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Structures of the N-linked carbohydrate of ascorbic acid oxidase from zucchini

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The *N*-glycan moiety of ascorbic acid oxidase from zucchini (*Cucurbita pepo*) has been described to be a core-pentasaccharide with a xylose [D'Andrea *et al.* (1988) *Glycoconjugate J* 5:151–7]. Ascorbic acid oxidase is sometimes used to characterize antibodies directed against carbohydrate determinants on plant glycoproteins. To prevent misinterpretations of immunological data, the structure of the *N*-glycan of ascorbic acid oxidase has been reinvestigated. The oligosaccharides were released by almond *N*-glycosidase and analysed as their pyridylamino derivatives by 2D-HPLC and exoglycosidase digestions. The main structure resembled the typical complex plant *N*-glycan consisting of a core-pentasaccharide decorated with xylose and 3-linked fucose. The other abundant species lacked the fucose residue. Small amounts of these glycans carried a GlcNAc residue on the 6-arm. Therefore, ascorbic acid oxidase will not only react with antibodies directed against the xylosylated region but also with those binding to *N*-glycans with 3-linked fucose.

***Keywords*: asparagine-linked oligosaccharides, plant glycoproteins, N-glycans**

***Abbreviations*: GnGn, GlcNAcβ1-Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc; MGn, Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc; M0XF^3, Manα1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc; MMXF^3, MMX, GnMXF^3 and GnMX, N-glycan structures depicted in Table 2.**

Introduction

Ascorbic acid oxidase (E.C. 1.10.3.3.) is a copper-containing enzyme widely found in plants. The commercially available enzyme from zucchini (*Cucurbita pepo* L.) is a glycoprotein with one N-glycosylation site at Asn-92 [1]. The primary structure of its N-glycan has been investigated by 500 MHz ^{1}H-NMR and found to be as shown in Figure 1 [2]. In this study, the carbohydrate was released from the glycoprotein by means of N-glycosidase F. However, since *N*-glycosidase F will not hydrolyse N-glycans with a fucose α1,3-linked to the asparagine-bound GlcNAc, it appears inappropriate for digestion of plant and insect glycoproteins [3, 4].

In a recent paper, van Ree *et al.* have characterised the IgE-binding properties of *Lol p* XI, a new allergen from the pollen of grass *Lolium perenne* [5]. *Lol p* XI was shown to carry an IgE-binding carbohydrate determinant. The binding of anti-carbohydrate IgE to *Lol p* XI could be inhibited by more than 90% by both honeybee venom phospholipase A_2 and ascorbate oxidase. The bee venom glycoprotein does not contain xylose. Rather it is characterized by the presence of α1,3-linked fucose [6]. This residue is essential for the binding of IgE antibodies from bee venom allergic persons [7]. However, according to D'Andrea *et al.* [2], the N-glycans of the oxidase contain xylose but no fucose. Possible explanations for the discrepancy could be that the immunoreaction with *Lol p* XI was inhibited by two different carbohydrate determinants or that the structure found by D'Andrea *et al.* comprised only a fraction of the N-glycans present on ascorbate oxidase. To resolve this problem, the structure of the N-linked carbohydrate of ascorbic acid oxidase from zucchini has been reinvestigated.

Materials and methods

Ascorbic acid oxidase was obtained from Boehringer Mannheim. On SDS-PAGE, it gave a major band of 70 kDa and a faint band of higher molecular mass (data not shown) which is consistent with the reported mass of the native dimer of 140 kDa [1]. To obtain the reducing oligosaccharides of ascorbic acid oxidase, the native enzyme was digested with pepsin and subsequently with N-glycosidase A (Boehringer Mannheim) [3]. The oligosaccharide/peptide mixture was diluted with 5% acetic acid and passed through a column of Dowex 1-X2. Free oligosaccharides were eluted with 5% acetic acid and lyophilised. The oligosaccharides were pyridylaminated [8] and subjected to two-dimensional HPLC as described [6, 9]. Individual peaks from the

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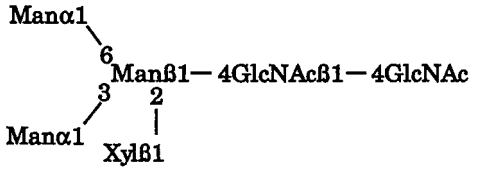


Figure 1. Reported structure of the N-glycan from ascorbic acid oxidase [2].

sizing column (MicroPak AX-5, Varian) were collected, lyophilized and subjected to reversed phase HPLC on 5 μm Hypersil ODS. The elution times on both columns are expressed in Glc-units by comparison with a series of pyridylaminated isomalto-oligosaccharides. Digestions with jack bean α-mannosidase ('low dose' = 10 mU in 20 μl) and jack bean *β-N*-acetylglucosaminidase (10 mU in 20 μl; both enzymes from Sigma) were performed as described [6, 9].

By a procedure similar to that described above, pyridylaminated reference oligosaccharides having the structure $XylMan_3GlcNAc_2Fuc$ ($MMXF^3$, see Table 2) and $XylMan_2GlcNAc_2Fuc$ ($M0XF^3$), where the terminal α-mannose is 6-linked to the β-mannosyl residue, were prepared from horseradish peroxidase and bromelain, respectively [10–12]. $XylMan_3GlcNAc_2$ (MMX, Table 2) was obtained by incubating peroxidase glycopeptides with concentrated trifluoroacetic acid for 24 h at room temperature before N-glycosidase digestion and derivatization of the oligosaccharide [10]. The structures of these standard compounds were confirmed by methylation analysis (data not shown). Pyridylaminated GnGn ($Gn_2Man_3Gn_2$) and MGn ($GnMan_3Gn_2$, the GlcNAc being linked to the 3-arm mannose) were prepared from bovine fibrin as in a previous study [14].

Results

The asparagine-linked oligosaccharides from ascorbic acid oxidase were released by N-glycosidase A and pyridylaminated. Four oligosaccharide fractions were obtained on the sizing column (Figure 2). Each fraction gave a single peak when chromatographed by reversed phase HPLC. The retention data of the four peaks and of the reference compounds are compiled in Table 1. The structural elucidation of the glycans, starting with peak '**3**' was based on the following observations.

On a two-dimensional map, peak '**3**' lay at the same position as the oligosaccharide $MMXF^3$ ($XylMan_3GlcNAc_2Fuc$) from horseradish peroxidase. Digestion with α-mannosidase increased the retention time on reversed phase by about 3 min and gave a product coeluting with $M0XF^3$ from bromelain (Table 1). For this study, an amount of mannosidase not sufficient to release the α1,6-linked mannose residue from the trimannosyl core was chosen. The rather large increase in retention on reversed phase of peak

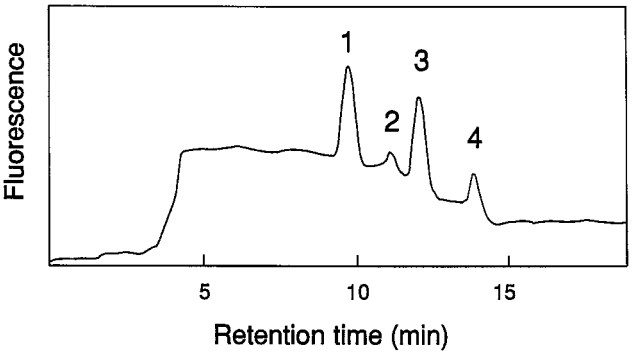


Figure 2. Fractionation of the pyridylaminated N-glycans from ascorbic acid oxidase on the sizing column (MicroPak AX-5). The numbers indicate the collected peaks.

Table 1. Elution positions of pyridylamino oligosaccharides.

Compound	*Elution position (Glc-units) on*	
	MicroPak AX-5	*Reversed phase*
Ascorbic acid oxidase		
Fraction **1**	5.6	7.4
post *α*-mannosidase	–	9.0
Fraction **2**	6.4	9.1
post *α*-mannosidase	–	> 10
post *β-N*-acetylglucosaminidase	–	7.1
Fraction **3**	6.8	4.9
post *α*-mannosidase	–	5.9
Fraction **4**	7.6	5.6
post *α*-mannosidase	–	7.0
post *β-N*-acetylglucosaminidase	–	4.8
Reference glycans (see Abbreviations)		
$MMXF^3$	6.8	4.9
$M0XF^3$	–	5.8
M0X	–	9.0
GnGn	–	8.8
MGn	–	6.8

'**3**' upon mannosidase digestion was consistent with the removal of an α1,3-linked mannose from a structure containing xylose. Removal of an α1,3-linked mannose residue from MMF^3 ($Man_3GlcNAc_2Fuc$, the fucose being α1,3-linked) would have led to a much smaller elution time shift on reversed phase. This behaviour gives, in addition to size, a valuable means of discriminating $MMXF^3$ from MMF^3, since their elution times on reversed phase are almost identical. The data strongly suggest the structure of peak '**3**' to be as shown in Figure 4, *ie* the typical 'complex' plant N-glycan containing xylose and fucose.

The other major component, peak '**1**', was smaller than '**3**' by about one glucose unit. Its higher elution time on reversed

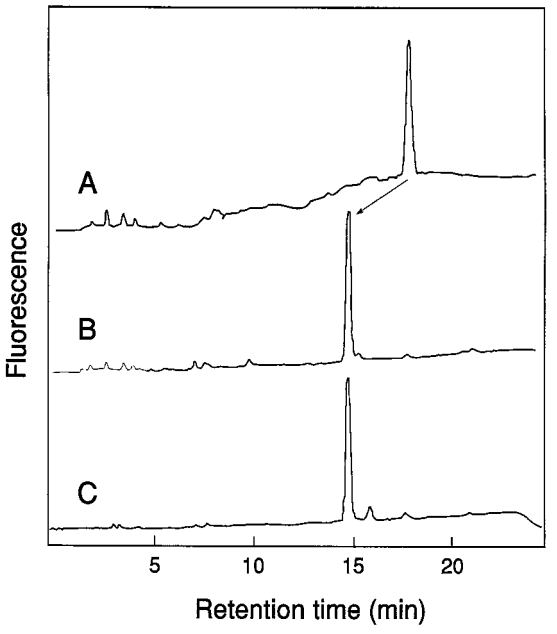


Figure 3. Analysis of peak '**4**' by digestion with jack bean *N*-acetyl-*β*-glucosaminidase and reversed phase HPLC. (A) peak '**4**', (B) peak '**4**' after digestion with *N*-acetyl-*β*-glucosaminidase, and (C) peak '**3**'.

phase indicated the absence of the 3-linked fucose residue, as this moiety has a strong impact on retention behaviour [6, 9, 13]. Peak '**1**' was also sensitive to α-mannosidase and gave a product (M0X) coeluting with a defucosylated bromelain glycan. Thus, peak '**1**' is concluded to be the non-fucosylated analogue of structure '**3**' and therefore corresponds to the structure reported by D'Andrea *et al.* [2].

Peaks '**2**' and '**4**' likewise gave a retention time shift of roughly 3 min upon digestion with α-mannosidase. Both peaks apparently had an unsubstituted 3-linked mannosyl residue. Both peaks eluted later than peaks '**1**' and '**3**', respectively; by 0.8 Glc-units on the sizing column and by 2.1 and 3.5 min on reversed phase. These data are consistent with a substitution of the 6-linked mannosyl residue by GlcNAc [9, 13, 14]. All four compounds were, therefore, incubated with jack bean *β*-*N*-acetylglucosaminidase. Indeed, peaks '**2**' and '**4**' were sensitive to this enzyme and gave products at the elution positions of '**1**' and '**3**' (Table 1; Figure 3). The elution times of peaks '**1**' and '**2**' resemble the difference between reference compound MGn and GnGn which likewise just differ by the absence or presence of a GlcNAc residue on the 6-arm (Table 1). Peaks '**1**' and '**3**' were insensitive to *β*-*N*-acetylglucosaminidase. According to these results, the structures of the four peaks were assigned as shown in Table 2.

Discussion

The structures found in this study comprise the frequently occurring 'vacuole-type' complex N-glycan from plants, MMXF^3 [see *eg* 10, 15–22] and less common structures lacking a fucosyl residue or having an additional GlcNAc residue linked to the 6-arm. Such structures have already

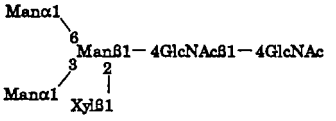
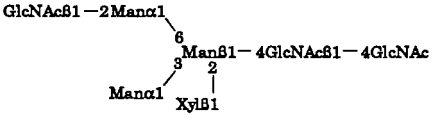
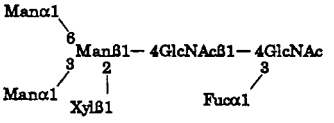
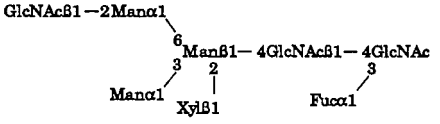
Peak No.	Abbreviation	Proposed structure	Amount (%)
1	MMX		38
2	GnMX		5
3	MMXF^3		43
4	GnMXF^3		14

Table 2. Proposed structures of the N-glycans from ascorbic acid oxidase from zucchini.

been found in other plant glycoproteins [5, 19, 22–25]. D'Andrea *et al.* [2] who just found MMX had obtained their ascorbate oxidase from a different source. Another difference is the use of N-glycosidase F instead of N-glycosidase A, as in the present study. Thus structures with a 3-fucosylated reducing GlcNAc present, would not have been released and detected.

The results of the present study provide an explanation for the similar carbohydrate-based immunological reactivity of ascorbic acid oxidase and phospholipase A_2 from honeybee venom, as both glycoproteins are shown to contain structures with a 3-fucosylated core. Garcia-Casado *et al.* investigated the reaction of IgE from baker's asthma patients with N-glycans [26] with various glycoproteins including ascorbic acid oxidase. They claimed that the major immunoreactive residue is the β1,2-linked xylose rather than the α1,3-linked fucose, mainly based on the structure of the oxidase's N-glycans as originally reported [2]. However, in the light of the present structural investigation the results of Garcia-Casado *et al.* would be entirely consistent with α1,3-linked fucose being the major immunoreactive carbohydrate epitope, as was the case with IgE from bee venom allergic patients and anti-horseradish peroxidase IgG [7, 10]. Batanero *et al.* [27] used, among other glycoproteins, ascorbic acid oxidase and honey bee phospholipase when investigating the reactivity of antibodies with an olive pollen allergen. However, their conclusion that the IgE antibodies were interacting with xylosylated structures was supported by the lack of reactivity with phospholipase of pooled patients' sera, as well as lack of reactivity of a rabbit antiserum against olive pollen, and is therefore, not called into question by the present study.

Acknowledgements

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