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Carbohydrate Research 337 (2002) 803–812

CARBOHYDRATE RESEARCH

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Studies on the glycosylation of wild-type and mutant forms of *Aspergillus niger* pectin methylesterase

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Received 31 October 2001; accepted 30 January 2002

Abstract

Pectin methylesterase (PME) is one of a number of enzymes released by the fungus *Aspergillus niger* that are involved in the degradation of specific plant cell-wall structures. PME is a glycoprotein with three potential sites for N-linked glycosylation. The glycosylation may affect the hydrolytic activity or the substrate specificity of PME. In this work, we investigate first the structures and the attachment sites of the glycans present on recombinant wild-type PME. Further, a series of PME mutants was created in which the three potential N-linked glycosylation sites were eliminated in all possible combinations. The glycosylation of the mutants and their activities were then studied. Mass spectrometric techniques tailored for carbohydrate analysis were applied to both characterize the glycan structures and to determine the specific sites of attachment. High mannose structures with variable numbers of mannose were found on the wild-type, as well as the mutant forms. Studies using the mutants suggest that glycosylation does not strongly influence the activity. Whether it may affect the substrate specify of the enzyme is unknown, and that aspect will be explored in future work.

Keywords: Pectin; Methylesterase; Glycosylation; Mass spectrometry; *Aspergillus niger*

1. Introduction

Proper growth and development of phytopathogenic and saprophytic fungi, such as the black filamentous fungus *Aspergillus niger*, requires absorption of nutrients from organic materials. Fungi must absorb rather than ingest their nutrients; therefore, the complex structures found in plants must first be decomposed to simpler compounds that the fungus can absorb and use.[1]

The absorption process is enabled by secretion of a diverse set of enzymes that specifically target the structures, such as pectin, which constitute the plant cell wall. Pectin is a set of complex heteropolysaccharides that are structurally related and include homogalacturonan, and rhamnogalacturonan I and II (RGI and RGII). The backbone of each pectic polysaccharide can be substituted in a variety of ways. Galacturonic acid (D-GalA) residues within the homogalacturonan regions may be acetylated at the C-2 and C-3 hydroxy groups and are likely to be esterified to various degrees at C-6 with methoxy groups. The methylation is thought to occur in a non-random order, with blocks of polygalacturonic acids completely methyl esterified.[2] RGII may also be methyl esterified, whereas RGI may be acetylated or substituted with neutral and acidic sugars, which results in branching of these structures.

The first and most abundant set of enzymes deployed by *A. niger* target the homogalacturonan regions of pectin. These enzymes include several types of polygalacturonases (PGs), pectic lyases (PLs), and pectic esterases. The pectic esterases such as pectin methylesterase (PME) play a subtle yet important role in pathogenesis. These enzymes catalyze the deesterification of methoxy groups within pectin to form regions

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0008-6215/02/$ - see front matter
PII: S0008-6215(02)00032-0

of homogalacturonan with degrees of esterification favorable towards PG and PL activity.[3]

The destructive nature of pectin-degrading enzymes comes at great cost to the agronomic sectors; however, other industries have exploited their properties. As a result, the fungal pectinases, most notably those from *A. niger*, have gained substantial commercial value. *A. niger* pectinases are widely used in the food processing, fruit juice, and wine industries. They are added to macerate fruit to increase product yield, and are also used to control the viscosity of juices to obtain a desired consistency and to ease handling and processing.[4] In 1995, the industrial enzyme market totaled one billion dollars of which approximately 10% resulted from sales of pectinases.[5]

A common structural feature in many of the fungal pectinases characterized to date is glycosylation. Attachment of the glycans to the protein occurs via the nitrogen of asparagine (N-linked) or the oxygen (O-linked) of serine and threonine. Both N-linked and/or O-linked carbohydrates have been observed in several homologously and heterologously expressed PG and PL genes of *A. niger*. Two PGs, endopolygalacturonase I and II, were shown to contain high mannose glycans attached to a single site, a feature that was maintained upon altering the growth conditions during the overexpression.[6–8] Other *A. niger* pectinases such as PGA, PGC, and PL also exhibit high-mannose N-linked as well as putative O-linked carbohydrates.[9–11] Researchers have placed considerable emphasis on characterizing the glycans of pectinases due to the potential effects of glycosylation on the protein structure and activity and for quality control of recombinant enzymes.

The research described herein focuses on the glycosylation of recombinant *A. niger* pectin methylesterase. *A. niger* PME is a protein composed of 331 amino acids. The molecular mass of the mature peptide is 33,297 Da as shown in the sequence in Fig. 1. Three sequons for potential N-linked glycosylation were found initiating at asparagine residues 95 (N95), 283 (N283), and 302 (N302). Mutation of the N-linked sequon, at the asparagine, serine or threonine, precludes glycosylation at that site and offers a way to measure the importance of glycosylation (by its absence) to specific properties of the protein such as activity. Prior to such activity studies, the absence of glycosylation at the mutated site must be confirmed, and the presence and structures of glycans on the remaining sites must be determined. MALDIMS and ESIMS were used to analyze seven mutants of PME that were altered at each of the potential mutation sites, singly and in combination.

2. Experimental

Preparation of PME: strains, plasmids, and growth conditions.—Wild-type and mutant PMEs from *A. niger* were prepared by homologous overexpression. The recombinant non-mutant form is herein referred to as wild-type PME. Mutant forms of PME resulted from mutation of asparagine to glutamine and are notated as

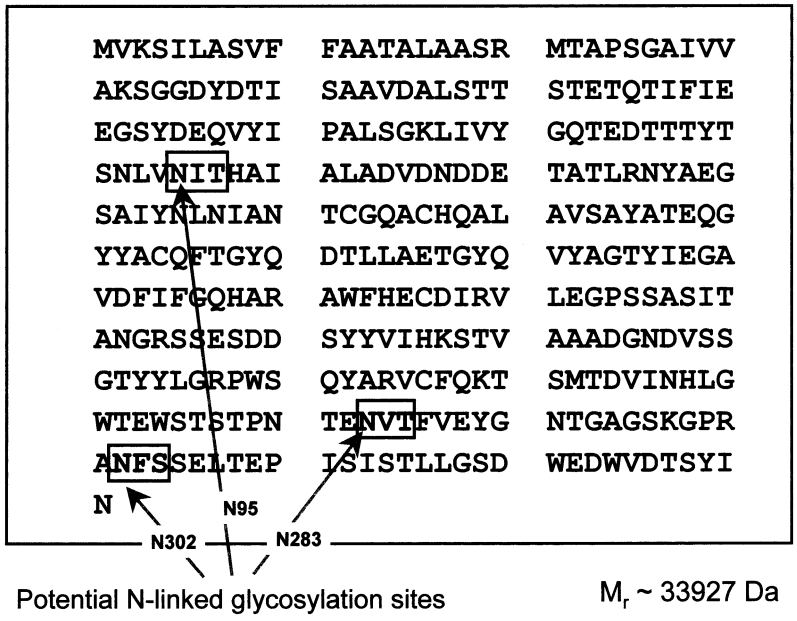

Fig. 1. Amino acid sequence of pectin methylesterase from *A. niger* and the potential sites for N-linked glycosylation. Asparagines contained in the glycosylation sequon NXS/T are numbered relative to their positions from the full protein sequence. The 'protein' molecular mass and theoretical tryptic fragment masses are measured using the sequence of the mature peptide (33,927 Da), which excludes the first 13 residues of the full protein sequence.

N(residue no.)Q, i.e. N95Q. The *A. niger* strain NW290 (*pyr*A6, *prt*F28, *gox*C17, *fwn*A12, Δ*arg*B, Δ*pga*B + argB nig, Δ*pga*A + *arg*B nid) was used for transformation. The strain was grown in minimal medium containing per liter: 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g KCl, and trace elements[12] supplemented with 0.5% yeast extract, 0.2% casein amino acids, 1.22 g uridine, and 2% glucose. The pH was adjusted to 6.0.

For a selection of (mutated) PME-producing transformants, the cultivation was performed in minimal medium supplemented with 0.1% yeast extract and 1% fructose, pH 6.0. As a nitrogen source NH_4Cl (4 g/L) was used instead of $NaNO_3$. For large-scale enzyme production of the transformants producing either wild-type or mutant PME forms, a minimal medium containing NH_4Cl as the nitrogen source, 0.005% yeast extract and 2.5% fructose at pH 4.5 was used. Cultivation was performed in 3-L jacketed stirred tank reactors (Applikon, Sweden). Air-saturated culture medium was inoculated with spores (see below). The spores were allowed to germinate for 6 h at low stirring speed (400 rpm) after which the cultivation was continued for 18 h at 750 rpm. The culture was maintained at pH 4 by the addition 5 M NaOH. The cultures were sparged with air (2 v.v.m.), and 0.5 mL polypropylene glycol (30% in EtOH) was added per liter of medium as antifoam. In all cases the liquid cultures were inoculated with spores to a final concentration of 10^6 spores/mL, and growth was performed at 30 °C.

Site directed mutagenesis of PME.—A transcriptional fusion between the promoter of the glycolytic gene *pkiA* (encoding *A. niger* pyruvate kinase) was constructed in a similar way as described previously for the endopolygalacturonase encoding genes[13,14] and resulted in plasmid pIM3850. This construct allows the expression of PME during growth on a glycolytic carbon source. Glucose or fructose represses the expression of other pectinolytic genes, including *pmeA*, at the wild-type locus. Desired mutations, N95Q, N283Q, N302Q were generated by PCR using the QuickChange™ site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA). For each mutation introduced, the *pki–pme* gene-fusion construct was sequenced entirely to establish the expected mutation and to check for undesired mutations. Subsequent rounds of mutagenesis introduced the various combinations of mutations (double and triple mutations).

Transformation and selection of PME-producing transformants.—*A. niger* strain NW 290 was transformed as described in previous work[15] using 1 μg of the selection plasmid pGW635 and 40 μg of the co-transforming plasmid encoding either wild-type or mutated PME. For each transformation 20 colonies were picked randomly and used to inoculate 1 mL of the minimal medium. After growth for 24 h at 30 °C, 40 μL of the culture fluid was mixed with 500 μL 1% (w/v) lemon pectin [degree of methyl esterification (DM) = 75%] in a solution containing 0.15 M NaCl, 10 mM $CaCl_2$, and the pH-indicator, Methyl Red. The pH was adjusted to 6.0 by adding 8 μL of 8% (w/v) NaOH. Removal of the methyl esters by PME activity results in formation of uronic acids, which results in a decreased pH. The transformants that produced the highest amount of (mutated) PME were selected by monitoring red coloration and gelation of the solution as a result of PME activity. Those transformants selected were subjected to a second screening in which the transformants were grown in 50 mL minimal medium for 24 h at 30 °C and 250 rpm. Enzyme production was monitored by SDS–PAGE with subsequent Coomassie Brilliant Blue R250 staining. For the wild-type and each mutated PME, the highest producing strain was selected and used for further enzyme purification.

Enzyme assay and protease sensitivity.—The activity of PME was routinely assayed using an automated titrator (Radiometer, Denmark) in 15 mL of a 1% lemon pectin solution containing 0.15 M NaCl at 40 °C and pH 4.8. The DM of the pectin = 75% (CPF, Copenhagen, Denmark). The principle of the assay is based on the acid released during hydrolysis of the methyl ester. A pH of 4.8 was maintained during the assay. After addition of the PME sample, the rate of titrant (0.0625 M $NaHCO_3$) consumption was monitored. Pectin esterase activity was calculated from the slope of the consumption of titrant. A titrant consumption of one μequiv base/min corresponds with one unit of PME.

The protein concentration of the purified enzymes was estimated by spectrophotometry, using the calculated molar absorption coefficient of 61740 M^{-1}/cm for wild-type pectin esterase.[16]

The purified PMEs were tested for protease sensitivity by incubation in a crude culture filtrate of *A. niger* N402 grown on wheat bran containing medium that has been shown to be optimized for protease production.[17] The enzymes were diluted (0.2 mg/mL) in 0.2 M NaOAc buffer, pH 4.5, or 0.2 M Na citrate buffer, pH 2.8, then 200 μL was mixed with 50 μL of the culture filtrate. The mixtures were incubated for 20 h at 30 °C, after which time the residual pectin esterase activity was determined. Blanks were treated identically but frozen immediately after mixing. *A. niger* pectin lyase B, which is sensitive to proteolytic degradation by *A. niger* extracellular proteases,[17] was used as a positive control.

Purification of the enzymes.—Culture fluid (2.5 L) was collected by filtration, diluted twofold with water and adjusted to pH 6.0. Proteins were collected by batchwise adsorbtion. For this, 50 g Streamline Q XL (Amersham Pharmacia Biotech, Sweden) was added, stirred for 2 h at 4 °C, followed by collection of the matrix by filtration. Bound proteins were eluted by 0.01

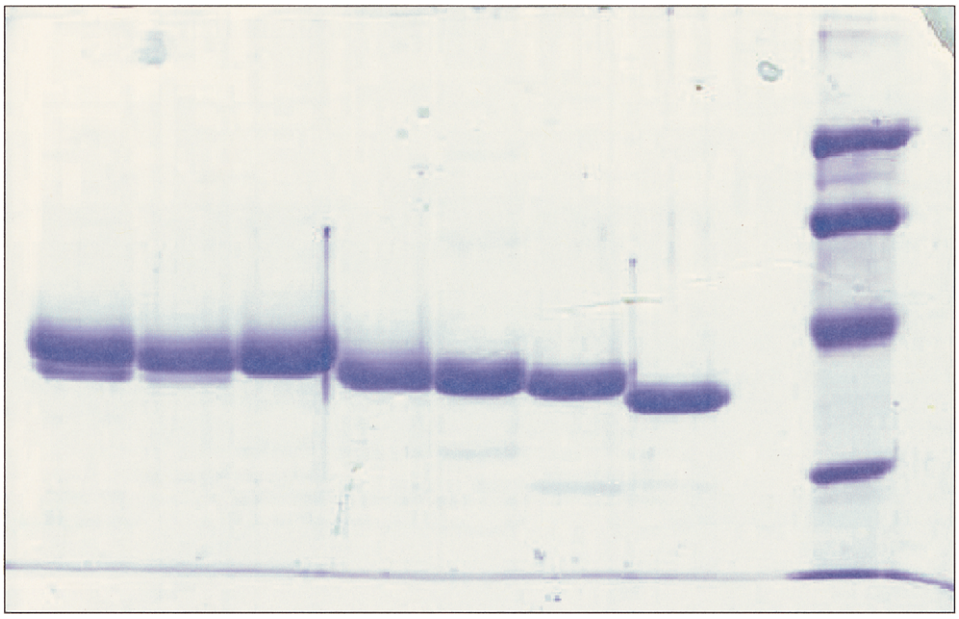

Fig. 2. SDS–PAGE of purified mutant pectin methylesterase. Lanes from left to right: N95Q, N283Q, N302Q, N95Q N283Q, N95Q N302Q, N283Q N302Q, N95Q N283Q N302Q. Markers: 92.5, 67, 45, and 29 kDa.

M piperazine·HCl, 1 M NaCl, pH 6.0, and dialyzed against 0.01 M piperazine·HCl, pH 6.0. Next, the dialysate was loaded onto a Source 30 Q column (Amersham Pharmacia Biotech, Sweden, 16 mL), equilibrated in 0.01 M piperazine·HCl, pH 6.0, and eluted with a 200 mL of a linear gradient of 0–0.4 M NaCl in buffer. The collected fractions (5 mL) were assayed for pectin esterase activity, and purity was tested by SDS–PAGE (Fig. 2). Fractions containing pure enzyme were pooled, dialyzed against 0.05 M NaOAc buffer (pH 5.0), and stored at -20 °C.

Strategies for glycan analyses.—Fig. 3 depicts the general experimental strategy for the glycan analysis of the wild-type and mutant forms of PME. The strategy utilizes MALDI-TOFMS, ESIMS on a quadrupole time-of-flight (QTOF) instrument, HPLC, and biochemical techniques such as glycosidic and proteolytic digestions. The analyses of both wild-type and mutant forms followed the course directed by the single-lined arrows. The proteins were first analyzed by MALDIMS to measure the molecular mass of the intact protein–glycoprotein. A portion of each sample was subsequently digested with an endoglycosidase. The mass of the deglycosylated protein was measured by MALDIMS to determine, by difference, the mass of the glycans removed and to ensure the absence of O-linked glycosylation. The remaining portions of the samples were treated with an endoprotease to digest the proteins into peptide–glycopeptide fragments. Finally, the resulting fragments were scrutinized by MALDIMS, as well as by LC–ESIMS on the QTOF. The wild-type PME sample diverges from this course by inclusion of an LC separation step as shown by the double lined-arrows. This step was necessary since the preceding MALDI analysis revealed that wild-type PME contained two glycoforms.

MALDI-TOF measurements.—MALDIMS data were obtained using a Hewlett–Packard G2030A (Palo Alto, CA) MALDI-TOF mass spectrometer. The instrument was operated at a pressure of $\sim 1 \times 10^{-6}$ Torr, 28 kV accelerating voltage and 7 kV extractor voltage. The samples were desorbed and ionized from the probe tip by UV radiation emitted from a nitrogen laser at an output wavelength of 337 nm. Sinapinic acid (Aldrich) was used as the matrix for the protein samples, while α-cyano-4-hydroxycinnamic acid was used for the tryptic digestions. Both matrices were dissolved in 70% aq MeCN (Sigma) with 0.01% trifluoroacetic acid (Aldrich) at a concentration of 10 mg/mL.

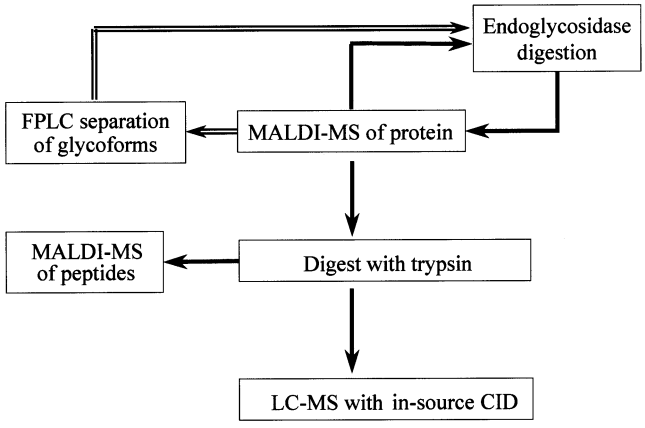

Fig. 3. Experimental outline used to study the glycosylation of wild-type and mutant pectin methylesterase.

Table 1
Results of the purification of *A. niger* wild type and mutant pectin methylesterase

Enzyme	Amount protein (mg)	Protein concentration (mg/mL)
Wild type	53	0.83
N95Q	41	2.05
N283Q	32	2.13
N302Q	94	3.75
N95Q, N283Q	58	2.9
N95Q, N302Q	21	2.07
N283Q, N302Q	23	1.56
N95Q, N283Q, N302Q	20	1.34

LC separation of the wild-type PME glycoforms.—The two glycoforms found in the wild-type PME sample were separated by liquid chromatography using a BioRad (Hercules, CA) Uno Q column (7 × 35 mm), buffers A (20 mM HEPES, pH 7.5) and B (20 mM NaOAc with 0.5 M sodium chloride) at a flow rate of 0.5 mL/min. The proteins were separated using an optimized gradient of 20–26% buffer B for 70 min. Fractions collected at 1-min intervals during the gradient were analyzed by MALDIMS for purity and pooled to contain only one glycoform/pool. The pools of the separated 38 and 40 kDa glycoforms were each concentrated by speed-vac and dialyzed against water using a Millipore (Bedford, MA) 5000 MWCO Microcon cellulose filter.

Endoglycosidase digestions.—To quickly screen for N-linked glycans, the samples were digested with specific endoglycosidases. In previous experiments using PGI and pectate lyase, we have proven that glycosidase digestions using both exo- and endoglycosidases can be performed directly on the MALDI sample target, thus facilitating the deglycosylation process and analysis.[8,18] Partial removal of the glycans from the protein was achieved by combining 1 μL of protein solution with 0.3 μL (1:8 dilution of purchased stock solution) of endoglycosidase-H (Endo-H) (Prozyme Inc., San Leandro, CA) on the MALDI target. To maintain solvation of the digestion mixture, 1 μL of water was added intermittently during the 20-min digestion. The matrix solution (0.5 μL) was then added to the mixture prior to MALDIMS analysis of the deglycosylated protein.

Proteolytic digestions.—Proteolytic digestion was carried out in 50 mM NH_4HCO_3 (Aldrich) using sequencing grade trypsin (Promega, Madison, WI) with ~20 μg of PME at a 1:50 ratio of trypsin to PME. The proteins were digested for 24 h at 37 °C.

LC–ESIMS using stepped-orifice voltages.—An aliquot of the digested sample (5 μg) was injected into a Phenomenex Jupiter C-18 column (50 × 1 mm). Reversed-phase separation was achieved by flowing buffers A [0.1% (v/v) HCOOH] and B [80% (v/v) MeCN, 0.1% HCOOH] at a gradient of 1% B/min from 10–60 % B. A Hewlett–Packard 1100 LC system delivered the solvents at a flow rate of 30 μL/min and measured the absorbance of the digest components at 214 nm. The effluent was sprayed into a quadrupole time-of-flight mass spectrometer (Micromass QTOF2; Beverly, MA) and ionized in positive-ion mode. Two acquisitions were programmed into the mass spectrometer software. In function 1 the cone voltage is set to a highly energetic value (70 V) to promote fragmentation of the glycopeptides and peptides in the effluent. A range of 50–500 m/z was detected during this function to monitor carbohydrate-containing species that fragment to form ions commonly observed at 163, 204, and 366 m/z.[19–21] During function 2 the cone voltage was lowered to a less energetic value of 30 V as the instrument detects an m/z range (m/z 501–2000), a range suitable for detection of intact peptides and glycopeptides.

3. Results and discussion

Transformation and purification of PME.—PME was the most abundant protein in the culture fluid; therefore, only one column chromatographic step was required to purify the enzyme. This is a direct result of growth of the transformed fungus on fructose, which facilitates a high expression of the pectin methylesterase gene under the control of the pyruvate kinase promoter and repression of other pectinases.[22]

Table 1 summarizes the purification of wild-type and mutant pectin methylesterase. As shown in Table 1, the amount of protein isolated from the culture fluid varies, possibly due to differences in the number of gene copies integrated into the genome for the different transformants. We have yet to investigate the relationship between copy number and expression level.

Glycan analysis of wild-type and mutant PME.—The first indication of the presence and extent of glycosylation was found by comparing the predicted protein mass calculated from the amino acid sequence (Fig. 1) to the measured masses obtained by MALDIMS (Table 2). The measured masses of the two forms of the wild-type and for the single and double mutants were substantially greater than the predicted mass. MALDIMS revealed the presence of two forms of the wild-type PME with measured masses of 38,192 Da (PME 38) and 40,487 Da (PME 40), which are approximately 4000 and 6000 Da greater than predicted. Such large mass differences are typically attributed to N-linked glycosylation. All N-linked glycans are composed of a pentasaccharide $Man_3GlcNAc_2$ core and are

typically found as extended structures of this core within a heterogeneous population. The mass of the core is 892 Da, and we have observed that a singly occupied site contains glycans with an average mass of ~2000 Da. Thus we hypothesize that the glycans on PME 38 and PME 40 occupy 2 and 3, respectively, of the three available sites for N-linked glycosylation.

In the case of the triple mutant form of PME, there was only a slight (120 Da) difference between the measured and predicted masses, suggesting an absence of glycosylation due to mutation of the asparagines at all three sites. The degree of difference is not large enough to suggest O-linked glycosylation and is within the error of the MALDI-TOF instrument. Only one protein form was observed for the double mutants. The measured masses of the double mutants averaged 2000 Da greater than the predicted mass, a difference proportional to the average mass of N-linked glycans occupying a single site. Unlike the double mutants, yet similar to the wild-type PME, two glycoforms were observed for each of the single mutants.

Further evidence of glycosylation was found by on-probe deglycosylation of wild-type and mutant PMEs. Each sample, except the triple mutant, was digested by Endo-H to produce a peak corresponding to the mass of the protein plus a residual GlcNAc from the glycan core, which is not cleaved by Endo-H. These results simultaneously confirm the presence of N-linked structures and narrow the possible type of glycan structures to either the high-mannose or hybrid types, for which Endo-H is specific.

In order to specifically identify which of the three potential N-linked sites were glycosylated, digestion of the protein was required so that each of the sites is localized within separate (glyco)peptides. Trypsin was chosen as the proteolytic agent based on results from *in silico* digestion of the PME sequence in which the three N-linked sites at residues 95, 293, 302 were contained within separate peptides consisting of residues 77–115 (4269 Da), 260–297 (4133 Da), and 301–331 (3488 Da), respectively.

Ninety-five percent of the predicted protein sequence was mapped using the tryptic peptide masses (from the triple mutant) measured by MALDI. In all cases except the triple mutant, we were also able to observe series of six or more ions increasing by 162 m/z units at higher m/z ranges (Fig. 4). These series are characteristic of N-linked glycosylated peptides with each unit of the series containing an additional mannose (m/z 162) residue as found in high-mannose or hybrid N-linked glycans. In the digests of the mutants, a peptide with a mass corresponding to that containing the mutated site(s) was also observed, thus confirming that the mutation was successful and that the mutated site is, not unexpectedly, free of glycosylation.

The specific carbohydrate structures and their sites of attachment to the protein were determined through LC–MS (with in-source CID) of the tryptic digests on the QTOF. Glycan-containing peptides were readily found during the chromatographic separation by using the stepped-orifice voltage technique originally described for a triple-quadrupole instrument.[19,20] It was suggested that this technique is transferable to all single quadrupole instruments, including the first mass analyzer of the hybrid QTOF. Extension of this technique for use on the QTOF was relatively straightforward

Table 2
The molecular masses of PME and the potential number of occupied N-linked sites as determined by MALDIMS

Sample	Observed molecular mass (Daltons)	Mass difference from predicted molecular mass (Daltons)	Potential number of glycosylated N-linked sites
Wild-type PME			
PME40	40,487	6560	3
PME38	38,192	4165	2
Mutant PME			
N95Q	38,484	4556	2
	36,625	2697	1
N283Q	37,890	3962	2
	35,565	1637	1
N302Q	37,671	3743	2
	35,850	1922	1
N95Q, N283Q	36,493	2565	1
N95Q, N302Q	35,951	2024	1
N283Q, N302Q	35,208	1280	1
N98Q, N283Q, N302Q	34,048	120	0

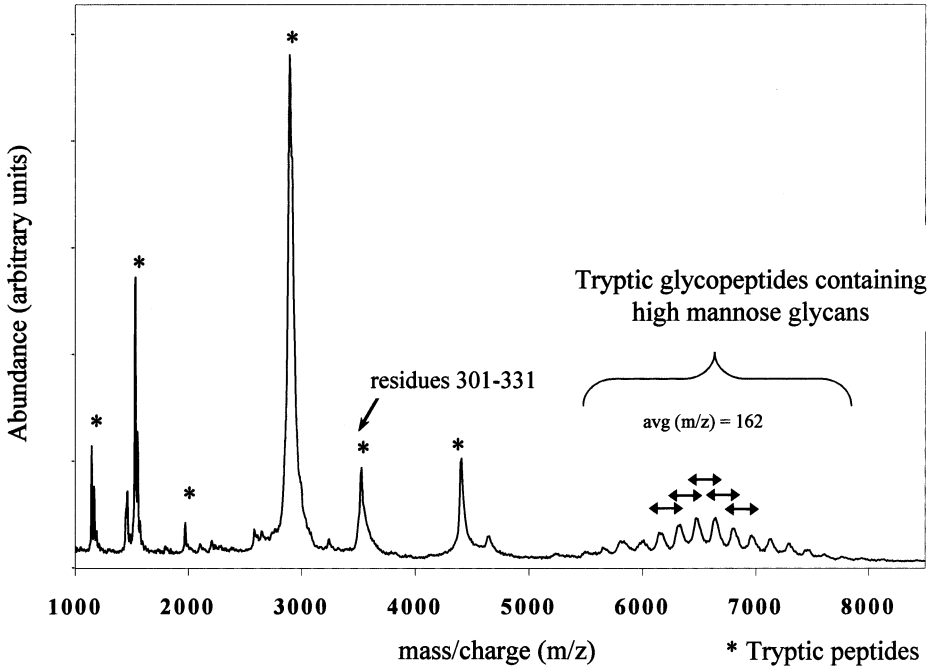

Fig. 4. The MALDI mass spectrum of the tryptic digestion of a single mutant of PME (N302Q). Approximately 1 pmol of sample was analyzed using α-cyano-4-hydroxycinamic acid as the MALDI matrix.

with only minor differences in the data acquisition and instrumental settings. Cone voltage settings were chosen from preliminary optimization tests on purified standard glycans and glycopeptides fragmented at variable cone voltages. At the optimal cone voltage value of 70 V, strong carbohydrate fragment ion signals were observed. Lower cone voltages resulted in a reduction in the fragment-ion signals, whereas higher cone voltages had either no effect on or degraded the signal intensity.

Data and results from a single sample, the mutant N302Q, are presented and outlined in Fig. 5(A–D). The data shown are representative of the wild-type and all of the mutant samples except the triple mutant. Ions detected by the mass spectrometer are plotted in two total-ion chromatograms (TICs), one for each cone voltage setting (Fig. 5(A)). The TICs share similar profiles and reveal digest components as peaks eluting between 29 and 80 min. An average spectrum of the high cone voltage TIC was obtained for this time frame and surveyed for the three specific fragment ions commonly observed for high-mannose and hybrid glycans found at m/z 163, 204, and 366 (Fig. 5(B)). The specific location of the glycopeptides within the experiment was then found by extracting the mass of each carbohydrate fragment ion from the high cone-voltage TIC. This results in ion chromatograms that revealed only the portions of the TIC (as peaks) that contained glycans, which in this case centered around 35 and 36 min (Fig. 5(C)). Masses of the intact glycopeptides were determined by obtaining an averaged spectrum of the low cone voltage TIC only at 35 and 36 min as shown in Fig. 5(D). Each averaged spectrum produced two series of peaks. Ions from the lower molecular mass series differed by m/z 40, while those of the higher molecular mass differed by m/z 32.5, corresponding to glycopeptides containing a series of mannoses with $+5$ and $+4$ charges, respectively. These masses were determined by deconvolution of the multiply charged species to their singly charged forms.

Once the glycopeptide masses were determined, the glycan structures and their sites of attachment were easily calculated. Initially, this information was found by manual calculation of the deglycosylated peptide mass. Both MALDI and QTOF data had consistently indicated that the attached glycans were of the high-mannose type. Furthermore, we had observed only oligomannose structures on other *A. niger* pectinases. Based on these assumptions the masses of known high mannose structures, i.e., Man 5–Man 10, were subtracted from the glycopeptide masses to obtain the mass of the deglycosylated peptide. One of the six resulting masses should match the mass of a theoretical tryptic fragment based on the amino acid sequence of PME. The matching mass also reveals the carbohydrate structure attached. These calculations are shown in the N302Q sample (Fig. 6). A series of glycopeptide masses was first determined by deconvolution of the ESI data. The average mass difference between adjacent glycopeptides was 162 Da. Therefore, only the first glycopeptide in the series was analyzed since each

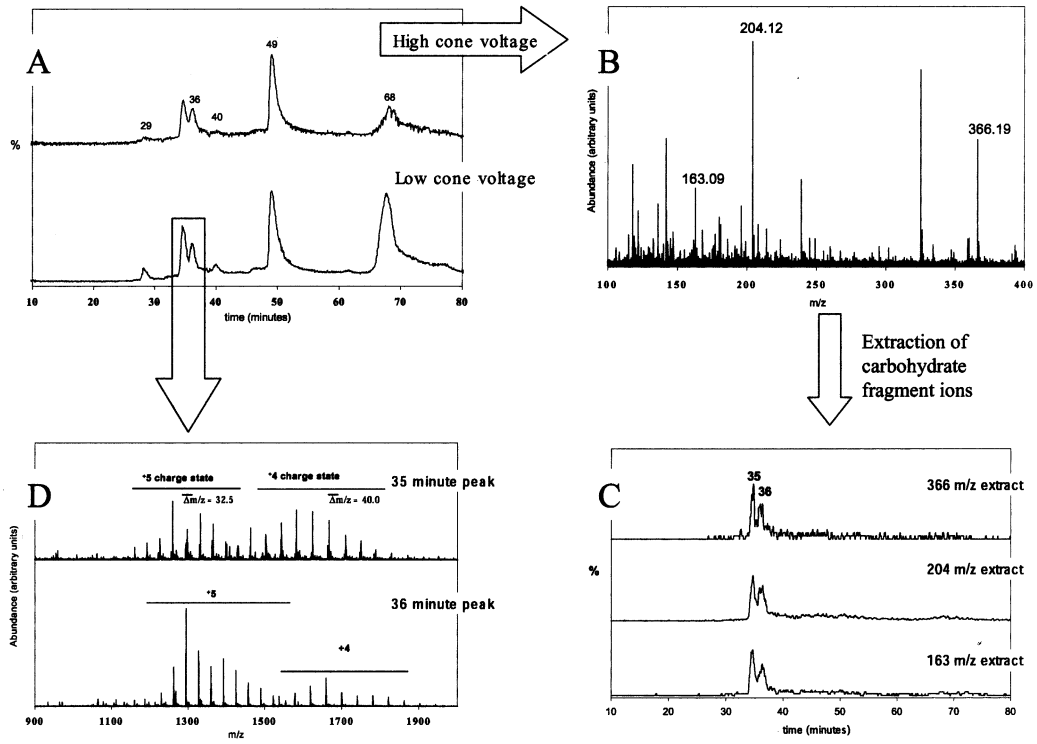

Fig. 5. The results of the LC–ESIMS experiment on the QTOF for mutant N302Q and the schematic of data analysis procedure used to determine the glycan structures and attachment sites. Figures shown are (A) total-ion chromatograms (TIC) at high and low cone voltages; (B) average spectra of high cone voltage scan; (C) ion chromatograms after extraction of carbohydrate fragment ions; and (D) ESIMS of 35 and 36 min peaks from low cone voltage. Refer to text for description of data analysis procedure.

subsequent glycopeptide contains an additional mannose residue. The masses of Man 5–Man 10 glycans were subtracted from the first glycopeptide mass (6148.85 Da) yielding six masses, one of which corresponds to an aglycosylated tryptic peptide of PME. The peptide with a calculated mass of 4446.85 Da matched a theoretical tryptic peptide (4447.15 Da) comprised of residues 260–300. This residue contains one (N283) of

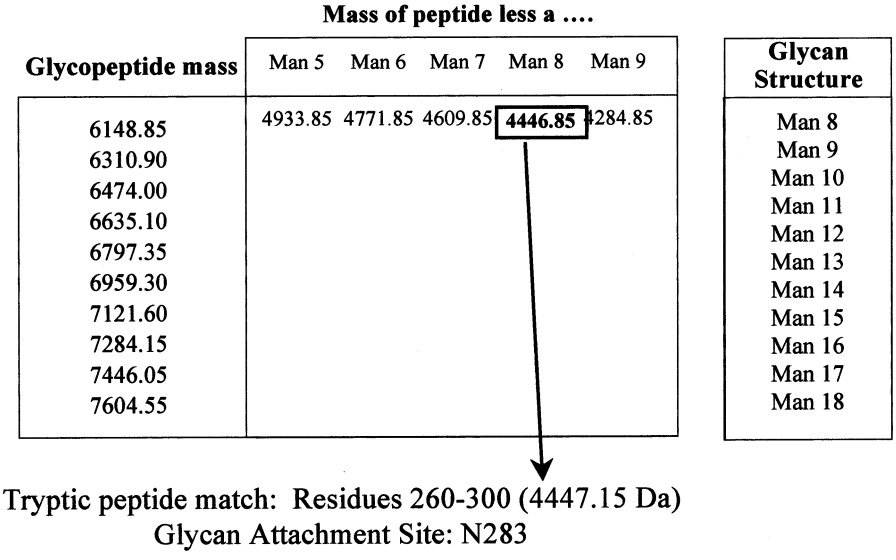

Fig. 6. Manual calculation of N302Q glycan structures and sites of attachment. Glycopeptide masses were determined by deconvolution of the ESI spectrum. The masses of the peptides attached to the glycans were determined by subtraction of Man 5 to Man 9 masses to yield masses that may correspond to a tryptic peptide of PME containing an N-linked site.

Table 3
The glycan structures and attachment sites of wild-type and mutant PME as determined by LC–ESIMS

Sample	Carbohydrate structure	Attachment site
Wild-type PME		
PME40	Man 5–9	N95
	Man 8–17	N283
	Man 7–15	N302
PME38	Man 8–16	N283
	Man 7–15	N302
Mutant PME		
N95Q	Man 5–14	N283
	Man 7–17	N302
N283Q	Man 5–9	N95
	Man 7–18	N302
N302Q	Man 7–17	N283
	Man 6–13	N95
N95Q, N283Q	Man 7–18	N302
N95Q, N302Q	Man 8–13	N283
N283Q, N302Q	Man 5–9	N95
N98Q, N283Q, N302Q	No evidence of glycosylation	

the two non-mutated asparagines. The matching peptide mass was that in which the Man 8 structure was subtracted from the glycopeptide mass. Thus, we deduce that this first glycopeptide contains a Man 8 structure attached to N283. Additional glycopeptides in the series are higher order mannose structures (Man 9–18).

The results from the manual calculation were confirmed through the use of the Glycomod Tool™ provided through the EXPASY website.[23] Glycopeptide masses were entered, and the program queried these masses against those of known carbohydrate structures within a database. Limitations such as type of linkage (N- or O-linked), potential modifications (acetylations, etc.), and derivatizations provided a more accurate and rapid search. We limited our searches to N-linked glycans and entered the protein sequence and the masses of the first glycopeptides. The results confirmed those obtained by the manual calculations. All of the PME samples were analyzed in this manner, the results of which are shown in Table 3. Only high-mannose glycans ranging from Man 5–Man 20 structures were observed. For the wild-type PMEs, PME 40 was glycosylated at all three potential N-linked sites with high mannose structures, whereas PME 38 contained similar glycans only on N283 and N302. The absence of glycans on N95 of the PME 38 form was also substantiated by the appearance of a tryptic peptide that contained the N95 residue. The glycans of the mutant PME samples were found attached only to the non-mutated residues, providing evidence that the asparagine mutations were successful, since this type of glycosylation occurs only on asparagine.

PME activity and protease sensitivity.—Comparison of the specific activity of the wild-type and the mutant enzymes, as shown in Table 4, demonstrates that the loss of one, two or all three N-glycosylation sites had almost no effect on the enzymatic activity. Furthermore, incubation of this set of PME at pH 4.5 with culture fluid containing a full spectrum of proteases did not have any effect on the activity of the PME. Even at pH 2.8, which is the optimal pH for acidic proteases abundantly produced by *A. niger*, both the wild-type enzyme and the fully deglycosylated enzyme were resistant to proteolytic degradation. In comparison, incubation of the pectin lyase B control at pH 2.8 with the culture fluid resulted in a 1% residual activity after 1 h.

4. Conclusions

Only slight differences in the activities and protease resistance of wild-type and mutant PMEs were observed, thereby suggesting that glycosylation of PME does not appear to play a major role in these properties. Despite these results, the information gained through the glycan analysis of PME wild-type and mutant forms can be useful for quality control in production processes.

Table 4
Wild type and mutant PME: results of activity and protease sensitivity assay (determined by residual activity)

Enzyme	Protein concentration (mg/mL)	Specific activity (U/mg)	Residual activity at pH 4.5 (%)	Residual activity at pH 2.8 (%)
Wild-type	0.83	968	102	101
N95Q	2.05	1020	105	n.d.
N283Q	2.13	906	104	n.d.
N302Q	3.75	976	103	n.d.
N95Q, N283Q	2.9	979	104	n.d.
N95Q, N302Q	2.07	967	102	n.d.
N283Q, N302Q	1.56	823	106	n.d.
N98Q, N283Q, N302Q	1.34	879	104	101

We have shown that wild-type PME is either fully glycosylated (PME 40) or is absent of glycosylation at the N95 site (PME 38). The fully glycosylated protein appears to be the major (~ 90%) product formed; thus, the activity of wild-type PME may be predominantly based on the activity of the fully glycosylated form.

Furthermore, we have characterized the glycans, if present, of all the forms present from the triple and double mutations and partially characterized the glycans of the single mutants. Mutation of all three sites results in the absence of N-linked glycosylation and no evidence of O-linked glycans. Mutation of two of the three possible N-linked sites produced one glycoform, which was glycosylated only at the non-mutated site. However, mutation of one of the three possible sites as in the single mutants produced two glycoforms that contained carbohydrates on one or both of the remaining non-mutated sites as shown in Table 3. Although we have determined that the mutated site is not glycosylated, and only high mannose glycans are present, we are unable to determine which of the two non-mutated sites is not glycosylated in one of these glycoforms. The presence of two glycoforms suggests that there is no preference for complete glycosylation at all possible sites. If this preference existed, we would expect to see only one glycoform for the single mutants. Interestingly, we did not observe aglycosylated forms of the single or double mutants. It appears that the enzyme prefers to retain glycans on at least one site.

Acknowledgements

The authors acknowledge and are grateful for financial support of this research provided by the National Institutes of Health (NIH grant number P41RRR05351), the North Atlantic Treaty Organization (NATO grant number CRG973086), the Department of Energy (DOE Center for Plant and Microbial Complex Carbohydrates, DOE grant number DE-FG02-93ER20097, and DOE grant number DE-FG02-96ER20221) and the National Science Foundation (NSF grant number 9626835).

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