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## Characterization of the peptide- $N^4$ -( $N$ -acetylglucosaminyl) asparagine amidase (PNGase Se) from *Silene alba* cells

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The peptide- $N^4$ -( $N$ -acetylglucosaminyl) asparagine amidase (PNGase Se) earlier described [Lhernould S., Karamanos Y., Bourgerie S., Strecker G., Julien R., Morvan H. (1992) *Glycoconjugate J* 9:191–97] was partially purified from cultured *Silene alba* cells using affinity chromatography. The enzyme is active between pH 3.0 and 6.5, and is stable in the presence of moderate concentrations of several other protein unfolding chemicals, but is readily inactivated by SDS. Although the enzyme cleaves the carbohydrate from a variety of animal and plant glycopeptides, it does not hydrolyse the carbohydrate from most of the corresponding unfolded glycoproteins in otherwise comparable conditions. The substrate specificity of this plant PNGase supports the hypothesis that this enzyme could be at the origin of the production of 'unconjugated N-glycans' in a suspension medium of cultured *Silene alba* cells.

**Keywords:** de-N-glycosylation, PNGase, white campion

**Abbreviations:** GlcNAc,  $N$ -acetylglucosamine; PNGase, peptide- $N^4$ -( $N$ -acetylglucosaminyl) asparagine amidase; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin layer chromatography; HPAEC-PAD, High pH anion exchange chromatography-pulsed amperometric detection.

### Introduction

We have recently demonstrated the presence of a peptide- $N^4$ -( $N$ -acetylglucosaminyl) asparagine amidase Se (PNGase Se) in the cell extracts of white campion (*Silene alba*) [1] that could explain the occurrence of previously described extracellular free complex N-glycans produced by this organism [1, 2]. As these and other plant 'unconjugated N-glycans' were recently qualified as oligosaccharins and their importance underlined [3], the N-glycosyl protein breakdown by PNGases appears to be physiologically important.

PNGases have proven useful for the elucidation of the structure and biosynthetic pathways of glycoproteins [4]. PNGase A from almond emulsin was the first glycopeptide amidase to be detected and characterized [5], followed by the discovery of the bacterial PNGase F from *Flavobacterium meningosepticum* [6].

Other PNGase-type activities were described in plants [7] and the common occurrence of this type of enzyme has been demonstrated [8]. A striking difference seems to exist between the bacterial PNGase F and plant PNGases. The former is unable to cleave plant N-glycans with a fucose residue  $\alpha(1-3)$ -linked to the innermost GlcNAc residue while PNGase A, for example, has a broader specificity being able to cleave N-glycans containing an  $\alpha(1-3)$  or an  $\alpha(1-6)$  or both  $\alpha(1-3)$  and  $\alpha(1-6)$  fucose residues linked to the reducing-end of the GlcNAc residue [9, 10]. This difference of specificity between PNGase F and the plant PNGase A has been efficiently used to selectively release N-glycans from honeybee venom phospholipase A2, bearing  $\alpha(1-6)$  or both  $\alpha(1-3)$  and  $\alpha(1-6)$  fucose residues [11].

In this paper we report the partial purification of PNGase Se. This enzyme was found to readily cleave the asparaginyl-glycan linkage of various glycopeptides and to potentially hydrolyse glycoprotein (or glycopeptide) substrates found in plants [12].

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## Materials and methods

**Partial purification of PNGase Se** White campion suspension culture (*Silene alba* (Miller) E. H. L. Krause) was obtained by the procedure of Dubois [13]. Subcultures were conducted in 500 ml flasks with 200 ml of medium as previously described [14]. Affinity chromatography on a Concanavalin A Sepharose-4B (Sepharose-Con A) column (1 × 10 cm) was carried out as described [15]. The cell extract was obtained from a 14 days culture [1]. It was fractionated by ammonium sulfate precipitation (20–80% saturation) and the precipitate was dissolved and dialysed against 5 mM sodium acetate, pH 5.2, containing 0.1 M NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 0.02% NaN<sub>3</sub> (Con A-buffer). Bound glycoproteins were eluted successively with 10 mM and 300 mM methyl- $\alpha$ -D-glucopyranoside ( $\alpha$ -Me-Glc) in the Con A-buffer. The PNGase activity was quantified using resorufin-labelled N-glycopeptide as described [1]. Fractions containing the PNGase activity were pooled. Protein concentration was determined by the Lowry method [16] with bovine serum albumin as a standard. Exoglycosidase activities were assayed using the appropriate p-nitrophenyl glycosides [17], and protease activity was determined using azocasein [18].

**Characterization of the substrate specificity** Glycopeptide and glycoasparagine stock solutions (2 mg ml<sup>-1</sup>) were made in 100 mM sodium acetate, 50 mM EDTA, pH 4.0. The enzyme (50  $\mu$ l, 2.5 mU) was added to the substrate (250  $\mu$ l) and incubated at 37°C. Oligomannoside glycoasparagines were obtained by pronase digestion of hen ovalbumin and purified as described [19]. Complex diantennary glycoasparagine was obtained by pronase digestion of human plasma proteins and purified by anion exchange chromatography [J. C. Michalski, unpublished data]. The monosaccharide composition was determined by GLC after methanolysis (methanol/0.5 M HCl for 24 h at 80°C) and trimethylsilylation [15]. The complex diantennary asialo glycopeptide and the glycopeptide from bromelain obtained from Dr F. Altmann were prepared as described [9]. Glycopeptides from horse radish peroxidase (HRP) and phytohaemagglutinin (PHA) were obtained by trypsin digestion [20]. GlcNAc-Asn-peptide-resorufin was prepared from the resorufin-labelled glycopeptide with Endo F digestion. Endo F and PNGase F were prepared as described [21]. The release of oligosaccharides from the corresponding glycopeptides due to the action of the enzyme was monitored by TLC or by analytical HPAEC-PAD. Man<sub>5</sub>GlcNAc<sub>2</sub>Asn-peptide-resorufin and Man<sub>7</sub>GlcNAc<sub>2</sub>Asn-peptide-resorufin were used in the fluorescent HPLC assay as described [22].

Glycoprotein stock solutions (1 mg ml<sup>-1</sup>) were made in 100 mM sodium acetate, 50 mM EDTA, pH 4.0. For native conditions the enzyme solution (10  $\mu$ l) was directly added to the reaction mixture which contained 10  $\mu$ g of the tested glycoprotein. For unfolding conditions 0.05% SDS and 100 mM  $\beta$ -mercaptoethanol were added, the samples were boiled for 5 min and Nonidet P-40 was added at the final concentration of

1%, before the addition of the enzyme. Incubations were carried out overnight at 37°C. Fetuin, asialofetuin, bovine  $\alpha$ <sub>1</sub>-acid glycoprotein, hen ovalbumin and phytohaemagglutinin were from Sigma (USA). Horse radish peroxidase was from Boehringer (Germany). The sample of human lactotransferrin [23] and jaccalin [24] were prepared as described. The deglycosylation of the glycoproteins tested was monitored by SDS-PAGE [25].

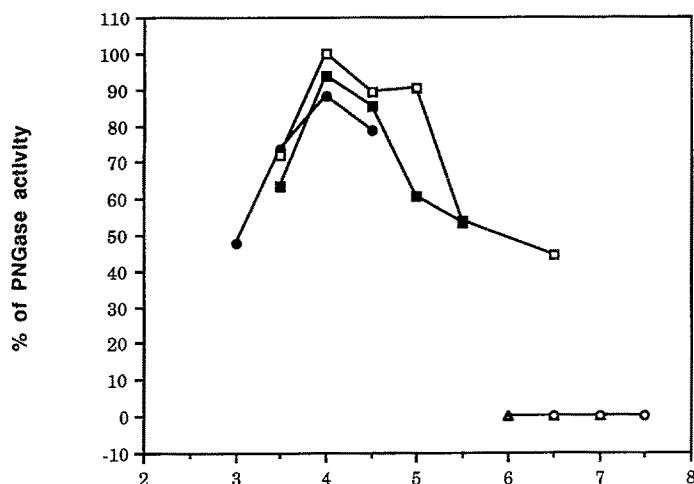
**TLC of oligosaccharides** was performed on Silicagel 60 plates (Merck, Germany) with n-butanol:acetic acid:water, 2:1:1 by vol for 1 h. The plate was dried and a second development was obtained with n-butanol:ethanol:acetic acid:pyridin:water, 10:100:3:10:30 by vol. Oligosaccharides were revealed with orcinol-H<sub>2</sub>SO<sub>4</sub> reagent (orcinol 0.1% in 20% H<sub>2</sub>SO<sub>4</sub>).

**Analytical HPAEC-PAD of oligosaccharides** Analytical HPAEC-PAD was performed on a Dionex DX500 system equipped with a GP50 quaternary gradient pump, a model ED40 detector and a CarboPac PA1 column (4.6 × 250 mm). Elution of the sample was carried out using a linear gradient from 0 to 100 mM NaOAc in 0.1 M NaOH at 1 ml min<sup>-1</sup> over 30 min.

**De-N-glycosylation of glycoproteins on nitrocellulose and lectin affino blotting** Proteins were separated by SDS-PAGE [25] with 12% gels and transferred to a nitrocellulose membrane [26] which was stained with Ponceau red to visualize the transferred proteins and incubated for 2 h in the blocking buffer (100 mM potassium acetate, pH 4.0, containing 2% BSA and 0.05% Tween 20). The blots were washed with the appropriate enzyme buffer (Tris-HCl, pH 8.6 (for PNGase F) and 100 mM potassium acetate, pH 4.0 (for PNGase Se), both containing 0.1% BSA and 0.05 Tween 20). Then, 2.5 mU of PNGase Se (or PNGase F) were added and the blot incubated overnight at 37°C. The fucose-containing glycoproteins were detected, by the use of *Aleuria aurantia* agglutinin as described [27].

## Results and discussion

In the present work, the PNGase Se was partially purified from the cell extract by ammonium sulfate precipitation and affinity chromatography, assuming that the enzyme was a glycoprotein as described for PNGase A [28]. Proteins were separated by affinity chromatography into three fractions: a fraction not retained (NR, 90%); a fraction weakly retained (WR, 4%) and eluted with 10 mM  $\alpha$ -Me-Glc; and a fraction strongly retained (6%) and eluted with 300 mM  $\alpha$ -Me-Glc. Eighty per cent of the PNGase activity was recovered in the SR fraction. The remaining activity was detected in the WR fraction. This result indicates that PNGase Se is a glycoprotein containing oligomannoside-type N-glycans allowing a convenient purification of this enzyme by affinity chromatography. At this stage of



**Figure 1.** Effect of pH on the PNGase Se activity. The enzyme activity was measured as described in the Materials and methods section in a buffer (100 mM) with different pH values. Different buffers used were sodium citrate (●), potassium acetate (□), sodium acetate (■), sodium phosphate (▲) and Tris-HCl (○).

purification, no  $\alpha$ - and  $\beta$ -mannosidase,  $\beta$ -D-galactosidase,  $\beta$ -D-xylosidase or  $\alpha$ -L-fucosidase activity were detected. Only a protease activity was present but this could be inhibited by 50 mM EDTA. The SR fraction (specific activity 260 mU per mg of protein, purification factor 13.3) was used for further studies of the specific activity of PNGase Se.

The pH dependence of the enzyme activity showed that PNGase Se has an optimum pH ranging between pH 4.0 and 5.0 using a resorufin-labelled N-glycopeptide as a substrate (Fig. 1). The activity was reduced by 50% at pH 3.0 and 6.0 and no significant activity was found when Tris-HCl or phosphate buffers were used. The stability of the enzyme was tested in the presence of several chemicals known to denature glycoproteins, since unfolding conditions may be useful to increase the accessibility of the glycans to the PNGase. The enzyme is 100% active in the presence of a few denaturants including 0.1% Triton X100, 0.1% Tween 20, 1% Nonidet P-40, 100 mM  $\beta$ -mercaptoethanol and 25 mM n-octylglucoside. However, 0.05% SDS, which is generally used to unfold glycoproteins, effectively inactivates this enzyme but this can be corrected by the addition of Nonidet P-40 (final concentration 1%) as suggested for PNGase F [29].

The studies of the substrate specificity of the partially purified PNGase Se were carried out using glycopeptide (or glycoprotein) fractions with well-characterized glycans, and the results are given in Table 1.

The de-N-glycosylation of native or unfolded animal and plant glycoproteins was monitored by the changes in the electrophoretic mobility on SDS-PAGE (Table 1). Only unfolded ovalbumin was partially cleaved. De-N-glycosylation of jacalin, a plant glycoprotein, was studied by direct action of PNGase Se on the glycoprotein transferred on a nitrocellulose membrane. The modification was monitored by detection of fucose-containing glycoproteins using a lectin affino blotting technique. While human lactotransferrin, used as a control,

**Table 1.** Hydrolysis of glycoproteins and glycopeptides by PNGase Se.

Substrates	PNGase Se		Method used
	Native	Unfolded	
<b>Glycoproteins</b>			
Fetuin	-	-	SDS-PAGE
Asialofetuin	-	-	SDS-PAGE
$\alpha_1$ -acid glycoprotein	-	-	SDS-PAGE
Ovalbumin	-	+	SDS-PAGE
Horse radish peroxidase (HRP)	nd <sup>a</sup>	-	SDS-PAGE
Phytohaemagglutinin (PHA)	nd <sup>a</sup>	-	SDS-PAGE
Jacalin	-	-	affino blotting
Human lactotransferrin	-	-	affino blotting
<b>Glycopeptides</b>			
XylMan <sub>2</sub> FucGlcNAc <sub>2</sub> -Asn-peptide (from bromelain)	+	-	TLC
Gal <sub>2</sub> GlcNAc <sub>2</sub> Man <sub>3</sub> GlcNAc <sub>2</sub> -Asn-peptide	+	-	TLC
Man <sub>5</sub> GlcNAc <sub>2</sub> -Asn	-	-	TLC
NeuAc <sub>2</sub> Gal <sub>2</sub> GlcNAc <sub>2</sub> Man <sub>3</sub> GlcNAc <sub>2</sub> -Asn	-	-	TLC
Man <sub>5</sub> GlcNAc <sub>2</sub> -Asn-peptide	+	-	Fluorescent HPLC
Man <sub>7</sub> GlcNAc <sub>2</sub> -Asn	-	-	Fluorescent HPLC
GlcNAc-Asn-peptide	+	-	Fluorescent HPLC
XylMan <sub>3</sub> FucGlcNAc <sub>2</sub> -Asn-peptide (from HRP)	+	-	HPAEC-PAD
PHA glycopeptides	+	-	HPAEC-PAD

<sup>a</sup>nd, not determined.

was efficiently deglycosylated by PNGase F but not by PNGase Se, jaccalin was not hydrolysed either by PNGase F or PNGase Se. These results are in agreement with previous data indicating that intact glycoproteins are poor substrates for plant PNGase A [28, 30] and PNGase J [7].

De-N-glycosylation of glycopeptides and glycoasparagines was visualized by TLC, by HPAEC-PAD or by fluorescent HPLC. Using TLC, we showed that the complex glycan from bromelain and an asialodiantennary complex glycan were both released by PNGase Se treatment. The Man<sub>3</sub>GlcNAc<sub>2</sub> and the sialylated diantennary complex glycoasparagines were not cleaved. We found, using fluorescent substrates and HPLC monitoring, that oligomannosides were hydrolysed by PNGase Se only when linked to a peptide. The enzyme also acted on a glycopeptide carrying a single GlcNAc residue. PNGase Se activity on plant substrates, HRP and PHA, was monitored by HPAEC-PAD. This enzyme was found to release the complex N-glycan from HRP glycopeptides bearing an  $\alpha(1-3)$ -linked fucosylated residue. The major compound obtained after separation by HPAEC-PAD (Fig. 2, compound 1) was identified as XylMan<sub>3</sub>FucGlcNAc<sub>2</sub> by <sup>1</sup>H-NMR spectroscopy (data not shown). PHA was reported to be N-glycosylated by both an oligomannoside and a fucosylated complex-type N-glycan identical to the HRP N-glycan described above [31]. Both N-glycans were quantitatively released from PHA glycopeptides by treatment with PNGase Se.

The substrate specificities of PNGase Se are similar to those described for PNGase A [28, 30, 32] and PNGase J [7]. This enzyme is able to cleave the asparaginyl-glycan linkage of N-glycans from animal and plant origins. However, intact glyco-

proteins are poor substrates. Glycoasparagine substrates are not hydrolysed by PNGase Se confirming that asparagine must be substituted as reported for other PNGases.

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 GlcNAcMan<sub>3</sub>XylGlcNAcFucGINAc [1] and Man<sub>3</sub>XylGlcNAcFucGINAc [2]. It was postulated that complex N-glycans isolated from medium of suspension cultured *Silene alba* cells [1], could be the product of a glycoprotein breakdown by a PNGase. The substrate specificity of PNGase Se supports this hypothesis since we demonstrated that both oligomannosides and complex N-glycans, which are reported to be linked to plant glycoproteins [10], are substrates for this enzyme isolated from cell extracts. Intact glycoproteins are not affected by treatment with PNGase Se. They must be degraded to glycopeptides in order to increase the accessibility of the asparaginyl-glycan linkage to the enzyme. However, the substrate specificity displayed by PNGase Se *in vitro* could be noticeably different from the specificity expressed *in vivo*. Indeed, the results obtained with all known PNGases indicate the importance of the peptide component on the overall substrate quality of the N-glycans. PNGase Se can contribute to the accessibility of the peptide part of N-glycosylproteins, which might be enhanced by separation of the oligosaccharide and protein moieties.

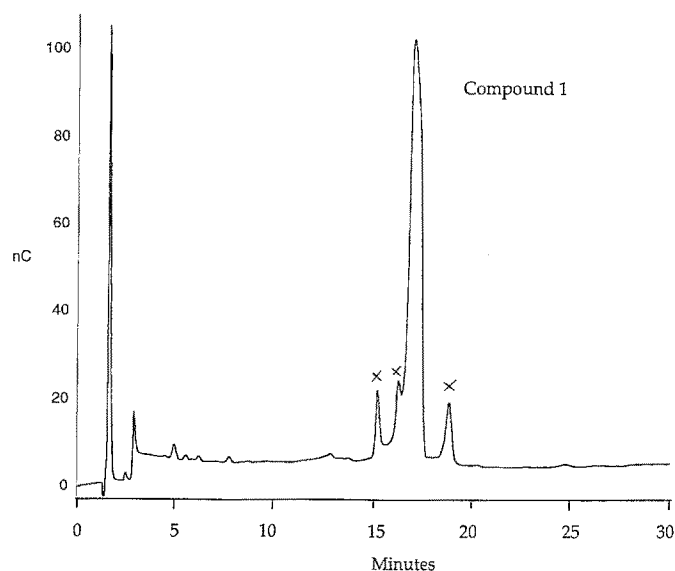
It is important to note that our results were obtained using a cell suspension culture which could be a useful method of studying the fate of N-glycans by, for example, pulse-chase experiments. In the other hand, the fact that free N-glycans were recently isolated from tomato fruits [33] suggests that the mechanism and the reasons for their release is not limited to isolated cells but also applies to tissues and/or organs. So it appears obvious to investigate other plant systems in order to confirm the occurrence and the properties of their PNGases.

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**Figure 2.** HPAEC-PAD chromatogram of the N-glycan XylMan<sub>3</sub>FucGlcNAc<sub>2</sub> released from horse radish peroxidase (HRP) glycopeptide by purified PNGase Se. Minor unidentified products are indicated by (x).

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