Pergamon

0031-9422(95)00001-1

Phytochemistry, Vol. 39, No. 3, pp. 481–487, 1995

0031-9422/95 $9.50 + 0.00

ENDO-*N*-ACETYL-β-D-GLUCOSAMINIDASE AND PEPTIDE-N^4-(*N*-ACETYL-GLUCOSAMINYL) ASPARAGINE AMIDASE ACTIVITIES DURING GERMINATION OF *RAPHANUS SATIVUS*

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(*Received* 28 *September* 1994)

Key Word Index—*Raphanus sativus*; Cruciferae; germination; deglycosylation enzymes; ENGase; PNGase.

Abstract—Endo-*N*-acetyl-β-D-glucosaminidase (ENGase, EC 3.2.1.96) and peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase (PNGase, EC 3.5.1.52) activities were monitored during germination and postgerminative development in *Raphanus sativus*. The PNGase activity was found in dry seeds and its level was constant during germination and postgermination. The ENGase activity was first detected about 18 hr after the start of imbibition (HAI) and displayed a maximum level at 36 HAI. After 36 HAI the production of both enzymes was constant until days 4–5. Both enzymes displayed substrate specificities corresponding to the potential glycoprotein substrates found in plants. They are in agreement (i) with the hypothesis that ENGase and PNGase are at the origin of the production of 'unconjugated N-glycans' and (ii) with the possibility that protein activity could be regulated by the removal of N-glycans.

INTRODUCTION

The enzymatic cleavage of the intracellular plant 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 biologically active oligosaccharides [1, 2] or for the regulation of protein activity [3, 4]. The 'unconjugated N-glycans' (UNGs) are now considered as a new class of oligosaccharins and their potential importance in regulating plant processes was recently underlined [5]. It was also shown that the inactive glycoprotein precursor of concanavalin A (ConA) can be converted to an active lectin by removal of the N-glycan (de-N-glycosylation) *in vitro* and thus its presence or absence determines the lectin activity of the precursor [3]. Whatever the need, two families of enzymes could be at the origin of the release of N-glycans from the carrier glycoproteins: (i) endo-*N*-acetyl-β-D-glucosaminidases (ENGases) which hydrolyse the di-*N*-acetylchitobiosyl linkage (Fig. 1) of the invariant pentasaccharidic inner-core of N-linked glycans giving an oligosaccharide presenting the *N*-acetyl-β-D-glucosamine (GlcNAc, **2**) residue in a terminal reducing position and a peptide (or protein) chain with GlcNAc **1** linked to the asparagine residue; (ii) peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidases (PNGases) which cleave the glycosylamine linkage between GlcNAc **1** and the asparagine residue, releasing the entire N-glycan and transforming the asparagine to aspartic acid with concomitant liberation of ammonia (Fig. 1).

A PNGase activity whose level correlated with the excretion of UNGs [6] as well as an ENGase were measured in *Silene alba* cells and it was shown that both activities were increased under conditions of carbon starvation which promoted the production of UNGs [7]. This result, suggesting a functional role of the two enzymes in plant physiology, was obtained using a cell suspension culture, but the recent demonstration of UNGs in tomato fruits [8] argues for a widespread phenomenon, even if in the latter case the enzymes potentially responsible for the occurrence of UNGs were not characterized. Furthermore, de-N-glycosylation seems to be a normal event during ConA processing *in vivo* [9] for *Canavalia ensiformis* in whose seeds PNGase and ENGase activities have been described [10, 11].

It was obvious to investigate the expression of PNGase and ENGase activities in the cells and to check whether these enzymes are regulated during particular physiological stages. Germination and postgerminative development represent critical transition periods in the sporophytic life cycle of higher plants. Germination starts with seed imbibition and ends with radicle protrusion through the testa, while postgermination represents the period

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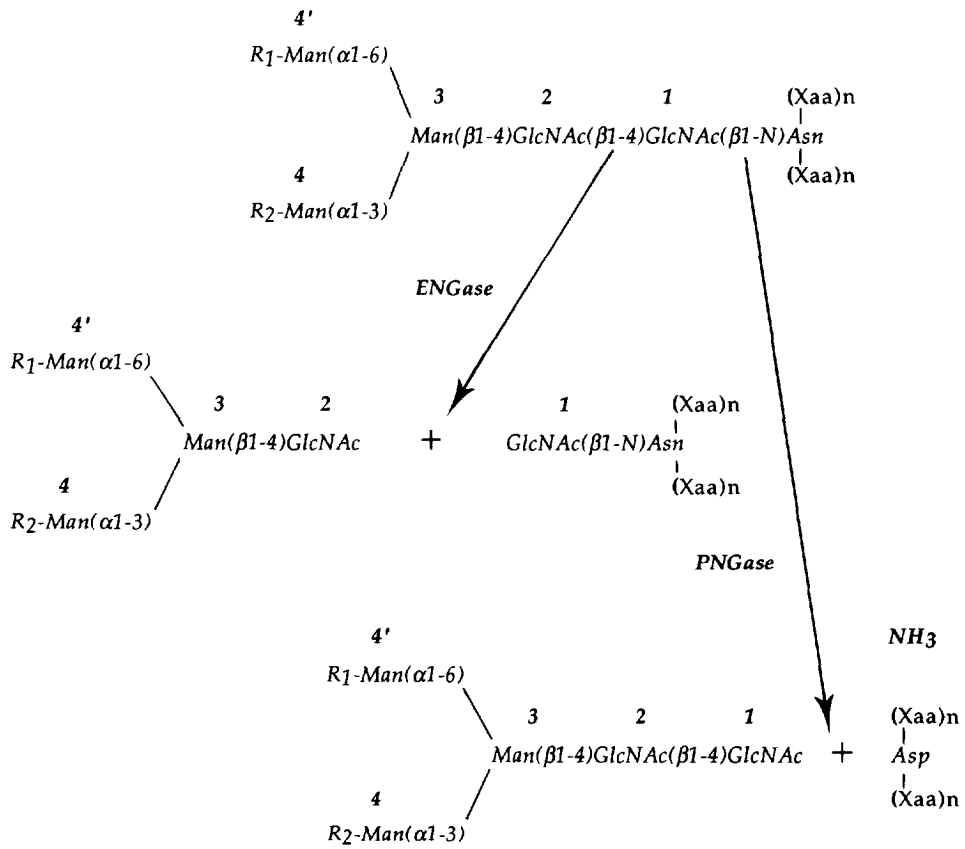


Fig. 1. Action of *endo*-*N*-acetyl-β-D-glucosaminidases (ENGases) and peptide N^4-(*N*-acetylglucosaminyl) asparagine amidases (PNGases) on the di-*N*-acetylchitobiosyl part of asparagine-linked glycans. (Xaa)n depict the peptide chain. R1 and R2 represent mannose (Man) or *N*-acetyl-β-D-glucosamine (GlcNAc) residues.

after emergence of the radicle during which events unique to the reinitiation of sporophyte growth occur [12].

In this study, we monitored the PNGase and ENGase activities during germination and postgerminative development in *Raphanus sativus* and demonstrated that the ENGase activity accumulated during postgermination, whereas the PNGase activity was found to be present at substantial levels in both stages.

RESULTS

Quantification of PNGase and ENGase activities

PNGase was quantified using the resorufin-labelled N-glycopeptide which has been previously succesfully used for the quantification of PNGase F from *Flavobacterium meningosepticum* [13] and PNGase Se from *Silene alba* [6]. ENGase was quantified using the $Man_7GlcNAc_2Asn$-resorufin glycoasparagine which was demonstrated not to be hydrolysed by several PNGases and thus to constitute a specific substrate for the detection of ENGase activities [14]. Neither substrate was incubated for more than 48 hr, since degradation may occur after this time. Under these conditions, the detection limit of the assays is 0.02 pkat ml^{-1} of extract. The crude extract was brownish and unfortunately pigments were shown to have the same retention time as the product of the action of ENGase. Nevertheless, this material could be removed by ethanol precipitation and assays could then be interpreted. The precipitation of the proteins from the crude extract was optimized in order to maintain maximum activity while eliminating pigments. The values obtained for ENGase with the retained conditions (final concentration 50%, v/v) were confirmed by the use of a dilution of the crude extract and an equivalent increase of the incubation period.

Evolution of the activities during germination and postgermination

Using the quantification approach described above it was possible to monitor the evolution of PNGase and ENGase activities. In crude extracts from dry seeds the PNGase activity was measurable whereas ENGase activity was not detectable. During the growth of radish, two phases can be distinguished for the patterns of production of the enzymes, the first from 0 to 36 hr after the start of imbibition (HAI) and the second after 36 HAI (Fig. 2). It is during the first phase, that germination take place. From the increase of the fresh weight of the seedlings (Fig. 2A), and owing to the change of the slope, we can say that germination was terminated at *ca* 15 HAI. Further-

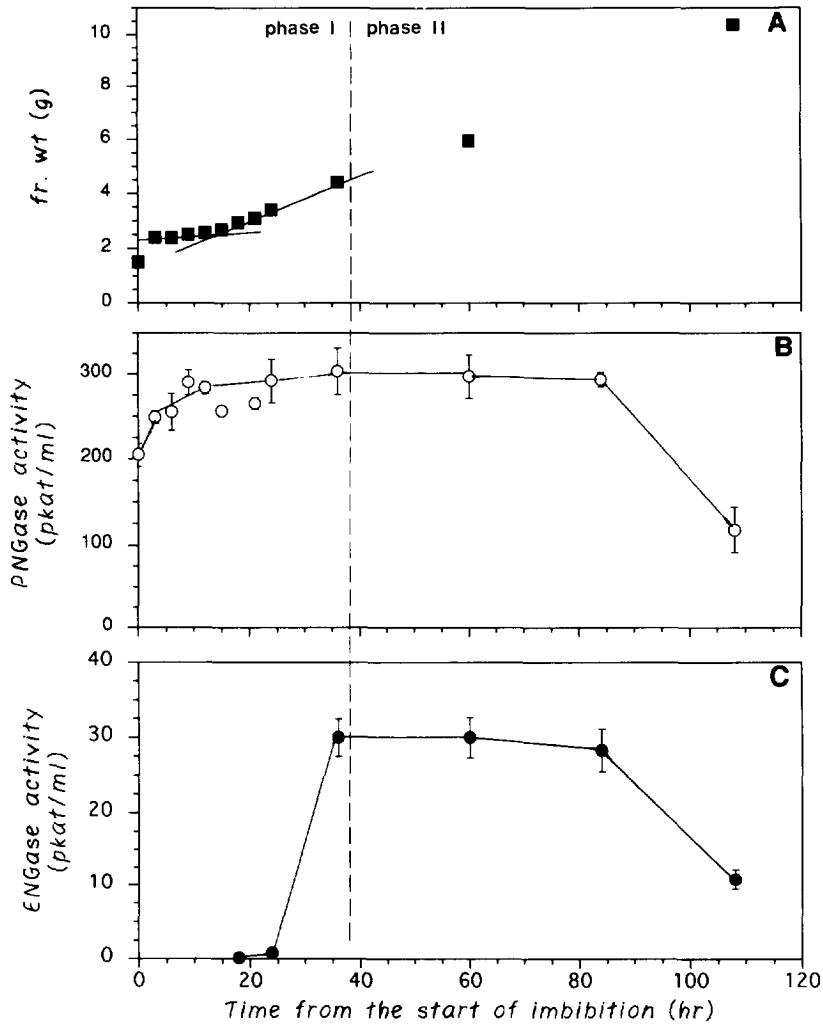


Fig. 2. Evolution of the enzyme activities during germination of radish. (A) Fresh weight of the seedlings; (B) evolution of PNGase; (C) evolution of ENGase. The enzyme activities were quantified with the resorufin-labelled substrates as described in the Experimental section. Bars represent standard deviations of the mean.

more, after 18 HAI, the radicle was visible for more than 95% of the seedlings. PNGase activity was maintained at an almost constant level between 3 HAI and day 4 (Fig. 2B). The ENGase activity was not detected before 15 HAI, but it was detected at 18 HAI, began to increase at 24 HAI and reached its maximum level at 36 HAI (Fig. 2C). A 100-fold increase can be seen between 24 and 36 HAI. During the second phase, after 36 HAI, the level of PNGase was always higher than ENGase and the ratio PNGase/ENGase was maintained constant (*ca* 10). Both activities declined at days 4–5. When cotyledons and hypocotyls were separately extracted, the activities were found essentially in the cotyledon fraction (85–90%).

Partial purification of the enzymes

Affinity chromatography on Sepharose–concanavalin A (Seph-ConA) was used as the first step for the purification of the enzymes. ENGase was eluted in the non retained fraction I, containing 84% of the loaded protein and PNGase activity was mainly recovered with 300 mM α-Me-Glc in the strongly retained fraction III containing 8.6% of loaded proteins.

The two enzymes were submitted to other purification steps until they were free of α- and β-mannosidase, β-galactosidase, β-D-xylosidase and α-L-fucosidase activities. A 239-fold purification was obtained for PNGase and a 26-fold purification for ENGase. The results of the partial purification are shown in Table 1. Throughout the procedure, only one peak of each activity was observed. At the degree of purification achieved, the enzymes were stable at 4°. No protease activity was detected in the purified PNGase while in the ENGase preparation a protease was still present that could be inhibited by addition of 1 mM PMSF or 0.5 mM Pefabloc-SC. The effect of pH on the enzymes was studied with the resorufin-labelled substrates. Maximum activity was obtained at pH 4–5 for both activities which decreased to 50% at pH 3 and 6.

Table 1. Purification of PNGase and ENGase from radish seedlings

	Protein (mg)	Total activity (nkat)	Specific activity (pkat mg^{-1} protein)	Enrichment (fold)	Yield (%)
PNGase R					
Crude extract	1610	780	483	1.0	100
Seph-ConA, fraction III	139	717	5150	10.7	92
CM-Biogel A	18.9	304	16100	33.3	39
Sephacryl S200	2.1	243	116000	240	31
ENGase R					
Crude extract	1610	81	50	1.0	100
Seph-ConA, fraction I	1360	81	55	1.1	91
DE-52	270	50	185	3.7	62
Sephadex G75	32.5	43	1320	26.5	53

Starting material: 170 g fr. wt of 3-day-old seedlings (*Raphanus sativus* L., cv Flamboyant).

Table 2. Substrate specificity of PNGase R and ENGase R from radish towards glycoasparagines and glycopeptides

		Relative hydrolysis rate (%)	
Substrate	Structure	PNGase R	ENGase R
Glycoasparagines			
Oligomannoside	$Man_7GlcNAc_2Asn$	0	100
Oligomannoside	$Man_9GlcNAc_2Asn$	0	100
Complex	$Gal_2GlcNAc_2Man_3GlcNAc_2Asn$	0	25
Glycopeptides			
Oligomannoside	$Man_5GlcNAc_2Asn$-peptide	30	100
N-Acetyllactosaminic	$Gal_2GlcNAc_2Man_3GlcNAc_2Asn$-peptide	100	45
Complex (from bromelain)	$(Xyl)Man_2GlcNAc(Fuc)GlcNAcAsn$-peptide	100	0

The concentration of the substrate (expressed in sugar content) was 1 mg ml^{-1}. The hydrolysis rate was estimated relative to the Man_5 glycopeptide.

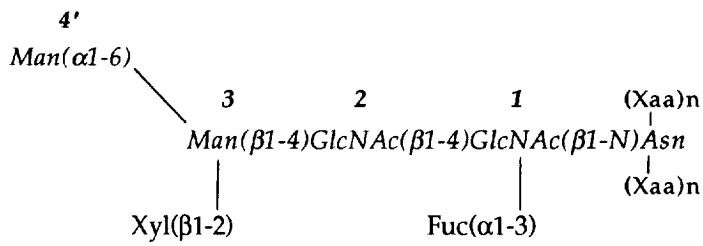
Substrate specificity

No oligosaccharide was released from glycoproteins containing N-glycans of the N-acetyllactosaminic type (bovine fibrinogen, bovine transferrin, bovine α1-acid glycoprotein and fetuin) or xylose containing complex glycans (jacalin) either by PNGase or ENGase. Only RNase B was de-N-glycosylated. This glycoprotein contains a single high-mannose type sugar chain composed of $Man_{5-9}GlcNAc_2$ with 48% of total glycoforms having five mannose residues [15]. No modification of the native glycoprotein was obtained. Under chaotropic-unfolding conditions the M_r of RNase B showed a decrease of *ca* 2 k, corresponding to the removal of the N-glycan, after the treatment with ENGase or PNGase. The de-N-glycosylation was much more efficient with ENGase than with PNGase (30-fold).

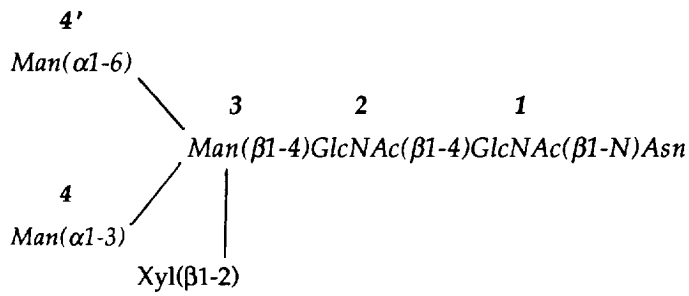
Results concerning the susceptibility of different glycoasparagines or glycopeptides to ENGase and PNGase were summarized in Table 2. PNGase hydrolysed the glycopeptide from bromelain (Fig. 3, structure A), bearing a fucose α(1-3)-linked to the innermost GlcNAc **1** residue. It also hydrolysed a biantennary glycopeptide of the *N*-acetyllactosaminic type and an oligomannoside, but was unable to cleave all the glycoasparagines tested (Table 2). ENGase was preferentially active on glycoasparagines and glycopeptides of the oligomannoside type with five to nine mannose residues but was inactive to the glycopeptide from bromelain.

DISCUSSION

We found that PNGase and ENGase from radish (PNGase R and ENGase R), both acting on the di-*N*-acetylchitobiosyl part of asparagine-linked glycans (de-N-glycosylation enzymes), are differentially produced by radish during postgermination, period corresponding to a critical transition in the sporophytic life cycle of higher plants. In fact, the ENGase activity, not detected in dry seeds or during germination, seems to be produced specifically during postgermination. Both activities were prevalent (85–90%) in cotyledons which serve as the major site of storage macromolecule mobilization while processes occurring in the hypocotyls are largely devoted to growth and differentiation. Thus, both enzymes are probably required for reserve mobilization, since de-N-glycosylation may occur before the action of proteases on



Structure A



Structure B

Fig. 3. Structure of two complex xylose-containing glycans used in this study for ENGase from radish (structure A) and for the study of ENGase P from *Phylostachys heterocycla* (structure B, ref. [20]).

the potential glycoprotein substrates. Enzymes involved in the hydrolysis of storage macromolecules can become prevalent in seedlings or be synthesized *de novo* following germination [16].

ENGase R and PNGase R were purified until no exoglycosidase was detectable. The fact that PNGase R was strongly retained on Seph-ConA suggested that it was a N-glycosylprotein, like PNGase A from almond meal [17], whereas the ENGase R was probably not N-glycosylated.

Both enzymes displayed substrate specificities similar to that of other PNGases and ENGases already found in plants [11, 17, 18]. Like PNGase A from almond meal [19], PNGase R was able to cleave N-glycans with a fucose residue α (1-3)-linked to the innermost GlcNAc **1** residue. It was previously shown that an ENGase, partially purified from *Phyllostachys heterocycla* (ENGase P) [20], was able to cleave oligosaccharides with a xylose residue linked β (1-2) to mannose **3** (Fig. 3, structure B), but not when a fucose α(1-6) substitution simultaneously occurred on GlcNAc **1**. ENGase R cannot cleave a substrate presenting the fucose α (1-3) substitution on GlcNAc **1**, a result suggesting that a free GlcNAc **1** residue is essential for the action of this enzyme.

The results on the substrate specificity suggest that both enzymes could act on the potential plant glycoprotein (and glycopeptide) substrates [21] and that their action is complementary. The two de-N-glycosylation enzymes can hydrolyse substrates in order to produce oligosaccharides like those found in the medium of suspension cultured *Silene alba* cells or in tomato fruits. PNGase can release the complex type N-glycans like $Man_3(Xyl)GlcNAc(Fuc)GlcNAc$ and $GlcNAcMan_3(Xyl)GlcNAc(Fuc)GlcNAc$ [1, 6]. ENGase is able to release the oligomannoside $Man_5GlcNAc$ from *Silene alba* [1] and also the oligomannosides $Man_{5-8}GlcNAc$ found in mature green tomato [8]. This is in agreement with the hypothesis that ENGases and PNGases are at the origin of 'unconjugated N-glycans', proven important molecules for the regulation of plant processes [5]. Among the glycoproteins tested, only RNase B was de-N-glycosylated and this was possible only in an unfolded state. However, it is possible that de-N-glycosylation occurs *in vivo* for native glycoproteins, and this could explain the possible control of protein activity by the removal of N-glycans observed, for example, for the maturation of the lectin Con A [3, 4]. Our results, and other observations, support the hypothesis that the maturation of Con A, *in vivo*, could be performed by ENGase: (i) Con A is synthesized as a glycoprotein precursor (pro-Con A) bearing a N-glycan of the oligomannoside type; (ii) glycoproteins containing N-glycans of the oligomannoside type are effective substrates for the two de-N-glycosylation enzymes isolated in this study, and also previously described [11]; (iii) de-N-glycosylation is more easily obtained with ENGase, and (iv) the

amount of ENGase dramatically increases just after germination, underlining the importance of the enzyme during this critical transition period.

Thus, in addition to their possible role for reserve mobilization, our results suggest that each of the de-N-glycosylation enzymes may have a specific role in plants.

EXPERIMENTAL

Plant material. Commercial dry seeds of radish (*Raphanus sativus* L. cv Flamboyant) were used for the experiments. Samples of dry seeds (1.5 g) were grown in Petri dishes on wet paper in a greenhouse under natural light conditions. Three samples were used for each experimental condition. At specified times after the start of imbibition, the extraction was done either with the entire seedlings or separately with dissected cotyledons and hypocotyls. The extraction was performed at 4° in a Waring Blendor, 2 min at the maximum setting, in 50 ml of 5 mM NaOAc buffer containing 50 mM EDTA (pH 5.2), 1 mM PMSF (or 0.5 mM Pefabloc-SC) and 1 mM 2-mercaptoethanol. Cell debris were spun down by centrifugation at 2600 *g* for 15 min and at 27 000 *g* for 30 min at 4°. The last supernatant constituted the crude extract.

PNGase and ENGase assay procedure. Both activities were quantified using fluorescent substrates in a HPLC assay. The PNGase activity was quantified using the $Man_5GlcNAc_2Asn$ (Ala, Thr, Ser) resorufin-labelled glycopeptide as in ref. [13]. The ENGase activity was quantified using a $Man_7GlcNAc_2Asn$-resorufin glycoasparagine as in ref. [14]. In both cases a 100 mM NaOAc buffer, pH 4.5 was used and the incubations were carried out at 37°. To remove the pigments, in the case of the crude extracts, proteins were precipitated by EtOH (stored at −20°) (final concn 50%, v/v). The ppt. was centrifuged (5 min at 12 000 *g*) and resuspended in the buffer and assayed for enzyme activity.

Assay of the activity of other enzymes. Exoglycosidase activities were assayed using the appropriate *p*-nitrophenyl glycosides [22]. The reaction mixture was incubated at 37° for 1 hr and the A_{400} was measured. Protease activity was determined by incubating azocasein with the enzyme prepn in 0.1 M Tris–HCl buffer, pH 7.6, at 37° [23]. The reaction was terminated by the addition of 5% TFA and the A_{440} of the supernatant was measured.

Partial purification of the enzymes. All the procedures were conducted at 4°. A crude extract was obtained as described above from *ca* 170 g of seedlings (3-days-old, originated from 50 g dry seeds, grown in the dark) in 5 mM NaOAc, 50 mM EDTA (pH 5.2), made up to 0.1 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.02% NaN_3 (ConA-buffer). It was directly loaded on a 30 ml Seph-ConA (Pharmacia) column, equilibrated in ConA-buffer. The column was washed with the same buffer to remove unbound proteins (non-retained fraction I). Bound glycoproteins were eluted [24] with 10 mM (weakly retained fraction II) or 300 mM (strongly retained fraction III) methyl-α-D-glucopyranoside (α-MeGlc) in ConA-buffer. Fraction III was loaded on a 10 ml CM-Biogel A (Bio-Rad) column, equilibrated and eluted (21 ml hr^{-1}) with 10 mM NaOAc pH 5.2 (acetate buffer) and with the same buffer containing 200 mM NaCl. The Sephacryl S200 column (720 × 15 mm) was equilibrated and eluted (21 ml hr^{-1}) with the acetate buffer. Fraction I from Seph-ConA was loaded on a 160 ml DE-52 column, and eluted (40 ml hr^{-1}) with 20 mM Tris–HCl buffer pH 7.5 (Tris–HCl buffer) and with a linear gradient of NaCl in the same buffer. The Sephadex G75 column (720 × 15 mm) was eluted (9 ml hr^{-1}) with the Tris–HCl buffer. After the CM-Biogel A and DE52 steps, the activity containing frs were concd by $(NH_4)_2SO_4$ pptn (80%) and the ppt. was resuspended in the buffer of the following step. During all the steps, protein elution was monitored by measuring the A_{280}. Protein concn was determined by the method of ref. [25] or with the BioRad protein assay kit [26] using BSA as standard.

Assay for optimum pH. To determine optimum pH of the enzymes, the substrate was incubated with 20 μl of enzyme soln previously suspended in 0.1 M appropriate buffer (Na citrate for pH 2.5–4.5; NaOAc for pH 3.5–6.5; Na-Pi for pH 6–7.5 and Tris–HCl for pH 7–9). The assay of the enzymes was then performed as described above.

Glycoprotein and glycopeptide substrates. Glycopeptide and glycoasparagine stock solns (2 mg ml^{-1}) were made in 100 mM NaOAc buffer, pH 4.5, containing 0.5 mM Pefabloc-SC. Glycoasparagines of the oligomannoside type were obtained by pronase digestion of hen ovalbumin and purified as described in ref. [27]. Complex biantennary glycoasparagines were obtained by pronase digestion of human plasma proteins and purified by anion exchange chromatography (J. C. Michalski, unpublished data). The asialo derivative of this glycoasparagine was obtained by treatment with 50 mM HCl for 1 hr at 80° and subsequent purification by gel filtration on a BioGel P-2 column. The monosaccharide composition was determined by GLC after methanolysis (MeOH/0.5 M HCl for 24 hr at 80°) and trimethylsilylation [24]. The complex biantennary asialo glycopeptide and the glycopeptide from bromelain obtained from Dr F. Altmann were prepared as described [19]. The release of oligosaccharides from glycopeptide due to the action of the enzyme was monitored by TLC carried out as described in ref. [28].

Glycoproteins (bovine fibrinogen, bovine transferrin, bovine α1-acid glycoprotein, fetuin and RNase B from Sigma, and jacalin given by P. Aucouturier) were diluted (1 mg ml^{-1}) in 100 mM NaOAc buffer, pH 4.5, containing 0.5 mM Pefabloc-SC. For native conditions the enzyme soln (10 μl) was directly added to the reaction mixture which contained 10 μg of the tested glycoprotein. Chaotropic-unfolded glycoproteins were obtained by treatment with 0.75 M NaSCN and 100 mM 2-mercaptoethanol [29]. Incubations were carried out for 6–24 hr at 37°. The modification of the glycoproteins tested was monitored by SDS–PAGE [30].

Acknowledgements—We are indebted to Alastair Balloch for linguistic advice, to Jean-Claude Michalski for the gift

of glycoasparagines, to Pierre Aucouturier for the jacalin sample and to Michel Delseny for helpful discussions.

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