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Kinetic comparison of peptide: N-glycosidases F and A reveals several differences in substrate specificity

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The initial velocities of hydrolysis of nineteen glycopeptides by peptide: N-glycosidase F and A were determined. Substrates were prepared from bovine fetuin, hen ovalbumin, pineapple stem bromelain, bovine fibrin and taka-amylase. From these glycopeptides, several variants with regard to peptide and carbohydrate structure were prepared and derivatized with dabsyl chloride, dansyl chloride or activated resorufin. Tyrosine containing glycopeptides were also used without an additional chromophore. Enzymatic hydrolysis of glycopeptides was quantified by narrow bore, reversed phase HPLC with turnaround cycle times of down to 6 min, but usually 15 min. K_M values ranging from 30 to 64 μM and from 4 to 36 μM were found for N-glycosidase F and A, respectively. Relative velocities of hydrolysis of the different substrates by each enzyme varied considerably. Little, if any, similarity of the performance of N-glycosidase F and A with the different substrates was observed. The minimal carbohydrate structure released by peptide: N-glycosidase F was a di-*N*-acetylchitobiose. N-glycosidase A could release even a single *N*-acetylglucosamine, albeit 3000 times slower than a di-*N*-acetylchitobiose or larger glycans. In general the structure of the intact glycan had little effect on activity, and with both enzymes the rate of hydrolysis appeared to be primarily governed by peptide structure and length. However, N-glycosidase F did not release glycans α1,3-fucosylated at the asparagine linked *N*-acetylglucosamine irrespective of the presence of xylose in the substrate.

Keywords: *N*-glycanase, glycopeptidase, deglycosylation, glycopeptides

Abbreviations: CAMCys, S-carboxamidomethyl cystein; CMCys, S-carboxymethyl cystein; Fib, Fet, Ova, Taa and Brl, glycopeptides derived from bovine fibrin, fetuin, ovalbumin, taka-amylase A, and bromelain, respectively; GlcNAc, *N*-acetylglucosamine; PLA, phospholipase A_2; PNGase, peptide N-glycosidase; RESOS, *N*-(Resorufin-4-carbonyl)piperidine-4-carboxylic acid *N'*-hydroxysuccinimide ester.

Introduction

Since their discovery about 15 years ago, peptide: N-glycosidases (PNGases) have become the probably most widely used agent for the deglycosylation of glycoproteins. A PNGase (glycopeptidase, N-glycanase, N-glycosidase, glycoamidase or, systematically, peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase) releases asparagine-linked oligosaccharides from glycopeptides by hydrolysing the amide of the asparagine side chain [1–4]. In addition to almond glycopeptidase (PNGase A), the first such enzyme to be discovered [1, 2], several other seeds have been shown to contain PNGases [5, 6]. The best known PNGase, however, was detected in the culture fluid of the facultatively pathogenic microorganism *Flavobacterium meningosepticum* where it occurs together with three different endo-*N*-acetyl-β-glycosidases [7, 8].

The glycosylated Asn must be in peptide linkage on both sides. Only PNGase A will also act, albeit very slowly, on glycopeptides where the glycosylated Asn residue is in a terminal position [6, 9]. Degradation of a protein to peptides is nevertheless a useful measure, because both enzymes act poorly, if at all, on native glycoproteins – exceptions may be observed depending on the three-dimensional structure of the particular glycoprotein. The tolerance of PNGase A towards KSCN was reported to be useful in certain cases [10], but no generally effective procedure for the digestion of glycoproteins with PNGase A has been described so far. In contrast, the stability of PNGase F towards SDS in the presence of nonionic detergent allows deglycosylation of virtually any glycoprotein by following a standard procedure [11, 12].

As far as glycoproteins from vertebrates only are concerned, both PNGases liberate all kinds of N-linked oligosaccharides irrespective of their structure, size or charge [see *e.g.* 13, 14]. However, N-glycans with fucose in α1,3-linkage to the Asn-

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bound *N*-acetylglucosamine, which are found in glycoproteins from plants and insects [15, 16], are resistant to PNGase F [17]. The ability to hydrolyse a substrate with a single *N*-acetylglucosamine residue has only been demonstrated for PNGase A [9]. Obviously, these enzymes differ by more than just size or pH-optimum.

Despite the wide use of these enzymes, there are only very few data on their substrate specificity in terms of initial rates and K_M-values [6, 18]. If PNGases are simply considered to be tools, such data may appear expendable in view of the rather tedious substrate preparation and enzyme activity determination. However, very recently the occurrence of PNGase activity in animals (Medaka fish, cultured mouse cells) has been reported [19, 20]. The data point to a possible involvement of PNGase in embryonal development. This finding emancipates PNGases as worthwhile objects of investigations e.g. on substrate specificity and enzyme kinetics.

In this report, we compare the kinetic parameters of PNGases A and F for three established and two new substrates by the use of narrow-bore, reversed phase HPLC. The influence of the size of the carbohydrate moiety was investigated with a series of glycopeptides which were degraded stepwise by exoglycosidases from a biantannery down to a GlcNAc-peptide. Likewise, the effects of alterations of the peptide moieties were examined. In addition, the stability of the PNGases towards SDS in the absence or presence of non-ionic detergent was reevaluated and the oligosaccharides released from an insect glycoprotein by either of the two enzymes were compared.

Materials and methods

Glycoproteins, enzymes and dyes

Bovine fibrin, bovine fetuin, hen egg albumin and crude pineapple stem bromelain were obtained from Sigma. The latter was purified as described [17]. α-Amylase from *Aspergillus oryzae* (Taka-amylase A) was generously donated by DI P. Dornhelm (Novo Nordisk A/S) and purified as described [21]. Pepsin, trypsin (TPCK-treated), thermolysin, and carboxypeptidase Y were purchased from Sigma. PNGase F, PNGase A, endo-β-*N*-acetylglucosaminidase D, and sialidase from *Clostridium perfringens* were obtained from Boehringer Mannheim. β-Galactosidase from *Aspergillus oryzae* and phospholipase A_2 from honeybee venom were prepared as described [16]. β-*N*-Acetyl-glucosaminidase and α-mannosidase from *Canavalia ensiformis* and snail β-mannosidase were purchased from Sigma. Bovine serum albumin (A 7906) was from Sigma. Dabsyl and dansyl chloride were obtained from Fluka and *N*-(Resorufin-4-carbonyl)piperidine-4-carboxylic acid *N'*-hydroxysuccinimide ester (RESOS) was bought from Boehringer Mannheim.

Analytical methods

Protein was determined with the Micro BCA assay (Pierce) with bovine serum albumin as standard. Carbohydrate in column effluents was determined with the orcinol-sulfuric acid reagent. Amino acids were analysed as their o-phthalaldehyde/2-mercaptoethanol derivatives [22] after acid hydrolysis or after treatment of glycopeptides (2 nmol) with carboxypeptidase Y (1 µg enzyme) in 50 mM sodium acetate at pH 5.0 for 16 h. Oligosaccharide structures were verified by pyridylamination of the glycans released from glycopeptides by PNGase A [16, 23].

Preparation of glycopeptides

Fetuin glycopeptides Carboxamidomethylated bovine fetuin [24] was digested with thermolysin and the glycopeptide containing the glycosylated Asn-81 [25] was purified by gelfiltration and ionexchange chromatography according to Plummer *et al.* [6]. The first and largest of the three major peaks obtained on Whatman DE-52 was further fractionated by HPLC using a 250 × 8 mm column of Hypersil ODS (Shandon). The column was equilibrated in 25 mM ammonium acetate at pH 6.0 and eluted with a gradient of 0 to 16% acetonitrile in 16 min at a flow rate of 6.0 ml min^{-1}. The first HPLC peak contained the glycopeptide from Asn-138, the second one that from Asn-81 as deduced from amino acid analysis (Table 1). The dabsylated derivatives of these sialylated glycopeptides, termed Fet^5 (Asn-81) and Fet^{5a} (Asn-138), were prepared and isolated as described below.

Additionally, the dansylated counterpart of Fet^5 (S-carboxamidomethylated) and the dansylated, S-carboxymethylated variant of Fet^5 were prepared.

Ovalbumin glycopeptides Ovalbumin was dissolved in 5% v/v formic acid and digested for 30 h with pepsin at an enzyme to substrate ratio of 1:30. The digest was purified by passage over Sephadex G50 superfine in 1% v/v acetic acid. The glycopeptide fraction was lyophilized and applied to Sephadex DEAE A25 in 50 mM ammonium acetate at pH 8.8. The column was eluted with a linear gradient from 0 to 1 M sodium chloride in buffer. The major peak was further fractionated by HPLC as described above. Again, several fractions were obtained from which the most abundant, termed Ova^9, had the peptide structure shown in Table 1 as ascertained by amino acid analysis and carboxypeptidase Y digestion. Because of the already high hydrophobicity of the underivatized peptide, it was labelled with the less polar resorufin group (see below). Because of its tyrosine residue, Ova^9 could also be used for HPLC without a dye. A portion of Ova^9 was further degraded to Ova^6 with trypsin using an enzyme to substrate ratio of 1:50 and a 0.1 M Tris/HCl buffer at pH 8.0.

Bromelain glycopeptide Bromelain glycopeptide was prepared as described [2, 17]. Again, final purification was achieved by HPLC. Incidentally, one of several preparations did not yield the usual undekapeptide Brl^{11} but a dekapeptide Brl^{10} (Table 1). Part of Brl^{11} was defucosylated by overnight incubation in trifluoroacetic acid at room temperature [26]. These bromelain peptides were dabsylated as described below.

Table 1. Hydrolysis of different glycopeptides by PNGase F and A.

Name of substrate	*Peptide sequence and glycan structure*	*PNGase F*			*PNGase A*		
		V ($\mu mol \times min^{-1} \times mg^{-1}$)	(V_{max})	k_M (μM)	*V* ($\mu mol \times min^{-1} \times mg^{-1}$)	(V_{max})	k_M (μM)
***d*-Fib^6**	*dabsyl*- Val-Gly-Glu-Asn(CHO)-Arg-Thr						
	CHO = Galβ4GlcNAcβ2Manα6 \ Manβ4GlcNAcβ4GlcNAc / Galβ4GlcNAcβ2Manα3	47.5	(110)	64	3.53	(5.0)	20
Glycoforms of *d*-Fib^6:							
***d*-Fib^6-core**	CHO = Manα6 \ Manβ4GlcNAcβ4GlcNAc / Manα3	38.9	(140)	120	4.08	(5.3)	15
***d*-Fib^6-tri**	CHO = Manβ4GlcNAcβ4GlcNAc	68.4	(280)	150	5.42	(7.9)	24
***d*-Fib^6-di**	CHO = GlcNAcβ4GlcNAc	0.92			3.65	(8.0)	60
***d*-Fib^6-mono**	CHO = GlcNAc	0			0.0012		
Peptide and chromophore variants of *d*-Fib^6:							
***d*-Fib^5**	*dabsyl*- Val-Gly-Glu-Asn(CHO)-Arg	2.3		80–110	0.41		50–90
***r*-Fib^6**	*resorufin*- Val-Gly-Glu-Asn(CHO)-Arg-Thr	29.7			1.85		
***d*-Fet^5**(Asn-81)	*dabsyl*- Leu-Ala-Asn(CHO)-CAMCys-Ser						
	CHO = NeuNAcα3/6Galβ4GlcNAcβ2Manα6 \ NeuNAcα3/6Galβ4GlcNAcβ4 \ Manβ4GlcNAcβ4GlcNAc [a] / Manα3 / NeuNAcα3/6Galβ4GlcNAcβ2	26.7	(55)	52	9.8	(16.4)	36
Glycoform of *d*-Fet^5:							
***d*-Fet^5-core**	CHO = Manα6 \ Manβ4GlcNAcβ4GlcNAc / Manα3	54.3			6.9		
Peptide and chromophore variants of Fet^5:							
	dansyl- Leu-Ala-Asn(CHO)CAMCys-Ser	40.1			11.9		
	dansyl- Leu-Ala-Asn(CHO)-*CMCys*-Ser	18.5			10.2		
***d*-Fet^{5a}**(Asn-138)	*dabsyl*- Leu-Asn(CHO)-Asp-Ser-Arg	0.56			0.59		
	CHO as for *d*-Fet^5						
***r*-Ova^9**	*resorufin*-Glu-Glu-Lys-Tyr-Asn(CHO)-Leu-Thr-Ser-Val						
	CHO = mixture of hybrid and oligomannose type structures	109.8	(180)	31	2.29	(2.6)	6.8
Peptide and chromophore variants of *r*-Ova^9:							
***u*-Ova^9**	Glu-Glu-Lys-Tyr-Asn(CHO)-Leu-Thr-Ser-Val	50.6			4.23		
***u*-Ova^6**	Tyr-Asn(CHO)-Leu-Thr-Ser-Val	25.7			5.66		

Table 1. Continued.

Name of substrate	*Peptide sequence and glycan structure*	*PNGase F*			*PNGase A*		
		V ($\mu mol \times min^{-1} \times mg^{-1}$)	(V_{max})	k_M (μM)	*V* ($\mu mol \times min^{-1} \times mg^{-1}$)	(V_{max})	k_M (μM)
***d*-Brl[11]**	*dabsyl*- Ala-Arg-Val-Pro-Arg-Asn-Asn(CHO)-Glu-Ser-Ser-Met						
CHO =	Manα6 Manβ4GlcNAcβ4GlcNAc Xylβ2 Fucα3	0			0.68	(0.73)	3.8
Glycoform of *d*-Brl[11]:							
***d*-Brl[11]-defuc**	CHO = Manα6 Manβ4GlcNAcβ4GlcNAc Xylβ2	35.2			0.62		
Peptide variant of *d*-Brl[11]:							
***d*-Brl[10]**	*dabsyl*- Ala-Arg-Val-Pro-Arg-Asn-Asn(CHO)-Glu-Ser-Ser	0			0.43		
***u*-Taa[8]**	Val-Ser-Asn(CHO)-Tyr-Ser-Ile-Asp-Gly						
	CHO = Manα6 Manα3 Manα6 Manβ4GlcNAcβ4GlcNAc Manα3	31.1	(50)	30	7.47	(8.3)	5.3
Peptide variant of *u*-Taa[8]:							
r-Taa[4]	*resorufin*- Val-Ser-Asn(CHO)-Tyr	0.43			2.76		

[a] major structures according to [39].

Fibrin glycopeptides Bovine fibrin was digested with pepsin (see above). The glycopeptides were purified similar as described for ovalbumin but prior to ion exchange the samples were desialylated with 50 mM sulfuric acid at 80°C for 1 h. The void fraction from Sephadex DEAE A25 contained, as the major product, an octapeptide derived from the β-chain of fibrin. This fraction was lyophilized, dissolved in 0.15 M Tris/HCl at pH 7.8 containing 3 mM $CaCl_2$ and incubated for 24 h with thermolysin at an enzyme to substrate ratio of 1:40. The resulting glycopeptide Fib^6 was purified by reversed phase HPLC as described above. The pentapeptide Fib^5 was obtained from the peptic octapeptide by digestion with trypsin (TPCK-treated; see above).

Taka-amylase glycopeptides Digestion of carboxamidomethylated [24] amylase from *Aspergillus oryzae* with trypsin (TPCK-treated) quite unexpectedly yielded the tetrapeptide Taa^4 (Table 1). Digestion with pepsin yielded three glycopeptides from which the nonapeptide Taa^9 was used for the experiments described herein (Table 1). For the HPLC separation of these peptic glycopeptides, 0.1% v/v trifluoroacetic acid was used as the aqueous solvent component while other parameters were as described above.

Labelling of glycopeptides with chromophores

Peptides were derivatized with dabsyl or dansyl chloride according to Tapuhi *et al.* [27]. Labelled glycopeptides were separated from excess reagent by passage over Sephadex G25 using 25% v/v methanol in water as the eluent. Final purification of dabsyl-labelled substrates was achieved by reversed phase HPLC on a 250 × 4 mm Hypersil ODS column. Conditions were similar to those given in Table 2, but solvent A was 10% v/v 2-propanol in 50 mM ammonium acetate at pH 4.0 and solvent B was 10% 2-propanol and 60% v/v acetonitrile in ammonium acetate. Absorption was monitored at 436 nm. The dansylated peptides had to be purified using 0.1% trifluoroacetic acid and an acetonitrile gradient.

To introduce the resorufin chromophore, 1 μmol of glycopeptide was dissolved in 0.5 ml dimethylsulfoxide containing 0.1 M imidazole. Then, 4.5 mg RESOS was added and allowed to react for 6 h at room temperature. The samples were diluted with an equal volume of water and passed over Sephadex G25 with 0.1 M sodium bicarbonate as the eluent. HPLC purification was carried out using an ammonium acetate buffer (50 mM, pH 4.0) as solvent A and 70% acetonitrile in buffer as solvent B. Absorption was monitored at 467 nm. The dabsyl, dansyl or resorufin labelled glycopeptides were lyophilized, redissolved in water and, upon quantitation by amino acid analysis, diluted to a concentration of 0.1 mM.

Table 2. Conditions for HPLC analysis PNGase digested glycopeptides. A 2 × 100 mm column of ODS-Hypersil was eluted at room temperature with a flow rate of 0.4 ml min^{-1}. The aqueous solvent component consisted of 50 mM KH_2PO_4 brought to pH 2.8 with phosphoric acid. This buffer was used as solvent A either as such or with 10% v/v iso-propanol. Solvent B consisted of 10% v/v iso-propanol, 60% v/v acetonitril and buffer.

Glycopeptide	*% iso-propanol in solvent A*	*% B*	*% B*
		at begin of a 10 min gradient	*at end*
All variants of			
d-Fib, *d*-Fet, *d*-Brl	10	25	55
r-Fib^6	0	10	40
r-Ova^9	10	20	40
u-Ova^9	0	5	30 (in 14 min)
u-Ova^6	0	15	25
u-Taa^8	0	10	40
r-Taa^4	0	22	50

Modification of the carbohydrate moiety of glycopeptides

The dabsylated asialo-biantennary nonasaccharide of *d*-Fib^6 was trimmed to a pentasaccharide (*d*-Fib^6-core) by the successive application of *Aspergillus* β-galactosidase and jack bean β-*N*-acetylhexosaminidase [21]. *d*-Fib^6-core was digested with α-mannosidase [16] to yield *d*-Fib^6-tri (see Table 1 for structure). Treatment of this glycopeptide with β-mannosidase (using the same buffer as for α-mannosidase) led to the chitobiosyl glycopeptide *d*-Fib^6-di and, due to a contaminating activity of the enzyme charge used, concomitantly to the GlcNAc-peptide *d*-Fib^6-mono. After each step, the product was separated from its precursor by HPLC as described below for the HPLC analysis of PNGase digests with one essential deviation: instead of a phosphate buffer the solvents contained 0.1% v/v trifluoroacetic acid. From Fet^5 the truncated variant Fet^5-core was prepared by the sequential application of neuraminidase, β-galactosidase and β-*N*-acetylhexosaminidase. Again, the intermediate products had distinct elution times (data not shown).

Hydrolysis of glycopeptides with PNGases

The protein content of the PNGase F and A stock solutions was 32 and 30 μg ml^{-1}, respectively. Enzyme doses were chosen to result in the hydrolysis of 5–15% of the substrate. For that, a series of dilutions of up to 1:50 000 for PNGase F and 1:8000 for PNGase A were prepared in buffers containing 0.1% bovine serum albumin to avoid adsorption losses. PNGase F was diluted in 50 mM Tris/HCl plus 20 mM EDTA at pH 8.0. The buffer for PNGase A consisted of 0. 1 M citric acid and 0.1 M NaH_2PO_4 brought to pH 5.0 with NaOH. Generally, 10 μl of enzyme were added to 10 μl of prewarmed substrate dissolved in water. All digests were kept at 37°C for 60 min. Enzymatic reactions were terminated by boiling the samples for 6 min after which they were mixed with 100 μl of water and centrifuged briefly to concentrate the entire liquid at the bottom of the tube. Finally, the samples were transferred to narrow autoinjector tubes and 90 μl were

subjected to HPLC analysis. As an exception, the total incubation volume was increased to 60 µl for the determination of the rather low K_M-values of PNGase A in order to ensure sufficient amounts of analyte.

HPLC analysis of PNGase digests

The HPLC module consisted of a Hewlett Packard 1050 series ternary gradient mixer, autoinjector and single wavelength UV/VIS monitor. A 2 × 100 mm column packed with 5 µm ODS Hypersil (ÖFZ Seibersdorf, Austria) was run at a flow rate of 0.4 ml min^{-1} at room temperature. The solvents and gradients used for the analysis of dabsyl-, resorufin-, or unlabelled peptides are given in Table 2. The dansylated peptides were analysed by a 10 min gradient from 17 to 40% acetonitrile only in potassium phosphate buffer, whereby glycopeptides eluted after 7 min, and peptides after 10 min. Dabsylated peptides were monitored at 500 nm, dansylated peptides at 254 nm, resorufin-labelled peptides at 467 nm and underivatized peptides at 220 nm. Fluorometric monitoring of resorufin-labelled peptides was of no advantage at the pH used.

Determination of enzyme kinetic parameters

Quantitative evaluation of HPLC chromatograms was based on the assumption that the molar detector response is the same for a glycopeptide substrate and its peptide product. For dabsylated Fib[6], resorufin-labelled Ova[9] and underivatized Taa[8], this assumption was verified by comparison of the total peak area of untreated and extensively hydrolysed samples (data not shown). The data used for the calculation of kinetic parameters were first inspected visually by V versus $[S]$ plots to show whether a useful concentration range had been chosen. The parameters themselves, however, were determined by computer using an arithmetical version of the direct linear plot method [28]. The acceptability of the obtained estimates was again examined visually by $[S]/V$ versus $[S]$ plots (Hane's plot).

Results

Preparation of glycopeptides Glycopeptides from five different glycoproteins were prepared for the study on substrate specificity of PNGases. Some of them were available with a differing peptide portion and some were modified in their carbohydrate moiety. The amino acid sequence of these glycopeptides was deduced from their amino acid composition and published sequence data: bovine fetuin [25], hen ovalbumin [29], pineapple stem bromelain [30], bovine fibrin [31], *A. oryzae* α-amylase [32, 33] (data not shown). In the case of Ova[9], Taa[8], and Brl[11] these structures were additionally examined by enzymatic release of the C-terminal amino acids by carboxypeptidase Y (data not shown). Amino acid analysis of dabsylated substrates indirectly identified N-terminal residues. The oligosaccharide structures were verified by 2D-HPLC of pyridylaminated glycans in the case of fibrin, α-amylase and bromelain glycopeptides (data not shown). The structures of all substrates used in this study are given in Table 1.

Fetuin and ovalbumin glycopeptides were chosen because they are used for the activity quantification of commercial PNGase F and PNGase A, respectively. Fet[5], a thermolytic peptide comprising glycosylated Asn-81 was prepared from S-alkylated fetuin. In accordance with Plummer *et al.* [6], a pentapeptide was obtained. S-carboxamidomethylation was found to allow the easiest separation of the several glycopeptides in the digest. It also avoids the introduction of: (1) a negative charge at the C-terminal side of Asn(CHO), which is said to render a glycopeptide a poor substrate for PNGase F [34]; and (2) a second primary amino group as would be the case with aminoethylation [6]. In the course of preparation, glycoforms other than the trisialylated structures were presumably eliminated. Thus, the glycan of Fet[5] may be regarded as quite homogeneous compared with that from the ovalbumin glycopeptide Ova[9] which is a mixture of several oligomannose and hybrid type structures [see e.g. 35]. However, for the dansylated fetuin glycopeptides a trifluoroacetic acid containing solvent system had to be used for their final purification and some desialylation may have taken place during this procedure. As a 'byproduct' of the preparation of Fet[5] another pentapeptide, Fet[5a], stemming from the glycosylation site at Asn-138 was obtained.

Ova[9] is identical with the glycopeptide described by Takahashi and Nishibe [29]. The third substrate with history is Brl[11], the undecapeptide from pineapple stem bromelain [29]. The defucosylated glycoform Brl[11]-defuc and a peptide variant lacking the C-terminal Met residue, Brl[10], were additionally available. To verify both these C-terminal structures and the presence of Glu in this peptide, the amino acids released by carboxypeptidase Y were analysed. With all of the glycopeptides tested, it proved difficult or impossible to release the amino acid adjacent to glycosylated Asn. If the sample was incubated with 30 ng of PNGase A for 1 h prior to the addition of carboxypeptidase, Glu was found, together with Asp (derived from Asn-CHO), Asn, Arg and some Val.

Two further substrates were prepared with the following intention: (1) they should be good substrates for both PNGases: (2) have a homogeneous peptide and, as far as possible, also a defined carbohydrate moiety; (3) the raw materials should be cheap; and (4) chromatographic separation of substrate and product should be simple. Moreover, they should allow the simultaneous determination of endo-*N*-acetylglucosaminidase activity and its discrimination from PNGase activity. To this end, the biantennary glycopeptide Fib[6] and the $Man_5GlcNAc_2$-peptide Taa[8] were prepared.

The primary product of pepsin digestion of bovine fibrin was an octapeptide with a Met-Thr extension at the C-terminus. While this peptide was a slightly better substrate than Fib[6], its dabsyl derivative turned out to undergo intermolecular reactions, possibly lactonization, which led to peak splitting (data not shown). The oligosaccharide moiety of Fib[6] was shown by 2D-HPLC to consist of a homogeneous asialobiantennary chain as shown in Table 1.

For the examination of the minimal carbohydrate structure released by PNGases A and F the glycan portion of dabsylated

Fib^6 (*d*-Fib^6) was modified by treatment with exoglycosidases. Surprisingly, all these dabsylated glycopeptides were separable by HPLC. The glycan structures of *d*-Fib^6-core and *d*-Fib^6-tri were verified by 2D-HPLC, and the structural assignment of *d*-Fib^6-di and *d*-Fib^6-mono was then unambiguous. As an additional confirmation, *d*-Fib^6-mono coeluted with the product obtained by incubation of *d*-Fib^6-core with endo-*N*-acetylglucosaminidase D (data not shown).

The glycan moiety of Taa^8 consisted of $Man_5GlcNAc_2$ with no detectable microheterogeneity (Table 1). However, depending on the raw material, varying amounts of $Man_6GlcNAc_2$ may be found in a Taka-amylase glycopeptide. Derivatization with RESOS gave low yields of product and dabsylation led to a peptide with too high an affinity to the ODS matrix. Fortunately, the tyrosine in Taa^8 allowed HPLC analysis of the underivatized peptide (*u*-Taa^8) with sufficient sensitivity.

Analysis of PNGase digestion of the glycopeptides Conditions were elaborated for each of the glycopeptide substrates which allowed the analysis and quantitation of their hydrolysis by PNGase. The use of a short narrow-bore column with a slightly acidic buffer met virtually all the needs: good separation of substrate and product, good detectability (resorufin would be discoloured in a more acidic solvent), speed and minimal consumption of organic solvents. Usually, the microheterogeneity of the carbohydrate moiety of a glycopeptide cannot be resolved by reversed phase HPLC, but the different glycopeptides derived by glycosidase trimming of the dabsylated substrates *d*-Fib^6 and *d*-Fet^5 were clearly separated (Fig. 1). Therefore, *d*-Fib^6 may be used for the simultaneous determination of PNGase and endo-*N*-acetylglucosaminidase activities, e.g. PNGase F and Endo F_2 [8]. Similarly, Endo D, H, F_1 or similar activities can be determined with *u*-Taa^8, where the GlcNAc-peptide also elutes between glycopeptide and peptide.

Dabsylated glycopeptides exhibited the best chromatographic characteristics with regard to peak shape and selectivity. For example, while *d*-Fib^6 gives excellent separation of

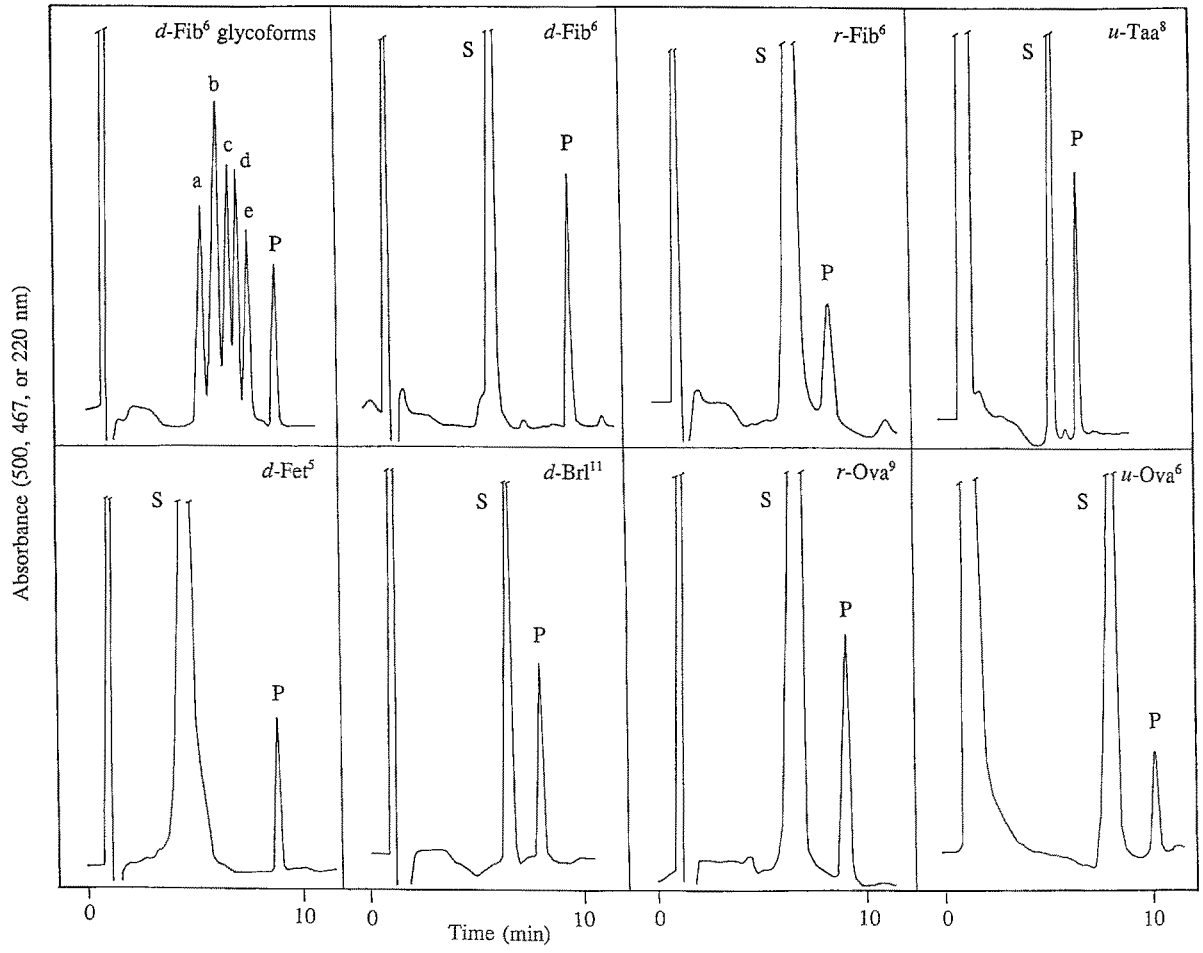

Figure 1. Examples for the analysis of PNGase digests by HPLC. The letters S and P denote the peaks for glycopeptide substrates and peptide products, respectively. The top left panel demonstrates the separation of *d*-Fib^6 with an asialo-biantennary glycan (a), *d*-Fib^6-core (b), *d*-Fib^6-tri (c), *d*-Fib^6-di (d), and *d*-Fib^6-mono (e) from the deglycosylated peptide (S).

substrate and product at acidic pH (Fig. 1) and still useful separation at pH 6.0, the resorufin-labelled peptides elute close together as rather broad peaks at pH 2.8. No separation of the resorufin-labelled substrates *r*-Fib^6 or *r*-Ova^9 and their respective products was possible at a more neutral pH, where the extinction of the chromophore would have been higher. The dabsyl group, on the other hand, confers considerable hydrophobicity to a peptide and it is therefore not suitable for peptides such as Ova^9, which by themselves already bind strongly to the reversed phase matrix. A noteworthy observation was made with some glycopeptides as *u*-Ova^9 or the dansylated fetuin glycopeptides which apparently complexed with Tris resulting in excessively broadened or split peaks. This problem could be overcome by trapping the glycopeptide on the column by a very low concentration of organic modifier (Table 2). The same problem arose when digests of *d*-Fib^6 were analysed by isocratic elution (42% solvent B). Here, the product peak eluted at 4 min after injection, and thus a turnaround time of 6 min was possible. Tris buffer would upset this separation and would have to be substituted by a phosphate buffer which gives identical measurements of PNGase F activity (data not shown).

Hydrolysis of the 'standard substrates' d-Fet^5, r-Ova^9, d-Fib^6 and u-Taa^8 The applied methodology permitted the reliable determination of V and K_M of several substrates (Table 1). As shown in Fig. 1, more than sufficient separation was obtained for all substrate product pairs. Analysis times were short enough to allow the processing of many samples and thus the determination of kinetic parameters. Values of reaction velocity were determined from two or three duplicate samples run on different days. Considering the many factors influencing these determinations, such as substrate concentration, enzyme dilution, pipetting of small volumes, peak integration and so on, the estimates may be associated with errors of up to ±15%. These values, however, are believed to apply to pure and active enzymes. This view is based on informations from the producer in the case of PNGase F and from enzyme purification in the authors' laboratory in the case of PNGase A (data not shown).

Definition of minimal carbohydrate size The enzymatic hydrolysis of artificial glycoforms of *d*-Fib^6 with the complete asialo-biantennary glycan, the pentasaccharide core, the core without the two α-mannosyl residues, the di-*N*-acetylchitobiose, and one *N*-acetylglucosamine only were compared (Table 1). Both enzymes release glycans containing the β-mannosyl residue at rates comparable to or even higher than that found for complete *d*-Fib^6. While removal of the β-mannose slows down the rate of hydrolysis by PNGase F, the glycopeptide having only a di-*N*-acetylchitobiose is still a good substrate for PNGase A. The almond enzyme can even release a single GlcNAc, albeit at only 0.033% of the rate observed with *d*-Fib^6-di. Although hydrolysis of *d*-Fib-mono by PNGase F could be observed, we can calculate from the enzyme dose that, if there were any enzymatic reaction, it would proceed by less than 0.00003% of the rate measured with *d*-Fib^6.

Effect of 'natural' structural elements of the carbohydrate moiety of substrates A comparison of the hydrolysis rates observed with *d*-Fib^6 and *d*-Fib^6-core as well as with *d*-Fet^5 and *d*-Fet^5-core does not indicate a substantial influence of structural features of the glycans non-reducing terminus on either of the two enzymes. Surprisingly, the plant enzyme PNGase A shows a slight preference for the intact triantennary glycopeptide *d*-Fet^5, which is its best substrate of those tested.

However, α1,3-fucosylation of the innermost *N*-acetylglucosamine residue of a bromelain glycopeptide impeded the action of PNGase F, as has already been reported in a previous paper [17]. The block could not be overcome by a several thousand-fold increase in the enzyme dose.

Conformational analysis of a similar plant glycan has indicated the close proximity of xylose and fucose [36]. The inaccessibility of bromelain glycopeptide to PNGase F may therefore be caused by sterical constraints only acting if α1,3-linked fucose and xylose are concomitantly present in one glycan. We have therefore compared the glycans released from bee venom phospholipase A_2 (PLA) by either PNGase A and PNGase F. Six of the fourteen oligosaccharides found on PLA contain α1,3-linked fucose but none of the glycans is xylosylated [16]. Three of the pyridylaminated derivatives of these α1,3-fucosylated glycans appear as separate peaks when analysed on MicroPak AX-5 [16]. These peaks were missing in the PNGase F digest of PLA (Fig. 2). This finding explains why it is not possible to achieve complete deglycosylation of

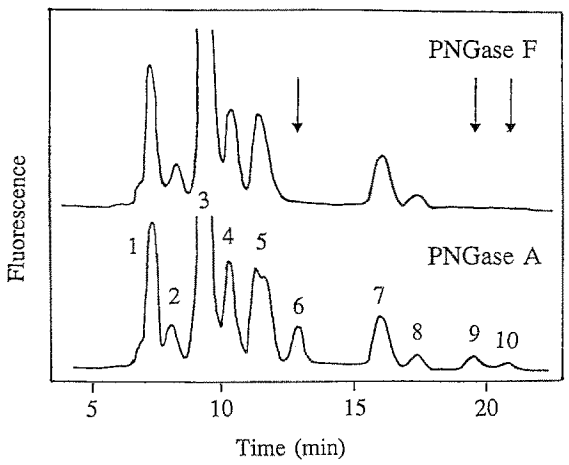

Figure 2. Oligosaccharides released from honeybee phospholipase A_2 by PNGase F and A. The peaks are numbered in accordance with Kubelka *et al.* [16]. The arrows point at three peaks which are missing in the sample of the PNGase F released oligosaccharides. These three glycans are α1,3-fucosylated at the innermost GlcNAc residue [16]. Other α1,3-fucosylated glycans are hidden under peaks 3, 4, and 5.

PLA by PNGase F, as can be deduced from SDS-PAGE (results not shown).

Effect of peptide length and chromophore Two variants of standard substrates were available in which only a single amino acid residue was attached to the C-terminal side of the glycosylated asparagine, namely *d*-Fib[5] and *u*-Taa[4]. Both substrates exhibited a drastically reduced reaction rate with PNGase F and an at least significantly lower rate with PNGase A. Reduction of the N-terminal amino acid chain to only one residue as obtained with *u*-Ova[6] had a much less pronounced effect on PNGase F and it even allowed faster deglycosylation by PNGase A. This may however not be the rule since the fetuin pentapeptide with Asn-138 was a very poor substrate for both enzymes. Generally, PNGase A exhibited a somewhat higher tolerance towards peptide shortening than PNGase F.

The presence and nature of a chromogenic label effected significant alterations in the cleavage rates of both enzymes, even though the chromophores appear to be well apart from the glycosylation sites. In conjunction with the distinctive effects of the dabsyl and the resorufin groups on the elution behaviour of glycopeptides and their related peptides, e.g. the much higher chromatographic selectivity observed with *d*-Fib[6] compared to *r*-Fib[6], this may indicate conformational changes induced by the chromophores. In this context it may be mentioned that even the presence of one additional amino acid separated from the glycosylation site by three amino acid residues can influence enzymatic hydrolysis as observed with *d*-Brl[11] and *d*-Brl[10] with PNGase A.

Detergents The long term stability of the PNGases in the presence of SDS was investigated using properly diluted enzymes, *d*-Fib[6] as the substrate and incubation times of 24h. Contrary to literature statements, the activity of both enzymes was destroyed at about the same concentration of SDS (Fig. 3). In another experiment, the glycopeptide substrate was dissolved in 3 µl of 1% SDS (mimicking the solution of denatured glycoprotein) and diluted with 7 µl of 2.4% non-ionic detergent prior to the addition of enzyme. Both enzymes were stabilized against SDS in the presence of non-ionic detergents such as Triton X-100 or Nonidet P40 (Fig. 3). However, attempts to deglycosylate honeybee phospholipase A_2 and hen ovalbumin with PNGase A upon denaturation with SDS and 2-mercaptoethanol (which has no negative effect on enzyme activity) failed miserably (data not shown). In contrast, PLA was readily hydrolysed by PNGase F even in its native form.

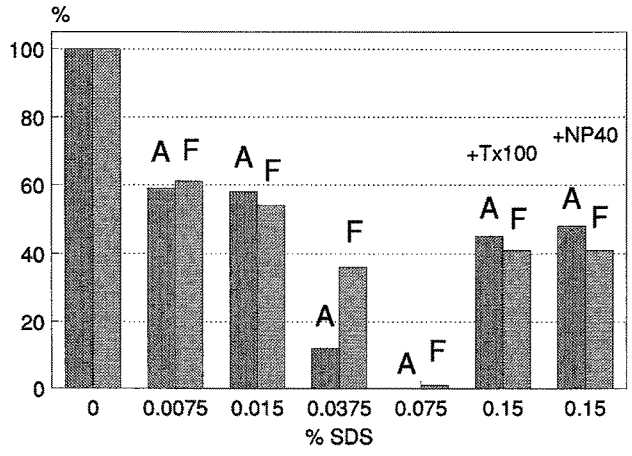

Figure 3. The activity of PNGase F and A was measured in the presence of various concentrations of SDS and additionally in the concomitant presence of SDS and the anionic detergents Triton X-100 or Nonidet P40 at a final concentration of 0.84%.*d*-Fib[6] was used as substrate and incubations were conducted at 37°C for 24 h.

Discussion

This work represents the first detailed kinetic characterization of PNGases with some of their most important substrates. Until now only three K_M-values have been published: pronase peptides of bromelain were found to have a K_M of 4 mM with PNGase A (Seigagaku, product specification sheet); the tryptic fetuin glycopeptide comprising Asn-138 had a K_M of 1.1 mM for PNGase A [14]; and 0.871 mM was estimated for a turkey ovomucoid glycopeptide with PNGase F [18]. These figures are in obvious discrepancy with the data obtained in the present study. In contrast, the hydrolysis rates approximately correspond with published data for PNGase A and Ova[9] [37] and PNGase F and Fet[5] [34]. Each of the two enzymes exhibits considerable differences in its rate of hydrolysis of different substrates. Remarkably, no correlation between these hydrolysis rates of the two PNGases can be seen. The data do not support the view that either of the two PNGases prefers glycopeptides of either the complex or oligomannose type. Generally, it is rather the structure and, included herein, the length of the peptide moiety which controls reaction velocity. Even seemingly small differences of substrate structure such as a different chromophore or S-alkylgroup etc. may influence reaction rate. Any specification of PNGase activity must therefore be accompanied by a thorough description of the substrate employed. Both for commercial and scientific purposes the use of standard substrates and methods would be desirable and hopefully this work is a step towards this goal.

While the finding that PNGase F and A differ in their limit glycan structure hardly has any practical consequences, the inability of PNGase F to release structures with α1,3-fucose linked to the Asn-bound GlcNAc constitutes a severe problem whenever the presence of this structural element cannot be excluded. Since this feature is now known to occur not only in plant glycoproteins but also in insect glycoproteins [16, 38] and maybe those from other arthropods (Tretter V, unpublished results), PNGase A should be chosen for work with glycoproteins from organisms other than vertebrates (or at least mammals and birds).

Unfortunately, PNGase A exhibits little competence towards glycoproteins even if the substrate has been denatured. However, this inability is apt to explain why most of the enzymes found in almond emulsin and even PNGase A itself carry N-glycans which obviously have not been removed

by the enzyme. It will be interesting to learn how far these differences in the substrate specificities of PNGase A and F reflect their particular physiological purpose.

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