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# Optimized Deglycosylation of Glycoproteins by Peptide-N$^4$-(*N*-acetyl-β-glucosaminyl)-asparagine Amidase from *Flavobacterium meningosepticum*

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**Peptide-N$^4$-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F) from *Flavobacterium meningosepticum* is a highly useful enzyme for the structural analysis of N(asparagine)-linked carbohydrate chains derived from glycoproteins. The enzyme was enriched using a published procedure [Tarentino AL, Gomez CM, Plummer TH, Jr (1984) Biochemistry 1985:4665-71; Tarentino AL, Plummer TH, Jr (1987) Methods Enzymol 138:770-78] and further purified by hydrophobic interaction HPLC on a weak hydrophobic TSK-Ether column from which it was eluted by a decreasing gradient of 1.7 M ammonium sulphate in 100 mM sodium phosphate, pH 7.0, containing 5 mM EDTA.**

**To determine the optimal conditions for a complete deglycosylation of glycoproteins by PNGase F, experiments were performed with human $\alpha_1$-acid glycoprotein, because the five complex type carbohydrate chains are quite resistant to enzymic hydrolysis. The influence of different detergents on the enzyme reaction was studied. Complete deglycosylation of human $\alpha_1$-acid glycoprotein was achieved by the use of 60 mU/ml PNGase F in 0.25 M sodium phosphate buffer, pH 8.6, containing 0.2% (w/v) SDS, 20 mM mercaptoethanol and 0.5% Mega-10.**

For structural analysis of N(asparagine)-linked carbohydrate chains of glycoproteins, the most widely employed procedure for cleaving all common classes of oligosaccharides is chemical hydrazinolysis [1]. This procedure, however, destroys the primary structure of the protein, and the released sugars have also been observed to undergo degradation and further chemical conversion [1-6].

In contrast, enzymic hydrolysis of asparagine-*N*-acetylglucosamine linkages by N-glycanases, yielding intact oligosaccharides and the intact deglycosylated protein, may permit structural analysis of the released carbohydrate chains as well as studies on the polypeptide moiety. Three N-glycanases from jack beans [7], human liver [8] and almonds [9-15] are active against glycopeptides only, while peptide-N$^4$-(*N*-acetyl-β-glucosaminyl)-asparagine

amidase (PNGase F) from *Flavobacterium meningosepticum* [16-19] was found to be especially suitable for the deglycosylation of intact glycoproteins. Acting on glycoproteins as well as on glycopeptides, this enzyme releases all common classes of N-glycans from the protein backbone [16, 17, 20]. In addition to PNGase F, the culture medium of *Flavobacterium meningosepticum* contains endo-β-*N*-acetylglucosaminidase (Endo F), which shows a limited substrate specificity; it hydrolyses the chitobiose linkage of oligomannose and certain hybrid and bi-antennary complex type sugars, leaving one *N*-acetylglucosamine residue bound to the protein backbone [16-19].

A purification procedure for PNGase F and Endo F from the bacterial culture medium has been described [16-19], and the enzymes are now commercially available from a number of sources. Since it has been suggested that PNGase F and Endo F differ in their hydrophobicity [16], we applied hydrophobic interaction HPLC for the further purification of PNGase F.

In many cases (ribonuclease B, ovomucoid, human transferrin, fetuin, lipase B and Fab fragment) deglycosylation by PNGase F was achieved using the native glycoprotein [16, 17]. However, by prior denaturation and use of detergents the rate of deglycosylation could be increased [16, 20-23]. It is known that the five complex type carbohydrate chains of human $\alpha_1$-acid glycoprotein are quite resistant to enzymatic hydrolysis. Therefore, this glycoprotein was chosen to determine optimal conditions for complete deglycosylation by PNGase F.

## Materials and Methods

### *Materials, Chemicals and Enzymes*

The following detergents were used in the experiments: Nonident P-40 and Triton X-100 were purchased from Sigma (Deisenhofen, Germany), Mega-10 (decanoyl-*N*-methylglucamide) and 3-[(3-cholamidopropyl)dimethylammonio]1-propanesulphonate referred to as "CHAPS" were from Calbiochem (Frankfurt/Main, Germany). LDAO (*N,N*-dimethyldodecylamino-*N*-oxide) and SDS (sodium dodecylsulphate) were obtained from Fluka (Neu-Ulm, Germany). Sodium borotritide ($NaB^3H_4$, 7.5 mCi/μmol) was purchased from Amersham Buchler (Braunschweig, Germany). All other chemicals of analytical grade were obtained either from Merck (Darmstadt, Germany) or Serva (Heidelberg, Germany).

*Flavobacterium meningosepticum* was a gift of Dr. V. Gross (Freiburg, Germany). Purified PNGase F as well as Endo F were kindly provided by Dr. W. Hösel (Boehringer Mannheim, Germany) for comparison. Resorufin-labeled $Man_5$-glycopeptide from soy-beans was purchased from Boehringer Mannheim. Bio-Gel P-4 (minus 400 mesh) was from Bio-Rad (München, Germany). A TSK-Ether 5PW column (75 x 7.5 mm) was kindly given by Dr. Kato (Toyo-Soda, Japan). A Zorbax (Bio Series) GF 250 and a GF 450 column, both 250 x 9.4 mm, were purchased from Du Pont (Dreieich, Germany).

### *Purification of PNGase F*

PNGase F was enriched from the culture medium of *Flavobacterium meningosepticum* by ultrafiltration, ammonium sulphate precipitation and finally by gel filtration on a TSK HW-55 (S) column as described [16, 19]. Fractions containing endoglycosidase activities were

pooled and submitted to hydrophobic interaction HPLC for further purification: 7 ml (12 mg) of partially purified PNGase F dissolved in 100 mM sodium phosphate buffer, pH 7.0, were mixed with 1.9 ml of 3.5 M ammonium sulphate at room temperature and submitted to hydrophobic interaction HPLC on the TSK-Ether 5PW column. The column was equilibrated with 100 mM sodium phosphate buffer, pH 7.0, containing 1.7 M ammonium sulphate and 5 mM EDTA, and afterwards it was washed for 30 min with equilibration buffer at a flow rate of 1 ml/min. Thirty min after injection, the ammonium sulphate concentration was decreased from 1.7 M to 0.9 M within 30 min, and finally from 0.9 M to zero within 15 min (Fig. 1). Fractions of 1 ml were collected and assayed for endoglycosidase activities using resorufin-labeled $Man_5$-glycopeptide as well as $^3$H-asialotransferrin at pH 8.6 and pH 5.5, respectively, as substrates. Protease activity was determined with Azocoll.

*SDS-Polyacrylamide Gel Electrophoresis*

Samples containing 25 µg of glycosylated or deglycosylated glycoprotein were diluted to 25 µl with distilled water and mixed with 25 µl of 62.5 mM Tris-HCl buffer, pH 6.8, containing 3% (w/v) sodium dodecylsulphate, 5% (v/v) mercaptoethanol, 10% glycerol and 0.01% (w/v) bromophenol blue. After ultrasonication for 3 min the mixture was heated for 3 min at 95°C and subjected to gel electrophoresis. SDS-PAGE was performed according to the method of Laemmli [25]. Gels were fixed and stained in a solution of Coomassie Brilliant Blue G-250 (0.1%) in water/methanol/acetic acid, 5/4/1 by vol, and finally destained in water/methanol/acetic acid, 175/10/15 by vol.

*Endoglycosidase Assay by Use of $^3$H-Asialotransferrin*

$^3$H-Asialotransferrin was prepared according to the method of Morell and Ashwell [26], and was finally purified by size exclusion HPLC on a GF 250 and GF 450 tandem column, which was eluted with 0.2 M sodium phosphate buffer, pH 7.0, at a flow rate of 0.5 ml/min. Fifty µg $^3$H-asialotransferrin ($9 \times 10^3$ Bq) were solubilized in 10 µl of 1% (w/v) SDS for 3 min at 90°C and mixed with 25 µl of incubation buffer containing 0.5 M sodium phosphate and 1% Mega-10 (w/v), pH 5.5 or pH 8.6, respectively. Fifteen µl of the respective sample containing endoglycosidic activity were added to the substrate solution and deglycosylation was performed at 37°C for 1 h. Endoglycosidic activity was estimated by measuring the radioactivity of enzymatically released oligosaccharides, after separation from unreacted as well as from deglycosylated glycoprotein by ultrafiltration in a Centricon microconcentrator tube (Amicon) at 8°C and 4,700 x *g*. In order to discriminate between the activities of PNGase F, Endo F and β-D-galactosidase, the composition of the hydrolysed carbohydrate chains was determined by size exclusion chromatography on a Bio-Gel P-4 column (150 x 1.6 cm) at 55°C according to the method of Yamashita *et al.* [27].

*Deglycosylation of Glycoproteins by PNGase F*

In a typical experiment, 50 µg of glycoprotein dissolved in 10 µl of a solution containing 1% (w/v) SDS and 100 mM mercaptoethanol, were denatured by ultrasonication for 25 min, followed by heating at 95°C for 1 min. To 10 µl of a solution containing 50 µg native or denatured glycoprotein, 25 µl of 0.5 M sodium phosphate, pH 8.6, containing 1% (w/v) Mega-10 were added. Concentrations of all other detergents used in our studies are given

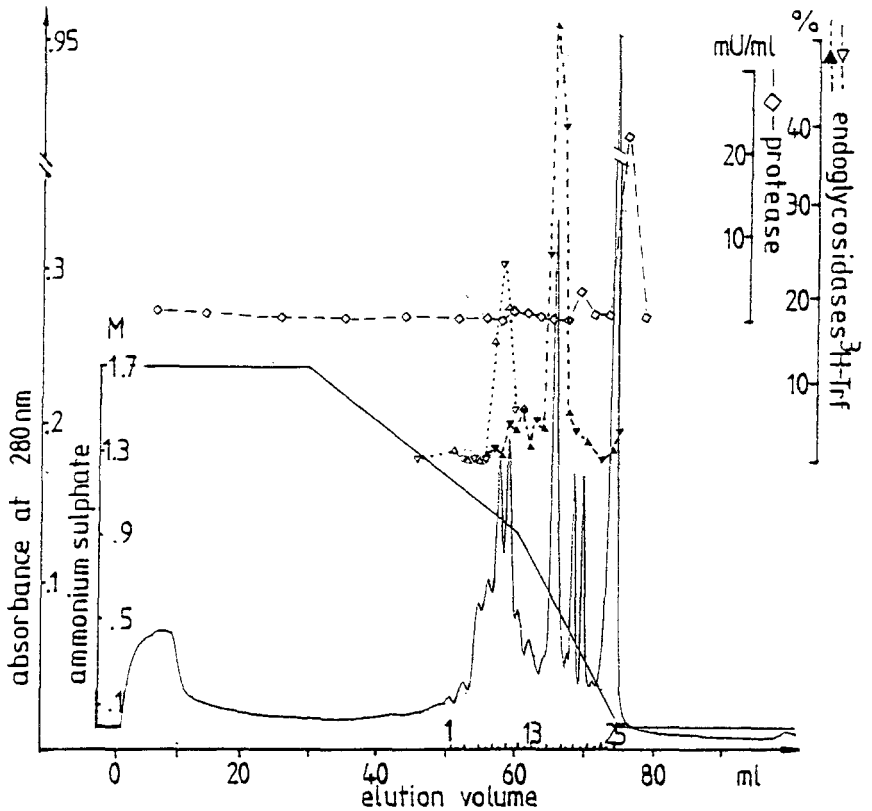


**Figure 1.** Purification of PNGase F by hydrophobic interaction HPLC on a TSK-Ether 5PW column.

7 ml of enriched PNGase F containing 12 mg of protein, obtained from gel filtration chromatography on TSK HW-55 (S), were adjusted to 0.75 M ammonium sulphate and applied to a TSK-Ether 5PW (75 mm x 7.5 mm) column. Elution was performed by a decreasing gradient of ammonium sulphate in 0.1 M sodium phosphate, pH 7.0, containing 5 mM EDTA as indicated. The profiles of enzyme activities were obtained by incubation of $^{3}$H-asialotransferrin ($^{3}$H-Trf) at pH 5.5 with fractions diluted 3-fold for Endo F (△) and at pH 8.6 with fractions diluted 20-fold for PNGase F (▲). Protease was determined with Azocoll (◇). Assays and chromatographic conditions are described in the Materials and Methods section.

in Figs. 2A and 2B. After a second ultrasonication for 25 min followed by a centrifugation at 1000 x *g* for 2 min, the pH value was checked and adjusted to pH 8.6 with up to 5 µl of 1 N NaOH if necessary. PNGase F (0.1 to 5 mU) was then added and the sample was diluted with water to a final volume of 50 µl. Five µl of toluene were added to inhibit bacterial growth. Deglycosylation was performed at 25 or 37°C for 2 up to 24 h. The reaction was terminated by freezing the assay sample at -30°C. The rate of deglycosylation was determined by SDS-gel electrophoresis as described above.

*Enzymic Assays*

β-D-Galactosidase activity was determined as described by Li and Li [28]. Endoglycosidase activity was determined with commercially available $Man_5$-glycopeptide as described previously [29]. Proteolytic activities were assayed with Azocoll in deglycosylation buffer, pH 8.6, as described by Chavira *et al.* [30].

## Results

PNGase F was enriched from the culture medium by ultrafiltration, ammonium sulphate precipitation and size exclusion chromatography on TSK HW-55 (S). However, if this enzyme preparation was employed for the deglycosylation of glycoproteins, partial degalactosylation of the released carbohydrate chains as well as degradation of the completely deglycosylated protein revealed the presence of further β-D-galactosidase and protease activities (data not shown). By use of hydrophobic interaction HPLC PNGase F was readily separated from the contaminating activities of Endo F, β-D-galactosidase and protease as well (Fig. 1). Hydrophobic interaction HPLC was found to be convenient and superior to further purification of PNGase F by rechromatography on TSK HW-55 (S) combined with cation exchange chromatography on SP-Trisacryl M.

In many cases deglycosylation by PNGase F was achieved with the native glycoprotein [16, 17]. By contrast, the five N-linked carbohydrate chains of native $\alpha_1$-acid glycoprotein are less susceptible to enzymatic hydrolysis at pH 8.6 (Fig. 1A, lane 2). Even by the addition of NP-40, Triton X-100 and CHAPS, only two sugar chains were partially removed from the glycoprotein at the high enzyme activity of 60 mU/ml (lane 3 to 5). If $\alpha_1$-acid glycoprotein was denatured in 1% SDS prior to enzyme addition (lane 6 to 8), the rate of deglycosylation was increased, even in the absence of a further detergent (lane 6), while the addition of NP-40 alone had no further effect (lane 7). Only in the presence of 100 mM mercaptoethanol NP-40 improved the rate of deglycosylation from the denatured glycoprotein (lane 10 to 13). However, substantial amounts of the monoglycosylated form were still detectable. Prolonged incubations for 20 h at 25°C or 37°C did not increase deglycosylation (lane 10 to 13). Nearly complete deglycosylation of denatured $\alpha_1$-acid glycoprotein was achieved by the use of CHAPS, even in the absence of mercaptoethanol (lane 8).

In order to obtain more detailed information on the effect of various detergents, the deglycosylation reaction was optimized at a relatively low PNGase F activity of 2 mU/ml. The results of the influence of SDS and mercaptoethanol, as well as the addition of various detergents, are given in Fig. 2B. Increasing the concentrations of mercaptoethanol (lanes 4, 5 and 8) and of CHAPS (lane 6 to 8) was less efficient, and prior denaturation in 2% SDS led to a total inhibition of the enzyme activity (lanes 3, 5). This problem can be overcome by the addition of 2% CHAPS (lane 8). By comparing the detergents CHAPS, CHAPS-O, Mega-10 and LDAO (lanes 2, 9, 10 and 11) the latter two compounds were found to be the most suitable for the deglycosylation of $\alpha_1$-acid glycoprotein after denaturation in 1% SDS containing 100 mM mercaptoethanol (lanes 10, 11). In the presence of these detergents the sugar chains were cleaved completely from the glycoprotein by 60 mU/ml PNGase F. These conditions are quite suitable tor the deglycosylation of membrane glycoproteins, such as DPP IV and Cell CAM 105 [31, 32] (data not shown).

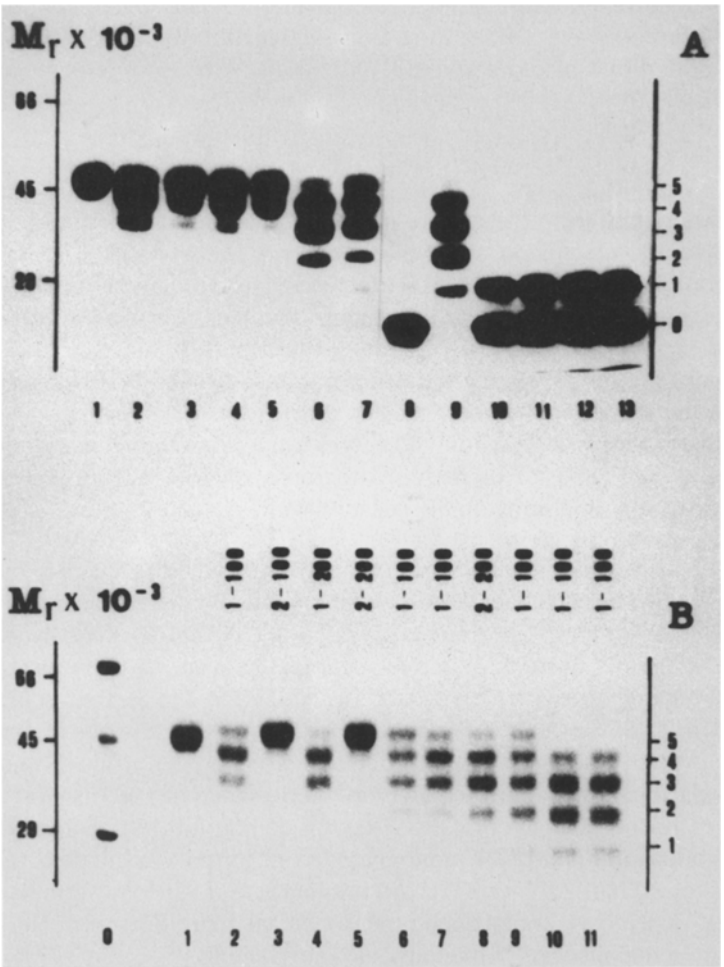


**Figure 2.** Deglycosylation of native and denatured human $\alpha_1$-acid glycoprotein by use of 60 mU/ml (2A) and 2 mU/ml (2B) PNGase F in the absence and presence of various detergents.

Deglycosylation was performed as described in the Materials and Methods section. The relative molecular weight of the gel markers are: carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa and bovine plasma albumin, 66 kDa. (A) Deglycosylation pattern of $\alpha_1$GP with 60 mU/ml PNGase F; SDS-PAGE on a 7.5% gel: untreated $\alpha_1$GP (lane 1); $\alpha_1$GP plus PNGase F, no detergent (lane 2); 1% NP-40 (lane 3); 0.5% Triton X-100 (lane 4) and 0.5% CHAPS (lane 5). $\alpha_1$GP previously denatured with 1% SDS (lanes 6 to 8); no detergent (lane 6); 1% NP-40 (lane 7) and 0.5% CHAPS (lane 8). $\alpha_1$GP denatured with 1% SDS, 100 mM mercaptoethanol (lanes 9 to 13); no detergent (lane 9); 1% NP-40 at 23°C (lanes 11, 12) and 37°C (lanes 10, 13) incubated for 4 h (lanes 10, 11) and 20 h (lanes 12, 13). (B) Deglycosylation pattern of denatured $\alpha_1$GP with 2 mU/ml PNGase F; SDS-PAGE on a 10% gel: $\alpha_1$GP (lane 1) was denatured in 10 µl by varying the ratio of denaturing agents as described in the Figure. 1-100 corresponds to 1% SDS and 100 mM mercaptoethanol. Deglycosylation was performed in a volume of 50 µl in the presence of different detergents at a final concentration of 0.5% to 2%: 0.5% CHAPS (lanes 2-5); 1% CHAPS (lane 6); 2% CHAPS (lanes 7, 8); 0.5% CHAPS-O (lane 9); 0.5% Mega-10 (lane 10) and 0.5% LDAO (lane 11).

## Discussion

PNGase F is a highly useful enzyme to obtain N-linked carbohydrate chains from glycoproteins for further structural studies. However, if high activities of the enzyme are required for a complete deglycosylation of the investigated glycoprotein, the duration of the reaction should not exceed 2 h at most, and the enzyme preparation applied should be essentially free of Endo F and β-D-galactosidase, because traces of contaminants may lead to a release of heterogeneous carbohydrate chains. Hydrophobic interaction HPLC may be a useful tool for a further purification of PNGase F. Residual traces of proteolytic activity may be inhibited by the addition of EDTA up to 25 mM .

In the case of human $\alpha_1$-acid glycoprotein and all membrane glycoproteins investigated in our laboratory, only small amounts of the carbohydrate chains were cleaved from the native glycoproteins. Complete deglycosylation was achieved only by prior unfolding of the glycoprotein using sodium dodecylsulphate, but increasing its concentration may completely inhibit PNGase F. However, prior unfolding of the glycoprotein in the presence or absence of mercaptoethanol *per se* as well as the use of detergents as Nonident P-40, Triton X-100 and CHAPS led to an enhanced but not complete enzyme reaction. Complete deglycosylation was achieved only if the glycoprotein was previously unfolded in sodium dodecylsulphate and the enzyme reaction was performed with up to 100 mU/ml of PNGase F in the presence of LDAO or Mega-10 as a further detergent, which may stabilize the enzyme. Prolonged incubations for up to 48 h did not lead to an increased deglycosylation reaction. Variation of the enzyme activity as well as the choice of appropriate detergents can effectively modulate the degree of deglycosylation.

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