2009. Proceedings of the Indiana Academy of Science 118(2):107–113

ACETYLATION LABELING MASS SPECTROMETRY: A METHOD FOR STUDYING PROTEIN CONFORMATIONS AND INTERACTIONS

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ABSTRACT. To support the ongoing effort to characterize and understand the specificity of phytopathogens for various crops, it has been necessary to develop a quick and robust method for observing the interactions of many pairings of pathogenic pectinases with plant defense proteins. Presented here is a modification of previously reported lysine acetylation protocols. The use of a slightly higher anhydride-to-protein ratio results in the acetylation of not only lysines, but serine, arginine, asparagine, histidine, tyrosine, glutamine, and cysteine side chains as well. These eight amino acids were found to react with acetic anhydride providing significantly broader surface mapping potential than single amino acid modification methods. Each residue that is acetylated increases the protein's mass by 42 Da, easily discernable by modest mass spectrometers. The technique called acetylation labeling mass spectrometry (ALMS) is used to observe and distinguish between native and denatured protein conformations. In addition to observing whole protein conformations, the stable nature of the mass tags allows for additional processes including enzymatic digestions and liquid chromatography to be performed without the loss of labels or inadvertent labeling. It is proposed that this technique could be ideally suited for investigating the interactions between phytopathogenic pectinases plant defense proteins.

Keywords: Mass spectrometry, protein interactions, acetylation, labeling

Protein interactions have significant influence over many biological processes including crop pathogenesis (Carpita & Gibeaut 1993; Jones et al. 1972). Recent studies of the interactions between phytopathogenic enzymes and plant defense inhibitor proteins have been valuable, but slow in coming, for a variety of reasons (Weis et al. 1992; Merritt 1994; Ehring 1999). The high degree of specificity of some phytopathogens for various crops has made it difficult to develop general understandings and predictions. As a result, it has been increasingly important to develop a rapid and robust method for detecting and observing protein interactions so that a large number of pathogenic enzyme and plant defense protein pairs can be studied.

Over the last decade hydrogen/deuterium exchange mass spectrometry has been used with great success to monitor protein conformations and binding interactions (Mandell et al. 1998, 1998; Deng & Smith 1999; King et al. 2002, 2002). Amide hydrogens along protein backbones are labile and will exchange readily with deuterium when a protein is dissolved in D_2O (Ehring 1999; Smith & Zhang 1994; Smith et al. 1996). The ability to isotopically label the exterior of a protein allows for the monitoring of protein folding and unfolding and detailed study of ligand binding (Akashi & Takio 2000; Mandell et al. 1998; Farmer & Caprioli 1998).

Despite the great successes realized through hydrogen/deuterium exchange there are several significant experimental limitations. In an effort to limit the loss of deuterium labels or inadvertent gain of deuterium during analysis, the pH (~2), temperature (~0 °C), and time of the experiment (< 20) must be closely controlled. Even under these conditions during analysis, more than 20–30% back-exchange is expected (Ehring 1999; Smith & Zhang 1994; Smith et al. 1996). Additionally, enzymes slow down at these low temperatures, and therefore relatively high enzyme-to-protein ratios are needed for digestion to occur quickly enough to avoid significant loss of deuterium.

Therefore, a permanent covalent mass tag to solvent accessible amino acids may prove advantageous in lieu of the inherent complica-

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tions experienced with hydrogen/deuterium exchange mass spectrometry. A number of covalent mass tagging procedures have been previously reported and demonstrated in the literature (Apuy et al. 2001; Zappacosta et al. 1997; Leite & Cascio 2002; Scaloni 1999; Ploug et al. 1995; Kussmann & Przybylski 1995). These techniques typically involve the labeling of one or a few specific amino acids. The specific and restrictive nature of these amino acid modifications, however, lacks the capability of thoroughly probing the entire protein surface. Protein binding, for example, can only be observed by these methods if the amino acids being modified exist in the active site. Thoroughly probing the protein surface is, after all, the primary advantage of hydrogen/deuterium exchange since every exterior amino acid except proline is capable of being tagged. To encompass the advantages of both approaches, we have modified a previously reported lysine-labeling procedure. The amine containing side chain of lysine has been shown to undergo acylation reactions in the presence of anhydrides (Fig. 1) (Turner et al. 2004; Glocker et al. 1994; D'Ambrosio et al. 2003; Suchau et al. 1992; Klotz 1967; Riordan & Vallee 1972). This procedure has been used with success to observe the solvent accessibility of lysine residues and in some circumstances may be used to elucidate a protein binding interaction if a lysine exists in the active site. In our modified approach, acetylation labeling mass spectrometry (ALMS), a slightly higher anhydride-to-protein ratio is used than previously reported. The elevated anhydride concentration results in the acetylation of not only solvent accessible lysine residues, but some guanidine, imidazole, amide, alcohol, and thiol containing amino acid side chains as well. Several anhydrides have been tested, and we report here the use of acetic anhydride as a potential reagent for creating acetylated mass tags. Stable, covalently bound tags to solvent accessible residues avoid many of the limitations of hydrogen/deuterium exchange mass spectrometry. The labeled molecules may be rinsed, digested, separated by LC, and analyzed by mass spectrometry without the concern of back-exchange or loss of the tag.

METHODS

Materials.—Cytochrome C from equine and bovine heart, normal and sickle cell hemoglobin, sinapinic acid, acetic anhydride, amino acids (serine, cysteine, tyrosine, threonine, asparagine, glutamine, lysine, arginine, histidine, and glycine), urea, pepsin, and *n*-butylamine were purchased from Sigma (St. Louis, Missouri). Methanol came from VWR Scientific (Batavia, Illinois). Sulfuric acid and hydrochloric acid were purchased from Fisher (Pittsburgh, Pennsylvania).

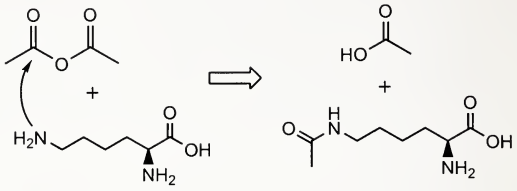

Figure 1.—The acetylation of the amine of the lysine side-chain is shown. The acetic anhydride is susceptible to nucleophilic attack by the amine resulting in an acetylated lysine and acetic acid.

Instrumentation.—GC/MS experiments were performed on an HP5890 gas chromatograph – HP5971 mass selective detector (Agilent, Palo Alto, California). Mass spectral analyses were performed on either an OmniFLEX MALDI-TOF MS (Bruker Daltonics, Inc., Billerica, Massachusetts) for standard MALDI-MS experiments or a Finnigan LCQ-Deca for LC-MS and MS^n experiments. The MALDI matrix was prepared by placing a saturated amount sinapinic acid in a 50% acetonitrile solution containing 0.05% trifluoroacetic acid. Liquid chromatographic separations were performed using a Hitachi L-7000 series LC with a 5 μm C-18 column from Supelco.

Free amino acids.—Saturated amino acid solutions were made by dissolving solid serine, cysteine, tyrosine, threonine, asparagine, glutamine, lysine, histidine, and glycine in 10 mM aqueous ammonium acetate. Except for glycine these amino acids were chosen due to their polar side chains and lone pair donating ability. Glycine was used as a negative control. Aliquots of each amino acid were placed in microcentrifuge tubes. Amino acid samples were reacted with approximately a 10,000-fold molar excess of acetic anhydride for 10 min. The samples were then esterified to prepare them for GC-MS analysis following a routine Fisher esterification method, and 10 μl aliquots of each solution were diluted into 1.0 ml of methanol. Then 5.0 μl of concentrated sulfuric acid was added to each tube and placed in an

oven at 45°C for approximately 10 hours. Two µl of each sample were analyzed by GC-MS, allowing 10 min to ramp from 70 °C up to 240 °C. The resulting mass spectra were used to determine the number of acetyl groups that were added. Since the N-terminus of every amino acid will react with acetic anhydride, only amino acids that acquire two or more acetyl groups are likely to react with anhydrides when they are found in a polypeptide chain.

Whole proteins: Protein unfolding.—Ten 1.5 ml microcentrifuge tubes were set aside for this protocol. Cytochrome C (equine) in 10 mM ammonium acetate was added to each test tube to a final concentration of 0.1 mg/ml after all reagents were added. Enough acetic anhydride was added to tubes 2–10 to yield a final anhydride-to-protein ratio of 10,000:1. Varying amounts of urea were added to each of tubes 3–10 such that the urea concentrations increased from 1.0 M to 4.5 M in 0.5 M increments, respectively. After 10 min 1.0 µl aliquots from each tube were spotted on MALDI targets along with sinapinic acid matrix for analysis.

Protein digests: Quenching acetylation.—Acetic anhydride (15 µl) and *n*-butylamine (30 µl) were combined in a plastic vial and allowed to react for 15 minutes. A 1 ml aliquot of 1 mg/ml cytochrome C (bovine) in 10 mM ammonium acetate was prepared in a separate plastic vial, and then added to the acetic anhydride and *n*-butylamine reaction vial. After 5 min, the solution was analyzed using an LCQ-Deca-MS.

Protein digests: Acetylated protein digested for LC-MS^n analysis.—Two 1 ml aliquots of 1 mg/ml cytochrome C (bovine) in 10 mM ammonium acetate were added to two plastic vials. Approximately a 2000-fold molar excess of acetic anhydride to protein was added to one vial and allowed to react for several minutes. Then *n*-butylamine was added in a 2:1 molar ratio and allowed to react for several minutes to quench any unreacted anhydride. HCl (1 N) was added to both vials to adjust the solution pH to about 2. Aliquots of 1 mg/ml pepsin were added to each vial to yield a 1:50 volume ratio of enzyme to protein. The reaction mixture was incubated in an oven at 37 °C for 1–2 h. The digested protein was analyzed by LC-MS/MS and evaluated using the online program Protein Prospector from UCSF.

RESULTS AND DISCUSSION

Free amino acids.—Previous reports have employed approximately a 1000-fold molar excess of acetic anhydride to acetylate solvent accessible lysine residues. It was also reported that "over-acetylation" was observed at greater concentrations of anhydride (Turner et al. 2004). In addition to lysines, other electron-rich guanidine, imidazole, amide, alcohol, and thiol functional groups within some amino acid side chains are expected to undergo acylation reactions when exposed to high enough concentrations of anhydrides. Initial tests were done with both propionic and acetic anhydrides. Being less limited by spatial constraints it is expected that smaller anhydrides will provide better mapping of protein surfaces, and therefore only the acetic anhydride data are reported here.

The anhydrides were predicted to react with the amino acid side chains of serine, threonine, arginine, asparagine, lysine, histidine, tyrosine, glutamine, and cysteine. To verify which potentially reactive amino acids actually do react with acetic anhydride, solutions of individual amino acids were reacted with acetic anhydride and analyzed by GC-MS. To make the acetylated amino acid samples more volatile for compatibility with GC-MS analysis, the labeled amino acids were methyl-esterified as described above. Each acetylation will result in a mass increase of 42 Da. It is important to note that the amine-terminus of free amino acids, with perhaps the exception of proline, will be acetylated as well. Therefore, the successful reaction of acetic anhydride with an amino acid side chain will be indicated by the addition of two or more 42 Da groups.

The candidates, serine, threonine, arginine, asparagine, lysine, histidine, tyrosine, glutamine, and cysteine as well as glycine, as a negative control, were analyzed. All but threonine and glycine showed an increase of 84 Da, and most showed a characteristic fragmentation pattern of two 42 Da mass losses as shown in Fig. 2 for lysine-methyl ester. Threonine and glycine had an increase in mass of 42 Da corresponding to the acetylation of only the amine-terminus. The authors are uncertain as to why threonine did not react similarly to the other alcohols, serine and tyrosine.

Whole proteins: Protein unfolding.—To test the capability of monitoring protein unfolding

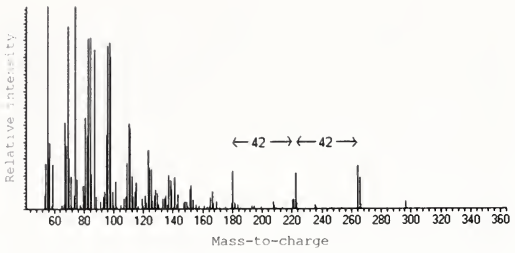

Figure 2.—The mass spectrum of the acetylated lysine methyl ester is shown. The two 42 Da peak intervals indicative of two acetylations are indicated.

by ALMS, samples of cytochrome C (equine), at various degrees of denaturation, were reacted with acetic anhydride for 10 minutes. The reaction of native cytochrome C with acetic anhydride added approximately 450 Da to the mass of the cytochrome C control, as shown in Fig. 3, suggesting the addition of between 10 and 11 acetyl mass tags. As cytochrome C was denatured more fully, with increasing amounts of urea, a second peak at higher mass appears corresponding to the fully denatured conformation. The mass spectrum of the acetylated cytochrome C in 4.5M urea is also shown (Fig. 3). The increase in mass from the native cytochrome C to the acetylated and denatured sample is approximately 1680 Da corresponding to approximately 40 mass tags. Equine cytochrome C contains 38 of the 8 potential anhydride reactive amino acids, serine (0), arginine (2), asparagines (5), lysine (19), histidine (3), tyrosine (4), glutamine (3), and cysteines (2). With the expectation that two cysteines may be bound to the heme group, the observed 40 labels is several more than the expected 36, but similar enough to strongly suggest that the eight amino acids that were observed to undergo acetylation as monomers also undergo acetylation reactions within a polypeptide. In addition to verifying the acetylation of an array of amino acids, the test demonstrates the capability of ALMS to observe two different protein conformations.

Protein digests: Quenching acetylation.—In order to perform more sophisticated ALMS experiments, including protein digestions, it is imperative to eliminate any excess anhydride so as not to inadvertently acetylate amino acids becoming accessible as a result of processes like enzymatic digestions. Quenching the acetylation reaction is crucial for realizing the full potential of this stable labeling method. Primary amines react well with anhydrides through a nucleophilic acyl substitution reaction, forming an amide and carboxylic acid, both of which are unreactive with amino acid side chains. We chose *n*-butylamine to react with any excess acetic anhydride, forming *n*-butylethanamide.

To test the ability of *n*-butylamine to fully react with acetic anhydride, cytochrome C (bovine) was added to a vial already containing a 2:1 molar ratio of *n*-butylamine to acetic anhydride. The sample was analyzed by electrospray mass spectrometry, LCQ-Deca, and

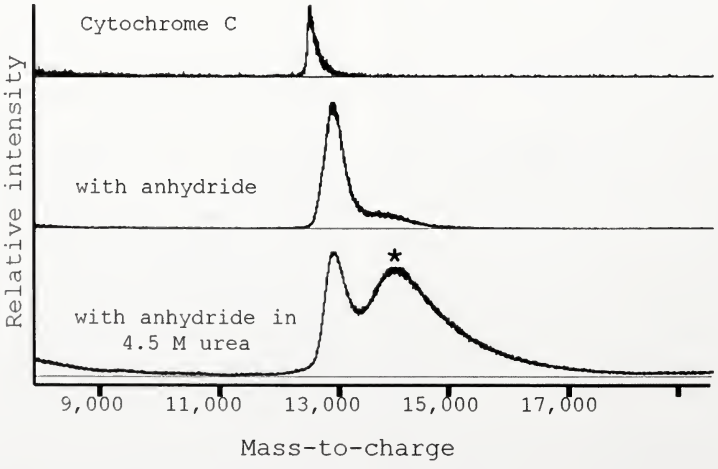

Figure 3.—Shown are the spectra of native cytochrome C (12,388 Da), native cytochrome C reacted with acetic anhydride for 10 minutes (12,837 Da), and cytochrome C in 4.5 M urea reacted with acetic anhydride for 10 minutes (*14,073 Da).

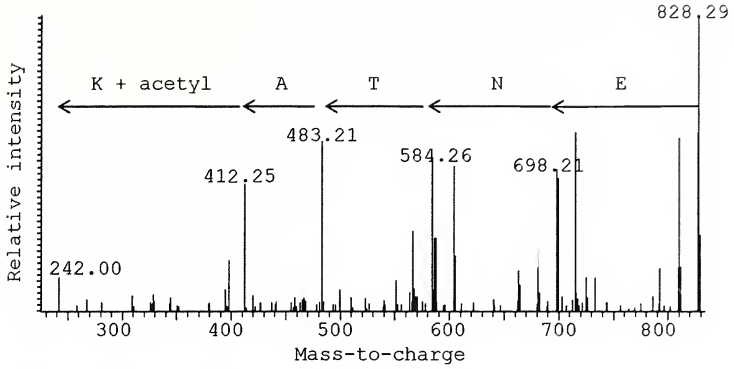

Figure 4.—The MS/MS spectrum of an acetylated peptide is shown along with the amino acids that correspond to each fragment loss. The mass difference between ions 412.25 Da and 242.00 Da corresponds to loss of lysine and an acetyl group, giving evidence for specific location of mass tag.

the resulting spectrum was compared to that of a control mass spectrum of cytochrome C. Both the experimental and control mass spectra of cytochrome C were identical, indicating that *n*-butylamine was successful in reacting with all of the anhydride originally present in solution.

Protein digests: Acetylated protein digested for LC-MS^n analysis.—It is possible to identify the specific protein regions or even specific amino acids that have been labeled by employing an enzymatic digest step followed by separation and identification by LC-MS/MS. In general, protein samples are exposed to acetic anhydride, and then the excess anhydride is reacted with *n*-butylamine. HCl is added to adjust the pH to be appropriate for the enzyme being used. A proteolytic enzyme is added and the samples can be incubated as desired. The resulting peptide digest solution can easily be separated by reversed phase LC and identified using online ESI-MS/MS. The addition of a digestion step not only allows users to observe global protein changes, but also to locate where those changes or interactions are occurring.

To demonstrate the use of ALMS to successfully identify acetylated peptides, cytochrome C (bovine) was exposed to acetic anhydride and, after quenching, digested with pepsin at approximately pH 2 for two hours. A control trial, without anhydride, was performed as well to validate the digestion process. Initially, the observed peptide m/z values from the control trial were compared to a theoretical digest using the online program Protein Prospector. Each identified peptide was then verified by evaluating the MS/MS fragmentation pattern. Because the presence of acetylations appeared to alter the digestion pattern to a small degree, only peptides observed in both the control and acetylated trials were thoroughly investigated. Only 14 peptides covering 86 of the 104 amino acids were present in both the control and acetylated trials. Of the 14 peptides, 8 appeared with one or more acetylations. Figure 4 illustrates the ability to both identify a peptide and locate the acetylation label through the use of MS/MS spectra. The sample MS/MS spectrum shown is the fragmentation of the C-terminal peptide, amino acid residues 99–105 (LKKATNE). The spacing between peaks corresponds to the standard incremental mass loss of each amino acid except for lysine 101. The spacing of 170 Da between peaks at 242 and 412 mass-to-charge indicates an acetylated lysine: the incremental mass of lysine (128 Da) plus acetylation (42 Da). Visual inspection of the protein crystal structure of cytochrome C reveals that lysine 101 is a member of a large alpha-helix at the C-terminus of the protein (Bushnell et al. 1990). The side chain of lysine 101 protrudes significantly away from the protein and is clearly solvent accessible. In this manner peptides can be identified, and, if the data are of sufficient quality, acetylations can be attributed to specific amino acids through MS/MS analysis.

ACKNOWLEDGMENTS

The authors would like to thank the Indiana Academy of Science for their generous support of this research through the Senior Research Grant program.

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Manuscript received 15 October 2008, revised 14 April 2009.