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Identification of the human Lewis[a] carbohydrate motif in a secretory peroxidase from a plant cell suspension culture (*Vaccinium myrtillus* L.)

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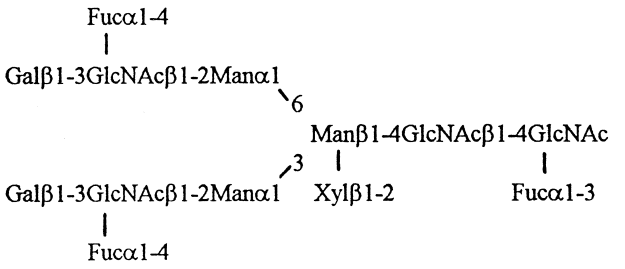
Abstract This paper reports for the first time the presence of the human Lewis[a] type determinant in glycoproteins secreted by plant cells. A single glycopeptide was identified in the tryptic hydrolysis of the peroxidase VMPxC1 from *Vaccinium myrtillus* L. by HPLC/ESI-MS. The oligosaccharide structures were elucidated by ESI-MS-MS and by methylation analysis before and after removal of fucose by mild acid hydrolysis. The major structure determined is of the biantennary plant complex type containing the outer chain motif Lewis[a]



A corresponding fucosyltransferase activity catalyzing the formation of Lewis[a] type structures in vitro was identified in cellular extracts of the suspension cultures.

Key words: Extracellular glycoprotein; Lewis[a]; Peroxidase; Plant complex oligosaccharide; *Vaccinium myrtillus*

1. Introduction

The studies on the biological roles of glycoprotein oligosaccharide moieties have been intensified due to increasing evidence of their importance as recognition determinants in host-pathogen interactions, in protein targeting and in cell-cell interactions. For example, the sialyl Lewis[x] and Lewis[x] determinants are found on glycoproteins and glycolipids at the surface of leukocytes and tumor cells [1] whereas the sialyl Lewis[a] and Lewis[a] determinants are mostly found at the surface of cancer cells of the digestive system and seem to be directly involved in the process of metastasis [2]. Molecules containing these motifs at their surface can be efficiently used for the inhibition of cellular interactions, and may be helpful in the therapeutic treatment of metastasis. The presence of Le[a] motifs seemed so far to be restricted to animal cells. In mung bean seedlings an enzyme with human-like Lewis fucosyltransferase activity was found [3], but not the corresponding oligosaccharide structures. The oligosaccharides from plant glycoproteins described so far are of the plant complex type defined by the Manα1–3,6(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc motif, or of the oligomannose type. The plant complex glycans of the vacuolar glycoproteins have one or two Man residues linked to the branching Man [4], whereas secreted glycoproteins have biantennary oligosaccharides [5].

Peroxidases (EC 1.11.1.7) are heme containing glycoproteins catalyzing the reduction of hydrogen peroxide by electron transfer, using a wide range of electron donor molecules. These enzymes are widely distributed in plants, and seem to play an important role in several plant physiological processes, including lignin biosynthesis, pathogen resistance and response to stress [6]. Previously we have described the establishment of a cell suspension culture of *Vaccinium myrtillus*, and the purification and characterization of the two major extracellular cationic peroxidases, VMPxC1 and VMPxC2 [7,8].

In the present article, we report a novel oligosaccharide structure of the biantennary plant complex type containing the Le[a] motif, from a plant secretory glycoprotein. A corresponding fucosyltransferase activity transferring L-Fuc from GDP-Fuc to the type I disaccharide Galβ1–3GlcNAc-OR [R = $(CH_2)_8COOMe$], but not to the type II Galβ1–4GlcNAc-OR acceptor has been identified in cellular extracts.

2. Materials and methods

2.1. Plant material and cell cultures

Seeds of *V. myrtillus* L. were kindly provided by the Botanical Garden of the University of Basel. Cell suspension cultures were established from callus of *V. myrtillus* and sub-cultured every 9 days, as previously described [7].

2.2. Peroxidase purification

VMPxC1 was purified from the culture medium of *V. myrtillus* cell suspension cultures, by cation exchange and molecular exclusion chromatography using a FPLC system (Pharmacia, Sweden) and checked for purity as previously described [7].

2.3. Release of N-glycans

N-Glycans were released from VMPxC1 (approximately 250 μg) by automated hydrazinolysis in a Glycoprep 1000 instrument (N mode, Oxford Glycosystems), or from the corresponding glycopeptide with 0.3 mU PNGase A as previously described [4]. Oligosaccharides were defucosylated by mild acid treatment using 0.2% trifluoroacetic acid at

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Abbreviations: ESI-MS, electrospray ionization mass spectrometry; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Le[a], Lewis[a]; Le[x], Lewis[x]; MALDI/TOF-MS, matrix assisted laser desorption/ionization time of flight mass spectrometry; Man, mannose; MS, mass spectrometry; PNGase A, peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase A; VMPxC1 or C1, *Vaccinium myrtillus* peroxidase C1; Xyl, xylose

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85°C for 1 h. Initial attempts to remove *N*-glycans with PNGase A from the native enzyme were not successful.

2.4. HPLC/ESI-MS

Tryptic peptide mixtures (approximately 100 pmol) were analyzed on an Applied Biosystems 172A microbore HPLC system using an Aquapore OD-300 C_{18} column (1.0 mm × 100 mm) at a flow rate of 40 μl/min and a linear gradient of 4–56% acetonitrile in 0.06% trifluoroacetic acid. Elution of peptides was monitored by UV absorption at 214 nm and by mass spectrometry (MS) on a TSQ 700 triple quadrupole instrument equipped with a Finnigan ES ion source connected on line to the HPLC system. Glycopeptides were selectively detected using MS conditions [9] favoring nozzle/skimmer fragmentation in the lower mass range by characteristic fragment ions of *m/z* 366 [Hex HexNAc+H] and 512 [Hex dHex HexNAc+H]. In a preparative run, the glycopeptide fraction was collected manually and subjected to Edman sequencing (Applied Biosystems, model 475A).

2.5. MALDI/TOF-MS

The intact glycoprotein was analyzed using a matrix of 22.4 mg 3,5-dimethoxy-4-hydroxycinnamic acid in 400 μl acetonitrile and 600 μl 0.1% (v/v) trifluoroacetic acid in H_2O, glycopeptides were measured using a matrix of 19 mg α-cyano-4-hydroxycinnamic acid in the same solvent mixture; native or reduced and permethylated oligosaccharides were analyzed using 2,5-dihydroxybenzoic acid as UV-absorbing material. For MALDI/TOF-MS, the sample solutions were mixed with the same volume of the respective matrix, 1 μl of sample was spotted onto the stainless steel tip and dried at room temperature. The concentration of the analyte mixture was 5–25 pmol/μl.

2.6. Carbohydrate compositional analysis and methylation analysis

Monosaccharides were analyzed by gas chromatography on a Carlo Erba Mega Series instrument after methanolysis, reacetylation and trimethylsilylation as the corresponding methyl glycosides on a 30 m DB1 capillary column [10]. For methylation analysis, oligosaccharides were permethylated according to Hakomori [11], purified, hydrolyzed, reduced and peracetylated as described [12]. Separation and identification of partially methylated alditol acetates were performed on a Finnigan gas chromatograph (Finnigan MAT Corp., San Jose, CA), equipped with a 30 m DB5 capillary column, connected to a Finnigan GCQ ion trap mass spectrometer.

2.7. Preparation of cell extracts

Cell extracts were freshly prepared prior to each assay. Suspension cells and aggregates were recovered after 4 days of culture by centrifugation at 5000 × *g*. The cells were immediately frozen with liquid nitrogen to disrupt cell walls, and homogenized at 0°C after resuspending in ice-cold extraction buffer (20 mM MOPS-KOH buffer pH 7.5 containing 1% Triton X-100). After 1 h incubation, the homogenate was centrifuged at 10 000 × *g* for 10 min. The supernatant was recovered and used within the same day for fucosyltransferase activity determinations, or used for fucosyltransferase purification. Fucosyltransferase activity determination and enzyme purification were performed as previously described [13]. Fucosylated 8-methoxycarbonyloctyl glycoside acceptors were defucosylated with almond α3,4-fucosidase (Oxford Glycosystems).

3. Results

3.1. Protein purification and preliminary characterization of oligosaccharides

VMPxC1 was purified from cell culture supernatants by cation exchange and molecular exclusion chromatography. The final protein preparation appeared as a single band of apparent molecular mass 33 000 in SDS-PAGE (Fig. 1). This is in good agreement with the broad molecular ion signal around 32 800 obtained for the native protein by MALDI/TOF-MS.

For the preliminary characterization of the oligosaccharides, the total glycans were released from the glycoprotein by automated hydrazinolysis at 95°C for 5 h. Compositional analysis of the total glycans revealed the presence of Xyl, Fuc, Man, Gal and GlcNAc in a ratio of 1:2.2:3:1.3:3.6. After reduction and permethylation, oligosaccharide mapping by MALDI/TOF-MS yielded major molecular ions at *m/z* = 2770 [Hex_5 $dHex_3$ Pent $HexNAc_3$ HexNAc-ol+Na], 2596 [Hex_5 $dHex_2$ Pent $HexNAc_3$ HexNAc-ol+Na], 2391 [Hex_4 $dHex_2$ Pent $HexNAc_3$ HexNAc-ol+Na] and 2146 [Hex_4 $dHex_2$ Pent $HexNAc_2$ HexNAc-ol+Na] suggesting the presence of complex type oligosaccharides. The detection of considerable amounts (30%) of ion signals compatible with loss of the proximal HexNAc-Fuc unit indicated partial degradation of oligosaccharides during the hydrazinolysis conditions. Methylation analysis (Table 1) confirmed the presence of 3-linked

Table 1
Methylation analysis of VMPxC1 oligosaccharides

Peracetylated derivative of	Substituted in position	Hydrazinolysis	PNGase A	
			before acid hydrolysis	after acid hydrolysis
Xylitol				
2,3,4-Tri-*O*-methyl-	terminal	0.9	0.8	0.9
Fucitol				
2,3,4-Tri-*O*-methyl-	terminal	2.4	2.4	0.9
Mannitol				
2,3,4,5-Tetra-*O*-methyl-	terminal	0.2	0.2	0.2
3,4,6-Tri-*O*-methyl-	2	1.8	1.8	1.8
3,4-Di-*O*-methyl-	2,6	trace	0.1	0.1
4-*O*-methyl-	2,3,6	1.0	1.0	1.0
Galactitol				
2,3,4,5-Tetra-*O*-methyl-	terminal	1.4	1.3	1.4
2-*N*-Methylacetamido-2-deoxyglucitol				
1,3,5,6-Tetra-*O*-methyl-	4	0.3[a]	n.d.	0.6
1,5,6-Tri-*O*-methyl-	3,4	0.7	0.9	0.4
3,4,6-Tri-*O*-methyl-	terminal	0.3	0.2	0.2
3,6-Di-*O*-methyl-	4	0.7	0.9	1.0
3,6-Di-*O*-methyl-	3	0.1	0.1	0.9
6-*O*-methyl-	3,4	1.5	1.5	0.6
3-*O*-methyl-	4,6	n.d.	n.d.	n.d.

n.d., not detected.
[a]This derivative, indicating a 4-monosubstituted reduced GlcNAc residue, was probably generated by the partial cleavage of the chitobiose bond of the oligosaccharides during hydrazinolysis. MALDI/TOF-MS of the native and permethylated oligosaccharides confirmed this partial degradation.

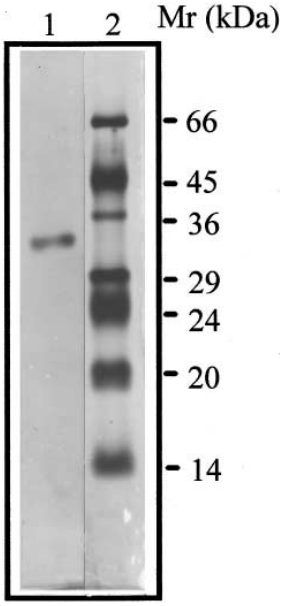

Fig. 1. SDS-PAGE of VMPxC1 peroxidase purified from *V. myrtillus* cell suspension cultures. Molecular mass standards are indicated on the right. Proteins were detected by silver staining.

proximal Fuc and Xyl linked to the O-2 of the branching Man residue. These structural characteristics have been previously reported for plant *N*-glycans [4]. The detection of large amounts of the monosaccharide derivative characteristic of 3,4-disubstituted GlcNAc, however, suggested the presence of either Le^a or Le^x type structural motifs on the outer antennae, which have not been observed before in plant glycoproteins.

3.2. *Identification of a single tryptic glycopeptide in VMPxC1*

Detailed carbohydrate structural information on the glycoprotein was obtained after tryptic digestion and HPLC/ESI-MS analysis of the resulting peptide mixture. Fig. 2a shows the background subtracted reconstructed total ion chromatogram after HPLC/ESI-MS with the glycopeptide peak indicated with an arrow. The presence of a single glycopeptide is conclusively shown in Fig. 2b which depicts the selective traces [9] of two glycan fragment ions [*m/z* 366: Hex HexNAc+H plus *m/z* 512: HexNAc Hex dHex] that could be expected from the preliminary carbohydrate analysis data. The low intensity of the glycopeptide peak is possibly due to low ionization properties since the signal in the UV showed the expected intensity. The mass range of the triple charged molecular ions of this glycopeptide fraction is depicted in Fig. 2c. Based on the amino acid sequence of the isolated glyco-

Table 2
Proposed structures and relative molar ratios of the oligosaccharides from the single *N*-glycosylation site in VMPxC1

	Oligosaccharide structure	Glycopeptide masses*	Relative molar ratios**
A	Manα1-6(Manβ1-4GlcNAcβ1-4GlcNAc) Galβ1-3GlcNAcβ1-2Manα1-3(Xylβ1-2)(Fucα1-3) Fucα1-4	3495.4 (3495.3)	0.17
B	GlcNAcβ1-2Manα1-6(Manβ1-4GlcNAcβ1-4GlcNAc) Galβ1-3GlcNAcβ1-2Manα1-3(Xylβ1-2)(Fucα1-3) Fucα1-4	3698.6 (3698.7)	0.23
C	Galβ1-3GlcNAcβ1-2Manα1-6(Manβ1-4GlcNAcβ1-4GlcNAc) Galβ1-3GlcNAcβ1-2Manα1-3(Xylβ1-2)(Fucα1-3) Fucα1-4	3860.7 (3859.9)	0.11
D	Fucα1-4 Galβ1-3GlcNAcβ1-2Manα1-6(Manβ1-4GlcNAcβ1-4GlcNAc) Galβ1-3GlcNAcβ1-2Manα1-3(Xylβ1-2)(Fucα1-3) Fucα1-4	4008.0 (4009.0)	0.49

*Calculated average masses are given in parentheses.
**Relative molar ratios were calculated based on peak heights obtained by ESI-MS of the glycopeptides and MALDI signal ratios of the native oligosaccharides.

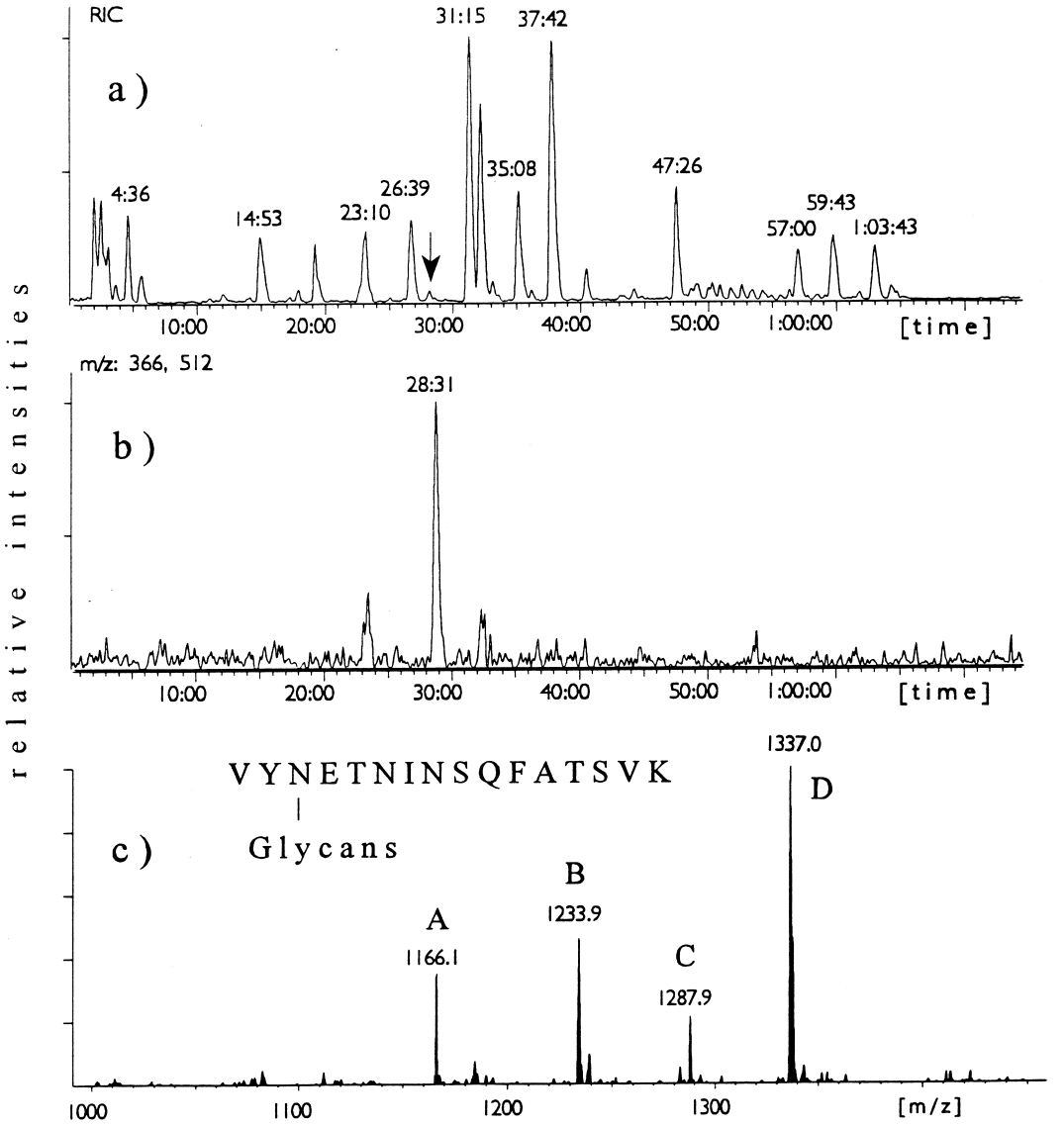

Fig. 2. HPLC/ESI-MS of tryptic peptides. a: Baseline subtracted reconstructed ion current chromatogram; the arrow indicates the elution position of the single glycopeptide from VMPxC1. b: Summed ion traces of selected glycan fragment ions (*m/z* 366 [HexHexNAc+H] and 512 [Hex dHex HexNAc+H]); the mass spectra of the lower intensity peaks showed no indication for the presence of other glycopeptides and are probably due to low molecular weight contaminants. c: Molecular ion mass region of the triply charged glycopeptide signals corresponding to molecular masses of A–D of 3495.3, 3698.7, 3860.7 and 4006.5 Da, respectively. The molecular masses are compatible with the monosaccharide compositions $Fuc_2GalMan_3XylGlcNAc_3$ (A), $Fuc_2GalMan_3XylGlcNAc_4$ (B), $Fuc_2Gal_2Man_3XylGlcNAc_4$ (C), and $Fuc_3Gal_2Man_3XylGlcNAc_4$ (D), respectively.

peptide resulting from automated Edman degradation (VYNETNINSQFATSVK) the signals $[M+3H]^{3+}$ at *m/z* 1166.1, 1233.9, 1287.9 and 1337.0 correspond to molecular masses that are in agreement with the modification by the different carbohydrate structures shown in Table 2.

3.3. ESI/MS-MS and methylation analysis of reduced and permethylated glycans

In order to determine the structures of the intact oligosaccharides these were enzymatically released from the tryptic glycopeptide by digestion with PNGase A.

After reduction and permethylation of the oligosaccharide mixture structural information was obtained by ESI/MS-MS analysis. Individual doubly charged (disodium adducts) molecular ion species (parent ions) were selected using the first mass analyzer and fragments were generated by collision induced dissociation with argon atoms. Fig. 3 shows the daughter ion spectrum of the largest oligosaccharide (*m/z* 1396: Hex_5 $dHex_3$ Pent $HexNAc_3$ HexNAc-ol+2Na) corresponding to the glycopeptide D in Fig. 2c. From the fragmentation pattern the following structural features were deduced (see scheme in Fig. 3). A fragment ion at *m/z* 490 [dHexHexNAc-ol+Na] indicated the linkage of Fuc to the proximal GlcNAc. The Fuc is linked at the O-3 of GlcNAc since a weaker signal at *m/z* 284 is generated by secondary elimination of Fuc from the primary fragment ion. This is not observed with proximal Fuc linked α1,6 characteristic of mammalian type oligosaccharides (M. Nimtz, unpublished result). The intense fragment ion at *m/z* 660 [dHexHexHexNAc+Na] and the complementary doubly charged ion at *m/z* 1077 (M-[dHexHexHexNAc]+2Na) confines the linkage position of additional Fuc residues to the outer *N*-acetyllactosamine anten-

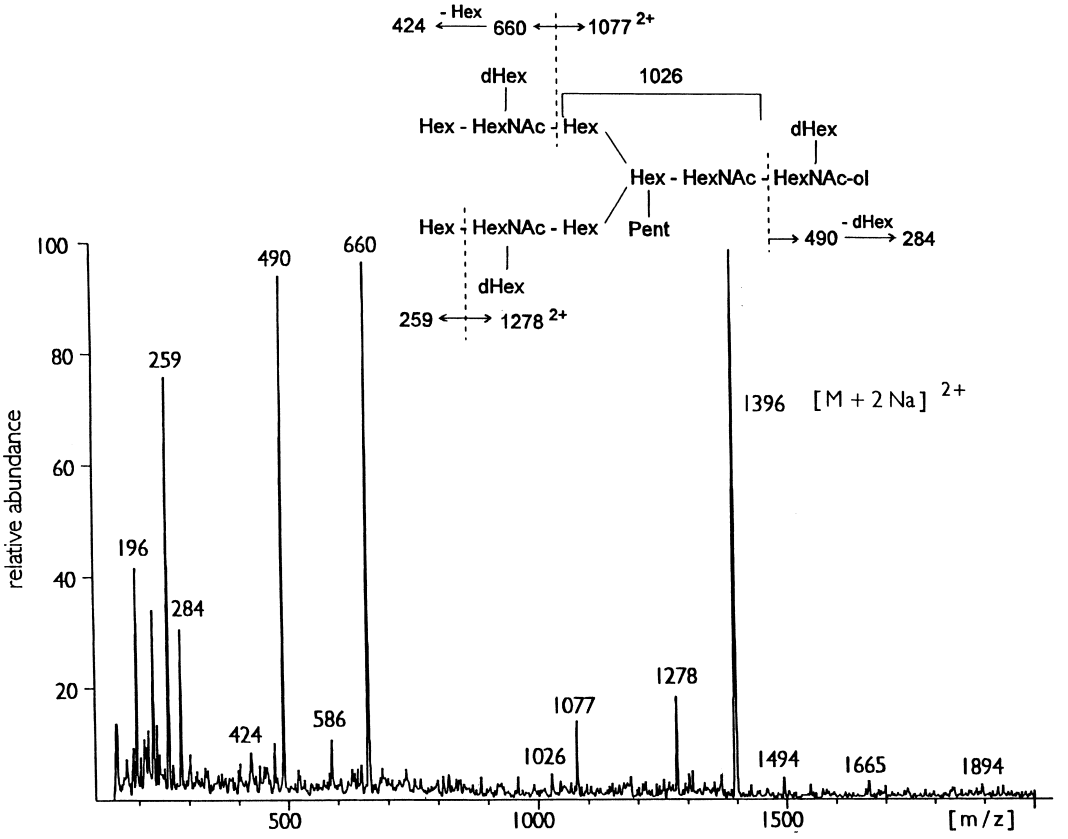

Fig. 3. ESI daughter ion mass spectrum of the largest reduced and permethylated oligosaccharide of VMPxC1 generated by collision induced dissociation of its doubly charged parent ion (disodium adduct). The inserted fragmentation scheme is explained in the text.

nae of a biantennary complex type structure. A signal at *m/z* 259 [Hex+Na] and the complementary doubly charged ion at *m/z* 1278 excludes Gal fucosylation. Therefore, Fuc should be linked to the GlcNAc residue of the antennae which indicates the presence of the Le^x or Le^a structural motifs. The preferential elimination of 3-linked substituents from GlcNAc residues compared to 4-linked isomers [14] suggests the presence of a Le^a motif with a 3-linked Gal and a Fuc residue 4-linked to GlcNAc, since the related fragment ions resulting from the loss of a hexose are present, whereas the fragments generated by elimination of Fuc residues were not observed. The linkage position of the plant specific Xyl residue to the branching Man was confirmed by a tertiary fragment ion at *m/z* 1026 comprising the xylosylated common core of Man and a GlcNAc residue. The position of the external α1,6-linked Man is proposed based on the known *N*-glycosylation biosynthetic pathway.

The glycopeptides A–C in Fig. 2c contain oligosaccharides similar to that described for glycopeptide D, where either Fuc (C), Fuc+Gal (B), or Fuc+Gal+GlcNAc (A) were absent from one antenna. This was deduced from the fragmentation patterns of the respective molecular ions and was further corroborated by the respective detection of 3-monosubstituted GlcNAc, terminal GlcNAc, and terminal Man in methylation analysis.

The linkage position of the Gal residues was unequivocally determined by subjecting the total glycan population to mild acid hydrolysis and subsequent methylation analysis of the resulting mixture. The amount of the 3-monosubstituted GlcNAc derivative increased significantly with a concomitant decrease of the 3,4-disubstituted derivative (Table 1).

3.4. Fucosyltransferase assays

The identification of Le^a structures in VMPxC1 led us to search for the related fucosyltransferase. Fucosyltransferase was assayed in culture medium and in cell extracts using 8-methoxycarbonyloctyl glycoside type I and type II acceptors. Cell extracts catalyzed the incorporation of Fuc into the type I acceptor at a specific incorporation of 0.4 nmol/min/mg protein, whereas no activity was detected towards the type II acceptor. The fucosylated substrate was incubated with almond α-fucosidase specific for 3-, 4-linked Fuc and it was observed that all the Fuc was removed from the substrate. These results indicate that the fucosyltransferase of cell extracts detected in vitro consist of Fuc transfer to the O-4 position of type I acceptors. No activity was detected in the culture medium. Partial purification of the cellular extracts by affinity chromatography in a GDP-Fractogel column [13] showed that the resin binds the α4-fucosyltransferase activity identified in the cellular extracts.

4. Discussion

The present work describes for the first time the elucidation of *N*-linked oligosaccharides with the Le^a type outer chain motif from a secreted plant glycoprotein. The Galβ1–3(Fucα1–4)GlcNAc-R structure is present in more than 90% of the glycan chains attached to a single glycopeptide.

Oligosaccharide mapping procedures by mass spectrometry techniques for characterization of natural and recombinant glycoproteins have been previously reported by our laboratory [15–17]. The detection of molecular ions by MALDI/TOF-MS or ESI-MS from native or permethylated oligosaccharides provides a rapid and highly sensitive 'mapping' procedure of oligosaccharides. However, this is an insufficient method for detailed structural analysis. In the present article we show that fragmentation patterns generated by MS-MS techniques can be successfully applied in the detection of novel structural carbohydrate motifs in reduced and permethylated mixtures of oligosaccharides from glycoproteins or glycopeptides. Furthermore, we show that this technique can be used to distinguish between the isomeric Le^x and Le^a structures which both give identical GlcNAc-derivatives in methylation analysis. The fucosylated type I antenna Galβ1–3(Fucα1–4)GlcNAc can be identified by an intense fragment ion from the 3-linked Gal by MS-MS whereas the signal obtained for Le^x type structures is significantly weaker. Furthermore, Lewis^a containing structures preferentially eliminate Gal, whereas Lewis^a type structures preferentially eliminate Fuc (M. Nimtz, unpublished results).

The increase of the 3-substituted GlcNAc derivative and the concomitant decrease of the 3,4-disubstituted GlcNAc derivative after acid hydrolysis observed from methylation analysis unequivocally demonstrated the presence of type I structures and consequently confirmed the Lewis^a motif as the dominant structure. Further supporting these results is the fact that the fucosyltransferase activity detected in cell extracts only transferred Fuc to the O-4 position of GlcNAc of the type I acceptor substrate.

The oligosaccharide structures characterized so far from plant cells are of the oligomannose type, e.g. soybean agglutinin, or of the plant complex type, when they contain Fuc α3-linked to proximal GlcNAc and Xyl β2-linked to the branching Man, e.g. horseradish peroxidase [18,19]. In most cases, the complex type oligosaccharides only have one or two Man residues α3- or α6-linked to the branching Man if the corresponding glycoproteins are targeted to the vacuole, or they are of the biantennary type with the Xyl, Fuc containing core when the corresponding glycoproteins are extracellular. It has been shown that a secreted glycoprotein, laccase, has peripheral Fuc linked to the GlcNAc in the biantennary chains, and it has been proposed that it would be α6-linked to a type II motif [5]. Our results rule out the presence of any α1,6-linked Fuc to GlcNAc in Galβ1–4GlcNAc antennae since no substitution of the amino-sugar in position O-6 could be detected and no increase of 4-substituted GlcNAc was observed after mild acid hydrolysis of the glycans. Preliminary results on the carbohydrate structures of another secreted peroxidase from *V. myrtillus* also suggest the presence of Le^a type structures. In mung bean and pea seedlings Crawley et al. [3] have reported the presence of a fucosyltransferase activity capable of synthesizing the Le^a determinant. Thus it is possible that this type of structure will be a general feature of secreted glycoproteins from plant origin.

The Le^a has been detected at the surface of cancer cells of the digestive system and seems to be directly involved in the process of metastasis mediating the adhesion processes with selectins [2]. The biological relevance of this structure in plant cells is not known but since it is present in secreted proteins it could be involved in stress responses through the interaction with pathogenic agents or it could mediate signaling between different cells. It will be most interesting to investigate the presence of selectin-like receptors in the plant kingdom which so far have only been described in animals.

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