MALDI and ESI mass spectrometry have been invaluable techniques for the analysis of a wide range of biomolecules including peptides, proteins, carbohydrates and glycoproteins. Although used extensively, the relatively new techniques still require improvements in the sample preparation process. These improvements are necessary to enhance the efficiencies and broaden the applications of MALDI and ESI towards problematic biomolecular samples. In addition, existing analytical techniques should be evaluated, modified and applied to study different biological systems. The scope of this dissertation involves an improvement to peptide and protein analysis by MALDI -MS and application of existing techniques of MALDI and ESI for studies involving a fungal enzyme.

Biomolecular samples can contain a variety of compounds, which at moderate or elevated levels interfere with their analysis by MALDI-MS. MALDI sample probes modified with self-assembled monolayers containing ionic terminal groups were developed and evaluated as an on-line purification or sample-cleansing device. Various types of probes were successful in removing several types of common buffer components or contaminants. These probes also exhibit useful characteristics for routine analysis.

MALDI and ESI- LC-MS were also used in studies of recombinant forms of Aspergillus niger pectin methylesterase. The specificity of the enzyme towards substrates, which contained multiple sites of potential activity was investigated. The specificity was
determined by structural analysis of the products formed as a result of the enzymatic activity of pectin methylesterase. Furthermore the enzyme itself is a glycoprotein. The structures and location of these carbohydrates were determined primarily using LC-ESI-MS as a carbohydrate-specific detection technique.

INDEX WORDS: Mass spectrometry, Tandem mass spectrometry, MALDI-TOF, ESI, QTOF, MS/MS, Glycosylation, Carbohydrate sequencing, Pectinases, Sample preparation, On-probe, On-target
BIOANALYTICAL APPLICATIONS OF MASS SPECTROMETRY:
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FOR ENHANCED MALDI-TOF-MS AND
EXAMINATION OF THE SUBSTRATE SPECIFICITY AND GLYCOSYLATION OF
A. NIGER PECTIN METHYLESTERASE BY ESI-MS

by

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DEDICATION

I dedicate this dissertation to the most important people in my life.

To Tony......................... my husband, best friend and the most intelligent person I know who inspires me to be a better person yet loves me for who I am.

To Hannah..................... my beautiful daughter whose smile and laughter remind me every day the true meaning of love and happiness.

To my parents.................. my role models whom I can never repay for the love they've given and the generosity they have shared.

To my brother & sisters.... my family and my friends. Thank you for all the love and encouragement you've given me. I share this blessing with all of you.
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CHAPTER I

INTRODUCTION
Perhaps there is no singular characteristic physical property of a biomolecule that is more important than its molecular mass. A simple, yet accurate, molecular mass harbors a wealth of information regarding the chemical and physical properties of all types of biomolecules. This information includes the inter and intramolecular interactions of proteins and/or nucleic acids,\textsuperscript{1,2} the stoichiometry and selectivity of metal-protein interactions,\textsuperscript{3,4} covalent modifications to proteins such as disulfide bonds, glycosylation and phosphorylation, as well as sequence information of natural, recombinant and synthetic biomolecules.\textsuperscript{5-8}

Mass spectrometry is an effective, efficient and the most accurate method to measure biomolecular masses. The technique has been effective in analyzing a broad range of biomolecules with masses up to 500,000 Daltons.\textsuperscript{9} Low sample requirements (femtomoles) and short acquisition times (\textmu seconds), provided the sample is relatively clean, contribute to the efficiency of mass spectrometric techniques. In terms of accuracy, a simple mass spectrometer such as a linear time-of-flight (TOF) instrument performs with a mass accuracy two orders of magnitude better than sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), an alternative method for measuring protein masses. Furthermore, high performance instruments such as ion cyclotron resonance mass spectrometers (ICR-MS) using Fourier transform (FT) mode of operation show astonishing unit resolution and accurate masses for proteins as large as 70kDa.\textsuperscript{10,11}

In practical terms the mass accuracy of a linear time of flight instrument is sufficient to determine peptide modifications such as acetylation or phosphorylation, which results in mass differences of 42 and 79 mass units, respectively. Furthermore, the
isotopic resolution provided by high performance instruments exceeds that needed to
discriminate a 2 Da mass difference due to disulfide bond formation between two
cysteines, and a 1Da mass difference due to deamidation of asparagine and glutamine
residues of modified peptides and proteins.\textsuperscript{4}

Mass spectrometry is a method that measures the mass to charge ratio (m/z) of gas-
phase ions. The key components of all mass spectrometers include an ionization source,
which converts analyte molecules into gas-phase ions, a mass analyzer for separation and
discrimination of the resulting ions and an ion collector or detector. A variety of mass
spectrometers with different capabilities have been created using various combinations or
configurations of these key components.

Of the possible mass spectrometric techniques available, those employing soft-
ionization such as matrix-assisted laser desorption/ionization\textsuperscript{12,13} (MALDI) and
electrospray ionization\textsuperscript{14-16} (ESI) are the most suitable for analyzing biomolecules,
particularly large biopolymers. In most cases formation of an intact molecular ion is the
desired effect. The gentleness of 'soft' ionization techniques limits the extent of
fragmentation yet supplies sufficient energy to ionize intact biomolecules.

Several instrumental and experimental factors account for the widespread use of
MALDI and ESI sources. From an instrumental perspective, each source has been easily
interfaced with a variety of mass analyzers. The most common MALDI mass
spectrometers have been created using time-of-flight, sector and ion-cyclotron resonance
mass analyzers. ESI sources are often interfaced with quadrupole, ion-trap, and ion
cyclotron resonance mass analyzers. In addition, ESI sources can be combined with two
or three mass analyzers configured in tandem as found in triple quadrupoles (QQQ), and in hybrid instruments such as the quadrupole/ion-trap (QIT) and quadrupole/time-of-flight (QTOF) instruments. From an experimental standpoint, the simple operation of most MALDI and ESI based mass spectrometers and their ability to analyze a broad range of analytes with high masses are additional factors for their growing popularity.

The growth of MALDI and ESI mass spectrometry as biomolecular probes requires constant development and evaluation of new instrumentation and experimental methods designed to analyze specific biomolecules obtained from various sources. The research presented in this dissertation involves bioanalytical mass spectrometry from two perspectives - method development and applications. As a method development the sample preparation process is improved for MALDI-MS by creating modified probes that act as on-line purification devices. The development and evaluation of these probes, their applications, and characteristics are discussed in Chapters III and IV. The second aspect involves the application of these two techniques to solve specific biological questions pertaining to a fungal enzyme, pectin methylesterase (PME). In Chapter V, mass spectrometry is used to examine the substrate specificity of PME using oligosaccharides as substrates. Furthermore, structural analysis of the carbohydrates attached to the protein were characterized the results of which are presented in Chapter VI.
REFERENCES


**Bioanalytical MALDI-Mass Spectrometry**

In the late 1980s, the concept of MALDI mass spectrometry originated from two independent research groups headed by Franz Hillenkamp and Koichi Tanaka. Each group discovered that compounds that strongly absorb radiation emitted from a laser source allows mass spectrometric analysis of large biomolecules such as proteins as intact species. For this purpose Tanaka, et al mixed cobalt powder in glycerol to obtain several multimeric ions of lysozyme with the largest ion observed at at 100,000 m/z.\(^1\) In lieu of a metal, Hillenkamp, et al used a small organic compound, nicotinic acid, and observed an ion at 200,000 m/z, which corresponds to the trimer of the protein bovine serum albumin.\(^2\)

The success of these experiments and others involving the contemporary technique of electrospray ionization revolutionized the fields of protein chemistry and mass spectrometry. Before the development of these techniques, mass spectrometry was limited to stable molecules of moderate size. Obtaining a mass spectrum of an intact protein greater than 10,000 Da was difficult due to its non-volatile nature and thermal instability.\(^3\)

The key discovery by Hillenkamp was the use of a compound which acts as a matrix to the biomolecule and assists in its desorption and ionization. The exact mechanism of this `assistance` is still under investigation. However, it is believed that the matrix acts as an energy buffer, absorbing the brunt of the energy delivered by the laser and transferring a small portion of this energy to the analyte molecule thus softening the impact of highly energetic laser pulse. It is also believed that the matrix acts as a source for protons during the ionization step.\(^2,4,5\) Another role of the matrix is to incorporate the
analyte molecules within the crystal lattice of the matrix and separate individual analyte molecules to prevent agglomeration.

Contemporary MALDI-MS experiments strongly resemble the original experiment described by Hillenkamp in 1998 albeit using different matrices and lasers. In the standard MALDI experiment, the analyte is mixed with excess matrix, using a 1:100 to 1:10,000 ratio of analyte to matrix which are then dried to a solid state. A laser fires a short pulse (1 to 100 ns) of radiation at the solid surface at an angle of incidence between 30 and 75 degrees. The matrix must have resonant absorption with the output wavelength of the laser, which is specific for the type of laser used. Currently, lasers emitting in the UV range, particularly the N₂ laser with an output wavelength of 337 nm, are most commonly found in commercial MALDI instruments. The explosive pulse of energy from the laser leads to desorption of analyte and matrix from the surface of the target as a jet or plume of gas-phase molecules or ions. The mechanisms in which ions are formed are still largely unknown and may occur either in the solid state or in the gas phase. Regardless of the physical state of the molecule ionization occurs by adduction of hydrogen or alkali metals in the formation of positive ions and by deprotonation to form negative ions. These ions are then directed towards the mass analyzer for discrimination.

Magnetic sectors, time-of-flight, quadrupolar ion-traps and FT-ICR instruments are among the mass analyzers that have been interfaced to MALDI sources. The most common mass analyzer for MALDI-generated ions are time-of-flight (TOF) instruments which discriminate mass/charge (m/z) based on ion mobility towards a detector in a field free vacuum or flight tube. Due to this popularity, the two general configurations of TOF
instruments, linear and reflectron, and their bioanalytical applications will be discussed in detail.

The simplest TOF configuration is the linear TOF shown in Figure 1. In all TOF instruments, the MALDI-formed ions are accelerated out of the source and into the flight tube by the influence of a strong electric field between a repeller and extractor plate. Due to this acceleration voltage each ion essentially contains the same final kinetic energy upon entering the flight tube. In this field-free and low pressure environment ions with the same kinetic energy but different m/z will arrive at the detector at different times. The time required for the ion to reach the detector is called the time-of-flight and is dependent on the velocity and mass of the ion. Smaller ions have greater velocity and will reach the detector earlier than larger, heavier ions. The mass of ion can then be correlated to its time-of-flight using the classical equations between kinetic energy, velocity, mass and time shown in Figure 1b.

Early studies revealed that the desorption/ion formation and acceleration processes are less than perfect. In an ideal desorption and ionization step, an analyte of a given mass yields a packet or population of ions on the same plane and hence each ion is equidistant to the flight tube. Also, during the ideal acceleration step, each ion within that population has the same final kinetic energy upon entering the flight tube. In reality, a given ion population has both spatial and kinetic energy distributions thus ions having identical masses will arrive at slightly different times and appear as slightly different masses. This phenomenon leads to increased in peak width and decreased resolution in the resulting spectrum.16
Figure II-1. Schematic of ion separation in a linear time-of-flight mass analyzer (a) and the equations used to calculate time-of-flight (b).
Many instrumental developments to time-of-flight instruments have focused on reducing the extent or effect of the spatial and KE distributions. The most significant development was the conception of Alikanov and Mamyrin in which the amount of time each ion spends in the flight tube is adjusted to compensate for their distribution in kinetic energies. This instrumental development called the reflectron TOF is a modified configuration of a linear TOF but contains, at the end of the flight tube, a series of rings or mirrors which act as ion reflectors to reflect ions towards a second off-axis detector. The voltage of each ring increases with distance from the flight tube entrance. The degree of reflection or penetration into the reflectors is based on the kinetic energy of a given ion. The more energetic ions within an ion population penetrate deeper and at higher reflectron voltages and thus travel a longer distance than less energetic ions that reflect at lower voltages and travel shorter distances so that each of these ion arrive at the detector at the same time. The overall effect is increased resolution as each ion arrives simultaneously at the detector. However, the benefit of increased resolution often comes at the expense of sensitivity and a lower mass range of less than 10,000 m/z. Another development to increase resolution is a method originally described by Wiley and McClarren for TOF based instruments in which ionization is followed by delayed acceleration. The method, called delay extraction (DE) is simply a means of cooling the ions before they are accelerated from the source. The cooling narrows the energy distributions of the ions and thereby increases the resolution. Simple, linear TOFs have low resolving power (450 to 600 for peptides) but when combined with DE, the resolution increases to 2000. It has been shown that
reflectron instruments when combined with delayed extraction can achieve very high mass resolutions \( \frac{m}{\Delta m} = 10,000\text{-}15000 \) of peptides up to 5000 Daltons.\textsuperscript{18,19}

Although the soft-ionization afforded by MALDI generally produces ions of intact species, decomposition of unstable molecules while in the source (prompt fragmentation or in-source decay) or while in the flight tube (metastable fragmentation or post-source decay) presumably due to collisions with neutral matrix molecules have been observed. Fragments produced by in-source decay can be discriminated by both linear and reflectron instruments since the fragments have equal KE but different velocities in the flight tube. Fragment ions produced as a result of post-source decay (PSD) have the same velocities but only a fraction of the KE of the parent molecule. Linear TOFs cannot discriminate these ions since they essentially arrive at the detector at the same time (Figure 2a). However, reflectron TOFs can discriminate the fragments due to their differences in KE and degrees of reflection as shown in Figure 2b. The production of metastable fragments and the ability of the reflectron to discriminate their masses have provided a means for sequence analysis. An ion gate selects the precursor ion, which is then allowed to decay in the flight tube. Fragments of the precursor ion that retain the charge can be discriminated by stepping the reflectron voltages to focus each fragment ion.

**Peptide and Protein Analysis by MALDI**

Studies involving peptides and proteins have dominated bioanalytical research using MALDI. The importance of MALDI for their analysis is stressed in the pivotal role MALDI has played in the revolutionary new field of proteomics. Proteomics involves
Figure II-2. Schematic of post-source decay of an ion (shown green) and the detection of the fragment ions in linear (a) and reflectron (b) TOF mass spectrometers.
characterization of the protein complement of a cell or subcellular component.\textsuperscript{20,21} This involves identification and in some cases quantitation of hundreds to thousands of proteins that are presented for analysis as semi-purified forms embedded within a polyacrylamide gel.\textsuperscript{22} Due to its speed, sensitivity, ease, and moderate tolerance to some contaminants MALDI-MS has been used to characterize the majority of the proteome in current proteomics research\textsuperscript{23} and is the method of choice for high throughput proteomics.\textsuperscript{24-26}

Peptides and proteins are obviously structurally related and the peptides studied often originate from a protein thus their analyses share common features and techniques. Nonetheless, there are differences in the types of matrices used, the level of laser power required and in the characteristics of their MALDI spectra that must be considered. A number of suitable UV absorbing matrices have been developed for peptide and protein analysis, most of which are derivatives of cinnamic and benzoic acids. Sinapinic acid (3,5- dimethoxy-4-hydroxy cinnamic acid), $\alpha$-cyano 4-hydroxycinnamic acid (CHHA) and 2,5-dihydroxybenzoic acid (2,5 DHB) are the three most commonly used peptide matrices while sinapinic acid is the preferred matrix for protein analysis reported in the literature.\textsuperscript{6,27,28} Proteins are generally more difficult to desorb than peptides and thus require higher laser powers, which can increase the peak width and result in decreased mass accuracy and resolution. Peptides in pure form or in mixtures ionize easily provided the sample is not riddled with non-peptide contaminants. The spectrum of a pure protein may contain several peaks representing multiple charge states and aggregates such as dimers and trimers for a single molecule. The predominant species observed in peptides contain either one or two charges.
Much of the literature describing peptide analysis by MALDI-MS involves analysis of peptide in mixtures that are often produced from enzymatic or chemical proteolysis (digestion) of a single or multiple proteins. The digests may be performed to identify the protein or to determine and locate potential modifications. Unlike other types of mass spectrometry using alternative ionization sources, MALDI-TOF lends readily to mixture analysis. As stated earlier, peptides produce mainly singly charged ions and to a lesser extent, doubly charged ions. The limited charge states for a given peptide allows easier interpretation of the mass spectrum of mixtures containing multiple analytes. Digestion of very large proteins of 100 kDa or greater may produce two or more peptides with similar masses (+/- 1 to 2 Da). In these cases reflectron instruments with resolutions greater than 3000 can resolve each peptide. Depending on their size and number of ionizable amino acids, certain peptides in the mixture may ionize more efficiently than others. This can lead to ion suppression of a weakly ionizing peptide by another peptide that has a high ionization efficiency. Large peptides with masses greater than 3000 Da typically have low ionization efficiencies.

Reflectron instruments have also been used to sequence peptides for quality control of synthetic peptides, identification of the protein from which the peptide originates, and determining the specific location of a potential modification. Fragmentation of the peptide via post-source decay occurs mainly between along the peptide backbone to produce sequence specific fragment ions. The fragment ions are named according to the nomenclature originally proposed by Roepstorff and Fohlman for collisionally-induced fragments by tandem mass spectrometry (Figure 3).
Figure II-3. Peptide fragmentation and nomenclature as proposed by Roepstorff, et al.
The degree of metastable fragmentation can be affected by the choice of matrix, the laser power and the acceleration field strength. Matrices with high proton affinities such as \(\alpha\)-cyano 4-hydroxycinnamic acid tend to produce extensive PSD fragmentation. Increased fragmentation has also been observed with increased laser power and extraction voltages.\(^{33-35}\) Presumably, these conditions desorb a greater amount of neutral matrix molecules, which upon colliding with the analyte ion, increases its internal energy and causes fragmentation.

**MALDI-MS Analysis of Carbohydrates**

Carbohydrate analysis constitutes the second major application of MALDI in bioanalytical studies. A variety of carbohydrates derived from natural sources as oligosaccharides, polysaccharides and glycoprotein glycans have been successfully analyzed by MALDI-TOF methods. In the first application of MALDI for carbohydrate analysis Karas, et al used nicotinic acid to enhance the signal for a simple neutral tetrasaccharide, stachyose.\(^{36}\) With the development of new matrices and techniques, the scope of MALDI has broadened to include more complex and structurally diverse carbohydrates.

Currently, the peptide matrix 2,5 DHB is the most widely used for most neutral carbohydrates as well as some acidic carbohydrates containing uronic acids. The spectra of these compounds produce cationized molecular ions primarily due to sodium adduction. Some carbohydrates such as those containing sialic acids can be problematic and require
use of esoteric matrices. These compounds form both negative and positive ions. This results in poor quality spectra as ions are split between the two modes. Matrices such as 6-aza-2-thiothymine and 2,4,6 tri-hydroxyacetophenone reportedly prevent salt adduction thus favoring formation of negative ions.

It is common practice to derivatize carbohydrates prior to analysis by mass spectrometry. The most common derivatives are mainly small, basic, aromatic compounds. The primary reasons for derivatization are to increase the signal yield in the mass spectrum and also to allow separation of carbohydrates by reverse-phase HPLC with UV or fluorescence detection. The desorption/ionization process is less efficient for carbohydrates than for peptides and proteins. This is improved by adding basic sites that are capable of accepting charge upon ionization. Furthermore, without these derivatives carbohydrates are typically separated by LC and detected at high pH by pulsed-amperometric detection. The high salt conditions of this type of separation are extremely unfavorable for direct analysis by mass spectrometry without extensive cleansing of the sample. Most derivatives are attached to the anomeric carbon (C-1) of the reducing end of the carbohydrates. These derivatives are incorporated into the carbohydrate structure using chemistries such as reductive amination and oxime formation. The common derivatives used for carbohydrates have been reviewed and compared.37

The sequence of carbohydrates has been determined by MALDI-PSD analysis. Two general types of fragmentation occur in carbohydrates. In the first type, the glycosidic bond between two monosaccharides is broken to produce a fragment containing the non-reducing end of the 'parent' ion and a second fragment containing the reducing
**Figure II-4.** Common fragmentation sites of carbohydrates and their notation as proposed by Domon and Costello.
end, one of which retains the charge of the parent ion. The nomenclature for these fragments follows that proposed by Bruno Domon and Catherine Costello (Figure 4). In glycosidic fragments of C and B types the charge is retained on the fragment containing the non-reducing end while Y and Z type fragments contain the non-reducing end.

Glycosidic fragmentation is the most common type of fragmentation and yields sequence information. Linkage information can be obtained by analysis of the second type of fragmentation. In this fragmentation referred to as cross ring cleavages two bonds are broken across the ring to form A and X type ions that contain the non-reducing and the reducing terminus, respectively.

The type and degree of fragmentation of carbohydrates appears to be dependent on form of the analyte ion. Ngoka, et al discovered that protonated forms of the carbohydrate decompose more readily than cationized forms. Furthermore, Orlando, et al observed that cationized forms undergo cross-ring cleavage more readily than protonated forms. Canchilla, et al also discovered that linear carbohydrates fragment more extensively than branched structures.

**MALDI-MS of Glycoprotein Glycans**

In carbohydrate analysis by mass spectrometry, particular emphasis has been placed on characterizing the carbohydrates or glycans that are attached to proteins. In these studies the occurrence, structures and changes in glycosylation of native and recombinant proteins are the major points of interest. Analogous to the relationship between peptide and protein analyses, carbohydrates released from glycoproteins can be
treated and analyzed in a similar manner to non-glycoconjugate carbohydrates as described in the previous examples. Thus the analytical techniques and chemistries developed for carbohydrates can be applied to glycoprotein-derived glycans. However, glycan analysis can also be performed on carbohydrates that remain attached to the protein or to a peptide. Glycan analysis via a glycopeptide or glycoprotein involves a number of biochemical, and purification techniques that are supported intermittently by MALDI-MS analysis.

Certain features of glycoproteins and their glycans must be considered for their analysis. Glycoproteins often exist as a heterogenous population of proteins called glycoforms that may contain structurally related carbohydrates attached to a single site or to multiple sites. The carbohydrate moieties are attached to the protein backbone through three specific amino acids and are classified based on these attachment sites. If linked to the protein through the nitrogen of an asparagine residue, the glycan is termed 'N-linked'. A further requirement for N-linked glycosylation is that the asparagine must belong to the tripeptide Asn-X-Ser or Asn-X-Thr where X can be any amino acid except proline.

O-linked glycans, the second class of glycoprotein carbohydrates, are attached to the polypeptide through the oxygen of serine or threonine. There are general structures for both N-linked and O-linked carbohydrates. N-linked glycans contain a biantennary pentasaccharide core consisting of three mannose and two N-acetylglucosamine residues (Man$_3$GlcNAc$_2$). The core structures may be elongated with additional monosaccharides such as mannose, galactose, N-acetylglucosamine and sialic acids. There are three general classes of N-linked glycans as shown in Figure 5. Glycans of the high mannose type contain only mannose residues while complex-type glycans are composed of various
Figure II-5. Examples of the three classes of N-linked glycans

**HIGH MANNOSE**

\[
\text{Man}\alpha(1-6) \quad \text{Man}\alpha(1-6) \quad \text{Man}\beta(1-4)-\text{GlcNAc}\beta(1-4)-\text{GlcNAc}\beta-N
\]

\[
\text{Man}\alpha(1-3) \quad \text{Man}\alpha(1-2) \quad \text{Man}(1-3)
\]

**COMPLEX**

\[
\text{Gal}\beta(1-4)-\text{Gal}\beta(1-2)-\text{Man}\alpha(1-6) \quad \text{Man}\beta(1-4)-\text{GlcNAc}\beta(1-4)-\text{GlcNAc}\beta-N
\]

\[
\text{Gal}(1-4)-\text{Gal}(1-2)-\text{Man}(1-3)
\]

**HYBRID**

\[
\text{Gal}(1-4)-\text{GlcNAc}(1-2)-\text{Man}(1-6) \quad \text{Man}(1-4)-\text{GlcNAc}\beta(1-4)-\text{GlcNAc}\beta-N
\]

\[
\text{Man}(1-2)-\text{Man}(1-3)-\text{Man}(1-3)
\]
monosaccharides. Hybrid structures are a combination of high-mannose and complex types, in which at least one antenna is composed solely of mannose residues.\textsuperscript{41} Six possible core structures have been determined for the structurally diverse O-linked glycans.\textsuperscript{42} N-linked carbohydrates are typically easier to process and characterize than the smaller O-linked glycans and thus much of the research reported in the literature and discussed in this introduction pertain to N-linked glycans.

Complete characterization of a glycan is a multifaceted process that requires determination of the presence of glycosylation, the sites of attachment to the protein, the glycan type and class, and the sequence and linkage of the oligosaccharide. Linear and reflectron MALDI-TOF mass spectrometry when used with ancillary techniques such as chromatography and/or proteolytic and glycolytic digestions have made these determinations for a variety of glycoproteins.\textsuperscript{43}

Most of the experimental procedures for N-linked glycan analysis that are reported in the literature follow one of two possible strategies shown in Figure 6 in which each strategy involves MALDI-MS. The glycans can be separated from the protein and analyzed independently using techniques previously discussed for free carbohydrates. The glycans can also be characterized while attached to a peptide or the protein.

Each strategy begins with MALDI analysis at the glycoprotein level. A linear MALDI-TOF measurement of the protein mass and \textit{a priori} knowledge of the polypeptide can confirm the absence or presence of glycans. Experimental masses that are at least 1500 Da greater than the mass based on the amino acid sequence signify the presence of N-linked carbohydrates. The mass differences is also an estimation of glycan mass.
Figure II-6. Experimental pathways for glycan analysis from glycoproteins and the information provided by MALDI-MS experiments at each structural level.
Furthermore the width of the measured protein can be used as a crude estimation of the
degree of heterogeneity in both linear and reflectron instruments. However, these
instruments do not have the sufficient resolution to resolve individual glycoforms
particular of glycoproteins greater than 15kDa.\textsuperscript{44}

Most of the research on N-linked glycans follows the course denoted by the blue
arrows, which allows for determination of the glycan attachment site. In this course the
glycoprotein is digested with a protease such as trypsin. The digest mixture is then
analyzed by MALDI to screen for glycopeptides. The spectrum of the digest mixture
typically contains several peptide ions and cluster of ions at higher m/z (>3000 m/z). Ions
in the cluster or series may differ by 162 m/z due to additional mannoses and represent
glycoforms of a particular tryptic peptide. Once the glycopeptide is located the
carbohydrate portion is removed to determine the mass of the glycan and simultaneously
identify the attachment site. If the amino acid sequence of the protein is known then a
virtual digest of such sequence provides masses for theoretical tryptic fragments. A match
between the theoretical and experimental peptide masses identifies the peptide fragment
and consequently the attachment site by locating the Asn-X-Ser/Thr sequence within the
matching peptide. This can be performed after off-line separation of the glycopeptide
from the digest mixture or can be performed directly on the mixture itself. Using the
glycopeptide as the analyte MALDI mass measurements can be used to determine the
attachment site, the glycan sequence and structure.

The carbohydrate sequence of glycan as a free glycan or glycopeptide can be
determined via PSD using a reflectron TOF. The characteristics of the PSD spectra of
glycoprotein glycans are similar to that described for carbohydrates in the previous section. In addition the linkage may be confirmed by using exoglycosidases which are highly specific for monosaccharides of a particular type and linkage.

**Sample Interferences to MALDI-MS**

A special consideration in biomolecular analyses by MALDI is the condition of and constituents within the sample. Proteins or glycoproteins obtained from biological sources often experience a series of purifications, which may include cell rupture, centrifugation to remove particulates, and potentially several stages of extraction. Additives such as detergents or buffering agents may also be required to maintain the activity, reduce absorption to synthetic surfaces or prevent precipitation of the biomolecule.

One of the main reasons for the popularity of MALDI is its tolerance to extraneous material that may contaminate the sample solution. There are however, limits to the amount and type of contamination. The limits are dependent on the type of matrix used and the type and amount of contamination present. In practice, sinapinic acid and 2,5-DHB matrices are more tolerant than CHHA of common contaminants. Common buffer components such as sodium chloride and tris (hydroxymethyl) aminomethane are tolerable up to 50mM but phosphates must be maintained below 10mM. Detergents such as Triton X-100, Np-40 and Tween are not a factor at less than 0.1% (v/v) while chaotropes such are guanidinium • HCl is limited to a concentration of 1M. In practice, it is often necessary to prepare sample solutions beyond these limits. For example, the tolerance of MALDI to
another chaotrope, urea, is 0.5M. However 6M urea is commonly used to enhance protein solubility in aqueous solutions.\textsuperscript{45}

The intolerance of MALDI to increased levels of contamination is expressed in poor quality spectra with low resolution, low signal yield and is furthermore complicated by the presence of strong adduct ions. In many cases there is global suppression of signal including those due to matrix ions.

It is thought that these contaminants affect the MALDI process in two ways; by adversely affecting the co-crystallization of the sample and matrix molecules and by competing for ionization with the analyte. These contaminants can be removed using standard purification methods such as chromatography, solid-phase extraction or dialysis but at the cost of increased sample preparation time and potential sample loss.

\textit{Improvements to Sample Preparation for MALDI-MS Studies}

Several research groups have developed techniques to improve sample preparation of contaminated samples and enhance their analysis by MALDI-MS. The unifying goal is to supplant the standard off-line purifications with quasi on-line methods by performing the purification directly on the MALDI target. The targets are made attractive to biomolecules either by chemical modification of the target itself\textsuperscript{46-48} or by depositing media such as labeled-agarose beads\textsuperscript{49,50} or hydrophobic membranes on the target surface.\textsuperscript{46-48,51-55} The targets are generally referred to as ‘bioaffinity probes’ and the method of purification is considered to be a form of solid-phase extraction.
Affinity-based purifications were the first of these techniques developed. Very fine agarose beads were covalently attached to molecular probes, deposited on the MALDI target and then layered with a contaminated sample containing the capture molecule. In this manner, Hutchens and Yip captured human lactoferrin from the urine of a pre-term infant using single-stranded DNA covalently immobilized to the agarose beads. Other groups have exploited the antibody-antigen and avidin-biotin relationships for similar extraction purposes. These affinity-based separations are very specific for a single or limited set of analytes.

A broader range of analytes can be extracted using hydrophobic-based purifications. These have also been performed on the MALDI target using synthetic polymeric membranes such as polyvinylidene difluoride (PVDF), polyethylene terathalate (PETE), polyethylene (PE), nylon, polypropylene (PP) and nitrocellulose (NC). The membranes may be physically attached to the target by clips or double-sided tape or may be applied as a thin layer by dissolving the membrane in acetone then quickly depositing the dissolved material on the MALDI target before the solvent evaporates. The membrane captures the analyte via hydrophobic interactions allowing removal of non-bound contaminants. Worrall, Cotter and Woods demonstrated that peptides and proteins could be desorbed from a variety of PE, PP, C8 and C18 surfaces with the added bonus of increased resolution when compared to using unmodified targets.

All of the examples described involve the use of external media. However, an alternative method is to chemically modify the surface to express the same characteristics of the media such as affinity and hydrophobicity. This has been the focus of past research
in our laboratory and our particular niche in this small field. For affinity-based separations
MALDI targets have been covalently linked to self-assembled monolayers, which in turn are attached to a molecular probe such as an antibody. Brockman, et al was able to extract 20 femtomole of lysozyme from the complex mixture of a human tear by using human anti-lysozyme, which were immobilized to a monolayer-modified MALDI target.  
Using the standard MALDI preparation, these strongly hydrophobic surfaces repel the aqueous sample solution. As a result very little interaction between the analyte occurs. Further growth of these monolayer-based surfaces for purification purposes requires development and evaluation of alternative surfaces that are compatible with the sample solution.

**Bioanalytical studies using Electrospray Ionization**

Dole, et al laid the foundations of bioanalytical ESI in the 1960s by developing an electrospray source capable of generating gas-phase ions of proteins. However successful interface between an electrospray source and a mass analyzer was not accomplished until the late 1980s when Fenn and Mann interfaced an electrospray source with a single quadrupole mass analyzer.

Electrospray refers to the process in which a fine spray or mist is created as a solution flows from a highly charged capillary. The spray and ensuing ionization results from downward potential and pressure gradients between a capillary, which is held at high voltage and at atmospheric pressure, and a counter electrode which is operated a near zero potential and in high vacuum (Figure 7). Use of high voltages generates highly charged droplets bearing the sample. As the solvent evaporates from the droplet the charge density
Figure II-7. A schematic of an electrospray source.
increases. At a particular size, the electrostatic repulsions are large enough to overcome the forces that keep the droplet intact. A "coulombic" explosion occurs and smaller droplets of reduced charged are formed in the process. This process continues until complete desolvation of the analyte occurs. Most of the current and past literature on ESI involves use of quadrupole mass analyzers either as single or triple quadrupoles (QQQ) although ESI using ion-traps and FTMS is experiencing rapid growth. Time-of-flight instruments and multi-staged sectors have also been interfaced with ESI but have not gained the popularity of the quadrupole-based instruments. The universal use of quadrupoles and triple quadrupoles warrant further discussion of these mass analyzers.

Quadrupole mass analyzers consist of four circular or parabolic rods aligned in parallel to form a cross section or core shown in Figure 8. Electrical connection is made between the rod pairs in the x plane and between those in the y plane of the cross section. Oscillating ions entering the quadrupole can pass through the core of the quadrupole or filter to a detector or may be destroyed when deflected by or in striking the rods. Dc and rf voltages applied to the rods determine the successful passage of an oscillating ion. The stability of an ion of a given mass is dependent on the frequency and the dc and rf voltages applied. At a particular dc or rf voltage ions of a limited m/z range will pass through the filter. However, at a fixed frequency, ions encompassing the entire m/z range can be transmitted if applied dc and rf voltages are varied but the ratio of rf to dc voltage is held constant at some optimal value.62

Single quadrupoles are primarily used for obtaining molecular masses with mass accuracy of 0.01%. Fragment ions can be formed and analyzed by single quadrupole
**Figure II-8.** Design of a quadrupole mass analyzer and illustration of a stable ion traversing through its core.
instruments by adjusting the voltage of the counter electrode. This type of fragmentation is non-discriminatory since all ions are subjected to this same field and thus all ions will fragment. However, for structural and sequence analysis of biomolecules in mixtures the ESI conditions and instrumentation must be capable of producing and discriminating fragment ions produced from a known or selected precursor ion. A better method of inducing fragmentation is by colliding the ions with neutral gas molecules. This method allows for selection of a precursor or parent ion. Furthermore the extent of fragmentation can be controlled by adjustment of the collision gas pressure. A triple quadrupole is the most common ESI configuration to achieve this goal. The standard configuration for a triple-quadrupole instrument is shown in Figure 9. For sequence analysis the first quadrupole is used as a mass filter, allowing transmission of only a specific m/z. In the second quadrupole collision gas is applied to induce the dissociation of a selected or precursor ion into fragments. The rods are supplied only with rf voltage allowing all fragment ions passage to the third quadrupole. The third quadrupole the masses of the fragment ions are scanned in the manner described earlier for the single quadrupole.

**ESI-MS and glycoprotein glycan analysis**

Glycan analysis using ESI may also proceed via the two courses described for MALDI-TOF and shown in Figure 10. ESI can be used at the glycoprotein, glycopeptide and glycan levels. MALDI and ESI are complementary techniques. In some cases the information regarding glycosylation provided by ESI can overlap that obtained by MALDI. Yet in others ESI-MS allows for greater flexibility and shows superiority in terms of mass
Figure II-9. A diagram of a triple-quadrupole mass spectrometer. MS/MS can be performed using the first quadrupole to select the precursor ion, Q2 as a cell for collisions between the selected (precursor) ion and applied gases, and Q3 to analyze the resulting fragments.
**Figure II-10.** Experimental paths for glycan analysis and the information provided by ESI-MS at each structural level.
accuracy and resolution. This is best illustrated in molecular mass measurements of a glycoprotein. As stated earlier a "pure" glycoprotein may in fact a mixture of glycoforms of one protein. In MALDI spectra, the glycoforms appear as a broad hump whereas in ESI individual glycoforms are observed.

There are many reasons to use ESI-MS exclusively for glycan analysis. The mass accuracy and resolution is superior to MALDI-TOF systems and fragmentation is more efficient especially when using collision gases. Furthermore because ESI introduces samples in solution liquid separations can be performed and the separated compounds sampled directly into the mass spectrometer.

The MS/MS capability of ESI-based instruments is more effective for sequencing glycans than MALDI-PSD. A number of N-linked as well as O-linked glycans have been characterized using ESI-MS/MS on triple quadrupole instruments. Perhaps the most significant contribution to characterization of N-linked glycan analysis by ESI is the stepped-orifice voltage technique introduced in 1993 by Steven Carr and Mark Bean. This technique is essentially an in-source fragmentation experiment in which the cone voltage is raised to induce fragmentation before the analyte reaches the first quadrupole. The novelty provided by Carr and Bean was the use of switched stepped-voltages during the chromatographic experiment. The orifice or cone voltage is switched from high to low (or normal) values at particular regions during the scan. The instrument is set to scan a low m/z range when the orifice voltage is high. Higher m/z ranges are scanned when orifice voltages are low. The overall effect is that intact ions and their fragments can be measured nearly simultaneously. This method is particularly important for carbohydrate
or glycan analysis since the common sugars found in glycans produce very specific ions of known m/z. For example, hexoses and N-acetylhexosamines produce ions at 163 and 204 m/z, respectively. One can simply look for the formation of these and other characteristic carbohydrate ions. Numerous papers report using this stepped-orifice voltage method to locate glycopeptides during LC-MS experiments using primarily single and triple quadrupole instruments.\textsuperscript{69,70} However, extending this technique towards other mass analyzers is promising. Developments in mass spectrometry such as the stepped-orifice method have contributed greatly to the explosive growth of the field and increase the appeal of mass spectrometry for bioanalytical studies.
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CHAPTER III

ON-PROBE SOLID-PHASE EXTRACTION/MALDI-MS USING ION-PAIRING INTERACTIONS FOR THE CLEAN-UP OF PEPTIDES AND PROTEINS


44
ABSTRACT

Biomolecular samples obtained from biological sources can be a complex mixture, which not only includes the analyte but also various inorganic salts, surfactants/detergents, buffers, chaotropic and other solubilizing agents. The presence of these extraneous compounds or contaminants typically destines the subsequent analysis of the sample by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to failure. Thus the analyst is required to clean-up the sample prior to analysis by MALDI-MS. This paper reports the development and use of a derivatized probe-surface that greatly simplifies this sample preparation process. MALDI probes possessing self-assembled monolayers (SAMs) terminated with ionic functional groups can quickly extract peptides via ionic interactions from contaminated solutions thus allowing sample clean-up directly on the MALDI probe. Our results show that probes modified in this manner are a practical solution for analyzing peptide samples containing extremely high levels of contaminating agents.
INTRODUCTION

Samples originating from biological sources often contain a complex mixture of inorganic salts, buffers, chaotropic agents, surfactants/detergents, preservatives and other solubilizing agents. These agents are employed by biologists for many reasons, such as the maintenance of a non-toxic cellular environment, the stabilization of solvated samples, and the preservation of enzymatic or other biological activity. Although their presence is critical for the above reasons, these same agents often cause problems for subsequent analyses, particularly within the domain of mass spectrometry.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is one of the most sensitive methods in the field of mass spectrometry and is now routinely used for biomolecular analysis. MALDI also shows the greatest compatibility with many biological buffers.\textsuperscript{1-3} However, MALDI-MS is not immune to interferences from the complexity of the biological media and is often crippled by the presence of inorganic salts, chaotropic agents, buffers and detergents. For example, the mass spectra of samples containing sodium at concentrations of 200 mM exhibit suppressed signals and multiple, intense sodium adducts.\textsuperscript{4} These adducts increase the analyte peak width and decrease the resolution, therefore desalting prior to MALDI analysis is beneficial to increasing mass accuracy. At significantly higher concentrations of sodium complete signal suppression can occur. Similarly, high concentrations of detergent result in reduction of ion yields and resolution. The current theories of the interference effects of these agents are that proper inclusion of the analyte into the matrix crystal lattice or total crystallization of the matrix itself is prevented. In addition, the interfering agents may compete with the analyte for
ionization thus leading to signal suppression of the analyte ion. The primary obstacle encountered in obtaining a MALDI-MS spectrum of biological samples is its cleanliness. Hence, the investigator is required to perform sample clean-up procedures before performing the analysis.

Several sample preparation techniques have been specifically designed for MALDI-MS analysis of contaminated biological samples. One strategy relies upon a thin film of matrix deposited in acetone. The peptide sample is applied to the surface of the matrix film and desalted using aqueous organic acids. Another development involves a re-crystallization method, which incorporates peptide molecules to a larger extent than the salt contaminants. The excluded salts can next be selectively washed away from the adhering crystals. Samples can also be freed of contaminants by depositing them onto polymeric membranes, such as porous polyethylene (PE) and poly(vinylidene difluoride) (PVDF). The analyte interacts with the membranes via hydrophobic interactions allowing non-binding salts to be washed from the membrane surface. The membrane containing the "cleansed" sample is attached to the MALDI probe, layered with matrix solution and then analyzed of its contents. All of these methods have been incorporated into the arsenal of sample preparative methods available to the MALDI analyst and have become valuable tools to battle the potential problems involved with MALDI analysis of unknown peptide samples.

We have developed an on-probe sample clean-up strategy based upon chemically modified MALDI probe surfaces designed with the capability of extracting biological polymers from solution while having little to no affinity for the contaminants in the
sample. The probe modifications or derivatizations that were used in this approach produce surfaces that have similar chemical characteristics as media used in solid-phase extraction (SPE), which has inspired us to name our approach SPE/MALDI-MS. For example, we have created MALDI probes with a hydrophobic self-assembled monolayer (SAM); a surface with similar chemical character to the C18 stationary phase commonly employed in SPE. We have shown that these hydrophobic SPE/MALDI-MS probes readily bind to peptides and proteins via hydrophobic interactions, and allow both inorganic salts and chaotropic agents to be removed with aqueous washes. A major limitation in hydrophobic SPE/MALDI-MS probes was the low degree of "wetability" of the surface. The hydrophobic nature of the surface combined with the aqueous solutions of the sample resulted in strong sample surface tension and the formation of near perfect sphere on the surface. The diminished contact area reduced the amount of peptide that could be extracted. Immersing the C-18 probe in the sample solution for an extended period provided greater area of contact and amount extracted. However, the volume of sample required to totally immerse the probe is too high and impractical for routine analysis of most samples.

The inherent limitation of the hydrophobic surfaces provided the drive to develop probes with "wetable" surfaces for SPE/MALDI-MS. Here, we describe the use of MALDI probes possessing SAMs that are terminated with ionic functional groups. MALDI probes modified with these functional groups have excellent "wetability" character and can rapidly extract peptides from solution via ionic interactions. We have
found that ion-pairing SPE/MALDI probes are a practical solution for analyzing peptide samples in a variety of contaminants.

**EXPERIMENTAL**

_Probe Preparation_

Self-assembled monolayers of each of the thiol reagents were prepared in an exact manner. Disposable commercial stainless steel rings with eight “mesas” were manufactured to fit a MALDI TOF probe tip (Hewlett-Packard). These were first etched with aqua regia (5.4 mL of 70% nitric acid (Aldrich) and 24.6 mL of 37% hydrochloric acid (Sigma). The etching serves to increase the tip’s surface area, and thus, its binding capacity. During etching, the aqua regia is “stirred” through the tips using a glass pipette. Subsequently, fine microcrystals of gold was sputtered onto each ring using a commercial sputtering instrument (SPI Module Coater).

SAMs were assembled in a similar fashion for each surface explored. The freshly sputtered gold tips were incubated for 30 minutes in an anhydrous ethanol solution saturated with the thiol of interest. Care was taken to stir the incubating tips using a shaker or rotator. After incubation, the tips were first washed in ethanol then in water to remove unbound thiolates. In this manner, strong cation-pairing (3-mercapto-1-propanesulfonic acid and 2-mercaptopethanesulfonic acid), strong anion-pairing (2-aminoethanethiol hydrochloride), and weak cation-pairing (3,3′-dithiodipropionic acid) monolayers were all assembled to further explore the separation properties of each type of surface.
The SAM forming species 3-mercapto-1-propanesulfonic acid (MPSA or “sulfonate-terminate”) and 2-aminoethanethiol $\cdot$ HCl (referred to as “amine-terminate) were purchased from Aldrich and 3,3’ dithiodipropionic acid (carboxylate-terminate) was purchased from Fluka. All reagents were used as purchased without further purification. Ethanol (Fisher), the thiol solvent, was dried over aluminate, filtered with a 0.22-micron filter, then de-aerated with helium for 30 minutes.

**Sample Preparation**

Stock solutions of the peptides, insulin, somatostatin, angiotensin (all from Sigma), and the proteins cytochrome-C (Sigma), and biotinylated-BSA (Pierce) were prepared using ultrafiltered de-ionized water to a concentration of 1 mg/mL. Appropriate aliquots of each solution were used to prepare working solutions of 20 pmol/µL of each peptide or protein. Triton-X 100 (Aldrich), Tween-20, urea (Fluka), guanidinium hydrochloride, and sodium acetate (Aldrich) were used in the experiments as representative nonionic detergents, chaotropes and salts.

**Loading and Washing of Samples**

A volume of 0.3 µL of the analyte is loaded onto the sample region of a derivatized probe tip. This is allowed to dry either under ambient conditions or by using vacuum crystallization. At these high concentrations of salt and detergents, drying at room temperature requires at least 10 minutes for each sample. The sample is then washed several times, dried, and 0.3 µL of matrix applied. In most of the experiments, de-ionized
water was used for washing. We also experimented with acidic (0.1%, 1% and 5% aqueous tri-fluoroacetic acid), basic (11mM sodium hydroxide) organic (70% aqueous acetonitrile and 70% methanol) and phosphate-buffered saline washes.

**MALDI TOF Measurements**

All mass spectra were obtained using a Hewlett Packard G2030A (Palo Alto, CA) MALDI TOF mass spectrometer. The instrument was operated at an accelerating voltage of 28 kV, and extractor voltage of 7 kV, and a pressure of ~1 x 10⁻⁶ Torr. The samples were desorbed/ionized from the probe tip using a nitrogen laser source with an output wavelength of 337nm. Calibration was performed using peptides with masses ranging from 300-7,000 Da (MALDI-Quality Standards Hewlett-Packard). Sinapinic acid (Aldrich), dissolved in 70% aqueous acetonitrile (Baker) with 0.01% trifluoroacetic acid (Aldrich), was used as the matrix for all samples.

“Conventional” MALDI refers to a typical MALDI preparation in which the analyst mixes a volume of matrix and a volume of sample and then dries the resulting mixture onto the probe surface. This method is the technique to which we wish to compare our method using SPE/MALDI-MS technologies. This procedure is referred to throughout the text as “conventional” MALDI.

**RESULTS AND DISCUSSION**

The basic steps of a SPE-MALDI/MS experiment are shown in Figure 1. In these experiments, a 0.3 µL aliquot of the analyte solution is placed on the modified probe and
Figure III-1. Schematic of the SAM formation on the MALDI probe and the subsequent removal of contaminating agents on the modified surface.
1. Etch surface
2. Sputter with Au

Au-covered probe
3. Incubate in thiol solution

SAM-derivatized probe
4. Apply sample

5. Wash away contaminants

6. Apply matrix

MALDI-TOF
allowed to interact with the surface. The sample is dried under vacuum then washed with water to remove the contaminating agents. A solution containing the matrix is then deposited onto the sample region and dried. The MALDI probe containing the sample and matrix is inserted into the mass spectrometer. The use of modified probes requires two extra steps not used in the conventional sample preparation. These extra steps add only a few minutes yet allowed the successful MALDI-MS analysis of samples which would have normally required extensive sample purification. Existing procedures used to eliminate these types of contamination can increase the sample preparation time by several hours.

The utility of ion-pairing surfaces for on-probe sample purification was evaluated with a series of SPE-MALDI experiments performed on peptide and protein solutions containing high concentration of salts. The on-probe desalting ability of this technique is clearly illustrated by the successful analysis of insulin (2 picomole/µL) dissolved in an aqueous solution saturated with sodium acetate (Figure 2). These spectra were obtained on modified probes possessing monolayers with either negatively (Figure 2a) or positively charged (Figure 2b) termini. Under these same sample conditions, conventional MALDI analysis did not provide any molecular weight information, as expected due to the high concentration of salt.

A series of experiments were performed to test these surfaces in the presence of detergents and chaotropes. Peptide and protein solutions contaminated with one of two types of chaotropes (8M urea and guanididium HCl) or a detergent (up to 20% (v/v) Triton X-100 and Tween-20) were analyzed. In each instance, the conventional
Figure III-2. Ion-pairing SPE-MALDI/MS spectrum of insulin (2 picomole/µL) from a solution of the peptide saturated with sodium acetate. These spectra were acquired with (A) a strong cation-pairing (3-mercaptopropanesulfonic acid) surface and (B) a strong anion-pairing (2-aminoethanethiol hydrochloride) surface.
MALDI experiment failed to obtain a mass spectrum as expected due to the high concentration of contamination. SPE-MALDI, on the other hand, provided intense molecular ions that were free from all of the interferences. These spectra are not included in this report for the sake of brevity. We proceeded to analyze samples with multiple interfering agents since the isolation of an analyte from a solution containing high concentrations of multiple contaminants is a better representation of the complexity of biological media and also better demonstration of the capabilities of SPE-MALDI.

To dramatically illustrate the sample cleanup prowess of ion-pairing SPE-MALDI, a series of peptides and proteins, ranging in molecular mass from 500 to 71,000 Da and dissolved in a solution containing 20%(v/v) Triton X-100, 8M urea, and saturated sodium acetate or sodium chloride were analyzed using each type of SAM surface and the previously described clean-up protocol. These solutions are probably more contaminated than most scientists will ever encounter, but they serve to demonstrate the level of interferences that can be successfully handled by ion-pairing SPE-MALDI. In each of these experiments, probes modified with the carboxylate, sulfonate and amine-terminated SAMs were successful. Figure 3 demonstrates the quality of the spectra that were obtained from the highly contaminated sample of insulin (2 picomole/µL). Conventional MALDI analysis of these samples, on the other hand, was unsuccessful in all of these instances, as expected, since a single contaminant at such a high level is detrimental to formation of the analyte ion.

We implemented various control experiments to ensure that the surface modifications were the cause of our successful isolation of the peptides and proteins in the
Figure III-3. Ion-pairing SPE-MALDI/MS spectra of insulin (2 picomole/µL) from a solution of the peptide containing containing 20%(v/v) Triton X-100, 8M urea, and sodium acetate at saturated concentration. These spectra were acquired with (A) a strong cation-pairing (3-mercaptop-1-propanesulfonic acid) surface, (B) a weak cation-pairing (3,3’-dithiodipropionic acid) surface, and (C) a strong anion-pairing (2-aminoethanethiol hydrochloride) surface.
above solutions. We were unsuccessful at obtaining spectra from peptides or proteins in solutions containing a single or multiple contaminants when an underivatized probe was substituted for a probe with an ion-pairing surface. These results suggest that an underivatized surface does not share the binding characteristics of the ion-pairing surfaces. We were also unable to acquire a spectrum from a peptide/protein dissolved in either the single or multiple contaminant solutions by conventional MALDI, i.e., mixing a 1 µL aliquot of sample with 1 µL of matrix, depositing this on the probe, and analyzing the sample directly. The absence of signal in these experiments confirmed that conventional preparatory methods are not suitable for this level of contamination.

At this point, our principle concern dealt with the nature of the peptide-monolayer binding. In particular, was the extraction actually a result of electrostatic interactions between the analyte and the surface or a result of other forces such as hydrophobic interactions? To lend evidence to the ion-pairing concept, we designed several experiments using a variety of wash solutions ranging from slightly to strongly acidic washes, and washes using organic solvents. Mild acid washes (pH 5.2) had no affect on the performance of the tips. Both insulin and cytochrome C were retained on the surfaces after mild acid washes on the carboxylate, sulfonate and amino tips. However, stronger acid washes (0.1% TFA pH 2.3) interrupted the ion-pairing between both insulin and cytochrome-C only on the carboxylate surface but not on the other negatively charged monolayer, the sulfonate. This can be attributed to the pKa of the two negatively charged surfaces. Specifically, under these conditions the carboxylate surface is expected to be fully protonated and hence have no charged groups available for ion-pairing, while the
sulfonate surface should still possess a negative charge. Washes with organic solvents had no effect on the signal suggesting that hydrophobic interactions play little to no role in the extractions of these surfaces. These results suggest that the peptides and proteins are indeed attracted and held to the surface via ion-pairing interactions.

Although the monolayer allows the sample to bind, it is the washing step that ultimately removes the contaminants from the probe, which in turn permits the mass analysis to occur in the absence of the interferences. Because the washing procedure used to remove the contaminants may also remove unbound peptides or protein, we were concerned with contamination of neighboring samples on the same probe tip during the wash. Theoretically, any unbound peptide removed in the wash could contaminate neighboring samples. Such migration, if unforeseen, could destroy the integrity of the samples involved particularly if different analytes were loaded onto adjacent sample regions on the same probe ring. To study the possibility of such migration, we loaded two different peptides, somatostatin and angiotensin (at a concentration of 2 picomole/µL in the multiple contaminant solution described above) onto adjacent sample regions of the amine-terminated SAM probe. There was no apparent migration or cross contamination from either peptide following the wash with de-ionized water (Figure 4). Consequently, sample migration does not appear to occur at concentrations above our limit-of-detection.

The spectra shown in Figure 4 also demonstrates the level at which the interferences are removed with this approach. The peptides analyzed and the detergent have similar molecular masses. However, the only signals observed in this m/z range are
Figure III-4. Ion-pairing SPE-MALDI/MS spectra of somatostatin and angiotensin (2 picomole/µL) in 20%(v/v) Triton X-100, 8M urea, and saturated sodium acetate), placed onto adjacent sample regions of an amine-terminated SAM probe. This experiment demonstrates that samples do not migrate between sample regions during the de-ionized water wash used to remove the contaminants.
due to peptide ions even though the detergent/surfactant was present at 20% (v/v) while the peptide was present at only 2 picomole/µL.

CONCLUSIONS

We have used MALDI-probe tips derivatized with self-assembled monolayers terminated with a variety of chargeable functional groups to separate both peptides and proteins from solutions containing salts, chaotropic agents, and non-ionic detergents or surfactants. Both negatively charged and positively charged surfaces allowed the separation of the sample and, effectively creating favorable conditions for MALDI-TOF analysis. However, due to the nature of the ion-pairing interactions, samples containing ionic detergents, such as SDS, are not amenable to ion-pairing SPE-MALDI at this time.

The preparation of the monolayers, samples and the subsequent analyses of the solutions are relatively easy to perform and yield spectra which would have been difficult if not impossible to obtain using conventional MALDI techniques. The ion-pairing nature of these surfaces offers the potential for a broad range of applications such as the analysis of oligonucleotides and oligosaccharides.
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CHAPTER IV

EVALUATING THE BINDING EFFICIENCY OF MALDI PROBES CONTAINING ION-PAIRING SURFACES

2 Warren, M.E., Orlando, R. To be submitted to Rapid Communications in Mass Spectrometry for publication at a later date.
ABSTRACT

We have previously developed a method in which 'dirty' or contaminated peptide and protein solutions can be cleansed directly on the sample stage of a MALDI instrument. The sample stage or probe was specifically modified to contain positively or negatively charged surfaces formed by monolayers. The probes function similar to solid-phase extraction media and was found useful as a decontamination device. In this work, we evaluate the effects of multiple uses, length of time in storage and probe preparation times on the binding efficiency of one type of modified surface. The binding efficiencies under these various constraints were measured using a quantitative MALDI approach in which ions formed from an internal calibrant were used to normalize the signal intensity of the analyte ions. We first evaluated the optimal length of time allowed for the formation of the monolayer on the probe surface. A thirty-minute reaction time appears to be sufficient period to form a monolayer with maximum binding efficiency. Shorter reaction times resulted in less binding and no improvement was gained using longer reaction times. The probes are also robust under ambient conditions. After 120 days in storage the modified probes exhibited the same binding efficiency as those freshly prepared. Each probe can also be used multiple times (up to 10 times) if a specific wash protocol is performed between uses. The protocol requires successive washes with methanol, water and dilute hydrochloric acid. The ability of these surfaces to be prepared quickly, reused, and stored are attractive features not only for potential commercial production but also on a smaller scale for routine analysis in small laboratories.
INTRODUCTION

Since it’s inception in 1988, biomolecular MALDI mass spectrometry has fulfilled its promise as one of the most powerful tools for biomolecular analyses.\(^1\) The wealth of information provided by an accurate molecular mass has helped answer numerous questions regarding the structure, function and interactions of peptides, proteins, lipids, carbohydrates and nucleic acids - the gamut of biomolecules.\(^2\) In a MALDI experiment the biomolecular sample is mixed with a UV absorbing matrix. A pulse of energy emitting from a laser, typically a nitrogen laser, irradiates a target on the probe, which contains the solid sample/matrix. The simplicity of this sample preparation, ease of operation and high mass range have made MALDI a favorite and reliable tool in the bioanalysts workbench.

Another attractive feature of MALDI is its tolerance to low levels of contaminants. However, most biomolecular samples are obtained by extraction from their biological sources. The levels of salts, buffers, chaotropes and detergents that may be used for the extraction often exceed this tolerance level. The presence of these contaminants either totally precludes MALDI analysis or results in low quality spectra. Consequently, extracted samples must often be purified prior to MALDI analysis. These samples are commonly purified off-line using chromatographic techniques or dialysis. Moreover, as a result of the purification the sample becomes so dilute that steps must be taken to concentrate the sample to a level detectable by MALDI. These additional steps further increase the sample preparation time and the likelihood of adsorptive sample losses.
Purification of the sample directly on the MALDI probe simplifies sample preparation and circumvents the tedious and time-consuming purification steps of HPLC and dialysis. This ‘on-line’ method requires modifying the existing MALDI metal probes or using media, which have an affinity for the biomolecule. In creating these modified probes there are two considerations: 1) the interaction of the media with the metal probe surface and 2) the desired interaction of the media with the biomolecule.

In the first consideration the separation/purification media can be covalently immobilized to or can be simply deposited on the probe surface. Several groups have experimented with each of these techniques generally for the purposes of using the probes as affinity media.3-9 In an example of a technique using deposited media, antigen-bound agarose beads deposited on the MALDI surface were used to fish for specific peptides of a growth factor that interacted with mouse monoclonal antibody.7 In a second application avidin-bound agarose beads were used to extract biotinylated peptides. As an example of the covalently bound media, Brockman, et al immobilized monoclonal antibody IgG1 directly on the probe to capture and subsequently analyze biotinylated insulin.9

The second consideration when preparing these modified probes is the desired interaction of the surface with the biomolecule of interest. The probe can be tailored to interact with a specific analyte or can be designed to have interactions with multiple analytes. Probes designed to interact with specific analytes typically involve antibody-antigen or avidin-biotin interactions in which one member of the pair is fixed directly to the surface or is fixed to media contained on the surface.3,9 Probe surfaces designed for multiple analytes typically contain hydrophobic or ionic groups that can capture a variety
of biomolecules without discrimination. Most of these surfaces are applied or deposited to the MALDI target. Various hydrophobic membranes such as polypropylene, nitrocellulose and polyethylene have been used in this manner as sample supports for peptides, proteins and nucleic acids.4,10-13

Our research has focused on creating MALDI targets with various covalently bound molecules that present hydrophobic and ionic groups at the probe surface. In our strategy, we use established chemistry between thiolates and gold to create self-assembled monolayers on the MALDI probe.14 The terminal functional groups of the monolayer constitute the exposed surface and thus interact directly with complementary functional groups of the biomolecular analyte. Simply by choosing thiolates with different termini we have created a variety of hydrophobic and ionic surfaces.

Although most of the modified probes described were designed specifically for extraction or purification purposes from mixtures of compounds they can also be used as a means for sample concentration and sample clean up. We have directed our modified targets towards this purpose. In previous work we produced MALDI targets with hydrophobic (CH₃) and hydrophilic surfaces and developed techniques for their primary use as sample clean up media.15-17 The more successful experiments involved probes containing monolayers with hydrophilic, charged functional groups. Probe surfaces containing terminal NH₃⁺, COO⁻ and SO₃⁻ proved successful in extracting both peptides and proteins from solutions with high concentrations of various types of salts, detergents and denaturing agents.16
Figure IV-1. Illustration of the MALDI probe ring with a close-up of a single probe target modified with amine-terminate self-assembled monolayers (inset). Note that the sulfur groups of the monolayer are covalently bound to the gold coating on the MALDI probe.
Efficient use of these probes for routine analysis requires their preparation in a batch, storage of the batch with minimal loss of function, and use and reuse when needed. Herein, we examine the reusability of the positively charged NH$_3^+$ probe (Figure 1) and the effect of continual use on the binding efficiency of the surface. The change in binding efficiency was also measured after storing the probes for up to four months. Furthermore, we determine the minimum reaction time in which a surface with acceptable binding efficiency can be formed. For all of the experiments a quantitative approach using an internal standard, added to the matrix, was used to analyze and compare the data under the different constraints.

**EXPERIMENTAL**

*Preparation and Modification of the MALDI Probe*

The unmodified MALDI probe is a thin, annular stainless steel ring (Hewlett-Packard, Palo Alto, CA) that can be threaded onto a probe holder prior to insertion into the mass spectrometer. Each ring contains eight platforms or stages that are used to contain and present the sample to the laser and the mass spectrometer. In this manuscript, we refer to a single sample stage as 'sample target or target' and refer to the entire ring as a 'probe'. This ring was chemically etched with an acid cocktail of 5.4 mL of 70% nitric acid (Aldrich, Milwaukee, WI) and 24.6 mL of 37% hydrochloric acid (Sigma, St. Louis, MO). The etching process creates pits on the normally smooth surface and thereby increases the surface area and consequently may increase the monolayer coverage and analyte binding. High purity gold (~400Å) was then deposited onto the surface of the clean rings using a
commercial sputtering device obtained from Denton Vacuum (Moorestown, NJ). To form the self-assembled monolayer on the probe surface, the gold rings were immersed in a saturated solution of 2-aminoethanethiol*HCl (Fluka, Buchs, Switzerland) dissolved in anhydrous ethanol, (Fisher, Fair Lawn, NJ). Anhydrous ethanol was prepared by drying the solvent over aluminate (Fisher), filtering the suspension using a 0.22-micron filter then degassing the dried ethanol with helium.

**Mass Spectrometry**

Mass spectra were obtained using a Hewlett Packard G2030A (Palo Alto, CA) MALDI TOF mass spectrometer. The instrument was operated at 28kV accelerating voltage, 7kV extractor voltage, and at a pressure of ~1 x 10^-6 Torr. We used peptide standards covering the mass range of ~ 500-6000 Da to calibrate the instrument and sinapinic acid (Aldrich)(10 mg/mL) dissolved in 80 % aqueous acetonitrile (Baker) as the matrix for all samples. UV radiation (337 nm) emitting from a nitrogen laser source was used to desorb and ionize the matrix and sample from the surface of the probe. In a typical analysis by MALDI, the laser power can be varied from shot to shot. Hence, the spectrum obtained is an average of ions with variable intensities produced at different laser power. To reduce this variation, we used a fixed laser power when obtaining data for the binding efficiency measurements. In the tests for surface reusability the laser fluence was varied until an optimum ion signal was achieved. The spectrum reported for each experiment is the average of 25 spectra.
**Tests for Reusability of the Modified Targets**

A single modified probe was prepared as described above allowing a 30-minute monolayer formation time. For these particular experiments, three peptide test samples, renin substrate, substance P fragment and angiotensin I (Sigma) were prepared at a concentration of 20 pmol/µL in a 0.5 M NaCl solution. To test the reusability of the surface, a single target of the probe was used repeatedly to bind each of the aforementioned peptides in succession.

The samples were prepared for MALDI analysis in the following manner. One microliter of the peptide solution was deposited on the modified surface then dried using a sample vacuum accessory fitted to the mass spectrometer. Contaminants such as salts were removed by washing the ring briefly (~30 seconds) in deionized water. The surface containing the cleansed sample was once again dried *in vacuo*, layered with 0.1 µL of the MALDI matrix, and then analyzed by MALDI-MS. Once the first peptide was analyzed the surface had to be cleansed in order to receive the next sample. A wash protocol was developed to remove the spent sample/matrix and regenerate a clean surface. The protocol requires successive immersion of the used ring in methanol, deionized water, 0.1 mM HCl and a final bath of deionized water. Each peptide was prepared in this manner onto the same region of the MALDI target.

**Preparation of Samples for Binding Efficiency Measurements**

The remaining experiments compare spectra using probes exposed to variable experimental conditions such as increasing reaction times for monolayer formation and
increased storage times. Comparative measurements in MALDI typically require use of an internal or internal calibrant to normalize the analyte ion intensities from spectra to spectra.\textsuperscript{18} We chose the neuropeptide angiotensin I to serve as the internal calibrant. For these experiments, the analyte, renin substrate, was deposited onto the modified surface in one-microliter volumes and dried. The ring was washed with water and dried again as described in the previous section. A solution containing the matrix and 10 picomoles of the internal standard was then deposited to a modified target prior to MALDI analysis. The signal intensities of the analyte and the internal standard were measured from the resulting spectrum. Herein we refer to the binding efficiency as the ratio of the renin-to-angiotensin ion intensities.

\textit{Surface Binding Efficiency: Effect of Monolayer Formation Time}

A batch of gold rings was immersed in a solution containing aminoethanethiol. Three rings were removed periodically after 15 and 30 minutes, 1, 2, 4, 8, and 16 hours into the reaction. The reactants were maintained in a sealed container and were gently shaken at room temperature (22\textdegree C). The binding efficiencies of the surfaces were evaluated immediately after removing the surface from the reactant solution using the procedure described above.

\textit{Surface Binding Efficiency: Effect of Storage}

A second batch of gold probe rings was simultaneously derivatized with aminoethanethiol for 30 minutes. The surfaces were rinsed with ethanol, dried at room
temperature then stored in a loosely sealed glass container at room temperature. The binding efficiencies of three surfaces were measured after 1, 30, 60, 90 and 120 days under these storage conditions.

**Surface Binding Efficiency: Effect of Reusing the Surface**

A single modified target was used in eleven successive experiments. For each experiment renin substrate was applied, desalted, dried then layered with the angiotensin-doped matrix solution. MALDI measurements were taken then the surface was cleaned using the previously described wash protocol. The binding efficiencies after each experiment were measured and compared to gauge the effects of repetitive and multiple uses.

**RESULTS AND DISCUSSION**

**Reusability of the Modified Targets**

The standard probes provided with commercial MALDI instruments are made of stainless steel or gold that are easily cleansed and reused for multiple analyses. Thus, if the modified surfaces are to be applied for 'routine' analysis, they should also be robust and reusable.

Reuse of the modified target requires removal of the dried sample and matrix used in the preceding experiment while maintaining the integrity of the positively charged monolayer. We developed a protocol (Figure 2) in which a series of washes is able to clean 'dirty' probes thus allowing them to be used for subsequent samples. The wash
Figure IV-2. A wash protocol used the remove the spent sample from the modified surface. The probes are washed by immersion into each of the wash solutions.
solutions were chosen to remove all non-covalently bound species from the MALDI surface. Methanol removed the dried organic matrix, which is soluble only in moderate to high concentrations of organic solvents. To remove the electrostatically bound analyte we adopted the acidic wash solution normally used in BIACORE instruments in which 100 mM HCl is used to disrupt the ionic interactions between proteins and a charged flow cell. Use of this particular wash releases the analyte from the probe surface. The surfaces were rinsed with deionized water after the methanol and HCl washes to remove the liberated species.

Figures 3a-c demonstrate the successful removal of spent sample using the wash protocol. The spectra shown as Figures 3a, 3b and 3c are MALDI spectra of three different peptides renin substrate, substance P fragment 2-11, and angiotensin, respectively. The experiments were performed separately on the same target of a modified probe. Each peptide was loaded, dried, layered with matrix, and then analyzed by MALDI. The probe was washed after each experiment using the wash protocol outlined in Figure 2. In the spectrum shown as Figure 3a the first peptide applied, renin substrate, produced a strong molecular ion peak at 1759 m/z. The minor peak at ~1650 m/z is a peptide contaminant, presumably a degradation product, that was found in the original stock solution and therefore is not a consequence of our experiments. In the spectrum shown as Figure 3b, the major peak produced by MALDI (1192 m/z) corresponds to the molecular ion of the substance P fragment 2-11- the second peptide tested. MALDI-MS of the final sample produced the molecular ion of angiotensin I at 1297 m/z. In each spectra only ion signals from the analyte currently being measured but not from the previous
Figure IV-3. MALDI-MS spectra of peptides applied in succession to a single target on the modified probe: (a) renin substrate, (b) substance P-fragment 2-11, and (c) angiotensin I. These spectra demonstrate the success of the wash protocol as evidenced by the absence of ions from the previous sample.
sample were observed. Strong molecular ion peaks were produced from each sample and cross contamination was not observed as a consequence of the desalting wash or the surface regeneration washes.\textsuperscript{16}

\textit{Surface Binding Efficiency: Effect of Reusing the Surface}

The previously described experiments confirmed that the modified probes could be reused without memory effects. However, repetitive use of the modified probes may in time change the surface characteristics and its capacity to bind analyte. To test this possibility a single modified target was repeatedly used in eleven experiments in which renin substrate was applied, cleansed, analyzed and removed from the target surface. The spectra taken after the first and final application are shown as Figures 4a and 4b, respectively. The presence of strong molecular ion signals for renin substrate was observed even after ten cycles of reuse. However, it appears that a decrease in analyte binding may be a consequence of repetitive use. The binding efficiency of the modified probes for the first experiment as measured from the renin-to-angiotensin ion ratio was 0.7 but by the eleventh use the efficiency decreased by 40\% to an efficiency of 0.3.

Repeated use of the same probe appears to degrade or damage the monolayer on the surface. The organic, acid, and aqueous washes should have no effect on the stability of the monolayer since the gold-thiol interaction is a strong covalent bond of \textasciitilde44 kcal.\textsuperscript{19} The monolayer may be damaged by repeated exposure energy from the UV laser used in the MALDI experiments. If this were the case the monolayer components (Au and aminoethanethiol) should also produce low mass ions. Ions in the low mass range (< 500
**Figure IV-4.** MALDI-MS spectra of 10 pmol of renin substrate and 10 pmol of angiotensin I analyzed on a single modified target upon the first (a) and eleventh (b) use. The signal intensity of the analyte, renin substrate decreases by 40% upon the eleventh use suggesting a decreased in surface binding capacity with increased use.
m/z) are in practice difficult to resolve and identify due to strongly interfering ions from the matrix and solvents. Therefore to truly test our hypothesis other analytical methods such as X-ray photoelectron spectroscopy or atomic force microscopy must be used to characterize the monolayer structures of these 'damaged' probes.

**Surface Binding Efficiency: Effect of Monolayer Formation Time**

Derivatization of the MALDI probe target involves reaction of the alkanethiol with the gold target to form the monolayer. The character of the monolayer and hence its binding efficiency can be affected by the length of this reaction which we refer to as the monolayer formation time. We analyzed the binding efficiency using probes derivatized for different time periods to study these effects. The results of these experiments are plotted in Figure 5. There is essentially no change in the binding efficiency of the surface between probes derivatized at 0.5 and 5 hours. The ratio of renin substrate: angiotensin I signal intensities averaged 0.79 +/- 7% during this period. The variability can be attributed to minor differences in surface area and monolayer coverage among the different targets tested. An incubation time of thirty minutes appears to be a sufficient period to generate a monolayer with acceptable binding ability. For reasons unknown at this time, a decrease in binding efficiency is observed at longer reaction times of 8 and 16 hours.

**Surface Binding Efficiency: Effect of Storage**

A desirable characteristic for any solid-phase extraction medium is stability under ambient conditions. This allows preparation of the media in batches to reduce the probe
Figure IV-5. Change in binding efficiencies of modified targets with increasing reaction time for monolayer (probe) preparation.
Figure IV-6. Change in binding efficiencies of modified targets with increasing time in storage.
preparation time. It also allows prepared media to be stored until needed. The stability of the modified targets under ambient storage conditions was tested by measuring the binding efficiency after 1, 30, 60, 90 and 120 days in storage. The results of these experiments are plotted in Figure 6. The binding efficiency was calculated as 0.8 after one day and 0.7 after 120 days of storage. Between these periods the binding efficiency changes in a random manner with a maximum value of 1.0 after 30 days, a minimum 0.7 after 120 days and an average of 0.8. The variability in the binding efficiency may once again be a consequence of using different probes. We tried to reduce this variability by using probes from the same batch and obtaining average measurements from experiments performed on three sample targets within the probe ring. There are probably differences even among the sample targets, differences that cannot be eliminated at this time. Although there is variability in the data the degree of variability is low and thus we conclude that the modified targets can be stored for up to 120 days and used with no significant decrease in performance.

CONCLUSIONS

The amine-terminate MALDI probe can be used routinely and efficiently as a decontamination device for 'dirty' peptide and protein samples. Creation of the modified probes is relatively easy, inexpensive and time efficient, requiring only 30 minutes. The probes are robust up to four months under ambient storage conditions. Therefore modified surfaces can be quickly made in batches rather than on an as needed basis. This further decreases the probe preparation time by a factor determined by the number of probes in the
batch. Each probe can be reused albeit with a 40% reduction in the binding efficiency after ten uses. However, if each probe is used only 4-5 times and the probes are made in batches then use of the probe is still both time and cost efficient especially when compared to conventional decontamination devices. The amine-terminate modified probes, which were characterized, constitute the foundation of expanding research on developing alternative ionic surfaces. The current focus of this research is in the creation of surfaces with greater binding efficiency and with increased applications.
ACKNOWLEDGMENTS

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CHAPTER V

TANDEM MASS SPECTROMETRIC ANALYSIS OF ASPERGILLUS NIGER
PECTIN METHYLESTERASE; THE MODE OF ACTION ON FULLY
METHYLESTERIFIED OLIGOGALACTURONATES

3 Warren, M.E., Kester, H.C.M., Benen, J.A.E., Visser, J., Orlando, R., Bergmann, C.,
Anker, D., and Doutheau, A. Reproduced with permission from Biochemical Journal
ABSTRACT

The substrate specificity and the mode of action of *Aspergillus niger* pectin methylesterase (PME) were determined using both fully methylesterified oligogalacturonates with degrees of polymerization (DP) 2-6 and chemically synthesized mono-methyltrigalacturonates. The enzymatic activity on the different substrates and a preliminary characterization of the reaction products was performed by high performance anion-exchange chromatography (HPAEC) at neutral pH. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was used to localize the methyl esters on the $^{18}$O-labeled reaction products during the course of the enzymatic reaction. *Aspergillus niger* PME is able to hydrolyze the methyl esters of fully methylesterified oligogalacturonates with DP > 2 and preferentially hydrolyzes the methyl esters located on the internal galacturonate residues, followed by hydrolysis of the methyl esters towards the reducing end. This PME is unable to hydrolyze the methyl ester of the galacturonate moiety at the non-reducing end.
INTRODUCTION

Pectin, a major constituent of the cell walls of higher plants, is a complex heteropolysaccharide composed of segments of “smooth” homogalacturonan regions and “hairy” rhamnogalacturonan regions. During plant ripening and other developmental processes as well as during attack by plant pathogens, pectin is degraded by an array of enzymes that can either depolymerize the pectin backbone (polygalacturonases, pectin and pectate lyases) or alter the structure of the intact backbone (pectin acetylesterases and methylesterase). Pectin methylesterases (PME) catalyze the hydrolysis of galacturonate methylesters in the backbone, producing pectate, which is vulnerable to the depolymerizing polygalacturonases and pectate lyases. 1 PMEs, produced by numerous plants, bacteria and fungi, 2-6 may differ with respect to their pH optima, iso-electric points and requirement of calcium for activity 2. Another notable difference is the mode of action by which methyl groups are hydrolyzed. Fungal PMEs have been shown in some cases to hydrolyze the methylesters of pectin in a random fashion, whereas in plant PMEs a block-wise de-esterification of pectin has been reported. 3,4

The activity of the pectin methylesterases is thought to be restricted to the “smooth” homogalacturonan regions of the pectin molecule, but absolute substrate specificity of fungal PME has not been firmly established. Recently it was demonstrated that PME from Aspergillus niger hydrolyzed only 50% of the methylesters of apple pectin. 5 Instead of using complex, poorly characterized pectin, well-defined oligogalacturonates may be more suitable substrates to examine the specificity of PME. This approach has been used to study the activities of tomato and fungal pectinases on
partially methylesterified oligogalacturonates. Although the results showed clear differences between the activities of the two enzymes only preliminary conclusions regarding the substrate specificity could be drawn since the reaction products were not fully characterized.\textsuperscript{6} Determining the substrate specificity of PME would require activity measurements on a series of fully characterized oligogalacturonates with subsequent analysis of the reaction products.

Mass spectrometry is becoming an important tool in the analysis of the substrate specificity of pectin-degrading enzymes. Information on substrate specificity of the pectolytic enzymes polygalacturonase and pectin lyase has been obtained using mass spectrometric techniques.\textsuperscript{7-9} Products resulting from the digestion of partially methyl-esterified pectin by endopolygalacturonase were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in conjunction with high performance anion exchange chromatography (HPAEC).\textsuperscript{7} Although this method effectively determined the number of methylesters on the products, it cannot be used to distinguish between methyl ester isomers in a mixture. More recently, tandem mass spectrometry using an ion-trap mass spectrometer was used to analyze the products of polygalacturonase II, exopolygalacturonase, and pectin lyase digestions of isotopically labelled, partially methyl-esterified pectin.\textsuperscript{9}

In this paper the mode of action of \textit{A. niger} PME is studied using fully methylesterified oligogalacturonates and chemically synthesized mono-methylesterified trigalacturonates.\textsuperscript{10} The mode of action of PME was established by determining its
activity on the different substrates and by ESI-MS/MS analysis of the reaction products formed during the course of the reaction.

**EXPERIMENTAL**

*Preparation of fully methylesterified and mono-methylesterified oligogalacturonates*

Oligogalacturonates of defined length (degree of polymerization (DP) 2 – 6) were prepared as described in previous work. The oligomers (50 mg) were dissolved in 5 mL methanol, 0.02 M HCl. After 14 days at 4 °C the esterification reaction was stopped by the addition of 80 mg (0.3 mmol) Ag₂CO₃. The silver chloride precipitate was removed by centrifugation and methanol was removed by evaporation under a stream of nitrogen. The dried material was dissolved in water (1 mL), loaded onto a 4 mL AG 501-X8 (Bio-rad Laboratories, Richmond, CA) mixed bed resin column, eluted with water and lyophilized to remove the partially methylesterified oligomers from the reaction mixture. The homogeneity of the oligogalacturonates with respect to the degree of polymerization and degree of esterification was analyzed by HPAEC at pH 12 and pH 5, respectively. The dried oligomer preparations were dissolved in water and the concentration was determined by measuring the uronate content. Mono-methylesterified trigalacturonates were synthesized chemically as described in previous work.

*Preparation of Pectin Methylesterase*

Pectin methylesterase was purified from the culture fluid of an *A. niger pkiA-pme* transformant grown on minimal medium with fructose as carbon source (J.A.E. Benen,
unpublished work). Prior to its use the enzyme preparation was checked for contaminating endopolygalacturonase (PG) activity. This was accomplished by mixing 0.05 mL with 0.5 mL of 1 % (mass/vol.) polygalacturonic acid in 50 mM Na-acetate buffer, pH 4.2, and incubating at 30 °C for 24 h. Potential endopolygalacturonase activity in the PME preparation was monitored by analyzing the oligomeric reaction products by HPLC as described in previous work. 12

**Measurement of PME activity on fully methylesterified oligogalacturonates (DP 2-6)**

Fully methylesterified oligogalacturonates with DP 2-6 (2.5 mM) were incubated with PME at 25 °C in 50 mM Na-acetate buffer, pH 5.0, in a total reaction volume of 0.2 mL. At timed intervals 35 µL of the reaction mixture was analyzed by HPAEC on a Carbopac PA-100 column (250 x 4 mm, Dionex, Sunnyvale, USA) at pH 5, as described previously. 10 The activity of PME on these substrates was calculated from the initial decrease in concentration of the fully methylesterified substrates in the reaction mixture with respect to time.

**Measurement of PME activity on fully and mono-methylesterified trigalacturonates**

Fully methylesterified trigalacturonate and the three mono-methylesterified trigalacturonates were incubated with PME at 25 °C in 50 mM Na-acetate buffer, pH 5.0. The substrate concentration was 0.5 mM in a total reaction volume of 0.2 mL. At timed intervals 20 µL of the reaction mixture was used to analyze the reaction products by HPAEC as described in the section above. For fully methylesterified trigalacturonate, the
PME activity was calculated as described in the previous section. The activity on the mono-methylesterified trigalacturonates was calculated from the initial increase of trigalacturonate, using trigalacturonate as an internal standard.

**Enzyme incubations and sample preparation for mass spectrometry analysis**

Fully methylesterified oligogalacturonates (DP 3-6) in 50 mM Na-acetate buffer, pH 5.0, were incubated with PME for 0.5 and 24 h. The substrate concentration was 5 mM in a 0.2 mL total reaction volume. The reaction was stopped by removal of the enzyme from the reaction mixture using a 0.2 mL Dowex 50W-X2 (H⁺) column from which the oligomers were eluted with 0.6 mL water and subsequently lyophilized. Use of the cation exchange column not only allowed removal of the enzyme but also converts the products from the sodiated species to the protonated form, which is more amenable to mass spectrometry.

**¹⁸O-exchange of the oligogalacturonate anomeric oxygen**

Sequencing carbohydrates by MS/MS requires the use of a label to differentiate between the reducing and non-reducing ends. Therefore, prior to mass spectrometric analysis, the lyophilized oligomeric reaction products were treated with ¹⁸O water. This exchanges the anomeric oxygen with ¹⁸O, providing a label on the reducing end of the oligogalacturonate. The exchange was accomplished by dissolving 50 µg of each sample in 50 µL of ¹⁸O water (Isotec, Miamisburg, OH USA). Formic acid (0.5% (v/v)) (J.T.
Baker, Phillipsburg, NJ USA) was added to catalyze the ring-opening and the reaction was allowed to proceed for 24 hours at room temperature.

**Mass Spectrometry**

Mass spectra were acquired using a PE-Sciex (Norwalk, CT, USA) API-III triple-quadrupole mass spectrometer equipped with an electrospray ion source. In a typical analysis 0.5 µg of the $^{18}$O-labeled sample was introduced into the mass spectrometer by flow injection through a 10 µL sample loop. The solvent, comprised of 49%: 49%: 2% methanol/water/acetic acid (v/v/v), was delivered by an Applied Biosystems (Foster City, CA, USA) 140B solvent delivery system at a flow rate of 5 µL/min. During MS experiments, the instrument scanned a range of 600 m/z, centered on the mass of either the fully methylesterified trimer, tetramer, pentamer or hexamer at a rate of 12 scans/min for an average of 20 scans. The precursor ion was selected in MS/MS experiments by the first quadrupole (Q1), collision-induced dissociation (CID) was performed in the second quadrupole (Q2), and the fragments were analyzed based on their m/z ratios in the third quadrupole (Q3). Argon was used as the collision gas at a thickness of approximately $3.00 \times 10^{14}$ molecules/cm$^2$. The third quadrupole scanned m/z ranges of 100-600, 100-800, 100-1000 and 100-1300 for the trimer, tetramer, pentamer and hexamer, respectively, at 12 scans/min.
RESULTS AND DISCUSSION

Activity of PME on fully methylesterified oligogalacturonates with DP 2-6

PME was active on the fully methylesterified oligogalacturonate trimer, tetramer, pentamer and hexamer but was not active on the dimer. Additionally, the initial rate increased drastically with the degree of polymerization of the substrate (Table 1), a phenomenon also observed for PME from *A. foetidus*. Specifically, the activity on the hexagalacturonate was 100-fold higher than on the trigalacturonate, 10.7 and 0.1 U.mg⁻¹, respectively. This indicates that the substrate binding site of pectin methylesterase is composed of multiple subsites, which is a common feature for pectin depolymerising enzymes. Oligomers with a higher degree of polymerization interact with more subsites during substrate binding. This leads to a higher affinity and also, as a consequence, an increase in the reaction rate with increasing chain length.

Calculation of the initial rates was based on the time-dependent decrease in concentration of the fully methylesterified substrates as analyzed by HPAEC. Since HPAEC analysis is based on charge differences and the products have the same degree of polymerization (DP), the separation is solely based on the net charge of the oligomer, which is dependent on the degree of esterification. Under the conditions used for the separation the fully methylesterified substrates were not retained by the column. In the same analysis the product progression was monitored. HPAEC analysis revealed that a limited number of early eluting, highly methyl-esterified products were formed. Prolonged incubation resulted in further de-esterification of the initially formed products. The
Table V-1. Initial reaction rates of *Aspergillus niger* pectin methylesterase on fully methylesterified oligogalacturonates with degree of polymerization (DP) 2-6. The reaction conditions were: 2.5 mM oligomer in 50 mM Na-acetate buffer, pH 5.0, 25 °C

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<th>Degree of polymeriation</th>
<th>PME activity (units/mg)</th>
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<tr>
<td>2</td>
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<td>3</td>
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products arising from fully methylesterified trigalacturonate (Fig. 1a, peak 2 and 3) were
assigned as di- and mono
methyltrigalacturonate, respectively. These peak assignments were made by comparing
the retention times of the products with those of a standard mixture of oligogalacturonates
(DP 1-3) and previously synthesized mono-methyltrigalacturonates (3 isomers,
methylesterified at position 1, 2 and 3, relative to the reducing end). Using the HPLC
method it was not possible to determine whether peaks 2 and 3 were composed of more
than one isomer with similar charge as the three synthetic mono-methyltrigalacturonate
isomers co-eluted at approximately 12 minutes and pure di-methyltrigalacturonate isomers
were not available.

Performance of PME on mono-methylesterified and fully methylesterified
trigalacturonates

As shown in the product progression profile (Fig. 1b), di-methyltrigalacturonate
was the initial product formed from fully methylesterified trigalacturonate. Prolonged
incubation with the enzyme resulted in further production of di- methyltrigalacturonate as
well as the formation of mono-methyltrigalacturonate as a second-generation product.
This latter product is rather resistant to de-esterification as evidenced by reaction rates of
PME on mono-methyl trigalacturonates (Table 2). Two of the mono-
methyltrigalacturonates showed reduced activity, whereas 3-methyltrigalacturonate was
not a substrate. This latter result, in combination with those from the MS/MS analysis of
the 24-h sample demonstrates that the second-generation product is indeed 3-
Figure V-1. (A) Time-course HPAEC analysis of products from incubation of fully methylesterified trigalacturonate with *A. niger* pectin methylesterase. The incubation conditions are: 0.5 mM substrate in 50 mM Na-acetate buffer, pH 5.0, at 25 °C. The products were eluted in the order: trigalacturonate with three (peak 1), two (peak 2) and 1 (peak 3) methylesters, respectively. (B) Progression of product formation from time-course HPAEC analysis of products on incubation of fully methylesterified trigalacturonate with *A. niger* pectin methylesterase. Traces are for trigalacturonate products with three (trace 1), two (trace 2) and 1 (trace 3) methylesters.
Table V-2. Performance of *Aspergillus niger* pectin methylesterase on fully and mono-methylesterified trigalacturonates. Incubation conditions were: 0.5 mM oligomer in 50 mM Na-acetate, pH 5.0, 25 °C. The position of the methyl ester is numbered from the reducing end. G* symbolizes a methylesterified oligogalacturonate. The reducing-end GalA is indicated in green

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>Specific activity (mU / mg)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully methylesterified trigalacturonate</td>
<td>G* - G* - G*</td>
<td>70</td>
</tr>
<tr>
<td>1- methyltrigalacturonate</td>
<td>G - G - G*</td>
<td>3.3</td>
</tr>
<tr>
<td>2- methyltrigalacturonate</td>
<td>G - G* - G</td>
<td>9.1</td>
</tr>
<tr>
<td>3- methyltrigalacturonate</td>
<td>G* - G - G</td>
<td>0</td>
</tr>
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</table>
methyltrigalacturonate. These results further demonstrate that, although the methyl ester of 3-methyltrigalacturonate is resistant to hydrolysis by PME, its presence is not essential for hydrolysis of the remaining two methyl groups.

**Analysis of Reaction Products by Mass Spectrometry**

ESI-MS/MS was used to characterise the products of the reaction of PME with fully methylesterified oligogalacturonates (DP 3-6) in order to understand the sequential de-esterification events. The incubation times were chosen, based on pilot experiments, to cover all events: the preferential de-esterification after 0.5 h of incubation and the later reaction products after 24 hours.

When subjected to tandem mass spectrometric conditions, oligosaccharides dissociate in predictable patterns that are used to determine the structure of the intact sugar. Fragmentation can occur across the carbohydrate ring (cross-ring cleavage) but typically occurs along the glycosidic bond. For detection by mass spectrometry, these fragments must be charged. Ions resulting from glycosidic bond cleavage are termed ‘Y and Z fragment’ ions if the charge is retained on the reducing end and ‘B and C fragment’ ions if the charge lies on the non-reducing terminus. The Y and B fragment ions lack one oxygen compared to the Z and C fragment ions, respectively. In a triple quadrupole mass spectrometer, dissociation of the oligogalacturonate occurs along the glycosidic bond to form primarily series of B and Y fragment ions with the occasional loss of water (Fig. 2). This is in contrast to results obtained using an ion-trap mass spectrometer, in which compounds of similar structure have been reported to also produce C ions, Z ions and ions
Figure V-2. MS/MS spectrum of di-methylesterified hexagalacturonate. ‘Precursor ion’ corresponds to the intact di-methylesterified hexagalacturonate. The diamonds, squares and circles represent the three isomers for this compound and $Y_n$ and $B_n$ correspond to fragment ions produced from the dissociation of each isomer.
due to cross-ring cleavages.\textsuperscript{9} The lack of cross-ring fragmentation in the triple quadrupole instrument leads to simpler analysis of the fragmentation patterns. This is extremely useful when analysing samples containing multiple isomers, as was the case in these samples.

MS/MS experiments on most of the precursor ions produced multiple series of Y and B fragment ions, with each series being produced by a single isomer of the precursor ion. For example, analysis of the MS/MS spectrum of a di-methylesterified hexagalacturonate revealed three series of Y fragment ions and some B fragment ions. Each series of fragment ions were produced by a different positional isomer of di-methylesterified hexagalacturonate. The sites of methylesterification were located in a straightforward manner by observing the mass differences between fragment ions, and between the precursor ion and the Y\textsubscript{5} ion of a given series, as illustrated in Figure 3. Mass differences of m/z 190 and 176 between the fragment ions correspond to methylesterified-GalA residues or GalA residues, respectively. This is illustrated for one isomer (symbolised by a circle) of the di-methylesterified hexagalacturonate (Figures 2 and 3), which produced a series of Y fragment ions (Y\textsubscript{2} to Y\textsubscript{5}) and a series of B fragment ions (B\textsubscript{1}, B\textsubscript{2} and B\textsubscript{3}). A mass difference of 190 m/z between the precursor ion and the Y\textsubscript{5} fragment ion identified a methylesterified GalA as the non-reducing residue. The remaining residues were identified based on mass differences between Y\textsubscript{n} and Y\textsubscript{(n-1)} fragment ions. Mass differences of 176 between the fragment ions Y\textsubscript{3}-Y\textsubscript{4} and Y\textsubscript{4}-Y\textsubscript{3} demonstrate that the 4\textsuperscript{th} and 5\textsuperscript{th} residue, relative to the reducing end, is a GalA. Furthermore, a mass difference of 190 between Y\textsubscript{3} and Y\textsubscript{2} fragment ions identifies a methylesterified GalA as the 3\textsuperscript{rd}}
Figure V-3. Illustration demonstrating the sequence analysis based on the fragment ions produced by MS/MS experiments.
residue. The calculations from the B-ion series confirm the results obtained from the Y-
ion series. The peak at m/z 191 corresponds to the B₁ fragment ion of a methylesterified
GalA on the non-reducing residue. Similarly, mass differences of 176 between the
fragment ion pairs B₁-B₂ and B₂-B₃ confirm that the 4ᵗʰ and 5ᵗʰ residue is a GalA.

The data from the MS/MS experiments are presented in Table 3. Examination of
the products produced from the oligomers with DP 4-6 during the initial stage of the
reactions (0.5-h incubations) demonstrates that the first event is the hydrolysis of a methyl
group located on an internal residue (Table 3). Hydrolysis of one methyl group from the
fully methylesterified tetramer results in 1,2,4-trimethyltetragalacturonate and probably
1,3,4-trimethyltetragalacturonate while the fully methylesterified pentamer yields 1,2,4,5-
tetramethyl- and 1,3,4,5-tetramethylpentagalacturonate. The position of the methylesters
is numbered from the reducing end of the oligogalacturonate. The methyl groups at
positions 4 and 5 and probably at position 2 are the first to be hydrolyzed in the fully
methylesterified hexamer. Under the MS/MS conditions used, we were occasionally
unable to observe fragment ions, which would allow location of all the methylesters. This
was especially true when the site of methylesterification was located at or near the
reducing GalA residue, as exemplified in isomer 2 of the di-methylesterified
tetragalacturonate (24 h) with the given structure G*-G-G?-G? (Table 3). The location of
one methyl ester, symbolised by G*, was determined to be on the non-reducing sugar.
However the location of the second methyl ester (G?) has been limited to either the
reducing residue or the residue adjacent to the reducing GalA residue. The trimer appears
to be unique in that initial hydrolysis of the methyl ester may occur at the internal residue
Table V-3. De-esterification of fully methylesterified oligogalacturonates with degree of polymerisation 3–6. Products analysed by tandem mass spectometry. For experimental details see experimental section. The reducing end is indicated in bold, Gn signifies the fully methylesterified substrate with DP = n, G* and G signifies methylgalacturonate and galacturonate, respectively. G? signifies that it is unclear whether the galacturonate is methylesterified.
<table>
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<th>time (h)</th>
<th>No. of methyls</th>
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<th>isomer 2</th>
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<td>G* G* G*</td>
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<td></td>
<td>0.5 h</td>
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<td>G* G* G*</td>
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<td></td>
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<td>2</td>
<td>G* G* G*</td>
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<td></td>
<td>24 h</td>
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<td>G* G* G* G* G* G* G* G*</td>
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</table>
or the reducing end residue, leading to 1,3 dimethyltrigalacturonate and 2,3-
dimethyltrigalacturonate, respectively (Table 3). HPAEC analysis or tandem mass
spectrometry without appropriate standards cannot establish the relative abundance of the
two isomers. Hence, it is not clear which of the two methylesters is preferentially
hydrolyzed by PME. Further de-esterification of the trimer leads to a residual methyl ester
at the non-reducing end galacturonate. The residual methyl ester is also located at the non-
reducing end galacturonate for the mono-methyl trimer, the tetramer and the pentamer.
This observation leads to the conclusion that PME does not hydrolyze the methyl ester at
this position. This is in agreement with data previously obtained for the trimer, in which
3-methyltrigalacturonate was not hydrolyzed by PME (Table 2) and supports the earlier
finding that PME is not able to hydrolyze all methylesters in the homogalacturonan part of
pectin. 5

In this paper it is shown that incubation of the fully methylesterified
oligogalacturonates with PME results in a mixture of partially methylesterified isomers. It
is further demonstrated that ESI-MS/MS is a very powerful technique by which the
positions of the methylesters can be determined without prior fractionation of the reaction
mixture. Based on this sequence information of the specific reaction products the mode of
action of A. niger pectin methylesterase has been established.
ACKNOWLEDGEMENTS

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CHAPTER VI

STUDIES ON THE GLYCOSYLATION OF WILD-TYPE AND MUTANT FORMS

OF ASPERGILLUS NIGER PECTIN METHYLESTERASE  

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Orlando, R. To be submitted to Biochemical Journal for publication at a later date.
ABSTRACT

Pectin methylesterase (PME) is one among a number of enzymes released by the fungus *Aspergillus niger* that are involved in the digestion or degradation of specific structures of plant cell walls. PME is a glycoprotein with three potential sites of N-linked glycosylation. The glycan portion may be important in the activity or the substrate specificity of PME. In this work, we investigate the structures and the attachment sites of the glycans present on recombinant wild-type PME. Furthermore, PME mutants were created in which the mutation was directed at the three potential N-linked glycosylation sites. The glycosylation of the mutants and their activities were then studied. MALDI and ESI mass spectrometry using techniques tailored for carbohydrate analysis were used to characterize the glycan structures and determine the specific sites of attachment structure. High mannose structures with variable numbers of mannose were found on the wild-type as well as the mutant forms. Although activity studies using the mutants suggest that glycosylation does not strongly affect the activity they may still affect the substrate specificity of the enzyme. This will be explored in future work.
INTRODUCTION

Proper growth and development of fungi, such as the black filamentous fungus, *Aspergillus niger* requires absorption of nutrients from organic materials typically provided by a plant source. 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 pathogenic 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 set of complex heteropolysaccharides that are structurally related and include homogalacturonan, and rhamnogalacturonan I and II (RGI and RGII). The backbone of homogalacturonan and RGII is composed primarily of a linear α−1-4-linked D-galacturonic acid (D-GalA) polymer/oligomer.² However in RGI the backbone is comprised of a repeating disaccharide of -(4)-α-D-GalA- (1,2)-α-L-rhamnose.³⁻⁵ The backbone of each pectic polysaccharide can be substituted in many ways. D-GalA residues within the homogalacturonan regions may be acetylated at the C2 and C3 hydroxyls and are likely to be esterified to varying degrees at C6 with methoxyl groups. The methylation is though to occur in a non-random order, with blocks of polygalacturonic acids completely methyl-esterified.⁶ RGII may also be methyl esterified while RGI may be acetylated or substituted with neutral and acidic sugars, which results in branching of the 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, pectin lyases, and pectin esterases. In *A. niger*, four pectin lyases, \(^7\) five types of polygalacturonases, and one pectin esterase \(^8\) have been reported. Of these enzymes the most destructive are the polygalacturonases (PG) and pectin lyases (PL) that degrade the backbone of pectin by cleaving the glycosidic bond between the \(\alpha-1-4\) linked D-GalA residues either by hydrolysis or by trans-elimination, respectively. PGs and PLs are specific for D-GalA and are active only in regions with low degrees of esterification.

The pectin esterases such as pectin methylesterase (PME) play a subtler yet equally important role in the pathogenesis. These enzymes catalyze the deesterification of methoxyl groups within pectin to form D-GalA thereby creating substrates and degrees of esterification favorable towards PG and PL activity. \(^9\)

Commercially, the destructive nature of pectin-degrading enzymes is costly for the agrarian sector, however other industries have exploited this property and thus fungal pectinases, most notably those from *A. niger*, have gained substantial commercial value. *A. niger* pectinases are widely used in many food processing, fruit juice and wine industries in which elimination of pectic substances is a requirement. They are added to macerate the fruit to increased product yield and are also used to control the viscosity of the juice solution to obtain a desired consistency and to ease handling and processing. \(^10\) Specifically, pectinases clarify apple and berry juices, maintain cloudiness of citrus products such as orange and lemon juices, and prevent gelling of grape juice. \(^11\) In 1995, the industrial enzyme market totaled one billion dollars of which approximately 10% resulted from sales of pectinases. \(^12\)
The significance of pectinases in plant pathogenesis and their role as industrial enzymes has encouraged basic research of their physical and biochemical properties. From a pathogenesis viewpoint, such information may be useful to prevent or inhibit fungal infestation and disease and to increase our understanding of plant cell-wall interactions. From a commercial standpoint, specific structural information may be beneficial for production of more efficient strains with optimal activities.

A common structural feature observed in many of the fungal pectinases characterized to-date is glycosylation. Glycosylation is one of several potential post-translational modifications to secreted proteins of eukaryotic cells. The carbohydrate moieties of glycoproteins share general structures, limited composition and are attached to the protein through specific residues. Attachment of the glycans to the protein occurs via an ether linkage only to nitrogen of asparagine (N-linked) and oxygen (O-linked) of serine and threonine. The asparagine of an N-linked glycan must be part of the consensus sequence NXS or NXT where X can be any amino acid except proline. The presence of this sequon is a signal for putative but not absolute glycosylation. N-linked glycans share a biantennary core structure of Man$_3$GlcNAc$_2$, which may be extended further with other carbohydrates such as mannose, GlcNAc, galactose and other sugars depending on the source organism. The extended structures are classified as high mannose, complex or hybrid. High mannose structures possess exclusively mannose residues attached to the core, whereas complex structures are composed of other carbohydrates. In hybrid structures at least one antenna must be composed only of mannose residues. O-linked glycosylation is less predictable since no consensus sequence has been observed. The
structures of O-linked glycans are more diverse, as evidenced by the existence of six core structures.\textsuperscript{14}

Both N-linked and/or O-linked carbohydrates have been observed in several homologously and heterologously expressed PG and PL genes of \textit{A. niger}. Two PGs, \textit{endopolygalacturonase I}\textsuperscript{15} and \textit{II}\textsuperscript{16} 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.\textsuperscript{17} Other \textit{A. niger} pectinases such as PGA, PGC and pectate lyase exhibit high mannose N-linked as well as putative O-linked carbohydrates.\textsuperscript{18,19} 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.

Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization mass spectrometry (ESI - MS) are the two most common mass spectrometric methods to characterize recombinant proteins.\textsuperscript{20,21} These techniques have been particularly useful for determining the structures and attachment sites of glycans of several fungal pectinases.\textsuperscript{15,16,19} The research described herein focuses on the glycosylation of recombinant \textit{A. niger} pectin methylesterase. \textit{A.niger} PME is a protein composed of 331 amino acids. The molecular mass of the mature peptide, sans signal peptide, is 33,297 Da as shown in the sequence in Figure 1. Upon survey of the sequence, three sequons for potential N-linked glycosylation were found initiating at the asparagines at residues 95 (N95), 283 (N283) and 302 (N302). Mutation of the N-linked site, either at the asparagine, serine or threonine, precludes glycosylation at that site and offers a way to
Figure VI-1. Amino acid sequence of pectin methylesterase from *Aspergillus 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.
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. MALDI-MS and ESI-MS were used to analyze seven mutants of PME that were mutated at each of the possible single mutations and in combination. MALDI-MS to determine the presence of N-linked glycans and the particular class of glycan attached while the glycan structures and attachment sites were characterized by ESI-MS using a glycan-specific experimental technique.

**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 aspartate and are notated as N(residue #)Q, i.e. N95Q. The *A. niger* strain NW290 (pyrA6, prtF28, goxC17, fwa12, ΔargB, Δpga argB nig, ΔpgaA+ argB nid) was used for transformation. The strain was grown in minimal medium containing per litre: 6.0 g NaNO₃, 1.5 g KH₂PO₄, 0.5 g MgSO₄.7 H₂O, 0.5 g KCl and trace elements²² 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 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 nitrogen source NH$_4$Cl (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$_4$Cl as the nitrogen source, 0.005% yeast extract and 2.5% fructose at pH 4.5 was used. Cultivation was performed in 3-liter 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 to a pH of 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 ethanol) 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$^{23,24}$ and resulted in plasmid pLM3850. 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 (doubles and triple mutations).

*Transformation and Selection of PME-producing Transformants*

*A niger* strain NW 290 was transformed as described in previous work\textsuperscript{25} 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 methylesterification (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 the 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. For this, 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**

Pectin esterase activity was routinely assayed using an automated titrator (Radiometer, Denmark) at 40 °C in 15 mL 1 % lemon pectin (CPF, Copenhagen, DM=75%) in 0.15 M NaCl, pH 4.8. The principle of the assay is based on the acid released during hydrolysis of the methylester. A pH of 4.8 was maintained during the assay. After addition of the PME sample the speed of titrant (0.0625 M NaHCO₃) consumption was monitored. Pectin esterase activity was calculated from the slope of the consumption of titrant. A titrant consumption of one micro-equivalent base per minute corresponds with one unit of pectin esterase.

The protein concentration of the purified enzymes was estimated by spectrophotometry, using the calculated molar absorption coefficient of 61740 M⁻¹.cm⁻¹ for wild type pectin esterase.²⁶ The purified PME 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.²⁷ The enzymes were diluted (0.2 mg/mL) in 0.2 M Na-acetate 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 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 proteases²⁷ was used as a positive control.
**Purification of the Enzymes**

The culture fluid (2.5 L) was collected by filtration, diluted twofold with water and adjusted to pH 6.0. Proteins were collected by batch wise adsorbtion. For this, 50 g Streamline Q XL (Amerham 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 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 (Amerham Pharmacia Biotech, Sweden, 16 mL) equilibrated in 0.01 M piperazine/HCl, pH 6.0 and eluted with a 200 mL linear gradient of 0-0.4 M NaCl in buffer. The collected fractions (5 mL) were assayed for pectin esterase activity and the purity was tested by SDS-PAGE (Figure 2). Fractions containing pure enzyme were pooled, dialysed against 0.05 M sodium acetate buffer (pH 5.0) and stored at –20°C.

**Strategies for Glycan Analyses**

Figure 3 depicts the general experimental strategy for the glycan analysis of the wild-type and mutant forms of PME. The strategy utilizes MALDI-TOF-MS, ESI-MS on a quadrupole time-of-flight, 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 MALDI-MS 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 MALDI-MS to determine, by difference, the mass
Figure VI-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
Figure VI-3. Experimental outline used to study the glycosylation of wild type and mutant pectin methylesterase.
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 MALDI-MS, as well as by LC-ESI-MS on the Q-TOF. 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**

MALDI-MS 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, 28kV 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 337nm. Sinapinic acid (Aldrich) was used as the matrix for the protein samples while alpha-cyano 4-hydroxycinnamic acid was used for the tryptic digestions. Both matrices were dissolved in 70% aqueous acetonitrile (Sigma) with 0.01% trifluoroacetic acid (Aldrich) at a concentration of 10mg/mL.

**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 x 35 mm), buffer A (20mM HEPES, pH 7.5) and B (20mM sodium acetate 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% to 26% buffer B for 70 minutes. Fractions collected at 1-minute intervals during the gradient were analyzed by MALDI-MS for purity and pooled to contain only one glycoform/pool. The pools of the separated 38 KDa and 40 KDa glycoforms were each concentrated and dialyzed against water on a 5K MWCO Microcon cellulose filter.

**Endoglycosidase Digestions**

To quickly screen for N-linked glycans, the samples were digested N-linked glycan 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.\(^{17,28}\) 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 (Prozyme Inc, San Leandro, CA) on the MALDI target. To maintain solvation of the digest mixture, 1 µL of water was added intermittently during the 20-minute digestion. The matrix solution (0.5 µL) was then added to the mixture prior to MALDI-MS analysis of the deglycosylated protein.

**Proteolytic Digestions**

Proteolytic digestion was carried out in 50 mM ammonium bicarbonate (Aldrich) using sequencing grade trypsin (Promega, Madison, WI) with ~20 ug of PME at a 1:50 ratio of trypsin to PME. The proteins were digested for 24 hrs at 37° C.
**LC-ESI-MS using Stepped-orifice Voltages**

An aliquot of the digested sample (5 µg) was injected into a Phenomenex Jupiter C-18 column (50 x 1mm). Reverse-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 to 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 Q-TOF2) (City, State) and ionized in positive ion mode. A voltage applied to the orifice of the instrument, also known as the cone voltage, creates a potential difference between the orifice and the first quadrupole. Two acquisitions or scan functions were programmed into the mass spectrometer software. During each acquisition the voltage is set so that the potential difference forms either an intact ionized species or causes the species to fragment within the instrument source as specific mass ranges are detected. In function 1 the cone voltage is set to a highly energetic value (70) to promote fragmentation of the glycopeptides and peptides in the effluent. A range of 50-500 m/z is detected during this function to monitor carbohydrate-containing species that fragment to form ions commonly observed at 163, 204, and 366 m/z.  

During function 2 the cone voltage is lowered to a less energetic or 'soft' value of 30 as the instrument detects a m/z range (501-2000 m/z), a range suitable for intact electrosprayed peptides and glycopeptides.
RESULTS AND DISCUSSION

Transformation and Purification of PME

Pectin methylesterase 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.\(^{32}\)

Table 1 summarizes the purification results 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 (Figure 1) to the measured masses obtained by MALDI-MS (Table 2). The measured masses of the two forms of the wild type and for the single and double mutants were substantially greater (1500 Da+) than the predicted mass. Such large mass differences are typically attributed to N-linked glycosylation since all other forms of post-translational modification result in smaller mass differences. MALDI-MS revealed the presence of two forms of the wild-
**Table VI-1.** Results of the purification *A. niger* wild type and mutant pectin

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>amount protein (mg)</th>
<th>protein concentration. (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>53</td>
<td>0.83</td>
</tr>
<tr>
<td>N95Q</td>
<td>41</td>
<td>2.05</td>
</tr>
<tr>
<td>N283Q</td>
<td>32</td>
<td>2.13</td>
</tr>
<tr>
<td>N302Q</td>
<td>94</td>
<td>3.75</td>
</tr>
<tr>
<td>N95Q  N283Q</td>
<td>58</td>
<td>2.9</td>
</tr>
<tr>
<td>N95Q N302 Q</td>
<td>21</td>
<td>2.07</td>
</tr>
<tr>
<td>N283Q N302Q</td>
<td>23</td>
<td>1.56</td>
</tr>
<tr>
<td>N95Q N283Q N302Q</td>
<td>20</td>
<td>1.34</td>
</tr>
</tbody>
</table>
Table VI-2. The molecular masses of PME and the potential number of occupied N-linked sites as determined by MALDI-MS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Observed molecular mass (Daltons)</th>
<th>Mass difference from predicted molecular mass (Daltons)</th>
<th>Potential number of glycosylated N-linked sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type PME</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PME 40</td>
<td>40487</td>
<td>6560</td>
<td>3</td>
</tr>
<tr>
<td>PME 38</td>
<td>38192</td>
<td>4165</td>
<td>2</td>
</tr>
<tr>
<td>Mutant PME</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N95Q</td>
<td>38484</td>
<td>4556</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>36625</td>
<td>2697</td>
<td>1</td>
</tr>
<tr>
<td>N283Q</td>
<td>37890</td>
<td>3962</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>35565</td>
<td>1637</td>
<td>1</td>
</tr>
<tr>
<td>N302Q</td>
<td>37671</td>
<td>3743</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>35850</td>
<td>1922</td>
<td>1</td>
</tr>
<tr>
<td>N95Q/N283Q</td>
<td>36493</td>
<td>2565</td>
<td>1</td>
</tr>
<tr>
<td>N95Q/N302Q</td>
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<td>1</td>
</tr>
<tr>
<td>N283Q/N302Q</td>
<td>35208</td>
<td>1280</td>
<td>1</td>
</tr>
<tr>
<td>N95Q/N283Q/N302Q</td>
<td>34048</td>
<td>120</td>
<td>0</td>
</tr>
</tbody>
</table>
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. All N-linked glycans are composed of a pentasaccharide Man\textsubscript{5}GlcNAc\textsubscript{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 \(\sim 2000\) 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 thereby suggesting an absence of glycosylation after 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 proportionate to the average mass of N-linked glycans occupying one site. Unlike the double mutants, yet similar to the wild type PME two glycoforms were observed for each of the single mutants. The glycans appear to occupy either only one or two sites.

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 not only confirm the presence of N-linked structures but also 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. The enzyme, 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 limited to separate tryptic peptides contained within 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. (Figure 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 glycan classes. 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.

Up to this point in our analysis of PME, MALDI-MS provided only limited information on the glycosylation. Only the presence, number and class of glycan had been determined. The specific carbohydrate structures and their sites of attachment to the protein were determined primarily through the LC-MS (with in-source CID) of the tryptic digests on the Q-TOF. Glycan-containing peptides were readily found during the chromatographic separation by using the stepped-orifice voltage technique originally described for a triple-quadrupole instrument (QQQ).\textsuperscript{29,30} It was suggested that this
Figure VI-4. MALDI mass spectrum identifying the components from the tryptic digest of a single mutant (N302Q) of PME. Approximately 1 pmole of sample was analyzed using alpha-cyano hydroxycinamic acid as a matrix.
technique is transferable to all single quadrupole instruments, which exists as the first mass spectrometer of the hybrid Q-TOF. Extension of this technique for use on the Q-TOF was relatively straightforward 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, strong carbohydrate fragment ion signals were observed. Lower cone voltages resulted in a reduction in the fragment ion signals, while higher cone voltages had either no effect on or degraded the signal intensity.

Data and results from only one sample, the single mutant N302Q, are presented and outlined in Figures 5A-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 (Figure 5A). The TICs share similar profiles and reveal digest components as peaks eluting between 29 and 80 minutes. 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 in high mannose and hybrid glycans found at 163, 204, and 366 m/z (Figure 5B). The occurrence of such ions indicated the presence of glycans at some point during the chromatographic separation. 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 37 minutes (Figure 5C). Masses of the intact glycopeptides were determined by
Figure VI-5. Schematic of data analysis procedure used to determine glycan structure and attachment site from the LC-ESI-MS experiment on the Q-TOF. 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) ESI mass spectra of 35 and 36 min peaks from low cone voltage. Refer to text for description of data analysis procedure.
obtaining an averaged spectrum of the low cone voltage TIC only at 35 and 37 minutes (+/- 0.5 min) as shown in Figure 5D. Each averaged spectra produced two series of peaks. Ions from the lower molecular weight series differed by 40 m/z while those of the higher molecular weight series differed by 32.5 m/z. Since electrospray ionization produces multiply charged species the low and high molecular weight series observed correspond to glycopeptides containing a series of mannoses with +5 and +4 charges, respectively. While the normal charge states for tryptic peptides/glycopeptides of ~4000 m/z are +2 or +3, the higher charge states we observed were expected since the PME glycopeptides had an average mass of ~6000 Da. 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 using two complementary methods. Initially, this information was found by manual calculation of the deglycosylated peptide mass. Both the MALDI and Q-TOF 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 (Figure 6). A series of glycopeptide masses was first determined by deconvolution of the ESI data. The average mass difference between
**Figure VI-6.** Manual Calculation of N302Q glycan structures and sites of attachment. Glycopeptide masses were determined by convolution of 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 a N-linked site.
adjacent glycopeptides was 162Da. Therefore, only the first glycopeptide in the series was analyzed since each subsequent glycopeptide contains an additional mannose residue. The masses of Man 5 thru 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 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 thru the EXPASY website. Glycopeptide masses are entered and the program queries 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 provide 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 confirm 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, PME40 was glycosylated at all three potential N-linked sites with high mannose structures while PME38 contained similar glycans only on N283 and N302. The absence of glycans on N95 of the PME38 form was
Table VI-3. The glycan structures and attachment sites of wild-type and mutant PME as determined by LC-ESI-MS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Carbohydrate Structure</th>
<th>Attachment Site</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild-type PME</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PME 40</td>
<td>Man 5 to 9</td>
<td>N95</td>
</tr>
<tr>
<td></td>
<td>Man 8 to 17</td>
<td>N283</td>
</tr>
<tr>
<td></td>
<td>Man 7 to 15</td>
<td>N302</td>
</tr>
<tr>
<td>PME 38</td>
<td>Man 8 to 16</td>
<td>N283</td>
</tr>
<tr>
<td></td>
<td>Man 7 to 15</td>
<td>N302</td>
</tr>
<tr>
<td><strong>Mutant PME</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N95Q</td>
<td>Man 5 to 14</td>
<td>N283</td>
</tr>
<tr>
<td></td>
<td>Man 7 to 17</td>
<td>N302</td>
</tr>
<tr>
<td>N283Q</td>
<td>Man 5 to 9</td>
<td>N95</td>
</tr>
<tr>
<td></td>
<td>Man 7 to 18</td>
<td>N302</td>
</tr>
<tr>
<td>N302Q</td>
<td>Man 7 to 17</td>
<td>N283</td>
</tr>
<tr>
<td></td>
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<td>N95</td>
</tr>
<tr>
<td>N95Q/N283Q</td>
<td>Man 7 to 18</td>
<td>N302</td>
</tr>
<tr>
<td>N95Q/N302Q</td>
<td>Man 8 to 13</td>
<td>N283</td>
</tr>
<tr>
<td>N283Q/N302Q</td>
<td>Man 5 to 9</td>
<td>N95</td>
</tr>
<tr>
<td>N95Q/N283Q/N302Q</td>
<td>No evidence of glycosylation</td>
<td></td>
</tr>
</tbody>
</table>
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 one hour.

**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 events. 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. We have shown that wild-type PME is either fully
Table VI-4. Wild type and mutant PME: Results of activity and protease sensitivity assay (determined by residual activity).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>protein concentration. (mg/mL)</th>
<th>specific activity (U/mg)</th>
<th>residual activity at pH 4.5 (%)</th>
<th>residual activity at pH 2.8 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.83</td>
<td>968</td>
<td>102</td>
<td>101</td>
</tr>
<tr>
<td>N95Q</td>
<td>2.05</td>
<td>1020</td>
<td>105</td>
<td>n.d.</td>
</tr>
<tr>
<td>N283Q</td>
<td>2.13</td>
<td>906</td>
<td>104</td>
<td>n.d.</td>
</tr>
<tr>
<td>N302Q</td>
<td>3.75</td>
<td>976</td>
<td>103</td>
<td>n.d.</td>
</tr>
<tr>
<td>N95Q N283Q</td>
<td>2.9</td>
<td>979</td>
<td>104</td>
<td>n.d.</td>
</tr>
<tr>
<td>N95Q N302 Q</td>
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<td>967</td>
<td>102</td>
<td>n.d.</td>
</tr>
<tr>
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<td>823</td>
<td>106</td>
<td>n.d.</td>
</tr>
<tr>
<td>N95Q N283Q</td>
<td>1.34</td>
<td>879</td>
<td>104</td>
<td>101</td>
</tr>
<tr>
<td>N302Q</td>
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</tbody>
</table>
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 absence of N-linked glycosylation and no evidence of O-linked glycans. Mutation of 2 of the 3 possible N-linked sites produced one glycoform, which was glycosylated only at the non-mutated site. However, mutation of 1 of the 3 possible sites as in the single mutants produced two glycoforms that contained carbohydrates on one or both of the remaining non-mutated sites. 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.
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Modified MALDI probe surfaces were developed and evaluated as sample cleansing devices. Self-assembled monolayers were used to impart ionic characteristics to the normally inert surface of the MALDI probe. The hydrophilic nature of the probes solved the initial problem with incompatibility between hydrophobic probe surfaces and aqueous samples. Positive and negative charges on the monolayer allow extraction of peptides and to a smaller degree, proteins from solutions with high and normal levels of contamination. The performance of these probes was tested using various contaminants. The usefulness of the surfaces extends to non-ionic detergents, urea, guanidinium HCl as well as several types of inorganic salts that are common to buffered solutions. The probes were unsuccessful with samples containing SDS. It is believed that the negatively charged SDS may itself bind to the modified probe. The probes have proven potential for use in routine analysis. Batches of modified surfaces can be created in less than an hour and can be used as needed for up to four months. Furthermore, each surface can be reused although with some loss in sensitivity.

MALDI and ESI mass spectrometry have been useful for studies of the substrate specificity and glycosylation of Aspergillus niger pectin methylesterase (PME). In the study of substrate specificity methylesterified galacturonates oligomers of variable length were digest by wild-type PME. LC-ESI-MS/MS determined the specific residues affected by the enzyme. The specificity was determined by comparing patterns of de-esterification among the substrates that varied in degree of polymerization. This form of PME preferentially removes and internal methyl esters first. Furthermore the non-reducing residue is unaffected by the enzyme. Glycosylation studies with PME revealed that only
high mannose structures are present in both the wild-type and mutated forms. Furthermore, wild-type PME exists as a mixture of two glycoforms. The major glycoform is glycosylated at all three potential N-linked sites while the minor form is absent of glycosylation at Asn<sup>95</sup>. Mutagenesis of PME was directed at the asparagines of the N-linked sites to ultimately test the effect of site-specific glycosylation on the enzyme activity and substrate specificity. Activity measurements using all the possible mutant combinations revealed that glycosylation does not significantly change the enzyme's activity. However, the effect of glycosylation on substrate specificity remains to be studied.