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Different Susceptibilities of Complex-, Hybrid- and High-mannose-Type α_1-Proteinase Inhibitor and α_1-Acid Glycoprotein to Endo-β-*N*-acetylglucosaminidase F and Peptide:*N*-Glycosidase F*

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Endo-β-*N*-acetylglucosaminidase F (endo F, EC 3.2.1.96) and peptide:*N*-glycosidase F (PNGase F, EC 3.2.2.18) from *Flavobacterium meningosepticum* were used for the deglycosylation of α_1-proteinase inhibitor and α_1-acid glycoprotein carrying oligosaccharide side chains of the complex-, high-mannose- and hybrid-type. High-mannose- and hybrid-type glycoproteins were obtained by the incubation of rat hepatocyte primary cultures with 1-deoxymannojirimycin or swainsonine, respectively. It was found that endo F cleaves hybrid- and high-mannose-type α_1-proteinase inhibitor and α_1-acid glycoprotein at pH 4.5 as well as at pH 8.5 in the presence or absence of 1% octyl-β-D-glucopyranoside. Complex-type α_1-proteinase inhibitor or α_1-acid glycoprotein were not cleaved by endo F even in the presence of octyl-β-D-glucopyranoside.

PNGase F was found to cleave complex-, hybrid- and high-mannose-type oligosaccharide side chains of α_1-proteinase inhibitor and α_1-acid glycoprotein at pH 4.5 and pH 8.5 in the presence of 0.75% octyl-β-D-glucopyranoside. The deglycosylation of both protein substrates was very poor without detergents.

Abbreviations. Endo F, endo-β-*N*-acetylglucosaminidase F (EC 3.2.1.96); PNGase F, peptide:*N*-glycosidase F (EC 3.2.2.18).

* Dedicated to Prof. Dr. Wolfgang Gerok on the occasion of his 60th birthday
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Elder and Alexander [1] first described a glycosidase activity produced by *Flavobacterium meningosepticum*. They named this activity, which cleaves glycans of high-mannose- and complex-type, endoglucosaminidase F. Subsequently, Plummer *et al.* [2] demonstrated that endo F preparations contain peptide:*N*-glycosidase F activity. These authors were able to discriminate the two enzyme activities by their different pH optima. Furthermore, Tarentino *et al.* [3] succeeded in the separation of both enzymes.

In a previous study from our laboratory [4], the mixture of both enzyme activities was used to study the deglycosylation of α_1-proteinase inhibitor, carrying high-mannose-, hybrid- or complex-type oligosaccharide side chains. In the present paper we describe the extension of these studies with pure endo F and PNGase F. To compare the action of both enzymes with respect to their substrate specificity, α_1-proteinase inhibitor and α_1-acid glycoprotein with oligosaccharide side chains of the high-mannose-, hybrid- and complex-type were used as substrates. It was found that endo F cleaves high-mannose- and hybrid-type α_1-proteinase inhibitor and α_1-acid glycoprotein even in the absence of detergent, whereas PNGase F hydrolyzes all three types of oligosaccharides side chains in α_1-proteinase inhibitor and α_1-acid glycoprotein, particularly in the presence of octyl-β-D-glycopyranoside.

Materials and Methods

Chemicals

L-[^{35}S]Methionine (1000 Ci/mmol) was purchased from Amersham-Buchler (Braunschweig, W. Germany), L-[5,6-^{3}H]fucose (56 Ci/mmol) was from New England Nuclear (Dreieich, W. Germany). Protein A-Sepharose CL-4B was obtained from Pharmacia (Freiburg, W. Germany). Kallikrein trypsin inhibitor (Trasylol®) and 1-deoxymannojirimycin (Bay N 1204) was a generous gift of Dr. G. Kinast, Bayer AG (Wuppertal-Elberfeld, W. Germany). Swainsonine was supplied by Dr. K. Vosbeck, Ciba-Geigy AG (Basel, Switzerland). Tunicamycin and phenylmethylsulfonyl fluoride was from Serva (Heidelberg, W. Germany). Leupeptin, chymostatin, pepstatin and antipain were obtained from Sigma Chemical Co. (München, W. Germany). Endo-β-*N*-acetylglucosaminidase F and peptide:*N*-glycosidase F from *Flavobacterium meningosepticum* were obtained from Boehringer Mannheim (Mannheim, W. Germany). One unit of PNGase F hydrolyzes 1 μmol of dansyl fetuin glycopeptide within 1 min at 37°C at pH 7.2. One unit of endo F hydrolyzes 1 μmol of dansyl-Asn-$(GlcNAc)_2(Man)_5$ within 1 min at 37°C at pH 5. Endo F contains 0.1% PNGase F; PNGase F contains 0.2% endo F.

Preparation of Rat Hepatocyte Monolayers and their Radioactive Labeling

Suspensions of rat hepatocytes were prepared and labeled as described [5-8].

Synthesis of Unglycosylated High-mannose-, Hybrid- and Complex-type α_1-Proteinase Inhibitor and α_1-Acid Glycoprotein by Rat Hepatocytes

Prior to the labeling with [^{35}S]methionine, rat hepatocytes were preincubated for 1 h with 3 μg/ml tunicamycin, 4 mM 1-deoxymannojirimycin or 2 μg/ml swainsonine in order

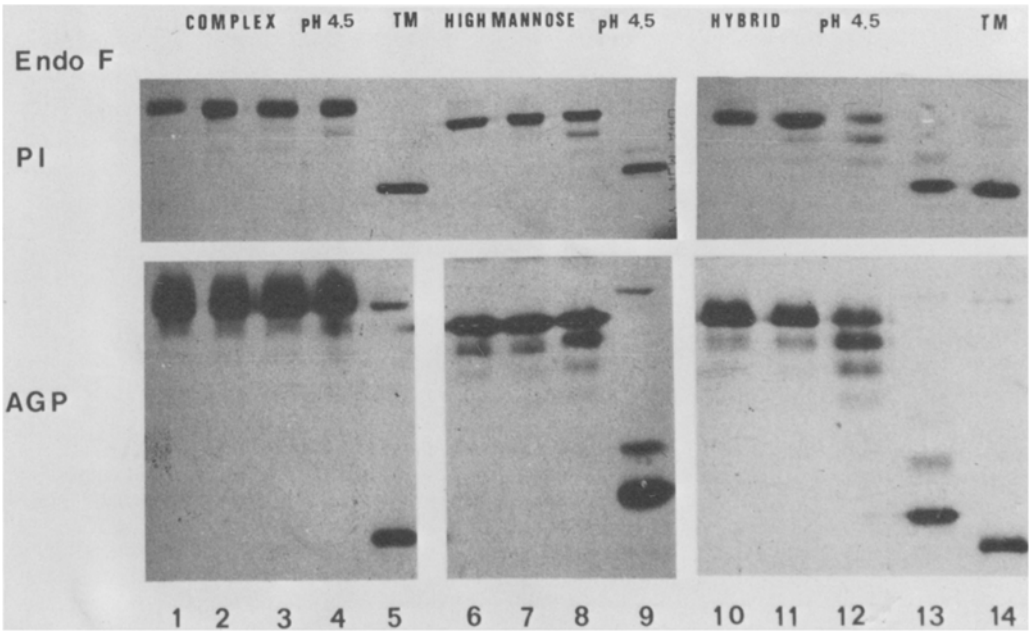

Figure 1. Deglycosylation of α_1-proteinase inhibitor (PI) and α_1-acid glycoprotein (AGP) by endo F in the absence of detergent. 250 μl of media from rat hepatocytes treated without (lanes 1-4) or with 4 mM 1-deoxymannojirimycin (lanes 6-9) or 2 μl/ml swainsonine (lanes 10-13) were incubated without (lanes 1, 6, 10), or with 0.08 milliunits (lanes 2, 7, 11), 0.33 milliunits (lanes 3, 8, 12) and 4.2 milliunits (lanes 4, 9, 13) of endo F at pH 4.5. Media of tunicamycin (TM, 3 μg/ml)-treated hepatocytes were used as controls (lanes 5, 14). α_1-Proteinase inhibitor and α_1-acid glycoprotein were immunoprecipitated from each sample as described in the Materials and Methods section.

to obtain the unglycosylated [6, 7], high-mannose- [9] or hybrid-type [10] glycoproteins from the respective hepatocyte media. Complex-type glycoproteins were synthesized from control hepatocytes.

Conditions for the Incubation with Endo-β-N-acetylglucosaminidase F and Peptide:N-Glycosidase F

250 μl of rat hepatocyte media containing different types of [^{35}S]methionine-labeled glycoproteins were mixed with 0.5 ml of either 75 mM sodium acetate buffer, pH 4.5, containing 50 mM EDTA; or 75 mM sodium phosphate buffer, pH 8.5, containing 50 mM EDTA with or without the addition of 1.5% octyl-β-D-glucopyranoside. The incubation mixtures also contained the following proteinase inhibitors (final concentration): phenylmethylsulfonyl fluoride (0.45 mg/ml), pepstatin (13 μg/ml) antipain (25 μg/ml) and chymostatin (13 μg/ml). Incubations were carried out at 37°C for 15 h with 0, 0.08, 0.33 or 4.2 milliunits of endo F or 0.25, 1.0 and 12.5 milliunits of PNGase F. To prevent evaporation, a layer of paraffin oil was placed on top of the incubation mixtures.

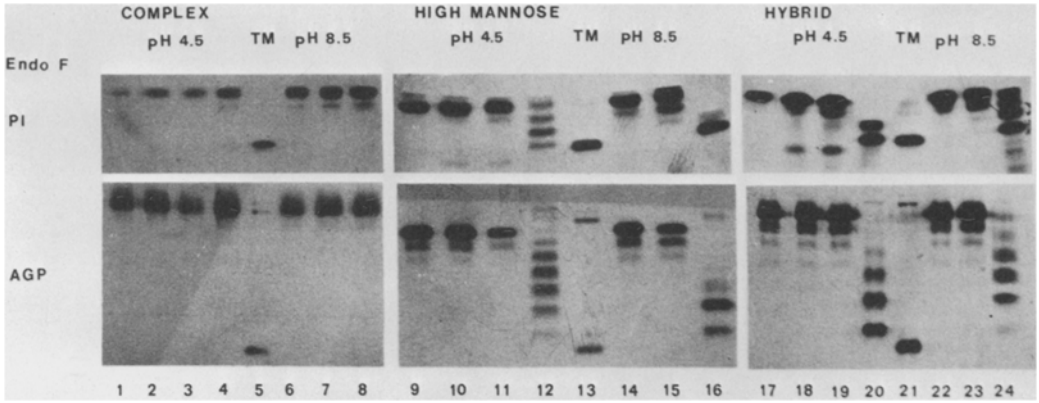

Figure 2. Deglycosylation of α_1-proteinase inhibitor and α_1-acid glycoprotein by endo F in the presence of octyl-β-D-glucopyranoside. 250 μl of media from rat hepatocytes treated without (lanes 1-4, 6-8) or with 4 mM 1-deoxymannojirimycin (lanes 9-12, 14-16) or 2 μg/ml swainsonine (lanes 17-20, 22-24) were incubated without (lanes 1, 9, 17), or with 0.08 milliunits (lanes 2, 6, 10, 14, 18, 22), 0.33 milliunits (lanes 3, 7, 11, 15, 19, 23) or 4.2 milliunits (lanes 4, 8, 12, 16, 20, 24) of endo F at pH 4.5 (lanes 1-4, 9-12, 17-20) or pH 8.5 (lanes 6-8, 14-16, 22-24) in the presence of 1% octyl-β-D-glucopyranoside. Media of tunicamycin (3 μg/ml)-treated hepatocytes were used as controls (lanes 5, 13, 21); α_1-proteinase inhibitor and α_1-acid glycoprotein were immunoprecipitated from each sample as described in the Materials and Methods section.

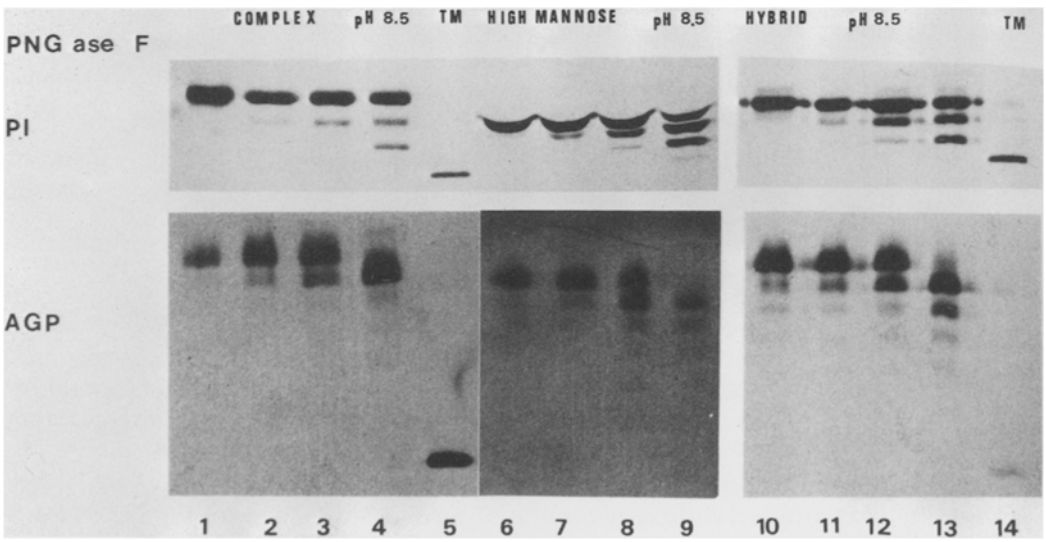

Figure 3. Deglycosylation of α_1-proteinase inhibitor and α_1-acid glycoprotein by PNGase F in the absence of detergent. 250 μl of media from rat hepatocytes treated without (lanes 1-4) or with 4 mM 1-deoxymannojirimycin (lanes 6-9) or 2 μl/ml swainsonine (lanes 10-13) were incubated without (lanes 1, 6, 10), or with 0.25 milliunits (lanes 2, 7, 11), 1.0 milliunit (lanes 3, 8, 12) and 12.5 milliunits (lanes 4, 9, 13) of PNGase F at pH 8.5. Media of tunicamycin (3 μg/ml)-treated hepatocytes were used as control (lanes 5, 14). α_1-Proteinase inhibitor and α_1-acid glycoprotein were immunoprecipitated from each sample as described in the Materials and Methods section.

Immunoprecipitation and Electrophoretic Separation of α_1-Proteinase Inhibitor and α_1-Acid Glycoprotein

For immunoprecipitation the reaction mixtures were added to 5 ml of buffer A (20 mM Tris-HCl, pH 7.6, 0.14 M NaCl, 5 mM EDTA, 1% Triton X-100, 2 mM methionine). After addition of 7.5 μl of a specific antiserum against rat α_1-proteinase inhibitor [11] or rat α_1-acid glycoprotein [12] and incubation at 4°C overnight, the antigen-antibody complexes were bound to 7 mg (dry weight) of protein A-Sepharose, washed four times with buffer A, then twice with 50 mM sodium phosphate buffer, pH 7.5. Elution was achieved using 0.1 M Tris-HCl, pH 6.8, containing 5% β-mercaptoethanol, 5% sodium dodecyl sulphate and 10% glycerol at 95°C for 5 min. The eluted proteins were directly subjected to sodium dodecyl sulphate polyacrylamide slab gel electrophoresis [13] and fluorography [14] using 10% and 12% polyacrylamide gels for α_1-proteinase inhibitor and α_1-acid glycoprotein, respectively.

Results

In a previous study [4] we compared the susceptibilities of differently glycosylated forms of α_1-proteinase inhibitor to an endo F preparation which contained both endo F and PNGase F activities. In order to discriminate between the effects of endo F and PNGase F, we have now used the purified enzymes for the deglycosylation of α_1-proteinase inhibitor and, in addition, α_1-acid glycoprotein carrying either complex-, high-mannose- or hybrid-type oligosaccharide side chains. Rat α_1-proteinase inhibitor has a carbohydrate content of 13.2% [15] and three oligosaccharide side chains [4, 7, 16]. α_1-acid glycoprotein contains 34-37% of carbohydrate [17] and six oligosaccharide side chains [18, 19]. α_1-proteinase inhibitor and α_1-acid glycoprotein carrying different numbers of oligosaccharide side chains can easily be separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis, where they form bands with different electrophoretic mobilities.

Deglycosylation with Endo-β-N-acetylglucosaminidase F

α_1-Proteinase inhibitor and α_1-acid glycoprotein with oligosaccharide side chains of the complex-, high-mannose- and hybrid-type were obtained from the media of control hepatocyte cultures or cultures treated with 1-deoxymannojirimycin or swainsonine, respectively. Fig. 1 shows α_1-proteinase inhibitor and α_1-acid glycoprotein immunoprecipitated from hepatocyte media incubated with different amounts of endo F at pH 4.5 in the absence of detergents. It is obvious that α_1-proteinase inhibitor and α_1-acid glycoprotein of the complex type cannot be deglycosylated by endo F under these conditions (lanes 2-4), whereas the high-mannose- (lane 9) and hybrid-type (lane 13) forms of the two glycoproteins were almost totally deglycosylated. Unglycosylated α_1-proteinase inhibitor or α_1-acid glycoprotein (lanes 5, 14) obtained from tunicamycin-treated hepatocyte cultures were used as standards to measure the degree of deglycosylation. It can be seen that the electrophoretic mobilities of α_1-proteinase inhibitor and α_1-acid glycoprotein deglycosylated by endo F (lanes 9, 13) are slightly slower than those of the unglycosylated proteins (lanes 5, 14). This small difference may be due to the fact that endo F cleavage leaves the first *N*-acetylglucosamine molecule of each oligosaccharide chain attached to asparagine.

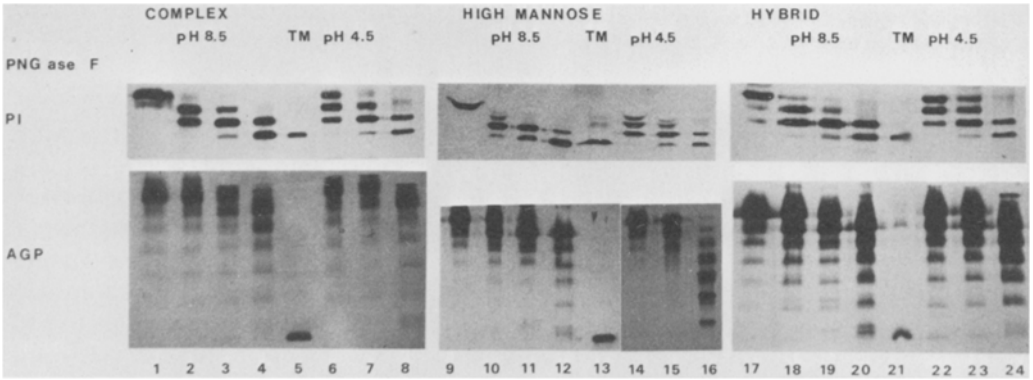

Figure 4. Deglycosylation of α_1-proteinase inhibitor and α_1-acid glycoprotein by PNGase F in the presence of octyl-β-D-glucopyranoside. 250 μl of media from rat hepatocytes treated without (lanes 1-4, 6-8), or with 4 mM 1-deoxymannojirimycin (lanes 9-12, 14-16) or 2 μg/ml swainsonine (lanes 17-20, 22-24) were incubated without (lanes 1, 9, 17), or with 0.25 milliunits (lanes 2, 6, 10, 14, 18, 22), 1.0 milliunit (lanes 3, 7, 11, 15, 19, 23) or 12.5 milliunits (lanes 4, 8, 12, 16, 20, 24) of PNGase F at pH 8.5 (lanes 1-4, 9-12, 17-20) or pH 4.5 (lanes 6-8, 14-16, 22-24) in the presence of 0.75% octyl-β-D-glucopyranoside. Media of tunicamycin (3 μg/ml)-treated hepatocytes were used as control (lanes 5, 13, 21); α_1-proteinase inhibitor and α_1-acid glycoprotein were immunoprecipitated from each sample as described in the Materials and Methods section.

To find out whether a more unfolded glycoprotein structure has a higher susceptibility to endo F, the incubations of α_1-proteinase inhibitor and α_1-acid glycoprotein with endo F were repeated in the presence of octyl-β-D-glucopyranoside. Octyl-β-D-glucopyranoside was chosen because in our previous deglycosylation study on α_1-proteinase inhibitor it was the most effective detergent [4]. Preincubation of either endo F or PNGase F did not lead to a reduction in the activities of either enzyme (not shown). Furthermore, the action of endo F was studied at two different pH values.

Fig. 2 shows that the presence of 1% octyl-β-D-glucopyranoside does not improve the degree of deglycosylation of both glycoprotein substrates. α_1-Proteinase inhibitor and α_1-acid glycoprotein of the complex type are completely resistant to endo F (lanes 1-4, 6-8) at pH 4.5 and 8.5. The incubation of α_1-proteinase inhibitor and α_1-acid glycoprotein of the high-mannose- and hybrid-type with endo F leads to similar cleavage patterns at pH 4.5 and pH 8.5 indicating that endo F is active over a rather broad pH range.

Deglycosylation with Peptide:N-glycosidase F

Since Plummer *et al.* [2] described that PNGase F has its pH optimum at 8.5, we used this pH for the deglycosylation of α_1-proteinase inhibitor and α_1-acid glycoprotein in the absence of detergent. Fig. 3 shows the results of these studies. α_1-Proteinase inhibitor carrying oligosaccharide side chains of the complex-, high-mannose- and hybrid-type exhibits similar susceptibilities to PNGase F at pH 8.5 in the absence of detergent. Mainly α_1-proteinase inhibitor with 1, 2 and 3 carbohydrate side chains (lanes 4, 9, 13) was detected. When α_1-acid glycoprotein was immunoprecipitated from the same reaction mixture, it was found to be much less susceptible to the action of PNGase F as compared to α_1-proteinase inhibitor.

In contrast to the results obtained with endo F, the addition of octyl-β-D-

Table 1. Susceptibilities of complex-, hybrid- and high-mannose-type α_1-proteinase inhibitor and α_1-acid glycoprotein to enzyme degradation.

Type of carbohydrate side chains	endo F			PNGase F		
	minus	plus detergent		minus	plus detergent	
	pH 4.5	pH 4.5	pH 8.5	pH 8.5	pH 4.5	pH 8.5
Complex	—/—	—/—	—/—	+/+	++/+	++/+
Hybrid	+++/+++	++/++	++/++	+/+	++/+	++/+
High-mannose	+++/+++	++/++	+++/+++	+/+	++/+	++/+

+++, total deglycosylation; —, no deglycosylation.
The symbols to the left of the diagonal line refer to α_1-proteinase inhibitor, and those to the right to α_1-acid glycoprotein.

glucopyranoside improved the degree of deglycosylation of both glycoproteins. The deglycosylation reaction was carried out at pH 4.5 and pH 8.5. It can be seen from Fig. 4 that neither the pH nor the type of the oligosaccharide side chains has an influence on the deglycosylation of α_1-proteinase inhibitor. In all cases α_1-proteinase inhibitor with no or one oligosaccharide side chain was detected after incubation with 12.5 units of PNGase F (lanes 4, 8, 12, 16, 20, 24).

Unlike α_1-proteinase inhibitor the oligosaccharide side chains of α_1-acid glycoprotein are less effectively removed by PNGase F in the presence of detergent. In addition, the degree of deglycosylation did not depend on the pH. When 12.5 milliunits of PNGase F were used for the deglycosylation of α_1-acid glycoprotein, the major deglycosylation products had five, four and three oligosaccharide side chains irrespective of their oligosaccharide type (lanes 4, 8, 12, 16, 20, 24).

Discussion

The results of the deglycosylation studies with α_1-proteinase inhibitor and α_1-acid glycoprotein carrying three different types of oligosaccharide side chains with endo F and PNGase F are schematically summarized in Table 1. It is evident from these data that both glycoproteins with hybrid- and high-mannose-type oligosaccharide side chains are most efficiently deglycosylated by endo F. The deglycosylation reaction was complete even in the absence of detergents. Thus, it is possible to deglycosylate native glycoproteins of the high-mannose- or hybrid-type by endo F. This might provide a useful approach for the investigation of the function of the carbohydate moieties of biologically active proteins. On the other hand, α_1-proteinase inhibitor and α_1-acid glycoprotein with complex-type oligosaccharide side chains could not be deglycosylated by endo F. This is in agreement with the findings of Plummer *et al.* [2] and Tarentino *et al.* [3]. A deglycosylation of complex type α_1-proteinase inhibitor and α_1-acid glycoprotein was achieved by PNGase F. Unlike endo F, the presence of detergent improved the extent of the deglycosylation by PNGase F. However, both glycoproteins could not be completely deglycosylated by PNGase F. Furthermore, no difference in their susceptibilities to PNGase F was found between complex-, hybrid- and high-mannose-type α_1-proteinase inhibitor and α_1-acid glycoprotein, although the deglycosylation of α_1-acid glycoprotein was less as compared to α_1-proteinase inhibitor.

Plummer *et al.* [2] were able to discriminate between endo F and PNGase F on the basis of their pH optima. They showed that PNGase F (assayed with a dansyl-fetuin pentaglycopeptide) is completely inactive at pH 4.0, whereas endo F is fully active. On the other hand, endo F (tested with a high-mannose glycopeptide) was only slightly active at pH 9.3, whereas PNGase F was highly active. We used the glycoproteins α_1-proteinase inhibitor and α_1-acid glycoprotein as substrates for both enzymes and could not detect such a pronounced pH-dependence of the deglycosylation reactions.

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