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Isolation and Characterization of Free Glycans of the Oligomannoside Type from the Extracellular Medium of a Plant Cell Suspension

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The oligosaccharides $Man_5GlcNAc$ and $Man_3(Xyl)GlcNAc(Fuc)GlcNAc$ presumed to originate from *N*-glycosyl proteins have been purified from an extracellular medium (concentration: 2-5 mg/l of 14 day cultures) of white campion (*Silene alba*) suspension culture. Their primary structures have been determined by ^1H-400-MHz NMR spectroscopy and FAB-MS spectrometry. They are probably the result of an autophagic process including protein catabolism due to sucrose starvation. Additional identification of digalactosylglycerol (galactolipid breakdown) argues for this hypothesis.

Oligosaccharides from both fungal and plant origin have been intensively investigated during the last decade. They are involved in host-pathogen interactions and in regulation of plant growth and development. In fact, chitin (or chitosan) and β-glucan oligomers, derived from fungal cell walls, as well as α-(1-4)-oligogalacturonide fragments, derived from plant cell walls, can induce active defense mechanisms [1]. Furthermore, in uninfected plant tissue, biological activity of oligosaccharides has been reported: a nonasaccharide originating from xyloglucans and generated by cellulase activity, presents a potent antiauxin activity in pea stems [2]. These oligosaccharides containing biological activities have been called "oligosaccharins" [3]. Efforts are made to extend the concept of oligosaccharins, and to find new oligosaccharides with biological properties. The glycans of plant *N*-glycosyl proteins have many influences: on protein stability, on the biological and physicochemical properties of those proteins and concerning recognition events [4]. In this paper we report

Abbreviations: Fuc, L-fucose; Man, D-mannose; Xyl, D-xylose; GlcNAc, *N*-acetyl-D-glucosamine; Gal, D-galactose; Glc, D-glucose; FAB-MS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance.

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for the first time the occurrence of glycans presumed to originate from *N*-glycosyl proteins, in a culture medium of a plant cell suspension. The origin of these compounds has not yet been investigated, but their presence is discussed.

Materials and Methods

General Methods

TLC of oligosaccharides was performed on Silica gel 60 plates (Merck, Darmstadt, W. Germany) with *n*-butanol/acetic acid/water, 2/1/1 by vol. Oligosaccharides were revealed with orcinol-H_2SO_4 reagent (orcinol (0.1% in 20% H_2SO_4).

Paper electrophoresis was carried out on Whatman no. 3 paper, at 25°C and 300 V, using the solvent system pyridine/acetic acid/water, 20/9/1971, pH 4.5. Sugars were detected by spraying aniline oxalate solution in water [5] and heating at 100°C for 5-10 min. Sugars were recovered from the paper by elution with water.

Total sugar was measured by the orcinol method [6]. The amount of uronic acids was estimated by the metahydroxybiphenyl-sulfuric acid method [7]. The monosaccharide composition of the different fractions was determined by GLC analysis of the alditol acetates, prepared [8] after acid hydrolysis (4 M trifluoroacetic acid, 4 h at 100°C). *Myo*-inositol hexaacetate was the internal standard. The alditol acetates were separated with a CP-Sil5CB (Chrompack) capillary column (0.32 mm x 50 m), using a flame ionization detector, an evaporator-injector, and N_2 as the carrier gas. Permethylation was carried out on oligosaccharide alditols as described [19]. The permethylated oligosaccharides were hydrolysed with 4 M trifluoroacetic acid, 4 h at 100°C, partially methylated monosaccharides were reduced by NaB^2H_4 and acetylated (pyridine/acetic anhydride, 1/1 by vol; 18 h at room temperature). The partially methylated alditol acetates were separated on a DB1 (J.W) capillary column (0.32 x 30 m). Mass spectra were recorded with a Riber 10-10 mass spectrometer using an electron energy of 70 eV and an ionization current of 0.2 mA.

Cell Culture

White campion suspension culture [*Silene alba* (Miller) E.H.L. Krause] was obtained by the procedure of Dubois *et al.* [10]. Subcultures were treated as previously described [11].

Purification of Oligosaccharides

Oligosaccharides were purified from the culture medium of 14-day-old cells (3 l). Cells were removed on a glass filter, the resulting medium was concentrated (5-fold) under vacuum and precipitated with ethanol (3 vol) at 4°C, 18 h.

The pellet (fraction F1, polysaccharides) was recovered by centrifugation (5,000 x *g*, 30 min). The supernatant (fraction F2) was concentrated to 20 ml and desalted on a column of Sephadex G-10 (Pharmacia, Uppsala, Sweden; 90 x 2 cm; distilled water), to separate the total oligosaccharide fraction (F3, void volume) from monosaccharides and salts (F4, total

Table 1. Preparation of neutral oligosaccharides.
Sugar composition was determined by the analysis of alditol acetate derivatives. F1 and F2 were obtained by ethanol precipitation: F1, pellet; F2, supernatant. F3 and F4 were recovered from F2 fractionation on Sephadex G-10: F3, oligosaccharides; F4, monosaccharides and salts. F5 was the neutral oligosaccharide fraction recovered after paper electrophoresis of F3.

Fractions		F1	F2	F3	F4	F5
Neutral sugars	%[a]	90.50	2.26	1.10	0.98	0.54
	(mg/l)[b]	(2626.7)	(65.7)	(32.0)	(28.3)	(15.7)
Uronic sugars	%[a]	7.06	0.17	0.06	0.10	-
	(mg/l)[b]	(205.0)	(5.0)	(1.8)	(3.0)	(-)
				molar %		
Rha		2.0	3.2	7.1	1.0	1.2
Fuc		-	3.6	1.6	-	3.4
Ara		11.5	21.4	10.7	4.5	13.8
Xyl		19.9	10.0	18.1	28.5	13.3
Man		15.7	18.0	17.0	11.2	30.7
Glc		32.2	37.6	19.0	42.3	13.5
Gal		18.5	6.2	26.4	12.5	15.0
GlcNAc		-	traces	traces	-	5.0

[a] As % of the total sugars in the cell culture medium.
[b] As total weight of sugars per volume of medium.

volume). Fraction F3 was subjected to paper electrophoresis. Then, the resulting neutral fraction (F5) was fractionated on a Bio-Gel P-2 column (Bio-Rad, Richmond, CA, USA; 129 x 2.7 cm; distilled water) and subfractions (A-E) pooled according to their TLC profile.

Subfractions C, D and E were further purified by HPLC on a column (1.6 x 50 cm) packed with HW40 gel (Merck, Darmstadt, W. Germany), using a Knauer differential refractometer. Acetic acid (0.5%) was used as the eluent at a flow rate of 0.5 ml/min.

Fast Atom Bombardment Mass Spectrometry

FAB-MS Spectra were obtained on a Kratos Concept II HH mass spectrometer. The compound (1 µl, 10-20 µg) in CH_3OH, 2 µl of AcONa (1% in CH_3OH) and 2 µl of matrix (thioglycerol) were put on a stainless steel target and bombarded with a fast atom beam of Xenon (1 mA, 8 kV). Spectra were recorded at a resolving power of about 2,500.

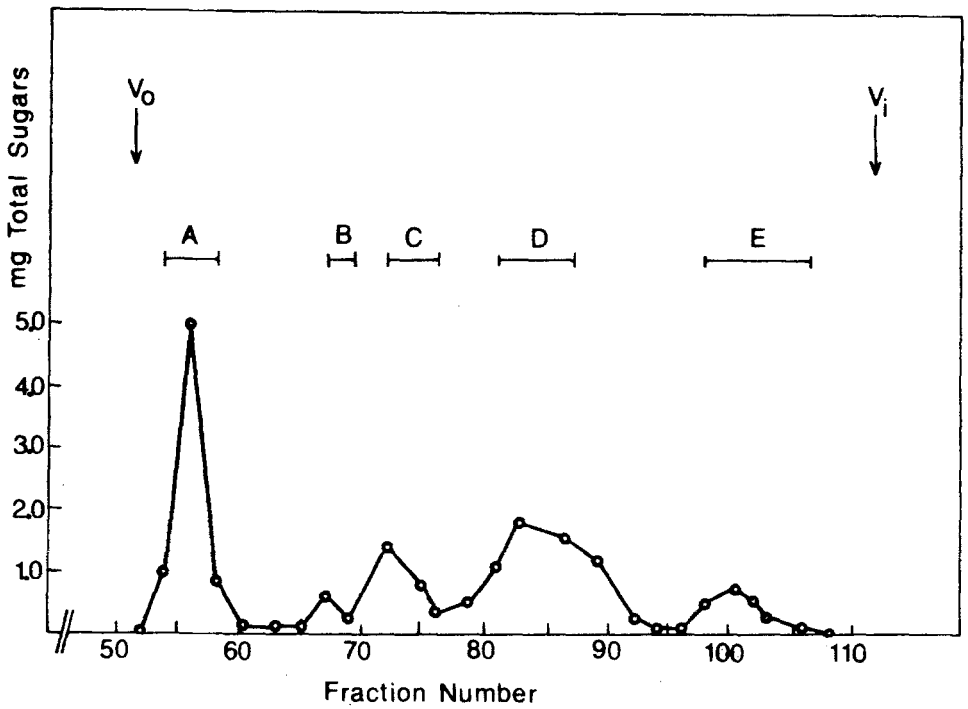


Figure 1. Fractionation pattern of the oligosaccharides from fraction F5.
Fraction F5 recovered after paper electrophoresis was lyophilized and dissolved in 10 ml of water. The Bio-Gel P-2 column was eluted with water at a flow rate of 30 ml/h and 4.2 ml fractions were collected. Sugars were estimated by the orcinol method. The subfractions (A-E) were pooled according to their TLC profile.

400 MHz ^{1}H-NMR Spectroscopy

Solutions of oligosaccharides were exchanged several times in ^2H_2O with intermediate lyophilisation. NMR Spectral analysis of 0.003 M solutions of the compounds in ^2H_2O (99.5%, Commissariat à l'Energie Atomique, France) was carried out on a Bruker AM-400 WB spectrometer operating in the Fourier-transform mode at probe temperatures of 300 K. Chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane 5-sulfonate (indirectly to acetone in ^2H_2O: $\delta = 2.225$ ppm). Resolution enhancement of the ^{1}H-NMR spectra was achieved by Lorentzian-to-Gaussian transformation as described [12].

Table 2. Sugar composition or subfractions recovered after chromatography of the neutral oligosaccharide fraction (F5).

Subfractions	A	B	C	D	E
Neutral sugars (mg)	13.9	1.0	4.7	10.9	6.0
Rha	7.6	4.7	-	-	-
Fuc	-	5.0	16.6	-	-
Ara	48.3	10.8	-	-	3.6
Xyl	7.6	16.4	15.5	9.6	13.1
Man	11.7	33.5	39.4	66.0	11.8
Glc	6.9	14.0	5.5	11.0	18.7
Gal	18.0	12.7	3.0	4.7	53.0
GlcNAc	-	3.1	20.0	7.1	-

Results

Ethanol precipitation of the culture medium (3 l) enabled elimination of the extracellular polysaccharides such as xylans, pectins and starch (fraction F1). Oligosaccharides and monosaccharides (197 mg) were recovered in the supernatant (fraction F2) and represented only 2.5% of the total medium saccharide content (Table 1).

Monosaccharides (i.e. high content of glucose and xylose, low content of mannose and absence of glucosamine) and salts (fraction F4) were removed by Sephadex G-10 chromatography. A total of 96 mg of oligosaccharides containing 5% of uronic acids (fraction F3) were recovered (Table 1). Acidic oligosaccharides and yellow pigments were removed from F3 by paper electrophoresis. The recovered neutral fraction (F5) was contained 47 mg of sugar.

After column chromatography of F5 on Bio-Gel P-2 (Fig. 1), five major subfractions (A, B, C, D and E) were obtained. Subfraction A contained arabinose and galactose as the major components (Table 2). According to the presence of 7.6% of rhamnose, typical of pectic material, A could be considered as arabinogalactans from hairy pectin zones [13]. Subfractions B, C and D were characterized by the presence of glucosamine and the high amount of mannose. Subfraction E contained galactose as the major sugar. A and B were not further purified. Purification of oligosaccharides C and D was achieved by HPLC. Each subfraction HPLC chromatogram exhibited a major sugar peak, at 50.1 ml (C) and 52.3 ml

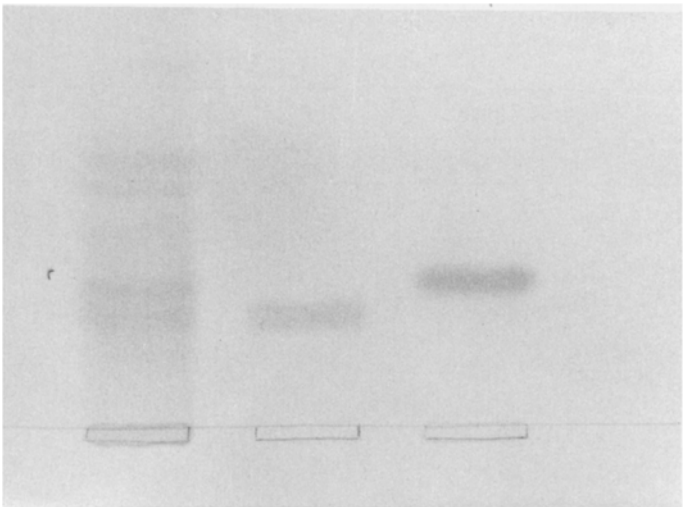


Figure 2. TLC analysis of oligosaccharides from fraction F5, and from purified oligosaccharides from subfractions C and D, obtained by Bio-Gel P-2 chromatography and HPLC.

(D). TLC Analysis showed that the purified subfractions C and D contained a single band corresponding to the predominant bands in fraction F5 (Fig. 2). The molar ratios of the oligosaccharides are presented in Table 3. The subfraction E was also purified in the same way. After the HPLC purification, galactose was the sole sugar of the major oligosaccharide (not reported in Table 3).

Structural Analysis

400-MHz ^{1}H-NMR Spectroscopy: To elucidate the primary structure of the oligosaccharides C and D isolated from the culture medium, 400-MHz ^{1}H-NMR spectra of the compounds in ^2H_2O were recorded. The expanded, resolution enhanced, structural-reporter-group regions of the spectra are presented in Fig. 3. Relevant NMR parameters for both oligosaccharides are listed in Table 4. For the interpretation of the spectra in terms of primary structural assignments, advantage was taken of the data previously obtained for reference compounds 1 and 2 (Table 4).

For the oligosaccharide C, the *N,N*-diacetylchitobiose structural element is characterized by the GlcNAc-1 H-1α, H-1β; GlcNAc-2 H-1α, H-1β; and NAc (α, β) signals found at δ = 5.162, 4.685; 4.561, 4.545; and 2.030, 2.025; respectively. The branching mannotriose core is reflected by the characteristic signals of Man H-1 and Man H-2 (Fig. 3 and Table 4). The β(1-2)-linked xylose residue attached to the Man-3 residue is recognized owing to the typical H-1, H-2, H-3 and H-5_{ax} reporter-group signals, of which the chemical shifts are in full agreement with the ^{1}H-NMR parameters reported earlier for the corresponding reference compound 1 [14]. The α(1-3)-linked fucose residue linked to GlcNAc-1 is characterized by

Table 3. Molar ratios of purified oligosaccharides after HPLC purification.

Purified oligosaccharides	Molar ratio Fuc	Xyl	Glc	Man	GlcNAc
Subfraction C	1.0	1.0	-	3.0	1.5
Subfraction D	-	1.2	0.9	5.0	1.1

the H-1, H-5 and CH_3 signals present at $\delta = 5.080$ ppm, $\delta = 4.721$ ppm and $\delta = 1.273$ ppm, which are comparable to those of reference compound 1 (Table 4), and differ considerably from those of α(1-6)-linked fucose residue linked to GlcNAc-1 [15]. Consequently, the structure of the oligosaccharide C was established as Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4 GlcNAcβ1-4(Fucα1-3)GlcNAc. The structure of the oligosaccharide D was established as Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc by comparison of its NMR spectrum with those previously established for reference compound 2 [16].

FAB-Mass Spectrometry: FAB-MS confirmed the structure of the reduced and permethylated oligosaccharides. The positive ion FAB-MS of purified $Man_{3(}$Xyl)GlcNAc(Fuc)GlcNAc displayed a molecular ion peak at m/z 1522.8 assigned to $(M+Na)^+$. The purified Man_5GlcNAc exhibited a molecular ion peak at m/z 1351.7 assigned to $(M+Na)^+$ and also molecular peaks at m/z 1337.7 $(M+Na)^+$ and m/z 1329.7 $(M+H)^+$ which can be ascribed to the xylose-glucose contaminant, see Table 3. According to the resulting molecular mass of 1328.7, the major contaminant could be a xyloglucan residue like Xyl_4Glc_3. Furthermore, among typical derivatives of the glycan D, permethylated hydrolysis yielded indeed 2,3,4-tri-*O*-methylxylitol, 3,4-di-*O*-methylxylitol, 2,3,4-tri-*O*-methylglucitol and 2,3-di-*O*-methylglucitol. These derivatives are characteristic for xyloglucans, which have side chains attached to position 6 [17]. The purified oligosaccharide E exhibited a molecular ion peak at m/z 565 assigned to $(M+Na)^+$ and also a molecular peak ion at m/z 543 (minor) assigned to $(M+H)^+$. After permethylation and hydrolysis, E yielded 2,3,4,6-tetra-*O*-methylgalactitol and 2,3,4-tri-*O*-methylgalactitol in a molar ratio 1/1. We deduced then by calculation the nature of the oligosaccharide E as Gal1-6Gal-glycerol. Fragment ions at m/z 219, 237, 307 and 423, corresponding to (B_1^+), (C_1^+), (Z_1^+) and (B_2^+), according to the nomenclature proposed by Domon and Costello [18], confirmed the digalactosylglycerol identification.

Discussion

The scheme proposed for the purification of the related oligosaccharides appeared to be efficient. Furthermore, detection and purification of free glycans was successful because other oligosaccharide types (such as xyloglucans) with similar molecular weights were present in lower amounts.

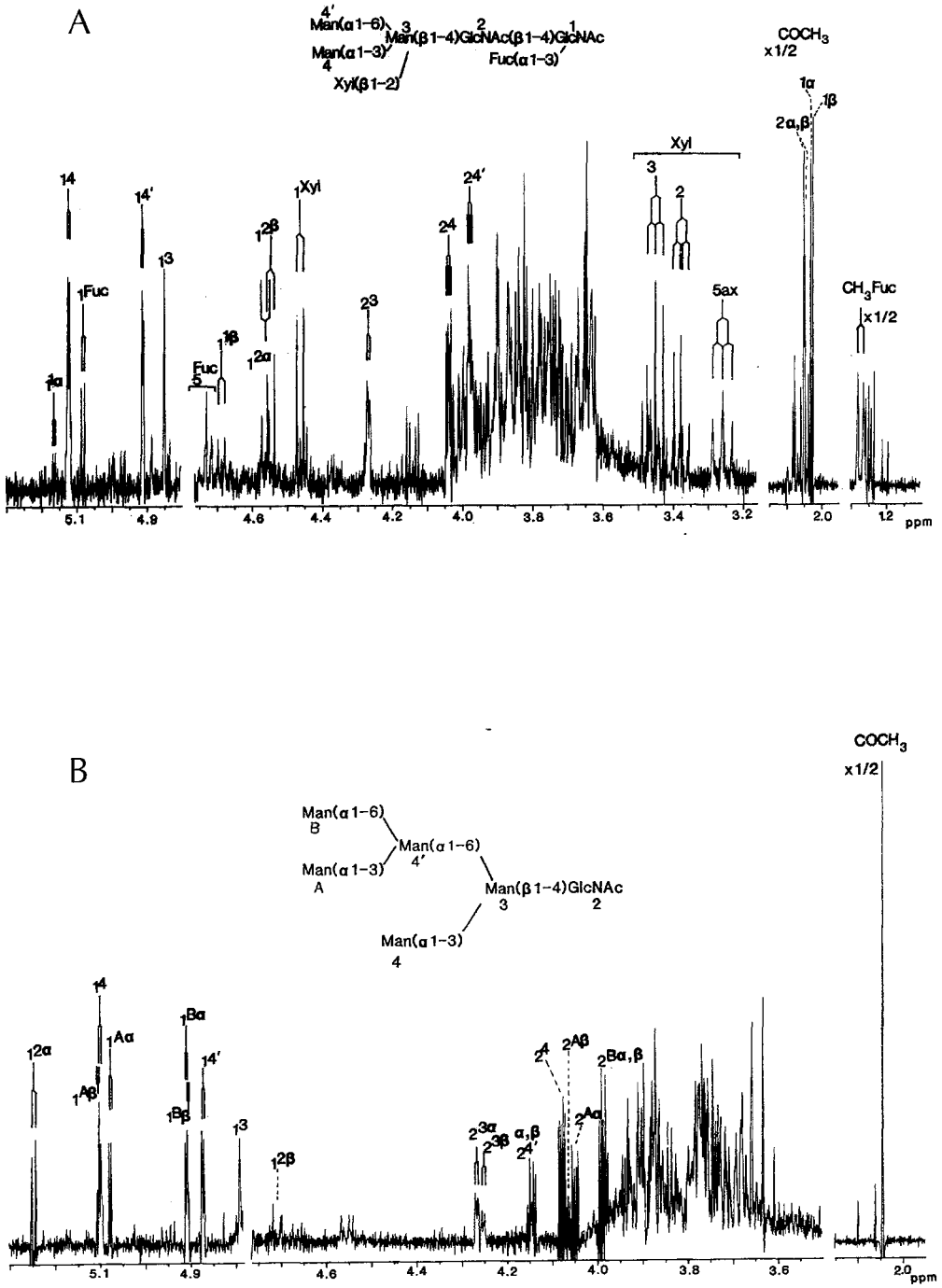
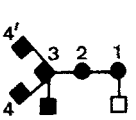
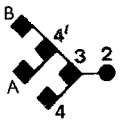
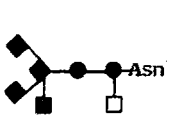
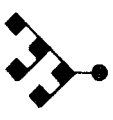


Figure 3. Structural-reporter-group region of the resolution-enhanced 400 MHz ^{1}H-NMR spectrum of the major oligosaccharides purified from subfractions C and D, panels A and B respectively.
Coding system: $1^{1\alpha}$ means GlcNAc-1, H-1α; 2^3 means Man-3 H-2. For NAc signals the group (NAc) is indicated above.

Table 4. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides for the oligosaccharides C and D, extracted from the extracellular medium of white campion suspension culture. Shifts were compared with those of the reference compounds 1 [14] and 2 [16].
Sugars: (□), Fuc; (●), GlcNAc; (◆), Man; (■), Xyl.

Residue	Reporter group	 C	 D	 1	 2
GlcNAc-1	H-1	α5.162,β4.685	-	5.082	-
	NAc	α2.030,β2.025	-	1.993	-
GlcNAc-2	H-1	α4.561,β4.545	α5.246,β4.710	4.568	α5.247,β4.709
	NAc	α2.048,β2.051	2.043	2.053	2.043
Man-3	H-1	4.849	α4.790	4.887	α4.787,β4.782
	H-2	4.265	α4.266,β4.252	4.265	α4.262,β4.249
Man-4	H-1	5.120	5.099	5.122	5.101
	H-2	4.038	4.076	4.037	4.076
Man-4'	H-1	4.910	4.872	4.910	4.872
	H-2	3.975	α4.146,β4.142	3.980	4.140
Man-A	H-1	-	α5.078,β5.104	-	α5.079,β5.105
	H-2	-	α4.048,β4.066	-	α4.048,β4.061
Man-B	H-1	-	α4.908,β4.904	-	α4.908,β4.904
	H-2	-	α3.988,β3.983	-	α3.987
Xyl	H-1	4.462	-	4.464	-
	H-2	3.375	-	3.372	-
	H-3	3.450	-	3.451	-
	H-5ax	3.257	-	3.258	-
Fuc	H-1	5.118	-	5.131	-
	H-5	4.721	-	n.d.	-
	CH_3	1.273	-	1.290	-

The chemical structures of the purified glycans were determined by component analysis, 400 MHz ^{1}H-NMR spectroscopy, and FAB-mass spectrometry of methylated derivatives. The $Man_5GlcNAc$ and Man_3(Xyl)GlcNAc(Fuc)GlcNAc chains have been reported in several plant glycoproteins (reviewed in [19]), but not in those from white campion.

The molecular mechanism by which the free oligosaccharides are generated remains unknown. At least two catabolic pathways can be involved: (i) enzymatic cleavage of the plasma membrane glycoproteins within the apoplastic area of the cell wall; or (ii) enzymatic

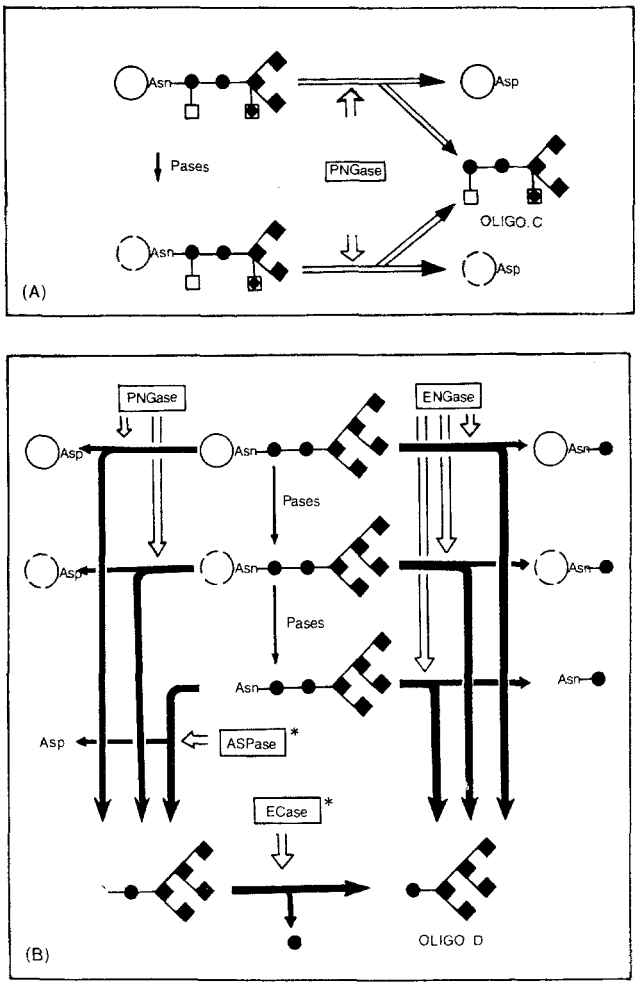


Figure 4. Different catabolic pathways hypothesized for the glycans C and D, panels A and B, respectively.
Enzymes: Pases, proteases; PNGase, peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase; ENGase, endo-β-*N*-acetylglucosaminidase; ASPase, aspartyl-*N*-acetylglucosaminidase; ECase, endochitobiase.
Sugars: (□), Fuc; (●), GlcNAc; (◆), Man; (■), Xyl.
(*) enzymes not reported in plants.

degradation of internal membranes, related to some conditions of stress, followed by excretion of waste products by exocytosis. The latter hypothesis corresponds to our experimental conditions. The oligomannosides are collected at the end of the culture, when cells are deprived of sucrose. Under these conditions, starch, proteins and lipid reserves are

consumed and internal membranes are destroyed, simultaneously with a cellular respiratory decrease corresponding to an autophagic process [20, 21]. In addition, a concomitant decrease of protein and galactolipid content in cells has been reported during sucrose deprivation [20]. The detected digalactosylglycerol (subfraction E), characteristic of a galactolipid breakdown, argues for the deprivation state of the white campion culture cells, and then, for the autophagic origin of the proteolysis. The details of the different catabolic pathways hypothesized for the occurrence of both glycans are summarized in Fig. 4. Several points have to be noted. The aspartyl-*N*-acetylglucosaminidase specific for the oligosaccharide-Asn substrate and the endochitobiase have been reported in human and rat lysosomes [22-24], but not in plants [25]. Next to the importance of determining the nature of the enzymes involved, it will be essential to know whether proteolysis precedes or follows deglycosylation. In the latter case, deglycosylation could play a role in the control/blocking of proteolysis

It is interesting to note the preservation of oligomannoside structures, which supposes a lack of the corresponding exoglycosidases. This could lead to the hypothesis that the remaining oligosaccharides transport information about the physiological state of the cells (i.e. nutritive deprivation) producing those oligosaccharides.

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