Molecular Cloning, Primary Structure, and Properties of a New Glycoamidase from the Fungus Aspergillus tubigensis*

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A new glycoamidase, peptide-\(N^\text{4}-(N\text{-acetyl}-\beta\text{-D-glucosaminyl})\text{asparaginase amidase (PNGase) At,} was discovered in the eukaryote Aspergillus tubigensis. The enzyme was purified to homogeneity, and the DNA sequence was determined by cloning in Escherichia coli. Over 80% of the deduced amino acid sequence was verified independently by Edman analysis and/or electrospray ionization-mass spectrometry of protease fragments of native PNGase At. This glycoamidase contains 12 potential asparagine-linked glycosylation sites, of which at least 9 sites are occupied with typical high mannose oligosaccharides. PNGase At consists of two non-identical glycosylated subunits that are derived from a single polypeptide gene precursor. Evidence is presented suggesting that autocatalysis is involved in subunit formation. PNGase At is an important new tool for analysis of asparagine-linked glycans; it can hydrolyze a broad range of glycopeptides, including those with core-linked \(\alpha-6\) or \(\alpha-3\) fucose, under conditions not favorable with existing glycoamidases.

Glycoamidases are an important class of deglycosylating enzymes, and their use has contributed greatly to our knowledge of the structure/function of asparagine-linked glycoproteins. Peptide-\(N^\text{4}-(N\text{-acetyl}-\beta\text{-D-glucosaminyl})\text{asparaginase amidase F (PNGase F)}\) is an amidase/ amidohydrolase that cleaves the \(\beta\text{-aspartylglycosylamine bond of peptide-bound asparagine-linked glycans, converting the asparagine residue to aspartic acid and releasing an intact 1-amino oligosaccharide (1). This enzyme has been purified to homogeneity from the Gram-negative bacterium Flavobacterium meningosepticum, and its substrate specificity has been well established (2). PNGase F has been cloned and sequenced (3), and its crystallographic structure and active center were determined to 2.2 Å (4, 5). Other glycoamidases catalyzing the same reaction have been reported in plants (e.g. almond PNGase A) (6, 7) and, more recently, in mammalian cells (8), where the enzyme is thought to have a regulatory role in glycoprotein maturation (9). However, only the bacterial glycoamidase PNGase F has been characterized at the molecular level, and an understanding of the primary structure of even the simplest eukaryotic glycoamidase is still lacking.

During the course of a glycoamidase survey, we discovered high levels of a new PNGase type enzyme in crude commercial pectinase extracts derived from Aspergillus tubigensis. Preliminary studies demonstrated that this glycoamidase, designated PNGase At, appeared significantly different from PNGase F in terms of its molecular weight, state of glycosylation, low pH optimum, and thermostability as to warrant further investigation into its protein structure and mechanism of action. In this report we describe the purification of PNGase At to homogeneity. The enzyme has been cloned and sequenced, and the deduced amino acid sequence verified independently to greater than 80% by ESI-MS. On the basis of the structural organization of the gene, PNGase At is initially synthesized as a single polypeptide of about 59,000 daltons. It acquires asparagine-linked glycans during post-translational processing and is cleaved into non-identical subunits. Subunit formation represents a fundamental departure from the structure of the corresponding bacterial enzyme and may reflect a different mechanism for catalysis of the \(\beta\text{-aspartylglycosylamine bond. This study represents the first description of a glycoamidase at the eukaryotic level.}

**EXPERIMENTAL PROCEDURES**

**Materials**

*Aspergillus tubigensis* strain 7 DNA, RNA, and Multifect PL enzyme were obtained from Genecor. *Pfu* polymerase, Bluescript KS+, SK+, and PCR script SK+ cloning vectors were obtained from Stratagene. Reverse transcriptase, RNase inhibitor, and RNase-free DNase were from Promega. T4 DNA ligase, polynucleotide kinase, and all the restriction enzymes used in this project were from New England Biolabs.

**Polymerase Chain Reactions**

PCR amplification reactions were performed using 100 ng of *Aspergillus tubigensis* total DNA, 100 pmol of each primer, 200 μM dNTP, 2.5 units *Pfu* DNA polymerase, and 1 × *Pfu* buffer (Stratagene) in a total volume of 100 μL. The reactions were carried out in a DNA thermal cycler (Perkin-Elmer) programmed for 30 three-step cycles of denaturation (1 min at 94 °C), annealing (1 min at either 45 °C for degenerate primers or 55 °C for specific primers), and elongation (1 min at 70 °C). A further elongation at 72 °C was performed for 10 min at the end of the program.

**Molecular Cloning of PNGase At**

Based on the peptide sequence analysis, two oppositely oriented degenerated oligonucleotides were designed, AnS1 (5′-ACAGAAATTC-GARGTNTRYGARGTNTRYCARCC-3′), amino acid residues 3–10) and AnA4 (5′-ACAAGATTTACGAAACGYTGCGTGAGCT-3′, amino acid residues 75–81) where N is either G, A, T, or C, R is either A or G, and Y is either C or T. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EIH Database with accession number(s) U96923. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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or T. Using these primers, a PCR product of 294 bp was obtained. This fragment was digested with EcoRI and HindIII restriction enzymes and cloned into pBlueScript (Stratagene) pre-digested with the same enzymes. The sequence reactions were performed using a Sequenase kit (U. S. Biochemical Corp.). To extend the sequence further, upstream and downstream regions of this fragment were amplified using RAGE (10) and rapid amplification of cDNA ends (11) protocols. Briefly, in vitro polyadenylated cDNAs (rapid amplification of cDNA ends) or restriction fragments of genomic DNA (RAGE) were used in PCR reactions with a specific primer and an oligo(dT) to amplify unknown sequences. Using this information, the whole gene was then amplified in two large fragments. For amplifying fragment A, the following primers were used, 5′-ATACTCTAGGGGGGAGCGCCGCTTGGTT-3′ and AnS18 (5′-GAAGGTTCACATCGACGAGCATC-3′) (5′-translated region, not shown). A second, nested PCR amplification was performed using primers AnA8 (5′-ATACTCTAGGGGGGAGCGCCGCTTGGTT-3′, nucleotides 669–693) and AnS18, resulting in a fragment of 1074 bp long. The same strategy was applied to amplify fragment B as follows: the first PCR reaction was carried out with primers AnS11 (5′-AACGCCCACGTGCAATATCCACGCT-3′, nucleotides 573–597) and AnA19 (5′-CTAGTCTCAGTATCCGAAACACGC-3′, nucleotides 1651–1677). A nested PCR reaction was performed using primers AnS6 (5′-ACAGATCCCTGGCCGAGGTTGGTC-3′, nucleotides 604–627) and AnA19. The PCR end product was 1074 bp long. Fragments A and B were cloned into PCRScript (Stratagene), and the nucleotide sequence for each of them was determined from two independent clones. The reconstruction of the gene from these two fragments was done in Bluescript SK+.

**Enzyme Purification**

All operations were performed at room temperature. **Sephadex G-75**—150 ml of the crude dark brown Multifeet PL extract (Genencor) was applied to a 7.6 × 58-cm column of Sephadex G-75 (65–88 μm) equilibrated in 10 mM sodium acetate, pH 5.2, containing 1% butanol. The column was developed at 190 ml/h, and 19-ml fractions were collected. PNGase A was eluted sharply after the void volume (tubes 42–64) and was well separated from most of the dark brown pigment material.

**Toyopearl DEAE 650S**—The PNGase A Pool from two gel filtration runs was applied to a 2.0 × 11-cm column of Toyopearl DEAE 650 (35 μm) equilibrated in 10 mM sodium acetate, pH 5.2. A linear gradient (0 to 0.4 M NaCl over 4 h) was used, and 10-ml fractions were collected. PNGase A Fractions were pooled from tubes 31 to 68 (0.05–0.15 M NaCl).

**Hydrophobic Interaction Chromatography**—Pooled fractions were diluted 10% by volume of 0.5 M sodium phosphate, pH 7.0, to bring to pH 7.3. This solution was equilibrated with 1 M ammonium sulfate. The extract was applied to a 1.6 × 47-cm column of Toyopearl butyl-650 M equilibrated in 100 mM sodium phosphate, pH 7.0, containing 1 mM ammonium sulfate and 10 mM EDTA. A decreasing linear gradient (Waters, curve 6) to 50 mM sodium phosphate, 10 mM EDTA was generated at 120 ml/h over 7 h, and 5-ml fractions were collected. The enzyme began eluting from the column at about 0.5 M ammonium sulfate.

**Protein-Pak DEAE**—Pooled fractions from the previous step were dialyzed against 20 mM sodium phosphate, pH 5.2, and the retentate was loaded on a 0.9 × 14-cm column of Macro-Prep ceramic hydroxyapatite (type 1, Bio-Rad) equilibrated in 10 mM sodium phosphate, pH 7.0. The column was washed at 15 ml/h at a flow rate of 30 ml/h and developed with a linear gradient from 0 to 0.1 M NaCl for 150 min.

**Hydroxyapatite Chromatography**—The pooled fractions were dialyzed against 10 mM sodium phosphate, pH 7.0, and the retentate was loaded on a 0.9 × 14-cm column of Macro-Prep ceramic hydroxyapatite (type 1, Bio-Rad) equilibrated in 10 mM sodium phosphate, pH 7.0. The column was washed for 15 min at a flow rate of 30 ml/h and developed with a linear gradient from 0 to 0.1 M NaCl for 150 min.

**Protease Digestions**

PNGase A in its native state was digested with trypsin, endoproteinase Glu-C, or endoproteinase Asp-N in ammonium bicarbonate, pH 8, according to the manufacturer’s recommendations (Boehringer Mannheim). Tryptsin-insoluble peptides were further digested with pepsin in 0.1% formic acid. Peptides were isolated from these digests by reverse-phase HPLC on 1 × 150-cm Vydac columns. Unfractionated digests or purified glycospptides were treated with PNGase F at pH 8.0 to release asparagine-linked oligosaccharides.

**Mass Spectrometry**

All spectra were acquired on a Finnigan TQF 700 equipped with a Finnigan ESI source. Samples were introduced into the mass spectrometer by infusion at a flow rate of 0.3 μl/min through a 20-μm inner diameter fused silica capillary. Solvents were various mixtures of water/methanol/acetonitrile with 0.1% formic acid or 0.04% trifluoroacetic acid. LC-MS was accomplished with a homemade 50 × 10 cm stainless steel column and a homemade 0.25 × 10 cm stainless steel column. Peptide fractions from LC-MS were automatically collected for sequence analysis. Methyl ester derivatives were prepared by exposure to 1 M methanolic HCl. MS/MS spectra were generated at 20–40 eV collision energy with argon as the collision gas.

**Edman Microsequence Analysis**

Automated Edman degradation was performed with a model 477A Applied Biosystem pulsed liquid sequenator equipped with a model 120A amino acid analyzer. Sequence analyses were conducted on samples isolated by HPLC or from Western blots on polyvinylidene difluoride membranes following SDS-PAGE (13).

**Deglycosylation of PNGase A with Endo F₁**

Native PNGase A (3.13 mg, 53 nmol) and equimolar Endo F₁ (1.77 mg, 56 nmol) were incubated at 37 °C in 50 mM ammonium acetate, pH 6.0, in a total volume of 15.3 ml. At 20 h the reaction was terminated by lyophilization. The sample was redissolved in 10 ml of water, and the pH was adjusted to 5.2 with 0.1 M acetic acid. The conductivity of the solution was equalized to 20 mM sodium acetate, pH 5.2, by addition of water. To separate Endo F₁ from deglycosylated PNGase A, the diluted sample was applied to a 0.5 × 10.5-cm column of Protein-Pak DEAE 15 HR (Waters) in 20 mM sodium acetate, pH 5.2, at a flow rate of 30 ml/h. The column was washed in starting buffer for 20 min to completely remove Endo F₁, followed by a gradient from 0 to 0.25 M NaCl over 30 min. Deglycosylated PNGase A was eluted in a stepwise manner with 0.5 M sodium acetate, pH 5.2, and then 0.5 M sodium acetate, pH 6.5. Carbohydrate analysis of deglycosylated PNGase A indicated a very low hexose content, consistent with essentially complete removal of the asparagine-linked oligosaccharides.

**Carbohydrate Analysis**

Total carbohydrate was determined on PNGase A samples by the phenol-sulfuric acid method (14). Monosaccharide and glucosamine analyses were done using 2 M trifluoroacetic acid at 100 °C for 5 h, and 4% HCl at 100 °C for 6 h, respectively (15). Parallel analyses were done using 2% HCl at 100° C for 2.5 h (16).

**Preparation of Glycopeptides**

Hen ovomucoid, GTN(CHO)ISK, was isolated from reduced carboxymethylated ovomucoid after tryptic digestion. Glycopeptides were purified on Sephadex G-50 followed by chromatography on Dowex 50-X2 as described for IgM glycopeptides (17). Other glycopeptides were purified as indicated: bovine fetuin, LANT(CHO)ecCS (where Aec is aminoethylcysteine) and LANCHOC-mCS (where Cmc is carboxymethylcysteine) (18); porcine fibrinogen, VEN(CHO)R, and VGEN(CHO)R (19). Pineapple bromelain glycopeptide was isolated from a thermolytic digest of reduced carboxymethylated enzyme after chromatography on Sephadex G-50 (17) and BakerBond Wide Pore C₁₈ (19). A modified glycopeptide containing a core GlcNAc with an α−6–9 linkage was produced as follows: DNS VGEN(CHO)R (0.78 pmol) was digested with Endo F₁ (15 μg) in 400 μl of 100 mM sodium acetate, pH 4.75, for 3 h at 37 °C. The digestion mixture was fractionated on a 0.9 × 130-cm column of Sephadex G-15 developed in 0.1 M acetic acid. The truncated glycopeptide eluting at 45–57 ml was the major peak and was lyophilized and quantitated for use in rat determinations. Glycopeptides were danylsated by the method of Tapuchi et al. (20).
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TABLE I
Summary of the purification of PNGase At

| Purification step       | Volume | Total A280a | Total milliunitsb | Purification factor | Yield |
|-------------------------|--------|-------------|-------------------|---------------------|-------|
| Sephadex G-75           | ml     | 5463        | 452,775           | 1                   | 100   |
| Toyopearl DEAE-650S     | 438    | 1138        | 597,616           | 4.8                 | 132   |
| Toyopearl Butyl-650M    | 217    | 108.5       | 257,833           | 50.4                | 57    |
| Protein-Pak DEAE        | 158    | 35.6        | 269,074           | 154                 | 59    |
| ConA-Sepharose          | 41.5   | 15.2        | 250,792           | 357                 | 55    |
| Hydorxapatite           | 6.8    | 6.2         | 160,965           | 881                 | 35    |

a The absorbance at 280 nm was used as an approximation to the protein concentration during purification. A 1% solution of pure PNGase At corresponds to an A280 of 1.66 based on its amino acid composition (12).

b 1 milliunit corresponds to 1 nmol of glycopeptide hydrolyzed per min per mg. The enzyme migrated on SDS-PAGE as a heterogeneous band with an apparent molecular mass of 43,000 (Fig. 1, lane 1). Peptide fragments derived from purified PNGase At were used to design oligonucleotides for cloning the PNGase At gene.

RESULTS AND DISCUSSION

PNGase Purification—Multifect PL enzyme is a highly concentrated commercial extract of the secretory enzymes from a proprietary strain (Genencor) of the fungus, A. tubigensis. This enzyme contains numerous glycosidases, carboxydases, and proteases, etc. and is widely used in the food industry. The purification of PNGase At to homogeneity from this crude material is summarized in Table I. Typically, 300 ml of multifect PL extract yielded 4 mg of pure PNGase At at an average final specific activity of 53,680 nmol of di-dansyl fluor glycopeptide hydrolyzed per mg per min at pH 5.2 and 37 °C.

Enzymes Assays
PNGase assays were performed by HPLC as described previously for Endo F2 and Endo F3 (2) but were done in 0.1 M sodium acetate, pH 5.2, for PNGase At and PNGase A and in 0.1 M HEPES, pH 8.8, for PNGase F. One milliunit corresponds to 1 nmol of glycopeptide hydrolyzed per min per mg. The enzyme migrated on SDS-PAGE as a heterogeneous band with an apparent molecular mass of 43,000 (Fig. 1, lane 1). Peptide fragments derived from purified PNGase At were used to design oligonucleotides for cloning the PNGase At gene.

Molecular Cloning and DNA Sequence Analysis—The PNGase At gene was cloned and sequenced in two fragments using PCR-based methodology. The gene was reconstructed as depicted in Fig. 2, and the complete sequence was confirmed. The complete nucleotide sequence and the deduced amino acid sequence of the PNGase At gene is presented in Fig. 3. Edman analysis identified the amino terminus of native PNGase At as LEVYFVEYQ, but a background sequence was also present in the data and identified as another subunit only in retrospect (see below). Assuming that the first methionine at position 21 is the translation start codon, as is the case with most fungal genes (21), then the open reading frame of the gene encodes a typical hydrophobic signal peptide of 21 amino acids as expected for a protein secreted by A. tubigensis. From the DNA sequence (Fig. 3) it is clear that the primary translation product of PNGase At is a single polypeptide with a predicted molecular mass of 59,335 Da. Post-transcriptional processing adds between 7 and 9 high mannose oligosaccharides. Man6GlcNAc2 was found almost exclusively at site 2. ESI-MS analysis of permethylated Endo F1-released oligosaccharides as well as Dionex PA-100 chromatographic analysis demonstrated that all other glycosylation sites were predominantly Man9GlcNAc (71%), with small amounts of Man9,GlcNAc at sites 3–5, 7, and 10, and peptides were identified by MS analysis with a mass consistent with the presence of a single GlcNAc residue (5–10%). This finding suggests the action of a trace endoglycosidase in the Multifect PL preparation that cleaves the di-N-acetylchitobiose linkage at accessible sites in PNGase At, but thus far such an activity has not been detected.

One O-linked glycosylation site at Thr352 was found by mass spectrometry; it contained mainly a single hexose (95%) and a small amount of a di-hexose (5%).

Subunit Structure—From the DNA sequence (Fig. 3) it is clear that the primary translation product of PNGase At is a single polypeptide with a predicted molecular mass of 59,335 Da. Post-transcriptional processing adds between 7 and 9 high mannose chains (average, Man9,GlcNAc) increasing the mass by about 9–11 kDa to over 70,000 Da. Because of the well-known anomalous behavior of large glycoproteins during SDS-PAGE, it was expected that PNGase At would migrate with an apparent Mr greater than its calculated mass of 70,000 daltons. However, six different preparations showed the same unusual result; PNGase At migrated on SDS-PAGE as a heterogeneous band with an apparent Mr slightly less than 43,000 (Fig. 1, lane 1). This result suggests that PNGase At consists of two glycosylated subunits that dissociate in SDS and co-migrate on SDS-PAGE with an apparent Mr around 43,000. After removal with high mannose oligosaccharides. Man9,GlcNAc2 was found almost exclusively at site 2. ESI-MS analysis of permethylated Endo F1-released oligosaccharides as well as Dionex PA-100 chromatographic analysis demonstrated that all other glycosylation sites were predominantly Man9,GlcNAc (71%), with small amounts of Man9,GlcNAc at sites 3–5, 7, and 10, and peptides were identified by MS analysis with a mass consistent with the presence of a single GlcNAc residue (5–10%). This finding suggests the action of a trace endoglycosidase in the Multifect PL preparation that cleaves the di-N-acetylchitobiose linkage at accessible sites in PNGase At, but thus far such an activity has not been detected.

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of the asparagine-linked oligosaccharides with Endo F1, deglycosylated PNGase At migrated as two well-resolved polypeptides (Fig. 1, lanes 4 and 6), an α-subunit with an apparent Mr about 38,000 and a lighter staining β-subunit with an apparent Mr of 28,000.

Additional evidence that PNGase At is composed of two non-identical subunits was provided by size-exclusion HPLC using the LKB Blue column. Under non-denaturing conditions PNGase At eluted in a high molecular weight form estimated to be 80,000 (Fig. 4A). However, after denaturation with SDS, the enzyme eluted as two subunits with an estimated Mr of 42,000 and 26,000, respectively (Fig. 4B). Both the glycosylated and deglycosylated form of PNGase At were resolved into their subunits by HPLC, whereas on SDS-PAGE only the deglycosylated form was resolved. Note that A and B are not directly comparable by size versus elution time because SDS shifts the molecular weight standard curve to the left.

The amino acid sequence of the two subunits was determined on deglycosylated PNGase At by SDS-PAGE and Western blotting of the subunits to polyvinylidene difluoride membranes. Edman analysis (Table II) of the deglycosylated α-subunit gave the amino-terminal sequence LLEVFEVY Q . . . , which corresponds to the amino terminus found for the intact protein. The deglycosylated β-subunit sequenced as two polypeptides, TGQAPEIYAPAP... (40%) and a truncated form APEIYAPA... (60%). Re-examination of the original sequence data on intact PNGase At (see above) indicated that the background sequence referred to in the cloning section corresponded to the amino-terminal sequence of the β-subunit and that the α- and β-subunit formation.

The nucleotide sequence of the PNGase At cDNA and the deduced amino acid sequence. The nucleotide and amino acid sequences are numbered from the left beginning with the first start codon. The sequence start sites at [mimus]21 and is underlined. Twelve putative asparagine-linked glycosylation sites are in bold. The double asterisks (**) indicate the cleavage site for α- and β-subunit formation.

Table II

| Amino termini | Trypsin | Pepsin | Endo-AspN |
|---------------|---------|--------|-----------|
| α-Subunit     | 69–81   | 51–57  | 28–50     |
| β-Subunit     | 336–353 | 124–133| 91–117    |
| α-Subunit     | 399–108 | 165–175| 116–122   |
| β-Subunit     | 289–282 | 186–191| 125–128   |
| α-Subunit     | 353–374 | 315–321| 208–212   |
| β-Subunit     | 406–424 | 240–265|           |

* Determined from Edman analysis of the isolated subunits after SDS-PAGE and Western blotting.
* As isolated as asparagine-linked glycosylation sites.
* Isolated from S. aureus V8 protease digest.

Table III

| Site | Sequon sequence | Glycoforms |
|------|-----------------|------------|
| 1 NCT 47–49 | 0 |
| NPT 56–58 | M₉N₂ |
| NTT 130–132 | M₉N₂, N |
| NAS 160–162 | M₉N₂, N |
| NAT 171–173 | M₉N₂ |
| NKS 284–286 | 0 |
| NGS 360–362 | M₉N₂, N |
| NLS 388–390 | ND |
| NKS 423–425 | M₉N₂, N, 0 |
| NKS 529–530 | ND |

* Asparagine-linked glycosylation sites determined from DNA sequence.

Glycopeptides derived from trypsin, pepsin, or endo-Asp N digests were analyzed by Edman or MS to determine the amino acid sequence (see Fig. 2), and by MS/MS for the carbohydrate at each site. Glycoforms are presented as 0, none present; or as oligosaccharides containing mannose (M) and N-acetylglucosamine (N) at the indicated composition. Single N-acetylglucosamine "stubs" are indicated by N.

* Not determined.
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**TABLE IV**

Rates of hydrolysis of asparagine-linked glycans by PNGase At and PNGase F

| Dansylated glycopeptide sequence | Oligosaccharide type | PNGase At | PNGase F |
|----------------------------------|---------------------|-----------|----------|
| LAN(CHO)AcCS                  | Triantennary         | 34,602    | 101,804  |
| LAN(CHO)CmCS                  | Triantennary         | 5,610     | 17,248   |
| GTN(CHO)ISIK             | GlcNAc hybrid with core Man,GlcNAc<sub>2</sub> | 2,289     | 6,483    |
| VEN(CHO)K                | Biantennary with core α-1→6 fucose | 3,211     | 1,238    |
| VGEN(CHO)K*            | Biantennary with core α-1→6 fucose | 378       | 1,161    |
| VGEN(CHO)R                | core fucose α-1→6 GlcNAc | 2,3       | 0        |
| ARVPRNN(CHO)ESS*        | Xyl,Fuc,Man,GlcNAc, with core α-1→3 fucose | 1,445     | 0        |

*a* Bovine fetuin; *Ac* is aminoethylcysteine.

*b* Chicken ovomucoid.

*c* Porcine fibrinogen.

*d* Porcine fibrinogen with Endo F<sub>3</sub> digestion.

*e* Pineapple stern bromelain.

*f* Previously reported (19).

β-subunits are present in approximately 1:1 ratio.

Subunit formation occurs in a Ser/Thr-rich hydrophilic region of PNGase At between residues Thr<sup>355</sup>–Thr<sup>356</sup> (Fig. 3, **). At this point it has not been established whether this cleavage is the result of a specific protease or is caused by an autoproteolytic reaction. As a working hypothesis we favor the latter, because of the similarity of PNGase At to a new class of amidohydrolase/amidohydrolase enzymes, designated Ntn-amidohydrolases (reviewed in Ref. 23). Ntn-amidohydrolases (e.g. glycosyl-asparaginase) are derived from a single inactive precursor gene product via an autoproteolytic reaction. This mechanism places a reactive Thr, Ser, or Cys residue on the amino end of the β-subunit, where it functions as a combined base-nucleophile catalyst. If autoproteolysis is responsible for the Thr<sup>356</sup>-subunit on the β-subunit of PNGase At, the enzyme should be only 40% active because 60% of the molecules have been degraded to the Ala<sup>355</sup>-form. This conversion did not result from deglycosylation by Endo F<sub>1</sub>, because native and deglycosylated PNGase At have the same specific activity. It is probably due to an aminopeptidase in the multifact FL extract.

Properties of PNGase At—PNGase At has no structural homology to PNGase F or to any other protein in the data bases. It has a 2-fold excess of acidic amino acids and a relatively low isoelectric point of 4.1. The pH activity curve is broad with a pH optimum at 5.0; at pH 3 and pH 6.6 the enzyme is 80 and 65%, respectively, of the maximum. In contrast PNGase F is less than 20% active at pH 5, and its pH optimum is between 8.6 and 9.0. Like many proteins secreted by *A. tubigensis*, PNGase At is relatively thermostable. It retains 65–70% of its original activity during incubation at 62 °C for 8 h, unlike PNGase F, which is inactive after 1 h at 62 °C. Glycopeptides can be hydrolyzed by PNGase At at 62 °C at over three times the rate at 37 °C. This is a useful property especially for glycopeptides with amino-terminal or carboxyl-terminal oligosaccharides, which are, in general, poor substrates for PNGase-type enzymes. Thus PNGase At is functional under conditions beyond the useful range for PNGase F.

A comparative analysis of the rates of hydrolysis of PNGase At and PNGase F toward selected glycopeptides is shown in Table IV. PNGase At has a broad substrate specificity hydrolyzing triantennary, biantennary, hybrid (Table IV), as well as high mannose-type (not shown) glycans. Triantennary glycans were preferred substrates for PNGase At and PNGase F. The negative charge of the carboxy-methyl group adjacent to the deglycosylation site adversely affects both enzymes compared with the corresponding amino-ethyl derivative. In general, the rates of hydrolysis by PNGase At were 3–4-fold slower at 37 °C than for PNGase F, except for the last two substrates in Table IV. As indicated in the preceding section, however, PNGase At may only be 40% active because of the loss of Thr<sup>356</sup> from the β-subunit. Interestingly, a truncated porcine fibrinogen glycopeptide (core fucα1→6GlcNAc) was hydrolyzed completely by PNGase At, albeit at a low rate, whereas it was completely resistant to PNGase F. This is an expected finding since a di-N-acetylchitobiose moiety is the minimum carbohydrate length required for PNGase F activity. Another indication of a difference in the active center of PNGase At and PNGase F is illustrated by the bromelain glycopeptide data in Table IV. PNGase At cleaved this glycopeptide at a very good rate, but it was totally resistant to PNGase F. This is an expected finding since a di-N-acetylchitobiose-binding domain in the active center of PNGase F allows for hydrolysis of glycans with fucose attached to the α1→6 position of the core proximal GlcNAc residue but lacks space for binding glycans with fucose in the α1→3 position (5).

PNGase At is more closely related in terms of substrate specificity to the almond enzyme PNGase A (glycopeptidase A)
than to the bacterial enzyme PNGase F. PNGase At and PNGase A (24) both hydrolyze a broad array of oligosaccharide types, including very short glycans, as well as oligosaccharides with α1→6 or α1→3 core-linked fucose (25, 26). PNGase At, like PNGase A, does not hydrolyze intact glycoproteins efficiently but prefers glycopeptide substrates.

For two enzymes performing the same catalytic function, it is striking that PNGase At and PNGase F are so dissimilar; they have no primary structural homology, their molecular weight and subunit structure are very different, and their physical properties and specificity are different. Among the large endoglycosidase family of enzymes, for example, one always sees clear indications of relatedness in structure and function, but the glycoamidases PNGase F and PNGase At appear to have developed along different evolutionary lines. What path a mammalian enzyme would follow is unclear at this time.

We are currently developing a high expression system to study PNGase At. Unresolved questions will require a demonstration of the putative primary translation product and its conversion to α- and β-subunits. X-ray crystallographic analysis of PNGase At and comparison with PNGase F will be important for investigating the development of the active center of these glycoamidases.

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