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Peptide-N^4-(*N*-acetylglucosaminyl)asparagine amidase (PNGase) activity could explain the occurrence of extracellular xylomannosides in a plant cell suspension

SABINE LHERNOULD[1], YANNIS KARAMANOS[2], SYLVAIN BOURGERIE[2], GERARD STRECKER[3], RAYMOND JULIEN[2] and HENRI MORVAN[1]*

[1]*Laboratoire de Biologie Cellulaire Végétale et Valorisation des Espèces Ligneuses, Université de Limoges, 123, Avenue Albert Thomas, 87060 Limoges Cédex, France*

[2]*Institut de Biotechnologie, Université de Limoges, 123, Avenue Albert Thomas, 87060 Limoges Cédex, France*

[3]*Laboratoire de Chimie Biologique de l'Université des Sciences et Techniques de Lille-Flandres Artois et Unité Mixte du CNRS No 111, 59655 Villeneuve d'Ascq Cédex, France*

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We have previously isolated mannoside and xylomannoside oligosaccharides with one or two terminal reducing *N*-acetylglucosamine residues from the extracellular medium of white campion (*Silene alba*) suspension culture. We have now demonstrated the presence of peptide-N^4-(*N*-acetylglucosaminyl)asparagine amidase (PNGase) activity in cell extracts as well in the culture medium that could explain the production of those compounds. An additional xylomannoside, (GlcNAc)Man_3(Xyl)GlcNAc(Fuc)GlcNAc, was characterized, and ^{1}H- and ^{13}C-NMR assignments for the oligosaccharide Man_3(Xyl)GlcNAc(Fuc)GlcNAc were obtained using homonuclear and heteronuclear spectroscopy (COSY).

Keywords: Xylomannoside, PNGase, white campion

Abbreviations: Endo, endo-β-*N*-acetylglucosaminidase; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; NMR, nuclear magnetic resonance; PNGase, peptide-N^4-(*N*-acetylglucosaminyl)asparagine amidase; Xyl, xylose.

Recently, free glycans such as xylomannoside Man_3(Xyl)-GlcNAc(Fuc)GlcNAc and oligomannoside Man_5GlcNAc, were isolated from the extracellular culture medium of white campion (*Silene alba*) suspension [1], which could be the products of *N*-glycosyl protein breakdown by enzymes acting on the di-*N*-acetylchitobiosyl part of asparagine-linked glycans. Several glycoproteins carrying this xylomannoside have already been reported: ricin from *Ricinus communis* [2], laccase from *Sophora japonica* [3] and the lectin jacalin [4]. Two distinct families of enzymes were characterized depending on whether they hydrolysed either the di-*N*-acetylchitobiosyl linkage (endo-β-*N*-acetylglucosaminidase, or Endo) or the glycosylamine linkage (peptide - N^4 - (*N* - acetylglucosaminyl)asparagine amidase, or PNGase) [5]. The hypothesis of the enzymatic breakdown of *N*-glycosylproteins is supported by the fact that several Endo and PNGase were described in plants: Endo was isolated from fig [6] and from jack beans [7] and PNGase purified from almond [8] and from jack beans [7]. In this paper, we demonstrate the presence of PNGase both in the cell extracts and the culture medium of *Silene alba* that could explain the occurrence of the earlier described xylomannoside [1] and also a new one identified by NMR spectroscopy.

Materials and methods

Cell culture

White campion suspension culture (*Silene alba* (Miller) E.H.L. Krause) was obtained by the procedure of Dubois [9]. Subcultures were made in 500 ml flasks with 200 ml of medium as previously described [10] except that they were extended to 30 days.

Cell extract

The cells were harvested by filtration on Whatman 41 paper with a Büchner funnel, suspended in 100 mM potassium

* To whom correspondence should be addressed.

phosphate buffer, pH 7.0, containing 0.1% phenylmethylsulfonylfluoride (PMSF, Sigma Chemical Co.), dispersed with a homogenizer (Ultra-turrax) and disrupted with a French pressure cell (1000 lb in^{-2}). The homogenate was centrifuged (15,000 × **g**; 15 min) to remove the cell debris. The supernatant, which constituted the cell extract, was adjusted to a final concentration of 15 mg dry weight per ml.

Endo and PNGase assay procedure

For the assay procedure, the $Man_5GlcNAc_2Asn$(Ala,Thr,Ser) resorufin-labelled glycopeptide was purified from the commercially available preparation (resorufin labelled *N*-glycopeptide; Boehringer Mannheim France, Catalog No. 1016776) by reversed phase chromatography and used as previously described [11]. 5 µl (75 µg dry weight) of cell extract were added to 25 µl (0.2 µg) of substrate in 50 mM sodium acetate buffer, pH 4.0. After incubation at 37 °C, the reaction was stopped by acidification (pH 1.5 with 10 µl trifluoroacetic acid, TFA) and the products were applied to a 3 µm Spherisorb ODS2 (10 cm × 0.46 cm, Colochrom) reversed phase column. Separation was achieved by an isocratic elution with 85% H_2O and 15% acetonitrile containing 0.07% TFA for 10 min at a flow rate of 1 ml min^{-1}. The products were detected with a Waters 470 scanning fluorescence detector using excitation and fluorescent emission at 467 nm and 559 nm, respectively. A WINner (Spectra-Physics) system was used for data acquisition.

PNGase F and Endo F were prepared as described [11].

One unit of PNGase was defined as the amount of enzyme required to convert 1 nmol (1939 ng) of substrate to the final product, resorufin-peptide, in 1 min at 37 °C in 50 mM sodium acetate buffer, pH 4.0.

Purification of oligosaccharides

Oligosaccharides were purified from the culture medium of 30-day-old cells. Cells were removed on a glass filter, the resulting medium was concentrated (twofold) under vacuum and precipitated with ethanol (3 vol) at 4 °C overnight. The pellet was recovered by centrifugation (5000 × **g**, 30 min). Ethanol was removed from the supernatant by rotary evaporation and the residue was desalted by chromatography on a dual column of Dowex 50-X8 (Sigma, 20–50 mesh, H^+ form; 3.5 cm × 40 cm) and Dowex 1-X8 (Fluka, 20–50 mesh, HCO_2^- form; 3.5 cm × 60 cm) pre-equilibrated with distilled water. Neutral oligosaccharides, eluted in the void volume were separated by preparative paper chromatography, performed on Whatman No. 3 paper, at 20 °C for three days, with the solvent ethyl acetate–pyridine–acetic acid–water, 5:5:1:3 by vol. Sugars were detected by spraying aniline oxalate reagent [12] and heating at 110 °C for 5–10 min. Sugars were recovered from the paper by elution with water.

NMR spectroscopy

The 400 MHz ^{1}H-NMR experiments were performed with a Brucker AM-400 WB spectrometer, equipped with a 5 mm ^{1}H-^{13}C mixed probe-head, operating in the pulse FT mode and controlled by an Aspect 3000 computer. After three exchanges with ^2H_2O (99.96 atom% ^{2}H, Aldrich, Milwaukee, IL, USA) and intermediate lyophilization, the products were analysed with a spectral width of 3000 Hz for 16 K frequency-domain points and time-domain points, giving a final digital resolution of 0.365 Hz per point. The chemical shifts are given relative to sodium 4,4-dimethyl-4-sila-(2,3-$^2H_4$)pentanoate (δ 0.0). The 2D-homonuclear COSY 45 experiments were performed with the standard Brucker pulse-programme COSY. The 2D-heteronuclear-correlated experiments were performed with simultaneous ^{1}H-decoupling by use of the standard Brucker pulse-programme XHCORRD.

Results

Purification and structure of the oligosaccharides

After paper chromatography, three major oligosaccharides (**1**, **2** and **3**) were obtained. Their concentration in the culture medium were 5 mg l^{-1}, 4 mg l^{-1} and 0.25 mg l^{-1}, respectively. Oligosaccharides **1** and **2** were shown to be $Man_5GlcNAc$ and Man_3(Xyl)GlcNAc(Fuc)GlcNAc.

Relevant NMR parameters for oligosaccharides **1** and **2** were previously reported [1]. In this study, assignments of all proton resonances were made from a homonuclear COSY experiment (Fig. 1 and Table 1), realized with one and two magnetization relays.

Starting from the anomeric protons of each monosaccharide, H-2, H-3 and H-4 resonances were successively assigned. From H-2 of Man-3 and Man-4, these correlations were extended to H-5. In the same way H-5_{ax} of xylose is used to assign H-4 and H-5_{eq}. Other resonances and, particularly, H-5 of Man-4′ and H-6 of all monosaccharides were assigned via heteronuclear ^{1}H-^{13}C chemical shift correlation (Fig. 2). In addition it also gave all the ^{13}C resonances (Table 2).

The 400 MHz ^{1}H-NMR spectrum of oligosaccharide **3** (Fig. 3) contains signals corresponding to three mannose, one fucose and one xylose residues with H-1 chemical-shifts identical to those observed for oligosaccharide **2**, except for H-1 of Man-4′ showing an upfield shift (−0.012 ppm) at $\delta = 4.898$ ppm. Moreover, an additional doublet is observed at $\delta = 4.548$ ppm, corresponding to an *N*-acetylglucosamine H-1. The H-2 resonance of Man-4 at $\delta = 4.039$ ppm demonstrates the presence of free hydroxyl at C-2, while H-2 of Man-4′ shows a downfield shift (+0.129 ppm) which is observed at $\delta = 4.104$ ppm. Thus, the additional *N*-acetylglucosamine (GlcNAc-5′) is linked to C-2 of Man-4′. On the basis of these results, the structure of oligosaccharide **3** was established (Fig. 3).

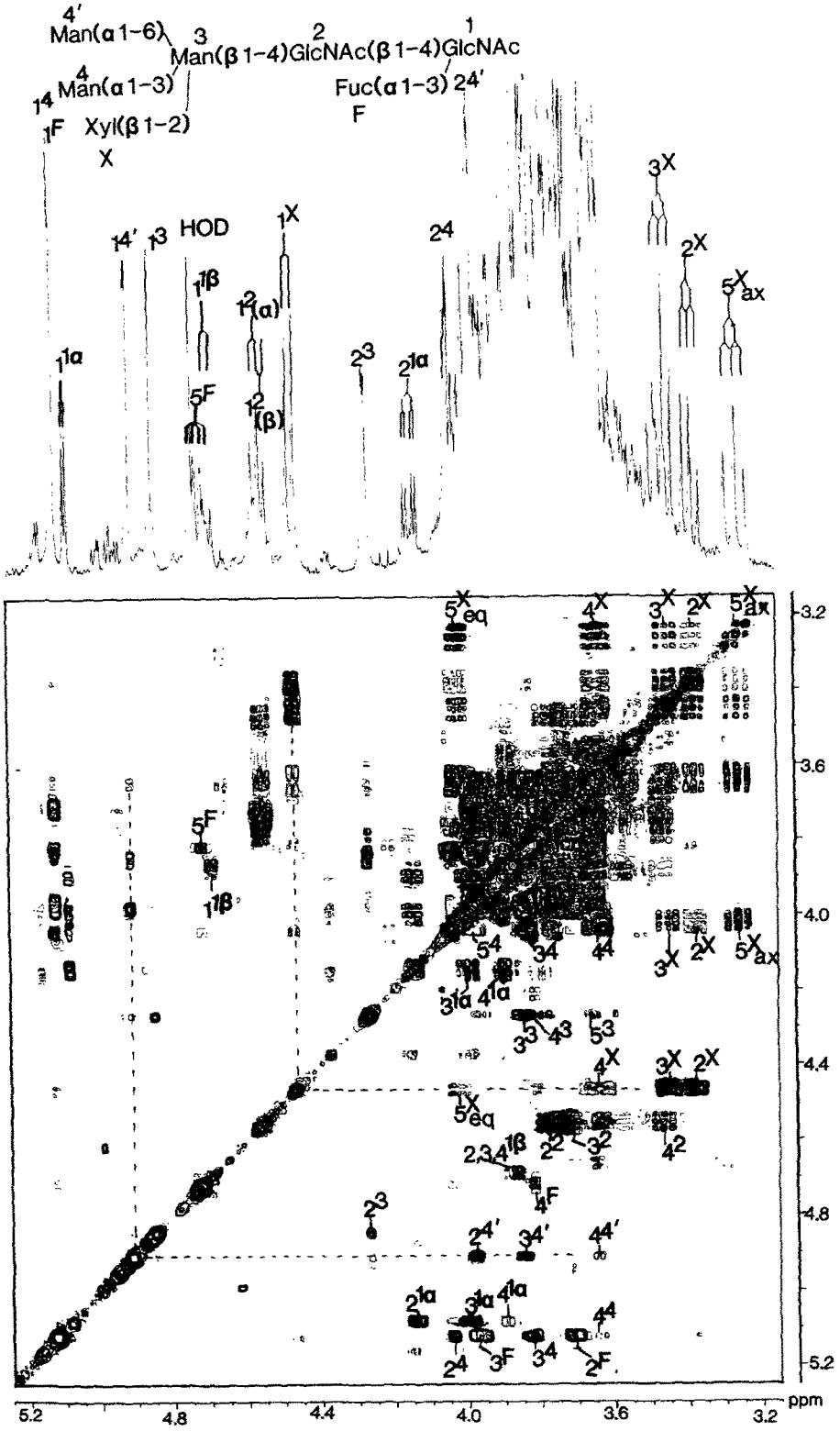

Figure 1. Contour plot of the COSY spectrum of oligosaccharide **2** isolated from the extracellular medium of white campion suspension culture. The numbers correspond to monosaccharide residues in Table 1. Key: 5^4, for example, corresponds to the H-5 resonance of the Man-4 residue.

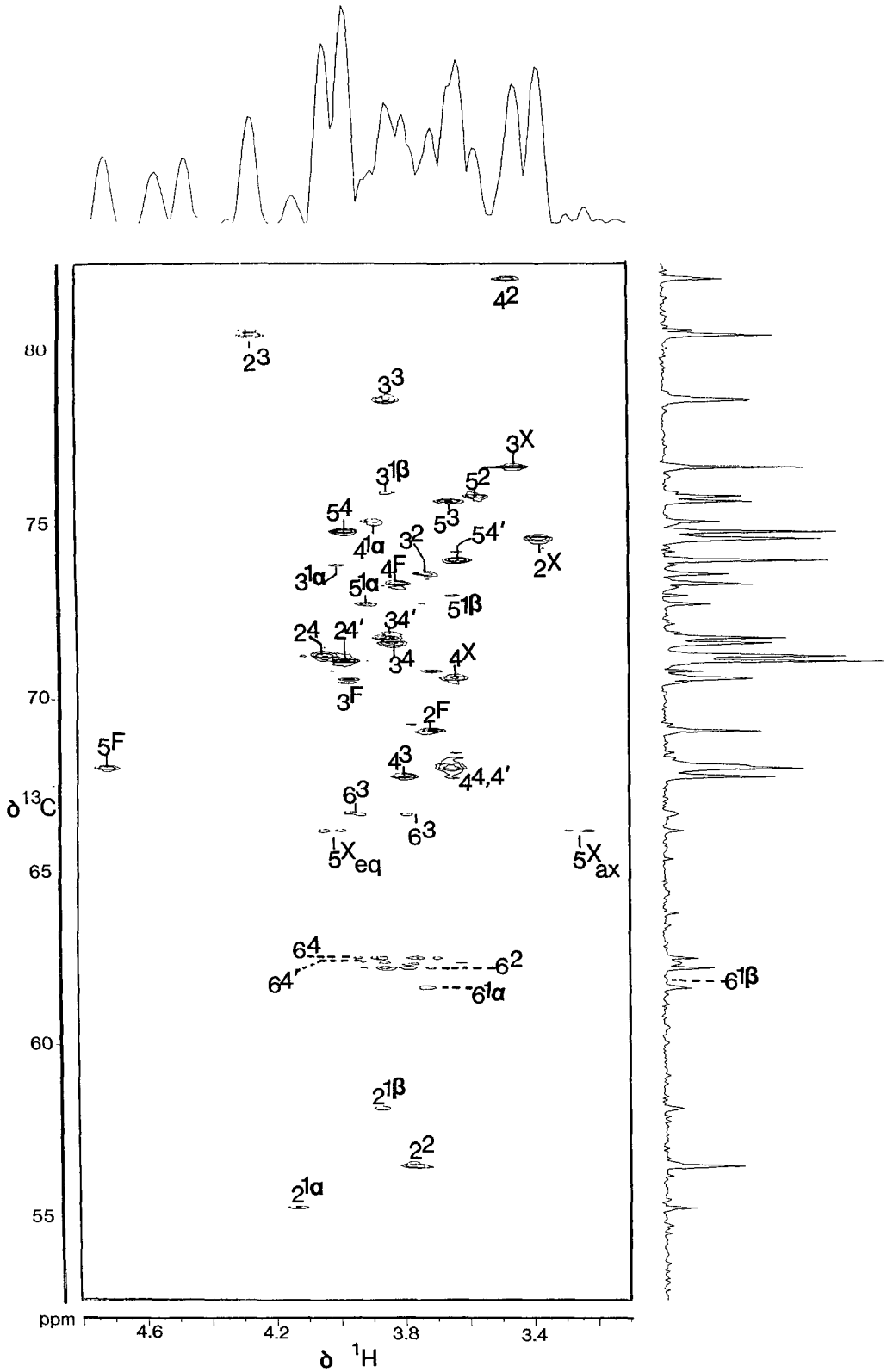

Figure 2. Contour map (3.2–4.8 ppm/52–82 ppm) of the ^{1}H-^{13}C shift correlation of oligosaccharide **2** isolated from the extracellular medium of white campion suspension culture. The numbers correspond to monosaccharide residues in Fig. 1 and the Tables.

Table 1. ^{1}H-Chemical shifts of the constituent monosaccharides for oligosaccharide **2** isolated from the extracellular medium of white campion suspension culture.

Residue		*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*	*CH3*
GlcNAc-1	(α)	5.084	4.139	3.996	3.891	3.574[a]	3.732[a]	3.732[a]	2.031
	(β)	4.689	3.872	3.846	3.890	3.644[a]	nd	nd	2.025
GlcNAc-2	(α)	4.561	3.770	3.723	3.469	3.574[a]	3.899[a]	3.749[a]	2.048
	(β)	4.545							2.051
Man-3		4.848	4.265	3.847	3.794	3.649	3.960[a]	3.758[a]	
Man-4		5.122	4.040	3.833	3.649	3.978	3.916[a]	3.723[a]	
Man-4'		4.910	3.975	3.847	3.641	3.626[a]	3.969[a]	3.820[a]	
Xyl		4.465	3.374	3.452	3.636	3.256 (ax) 4.018 (eq)			
Fuc	(α)	5.122	3.706	3.969	3.811	4.714	1.272		
	(β)	5.118[a]							

[a] Chemical shifts extracted from the ^{1}H-^{13}C COSY spectrum.

Table 2. ^{13}C-Chemical shifts of the constituent monosaccharides for oligosaccharide **2** isolated from the extracellular medium of white campion suspension culture.

Units		*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*CO*	CH_3
GlcNAc-1	(α)	92.12	55.22	73.87	75.14	72.76	61.04	175.53	23.35
	(β)	96.02	58.09	75.94	75.08	73.01	61.25	175.71	23.58
GlcNAc-2	(α)	101.70	56.46	73.64	82.11	75.85	62.22	175.96	23.46
	(β)		56.43	73.61					
Man-3		101.87	80.47	78.62	67.77	75.72	66.69		
Man-4		103.51	71.28	71.64	68.08	74.84	62.54		
Man-4'		100.97	71.13	71.81	68.02	74.02	62.35		
Xyl		106.32	74.64	76.70	70.61	66.22			
Fuc	(α)	99.72	69.08	70.57	73.36	68.02	16.80		
	(β)	99.59		70.51					

Occurrence of PNGase in the cell extracts

The products obtained by the action of the cell extract (Fig. 4c) to the $Man_5GlcNAc_2Asn$(Ala,Thr,Ser)-resorufin labelled glycopeptide (S) was compared to those obtained by PNGase F (Fig. 4a) and Endo F (Fig. 4b). After comparison with the chromatogram of the cell extract alone, it is clear that only one product was obtained and this had the same retention time as the resorufin-peptide (p) obtained by the action of PNGase F.

Monitoring the PNGase activity during the culture cycle

The oligosaccharides are continuously produced and reached a level of 0.5 mg per culture flask while intracellular PNGase activity increased sixfold during the first 15 days (Fig. 5). During the second part of the culture cycle, the total oligosaccharide concentration in the extracellular medium remained stable. PNGase activity was finally stabilized at the maximum range. Three experiments have shown that this activity in cells could be correlated with the course of the oligosaccharide excretion. PNGase activity was also found in the extracellular medium between 10 and 30 days at an average value of six units per culture flask.

Discussion

The enzymatic cleavage of the intracellular glycoproteins by enzymes acting on the di-*N*-acetylchitobiosyl part of asparagine-linked glycans was proposed as a possible mechanism for the release into the medium of Man_3(Xyl)GlcNAc(Fuc)GlcNAc and $Man_5GlcNAc$ oligosaccharides [1].

Using a simplified purification procedure based on ion exchange and paper chromatography, we have isolated an additional xylomannoside (GlcNAc)Man_3(Xyl)GlcNAc-(Fuc)GlcNAc. The occurrence of this xylomannoside, closely related to the already described Man_3(Xyl)GlcNAc-(Fuc)GlcNAc, confirms the hypothesis of degradation phenomena in white campion suspension culture. Oligosaccharide **3** with the additional GlcNAc-5' residue could be the precursor of oligosaccharide **2**. The structure

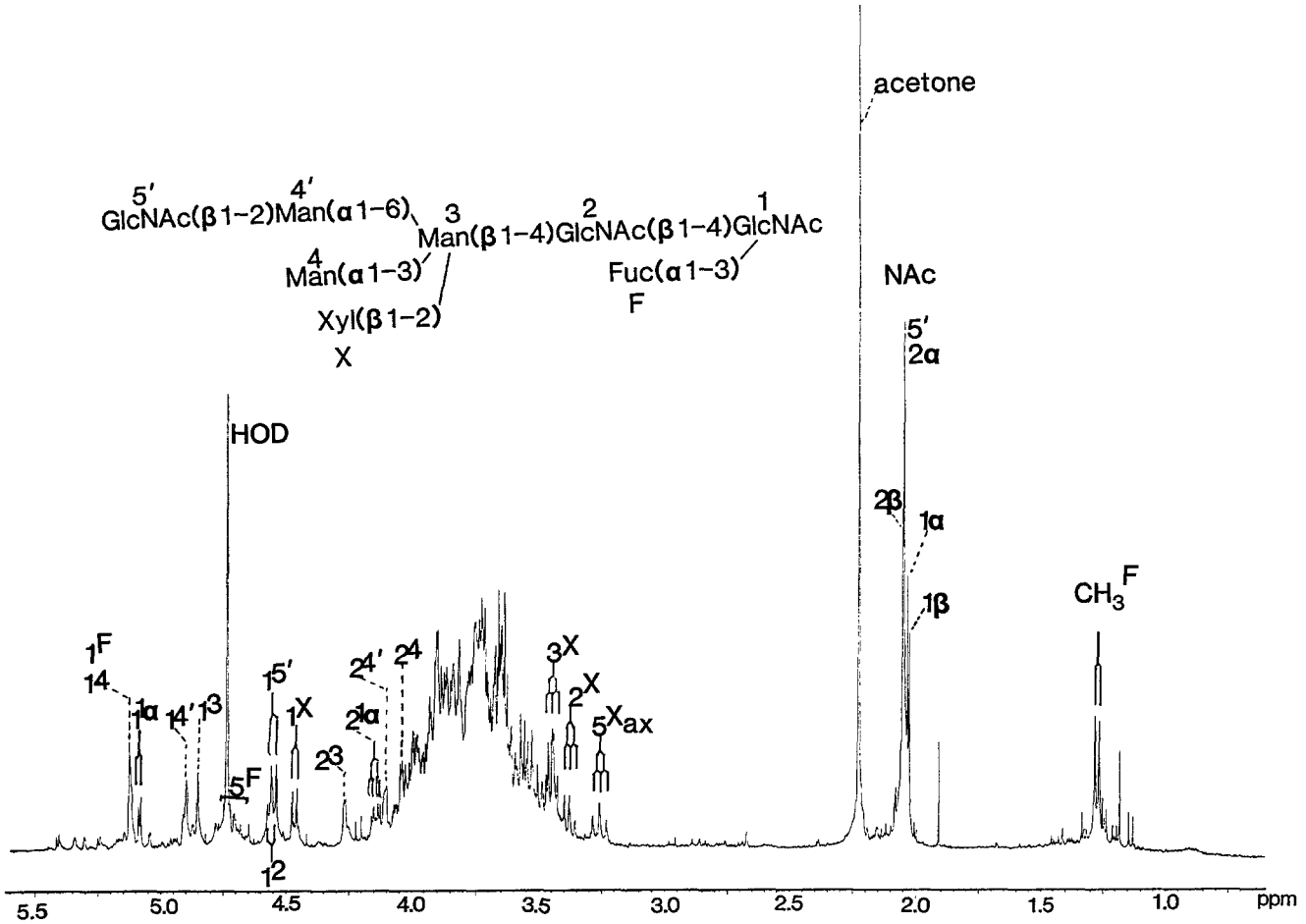

Figure 3. ^{1}H-400-MHz NMR spectrum of oligosaccharide **3**.

of those glycans, characterized by NMR spectroscopy, are similar to the oligosaccharidic portion of many plant glycoproteins [2–4].

Demonstration of enzyme activities acting on the di-*N*-acetylchitobiosyl part of asparagine-linked glycan was a prerequisite for the validation of the hypothesis of the enzymatic breakdown of *N*-glycosyl proteins. No endo-β-*N*-acetylglucosaminidase (Endo) activity, that could explain the occurrence of oligosaccharides with only one *N*-acetylglucosamine residue, was found. This type of oligosaccharide can be obtained also by the action of an endochitobiase as suggested by Priem *et al.* [1]. Nevertheless, this enzymatic activity has not yet been described in plants. We now demonstrate the presence of a PNGase activity in the cell extracts as well as in the culture medium. The connection between the amount of this activity and the release into the medium of oligosaccharides is supported by the observation that the level of the enzymatic activity correlates with the excretion of oligosaccharides (see Fig. 5). In addition, this activity can explain the occurrence of the two fucosylated xylomannosides.

Interestingly, Tretter *et al.* [13] recently demonstrated that PNGase F from *Flavobacterium meningosepticum*, the most widely used enzyme for removing *N*-glycosidically linked carbohydrate chains from glycoproteins, was unable to cleave glycans with fucose α(1-3)-linked to the innermost *N*-acetylglucosamine residue. PNGase A is capable of doing so [8] and we observe in the present study that PNGase from *Silene alba* (PNGase Se) is able to cleave these fucosylated glycopeptides. Thus, the PNGase Se described in the present communication will be an additional tool in the study of glycoproteins.

Acknowledgements

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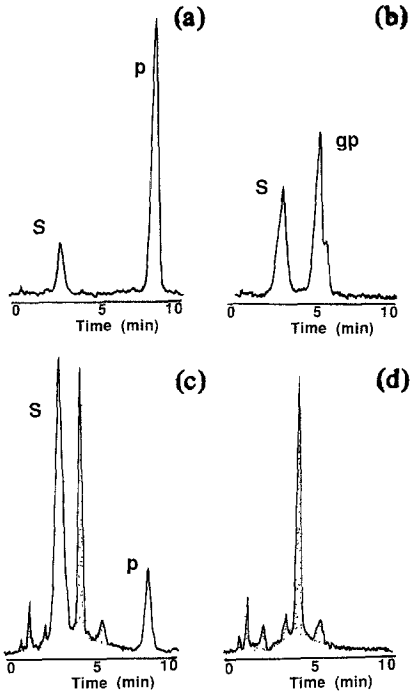

Figure 4. HPLC separations of the resorufin-labelled Man_5-$GlcNAc_2Asn$(Ala,Thr,Ser) glycopeptide (S) and its hydrolysis products, peptide-resorufin (p) and GlcNAc-peptide-resorufin (gp). (a) Action of PNGase F on the substrate; (b) action of Endo F on the substrate; (c) action of cell extract on the substrate; (d) chromatography of 5 µl of the cell extract without the substrate.

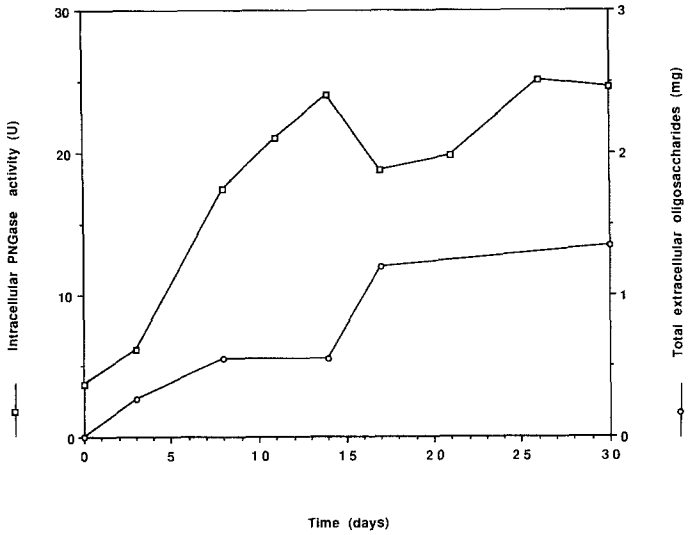

Figure 5. Evolution of PNGase activity (U) and the total oligosaccharides (mg) during the culture cycle. Both results are expressed per culture flask.

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