1 through 10 indicate the elution volumes of dextran oligomers composed of 1–10 glucose units. The elution positions of oligosaccharide standards are indicated by roman numerals: I, Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc-ol; II, Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-ol; III, Man(β1-4)GlcNAc(β1-4)GlcNAc-ol; IV, GlcNAc(β1-4)GlcNAc-ol; V, GlcNAc.

TABLE II

Proposed structures of N-linked oligosaccharides isolated from bee venom phospholipase A_2

Structure	Relative amount
Manα1→6 / Manα1→3 Manα1→6 / Manα1→3 Manβ1−4GlcNAcβ1−4GlcNAc	9.6
Manα1→6 / Manα1→6 / Manα1→3 Manβ1−4GlcNAcβ1−4GlcNAc (Fucα1→6)	9.2
Manα1→6 / Manα1→3 Manβ1−4GlcNAcβ1−4GlcNAc (Fucα1→6)	43
Manα1→6 / Manα1→3 Manβ1−4GlcNAcβ1−4GlcNAc	38

charides[12]. The absence of 2,4,6-tri-*O*-methylated mannose suggests that the mannosidase(s) responsible for processing of the high-mannose precursor(s) in insect cells cleaves the (1 → 3)-linked mannose residue on the (1 → 6) arm of the core before the (1 → 6)-linked mannose.

Table II summarizes the oligosaccharide structures recovered from bee venom phospholipase A_2. The molecule appears to contain four types of oligosaccharides: Man_5 and Man_3 structures without a core fucose and core-fucosylated Man_4 and Man_3 structures. Approximately half of the oligosaccharides on the molecule are core-fucosylated and the results from the methylation analysis suggest that the fucose residues are invariably linked to the 6 position of the reducing *N*-acetylglucosamine. This substitution position has previously been reported for the core fucose residues on a glycoprotein synthesized by the insect cell line Sf9[8]. Interestingly the largest core-fucosylated structure on phospholipase A_2 is Man_4. Previous work has positively identified core-fucose residues only on Man_3 structures[7,8], although methylation analysis data indicating the existence of core-fucosylated Man_4 and Man_5-GlcNAc structures have been presented for bee venom phospholipase A_2 (ref. 5). The substrate specificity of the insect core fucosyl transferase is not known, however it clearly differs from the mammalian counterpart since this enzyme has an absolute requirement for the addition of an *N*-acetylglucosamine to the Man_3 core prior to transfer of fucose[13]. Furthermore, a recent report by Staudacher et al.[14] suggest the presence of more than one core fucosyl transferase in the bee venom gland. These investigators found only two structures when characterizing the oligosaccharides on bee venom phospholipase

A_2: a core-fucosylated Man_3 structure with the fucose residue linked α-(1 → 6) to the reducing *N*-acetylglucosamine and a core-difucosylated Man_3 structure with one fucose residue linked α-(1 → 3) and another linked α-(1 → 6) to the reducing *N*-acetylglucosmine. We found no evidence for di-fucosylated oligosaccharides in our work, in fact ~ 50% of the structures characterized in our investigation are not fucosylated at all. Also different from the results reported by Staudacher et al.[14] are the almost 20% of the oligosaccharides recovered in our investigation which contain more than three mannose residues. A possible explanation for these differences is the use of different working material. We have noticed considerable differences in the distribution of oligosaccharide structures on phospholipase A_2 from different sources (Hollander and Elhammer, unpublished observations). In this context it is of interest to note that we found no evidence for the terminal *N*-acetylglucosamine containing truncated high-mannose structures, reported on bee venom phospholipase A_2 by Weber et al.[5], nor did we find any indication of other processed (complex or hybrid type) oligosaccharides; recent reports have demonstrated the presence of sialylated complex type oligosaccharides on human plasminogen synthesized by *Spodoptera frugiperda* as well as *Mamestra brassicae* cells[15–17]. Instead our findings are in agreement with reports suggesting that insect cells do not synthesize these kinds of structures[6–8,18,19], most likely due to the fact that they appear to lack the necessary glycosyltransferases[20].

REFERENCES

1 E. Habermann, *Science*, 177 (1972) 314–318.
2 R.A. Shipolini, G.L. Callewaert, R.C. Cottrell, and C.A. Vernon, *Eur. J. Biochem.*, 48 (1974) 465–476.
3 R.A. Shipolini, S. Doonan, and C.A. Vernon, *Eur. J. Biochem.*, 48 (1974) 477–483.
4 K. Kuchler, M. Gmachl, M.J. Sippl, and G. Kreil, *Eur. J. Biochem.*, 184 (1989) 249–254.
5 A. Weber, H. Schröder, K. Thalberg, and L. Märtz, *Allergy*, 42 (1987) 464–470.
6 P. Hsieh and P.W. Robbins, *J. Biol. Chem.*, 259 (1984) 2375–2382.
7 K. Kuroda, H. Geyer, R. Geyer, W. Doerfler, and H.-D. Klenk, *Virology*, 174 (1990) 418–429.
8 M. Wathen, P.A. Aeed, and Å.P. Elhammer, *Biochemistry*, 30 (1991) 2863–2868.
9 S. Takasaki, T. Mizouchi, and A. Kobata, *Methods Enzymol.*, 83 (1982) 263–268.
10 K. Yamashita, T. Mizouchi, and A. Kobata, *Methods Enzymol.*, 83 (1982) 105–126.
11 P.A. Aeed, D.M. Guido, W.R. Mathews, and Å.P. Elhammer, *Biochemistry*, 31 (1992) 6951–6961.
12 R. Kornfeld and S. Kornfeld, *Annu. Rev. Biochem.*, 54 (1985) 631–664.
13 J.R. Wilson, D. Williams, and H. Schachter, *Biochem. Biophys. Res. Commun.*, 3 (1976) 909–916.
14 E. Staudacher, F. Altmann, L. März, K. Hård, J.P. Kamerling, and J.F.G. Vliegenthart, *Glycoconjugate J.* 9 (1992) 82–85.
15 D.J. Davidson, M.J. Fraser, and F.J. Castellino, *Biochemistry*, 29 (1990) 5584–5590.
16 D.J. Davidson and F.J. Castellino, *Biochemistry*, 30 (1991) 6167–6174.
17 D.J. Davidson and F.J. Castellino, *Biochemistry*, 30 (1991) 6689–6696.
18 D.L. Domingo and I.S. Throwbridge, *J. Biol. Chem.*, 263 (1988) 13386–13392.
19 W. Chen and O.P. Bahl, *J. Biol. Chem.*, 266 (1991) 6246–6251.
20 T.D. Butters, R.C. Hughes, and P. Vischer, *Biochim. Biophys. Acta*, 640 (1981) 672–686.