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Aspects of Oligonucleotide and Peptide Sequencing with MALDI and Electrospray Mass Spectrometry

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Abstract—Biopolymer sequencing with mass spectrometry has become increasingly important and accessible with the development of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). Here we examine the use of sequential digestion for the rapid identification of proteolytic fragments, in turn highlighting the general utility of enzymatic MALDI ladder sequencing and ESI tandem mass spectrometry. Analyses were performed on oligonucleotides ranging in size from 2 to 50 residues, on peptides ranging in size from 7 to 44 residues and on viral coat proteins. MALDI ladder sequencing using exonuclease digestion generated a uniform distribution of ions and provided complete sequence information on the oligonucleotides 2–30 nucleic acid residues long. Only partial sequence information was obtained on the longer oligonucleotides. C-terminal peptide ladder sequencing typically provided information from 4 to 7 amino acids into the peptide. Sequential digestion, or endoprotease followed by exoprotease exposure, was also successfully applied to a trypsin digest of viral proteins. Analysis of ladder sequenced peptides by LCMS generated less information than in the MALDI-MS analysis and ESI-MS^2 normally provided partial sequence information on both the small oligonucleotides and peptides. In general, MALDI ladder sequencing offered information on a broader mass range of biopolymers than ESI-MS^2 and was relatively straightforward to interpret, especially for oligonucleotides.

Introduction

An important goal of mass spectrometry is the routine acquisition of complete sequence information from biopolymers. The realization of this goal has significantly progressed with the development of both electrospray ionization[1] (ESI) and matrix-assisted laser desorption/ionization[2] (MALDI). More specifically, ESI tandem mass spectrometry (MS^2) has been routinely used to generate fragment ions from a selected precursor ion by initiating ion/molecule collisions (a process known as collision-induced dissociation) which can then be mass analyzed and used to obtain sequence information. Perhaps the most well-known applications of ESI-MS^2 are the work performed by Hunt and colleagues to identify major histocompatibility complex (MHC) bound peptides[3–6] and the pioneering work of Mann et al. to map proteins.[7] ESI-MS^2 has also been successfully applied to the sequencing of small oligonucleotides.[8–12]

MALDI-MS is playing an equally exciting role in biopolymer sequencing, used in conjunction with enzymatic[13,14] or chemical digestion[15] to generate sequence-specific ladders for proteins and oligonucleotides. This process, also known as 'ladder sequencing', was first pioneered by Chait et al.[15] for proteins and further developed by Pieles et al.[16] for oligonucleotides. Protein ladder sequencing involves the analysis of a peptide/protein that has undergone a stepwise degradation in which ladder-generating chemistry produces a family of sequence-defining peptide fragments that differ from the next by one amino acid. Once the mixture of peptides is obtained, MALDI-MS analysis is performed to generate a mass spectral sequence ladder. The implementation of MALDI ladder sequencing to oligonucleotides with the exonucleases, namely snake venom phosphodiesterase (SVP) or bovine spleen phosphodiesterase (BSP), offers

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significant advantages[14,17] over the current, widely used radio labeling method developed by Maxam and Gilbert[18] for relatively small oligonucleotides.[19,20]

In this report we have analyzed both oligonucleotides and peptides with MALDI ladder sequencing using the exonucleases—bovine spleen phosphodiesterase (BSP) and snake venom phosphodiesterase (SVP)—and exoprotease carboxypeptidase Y (CPY). The results were compared to analyses performed with ESI-MS^2 and liquid chromatography ESI-MS. We also explored the use of sequential digestion on viral capsid proteins where endoprotease digestion was followed by exoprotease exposure to generate sequence information directly from enzymatic digests.

Results and Discussion

Oligonucleotide analysis

MALDI-MS ladder sequencing generated complete sequence information for the 30 mer and 20 mer (Fig. 1) oligonucleotides by combining the MALDI-MS results from different SVP and BSP digestions. The first exposure contained the highest SVP and BSP concentration and thus generated significant digestion of the oligonucleotide. Subsequent digests of the material at lower SVP and BSP concentrations generated enough information to sequence the entire oligonucleotide. As expected, the successively weaker solutions produced less digestion and therefore more information about the initial residues on the oligonucleotide termini. The signal-to-noise ratio was typically greater than 15 and the presence of salt adducts was less significant with these smaller oligonucleotides. In these and all other MALDI ladder sequencing experiments, no signal was observed from SVP or BSP. The total amount of sample consumed was typically 2 nanomoles for the entire set of analyses, which included nine digests.

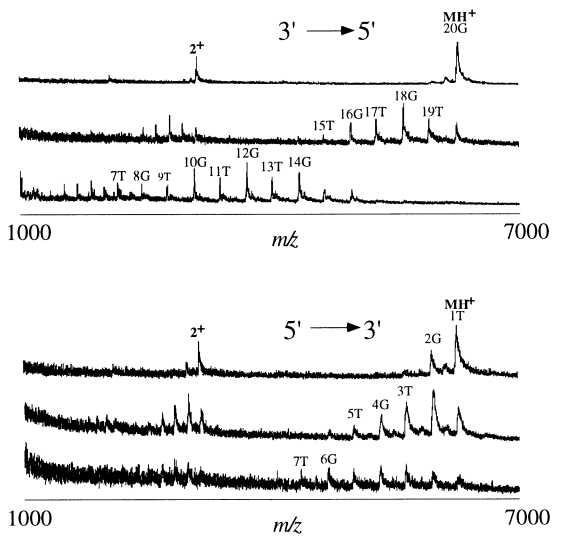

Figure 1. Positive ion MALDI-MS of a ladder sequence from a 20 mer oligonucleotide from the 3′ to 5′ end with SVP (top) and 5′ to 3′ end with BSP (bottom). The order of the peaks corresponds to the sequence. Snake venom phosphodiesterase (SVP) digests the DNA from 3′ to 5′. Bovine spleen phosphodiesterase (BSP) digests the DNA from 5′ to 3′.

MALDI-MS ladder sequencing of 50 mer (Fig. 2) and 40 mer (data not shown) oligonucleotides produced sequence information on approximately five residues from either the 5′ or 3′ end of the molecule. The amount of information was directly related to the exposure to the respective enzymes, however extensive exposure did not allow for complete sequencing of the oligonucleotides. The signal intensities from the 50 and 40 mer were typically less than those generated for the smaller oligonucleotides which may account for the inability to detect more complete sections of the ladders. In addition, a significant amount of salt adducts appeared on the molecular ion which caused peak broadening, even after extensive desalting.

Experiments with ESI-MS^2 using collision-induced dissociation (CID)[21] were performed on the 50, 40, 30, and 20 mer oligonucleotides (data not shown) using both a triple quadrupole and an ion trap mass spectrometer. Initial ESI-MS analysis on both the quadrupole and ion trap instruments gave excellent molecular ion signals corresponding to multiply charged states in the negative ion mode and provided accurate total molecular weight

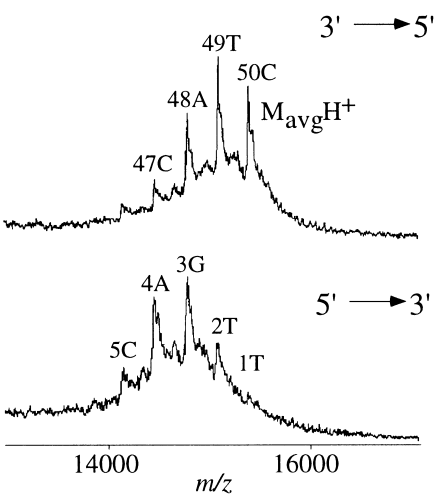

Figure 2. Positive ion MALDI-MS ladder sequence from a digest of a 50 residue oligonucleotide. It was sequenced from the 3′ to 5′ and from the 5′ to 3′.

information; however, ESI-MS^2 provided virtually no sequence information. These results were not surprising given the size of the molecules and the low energy collisions generated in the ion trap and triple quadrupole mass spectrometers.

Both ladder sequencing and ESI-MS^2 were then performed on smaller oligonucleotides (Figs 3–6) including an 18, 11, 6 (Figs 3 and 4), 4 (Figs 5 and 6), 3, and 2 mer. Similar to the ladder sequencing of the 20 mer (Fig. 1) the 18, 11, and 6 mer (Fig. 3) was straightforward and easily generated complete sequence information, with excellent S/N and mass accuracy. It was possible to generate complete sequence information for the 4 mer (Fig. 5), 3 mer and 2 mer as well, however the data was more challenging to interpret due to the background matrix interference in the low mass region.

When analyzed by ESI-MS and ESI-MS^2, the 18, 11, 6, 4, 3, and 2 mer produced excellent molecular ion signals in negative ion mode, while complete sequence information was possible only on the 6, 4, 3, and 2 mer (the entire sequence and cleavage site information of the 6 mer and 4 mer are shown in Figures 4 and 6). It should be noted that interpretation of the ESI-MS^2 results was typically more labor intensive (as compared to ladder sequencing) because fragmentation can occur at multiple sites on the phosphate, sugar, and base of the oligonucleotides. It should also be noted that computer programs designed to deconvolute MS^2 data on oligonucleotides are being developed to facilitate future data interpretation. In the oligonucleotide sequencing experiments neither ESI-MS^2 nor MALDI ladder sequencing were rigorously optimized to increase sensitivity and in general the amount of material consumed

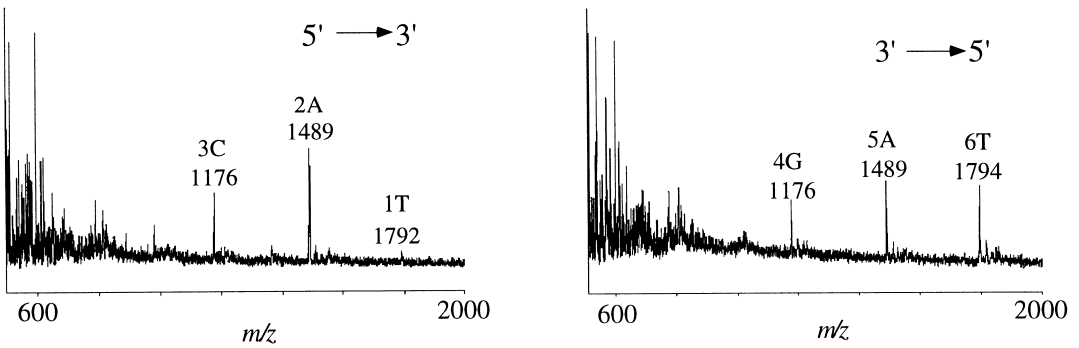

Figure 3. Positive ion MALDI-MS data generated from the oligonucleotide 5′-TACGAT-3′ using the ladder sequencing approach. The BSP digest is shown on the left, while the SVP digest is shown on the right.

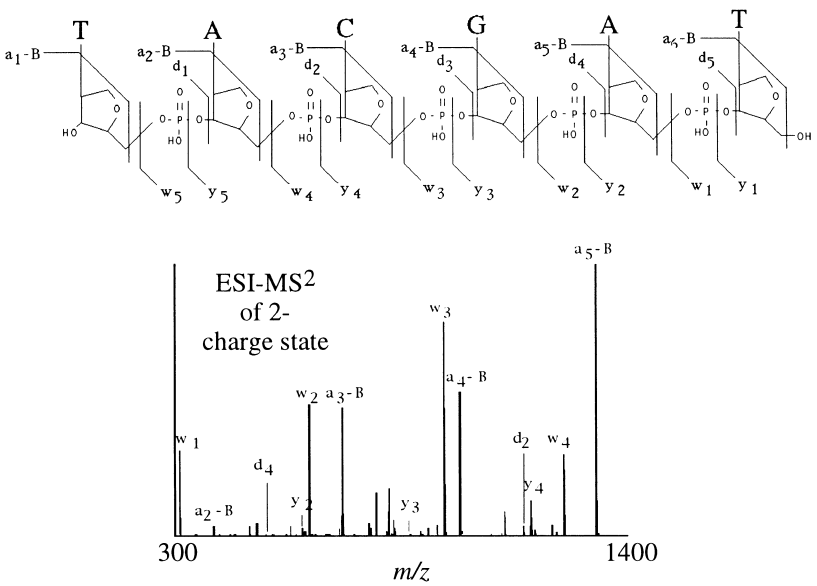

Figure 4. Negative ion ESI-MS^2 data generated in the oligonucleotides 5′-TACGAT-3′ using the collision-induced dissociation.

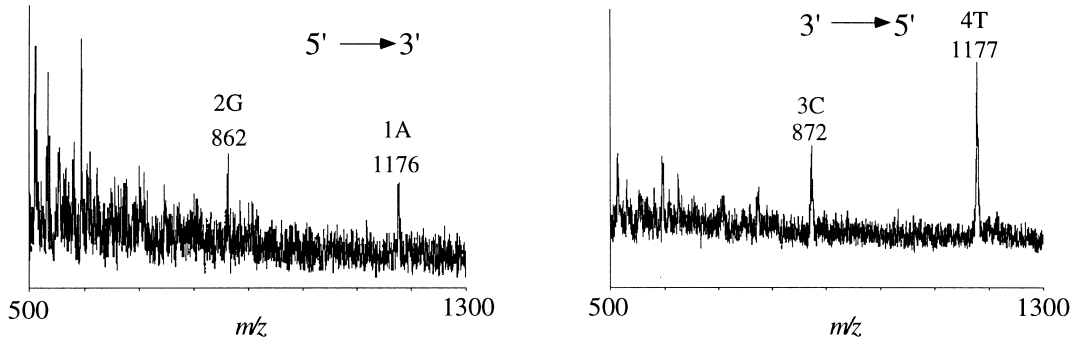

Figure 5. Positive ion MALDI-MS data generated from the oligonucleotide 5′-AGCT-3′ using the ladder sequencing approach. The BSP digest is shown on the left, while the SVP digest is shown on the right.

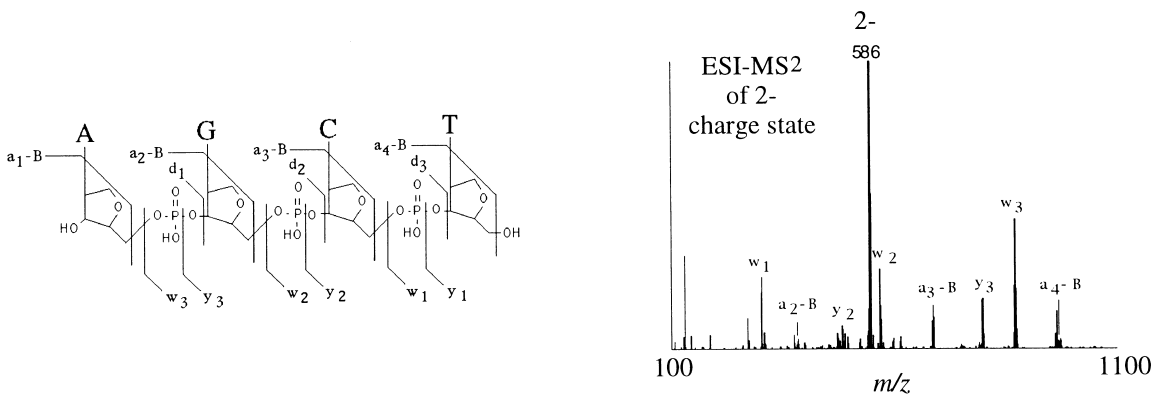

Figure 6. Negative ion electrospray ionization-MS^2 data generated in the oligonucleotide 5′-AGCT-3′. Cleavage of base, sugar, and phosphate were all observed in these experiments.

in the ladder sequencing experiments (~2 nmol) exceeded that of ESI-MS^2 (~500 pmol).

Peptide and protein analysis

Our sequencing efforts with peptides and proteins focused on MALDI-MS ladder sequencing[15] using C-terminal enzyme digestion[22] with CPY. Here we compared its utility to LC-ESI-MS, as well as ESI-MS^2 which has been extensively studied[23] for peptide analysis. We also examined the sequential use of endopeptidase and exopeptidase digestions to allow for one-step sequencing of endoproteolytic fragments and to thus improve the efficiency of protein identification.

Consistent with previously reported work,[22] the enzyme digestion MALDI-MS ladder typically provided sequence information on 5–7 residues of a peptide (Fig. 7), however extended exposure provided as much as 15 residues. The primary obstacle in these analyses was significant fluctuation in ion intensity from peptide to peptide in the ladder which could be partially due to differences of digestion kinetics. Analogous to the oligonucleotide work, the smaller (< 400 Da) peptides presented a challenge because the presence of matrix ions coincided with the smaller peptide fragments. The results from the smaller peptides highlight the complementary nature of MALDI-MS and ESI-MS^2 because information obtained from MALDI ladder sequencing could either be corroborated or even extended using ESI-MS^2, which does not suffer from matrix interference. Still, the strength of ladder sequencing appears to be more pronounced with larger peptides (> 15 residues) on which ESI-MS^2 can only generate limited fragmentation information (Fig. 7).

Because of the limited capacity of ESI-MS to analyze some mixtures, liquid chromatography was also combined with ESI-MS to analyze the sequence of peptides after a CPY peptide digest. Data from these experiments indicated that LCMS will not provide a practical alternative to MALDI-MS. The LCMS was significantly more time consuming, typically requiring runs over 20 min during which considerable time was spent searching for and identifying the digest fragments. In the analysis of five peptides, from which MALDI-MS

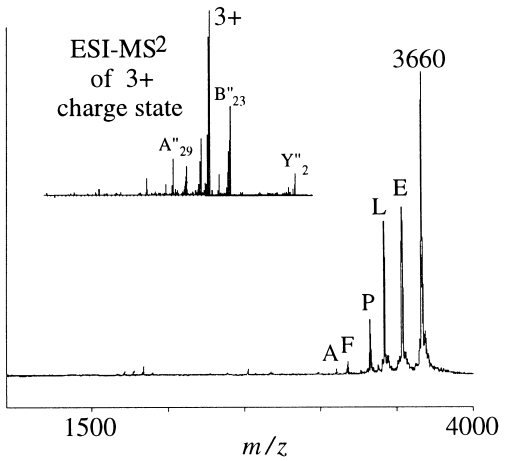

Figure 7. The positive ion MALDI-MS ladder sequencing data using the CPY digestion of ACTH (7–38) provides some C-terminal sequence information while positive ion ESI-MS^2 data (inset) is less informative unless the sequence is already known.

ladder sequencing generated information on 5–7 residues, only 2 or 3 could be observed with LCMS for the same sample. In an additional set of experiments, sequential digests using endoproteolytic digestion were combined with exoprotease MALDI ladder sequencing.[24] Tryptic fragments were first generated by exposing the viral capsid proteins of Flock House virus to the endoprotease trypsin. In order to identify the trypsin fragments, the entire digest was then exposed to the exoprotease CPY directly on a MALDI sample plate, which produced sequential fragments of the endoproteolytic tryptic fragments. After the matrix was added the MALDI sample plate was then inserted into the mass spectrometer for analysis. The mass spectral data allowed us to verify the identity of the trypsin fragments by providing partial sequence information (Fig. 8).

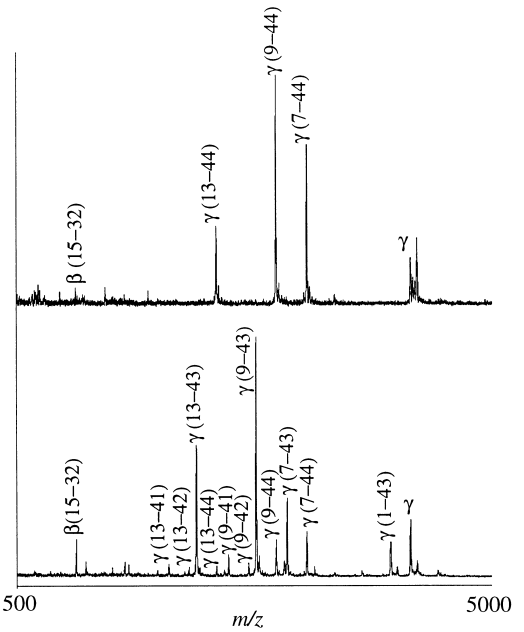

Figure 8. Sequential digestion of a viral capsid protein, γ (1–44), performed directly on the virus. (top) Positive ion MALDI-MS data generated from the trypsin digest and (bottom) data generated from the CPY digest of the trypsin digest (or *endolexo* sequential digestion) of the viral capsid protein.

Conclusion

These results provide insight into the general utility of ESI-MS^2 and MALDI ladder sequencing, both of which offer useful means of generating sequence data on oligonucleotides and peptides (summarized in Table 1). ESI-MS^2 played a useful role in sequencing small oligonucleotides[12] (<6 residues) and produced no matrix interference. However, the larger oligonucleotides generated much more complex spectra and the low-energy collisions in the ion trap and triple quadrupole mass spectrometers generated little fragmentation (data not shown), making it difficult to generate any sequence information. MALDI-MS ladder sequencing of oligonucleotides offered greater mass range and was comparatively straightforward to analyze. An inherent feature in oligonucleotide (DNA) ladder sequencing is that the mass differences between residues are significant enough (≥ 5 Da) to obtain sequencing information on DNA fragments as long as 50 residues. Analyzing the digested oligonucleotides with the MALDI-MS instrument routinely provided complete sequence information for up to 30 mers. Application of this method on oligonucleotides containing as few as six bases was relatively straightforward, however smaller oligonucleotides presented a problem due to matrix interference peaks.

The results of the peptide sequencing experiments highlighted common and distinguishing features of both ESI-MS^2 and MALDI ladder sequencing.[21–23,25] Both techniques offer good sensitivity,[3–6,22] however the primary limitations of both methods are due to incomplete ion series. In the case of ESI-MS^2, the generation of ions from both the N- and C-terminal of a peptide and internal fragments complicated the fragmentation patterns. ESI-MS^2 experiments were most useful for small peptides while the use of ESI-LCMS for peptide ladders did not offer any significant advantage over MALDI-MS. C-terminal MALDI ladder sequencing typically cleaved 5–7 residues into the peptide, results consistent with previous work done with N-terminal[22] exoprotease digestions. Lastly, the sequential digestion with MALDI-MS offers an approach for generating sequence information on viral protein digests and other complex protein systems. In the case of viral proteins, little sample preparation was required and sequencing was

Table 1. Characteristics of mass spectrometry-based sequencing approaches based on the experiments performed in this report and previously reported data.[8,9,12,13,17,20,23,26] It should be noted that where nano ESI-MS^2 is used to perform the ESI studies, considerably less material (femtomoles) would have been consumed.[7,27]

ESI Tandem Mass Spectrometry	MALDI-MS Ladder Sequencing
Oligonucleotides:	**Oligonucleotides:**
Picomole sensitivity	Picomole to nanomole sensitivity
Range to about six residues for complete sequence	Range to about 30 residues for complete sequence
Usually only partial sequence information obtained	Often full sequence information obtained
Interpretation of data is involved although computer deconvolution programs are becoming available	Interpretation of data is straightforward
This study*: no sequence on the 18–50 mer partial sequence on the 11 mer complete sequence on the 2–6 mer	This study*: partial sequence on the 50 mer and 40 mer complete sequence on the 2–30 mer
Peptides:	**Peptides:**
Picomole sensitivity	Picomole sensitivity
Range to about 25 residues	Range to 7–150 residue peptides
Usually partial sequence information obtained	Usually partial sequence information obtained
Interpretation of data is involved although computer deconvolution programs are available	Interpretation is relatively straightforward
This study*: no sequence on the 32 mer and 44 mer partial sequence on the 22 mer complete sequence on the 9 mer and 11 mer	This study*: partial sequence on the 9–44 mer

*Sequence of oligomers listed in the experimental section.

carried out directly on the MALDI sample plate in under 5 min. In conclusion, either technique can have significant advantages over the other, advantages that must be carefully evaluated based on size and sensitivity requirements, and the biopolymer's ionization characteristics.

Experimental

Oligonucleotides and peptides

The oligonucleotides were obtained (~50 nanomoles each) from GibcoBRL, The Scripps Research Institute's core facility, and La Jolla Pharmaceuticals. MALDI-MS ladder sequencing and ESI-MS^2 were used to perform sequencing on the following oligonucleotides: two 2 mers, (5′-CA-3′, 541 Da) and (5′-AG-3′, 581 Da), two 3 mers (5′-CAG-3′, 871 Da) and (5′-TGA-3′, 886 Da), two 4 mers (5′-AGCT-3′, 1175 Da) and (5′-ACGT-3′, 1175 Da), two 6 mers (5′-TACGAT-3′, 1792 Da) and (5′-AGATCA-3′, 1801′Da), an 11 mer (5′-AACGATACGCA-3′, 3335 Da), a modified 11 mer, an 18 mer (5′-GTTTCCCATACCCCAGGA-3′, 5421 Da), a 20 mer (5′-TGTGTGTGTGTGTGTGTGTG-3′, 6273 Da), a 30 mer (5′-CTGATTGATCAGCCCGACATCTGTGCGAAG-3′, 9208 Da), a 40 mer (5′-ACGTAATGACGTCCAGTCCATTACTACCCCGTTAGTGATT-3′, 12,207 Da), and a 50 mer (5′-TTGACTGGCCATTTGAGCTCGTAGGACAGTTACGATGTACTGACTTCATC-3′, 15,383 Da).

The peptides were obtained from SIGMA, The Scripps Research Institute's core facility, and Professor Jack E. Johnson. These include adrenocorticotropic hormone fragment ACTH7-38 (H_2N-FRWGKPVGKKRRPVKVYPNGAEDESAEAFPLE-CO_2H, M_{AVG} = 3659.2), ACTH18-39 (H_2N-RPVKVYPNGAEDESAEAFPL EF-CO_2H, M_{AVG} = 2465.7), bradykinin (H^2N-RPPGF SPFR-CO_2H, M_{mono} = 1059.6), an N-terminally blocked peptide (Ac-GLYQAKRFKVG-amide, M_{mono} = 1307.8) and Flock house virus capsid peptide (H_2N-ASMWERVK-SIIKSSLAAASNIPGPIGVAASGISGLSALFEGFGF-CO_2H, M_{AVG} = 4396.1) sequenced directly from an intact virus and six other capsid protein fragments.[28]

MALDI-MS and analysis

The MALDI-MS measurements were performed in a Voyager-Elite, time-of-flight mass spectrometer with delayed extraction (PerSeptive Biosystems, Inc., Framingham, MA). Samples were irradiated with a nitrogen laser (Laser Science Inc.), operated at 337 nm and attenuated with a neutral density filter. Ions produced by laser desorption were energetically stabilized during a delayed extraction period of 150 nanoseconds and then accelerated through the linear time-of-flight mass analyzer by 20 kV potential pulse. Spectra shown were typically an average from 128 laser pulses. The MALDI-MS measurements were performed in positive ionization mode principally due to the work of Weinberger et al.,[29]

who observed no significant difference in oligonucleotide signal when they used analyzed positive or negative ionization modes. Because subsequent comparative studies performed in our lab were consistent with Weinberger's results, the positive ionization mode was adopted.

Digestion of oligonucleotides and peptides for MALDI-MS

For analysis performed directly on the MALDI sample plate, each oligonucleotide and peptide were digested with an Oligonucleotide Sequencing Sequazyme Kit and C-Peptide Sequencing Sequazyme Kit, respectively (PerSeptive Biosystems). The time-dependent exonucleases, bovine spleen phosphodiesterase (BSP) and snake venom phosphodiesterase (SVP), were used in the ladder sequencing experiments in conjunction with MALDI-MS. The BSP digested the oligonucleotide from the 5′ to the 3′, while the SVP digested the oligonucleotide from the 3′ to 5′. Oligonucleotide samples were prepared at a concentration of 100 pmol μL and 1 μL of the sample solution was added to the plate followed by 2 μL water, 1 μL enzyme, and 1 μL buffer. The solution was allowed to incubate for 20 min at 37 °C after which 5 μL of the matrix was added. All samples were then dried using a heat gun and promptly analyzed. 3-hydroxypicolinic acid[30]/ammonium citrate (3-HPA/AC) (50 mg/mL in 50:50 water/acetonitrile, plus 30 mg/mL diammonium citrate in water) was used as the matrix in these studies, however an alternative matrix system, trihydroxy acetophenone (THAP), may also be used.[31] Our experience with THAP is that it provides no significant improvement in sensitivity over 3-HPA/AC, although in preliminary results from our lab indicate that THAP may improve accuracy. Following the protocols of the Sequazyme Kit, six dilutions of the SVP and three dilutions of BSP were created with stock solutions of the exonucleases individually reconstituted in 100 μL of HPLC-grade water. Five consecutive SVP dilutions of the original SVP solution were used (1:2.5, 1:5, 1:10, 1:25, and 1:50), as were two BSP dilutions (1:3 and 1:9) of the original solution (all in milli-Q water).

Following the protocols of the Sequazyme kit, peptide samples (1 pmol μL) were exposed to the enzyme, carboxypeptidase Y (CPY), which hydrolyzes the C-terminal residues of peptides. 0.5 uL of sample solution was applied to each sample well followed by 0.5 uL of a CPY solution. Samples were allowed to dry at room temperature and then 0.5 μL of alpha-cyano-4-hydroxy cinnamic acid matrix solution was added to each well and allowed to dry before analyzed. We were also able to perform CPY peptide digests (CPY provided by Sigma) simply by reconstituting the CPY in water at a 1 mg/mL concentration.

Sequential digestion of peptides for MALDI-MS

~10 pmol of Flock house virus (FHV) was digested with 1 pmol trypsin for 1–45 min before being inhibited by the addition of TLCK. One microliter of each sample was pipetted into two MALDI sample wells, after which the second well was exposed to 1 μL containing 1.0 pmol to 0.06 pmol of CPY, and then allowed to dry. After addition of sinapinic acid (the MALDI matrix) the sample was analyzed.

ESI-MS^2 and analysis

Tandem mass spectrometry (MS^2) was carried out in an API III Perkin–Elmer Sciex triple quadrupole mass spectrometer and a Finnigan LCQ ESI ion trap mass spectrometer. Samples were introduced into the source at a flow rate of 4.0 μL/min and a potential of 3500 to 4200 V was applied to the interface sprayer. Ions generated were then electrostatically directed through the vacuum interface. The samples were typically prepared in a 50:50 water:methanol solution by initially adding water to the oligonucleotide followed by addition of the methanol.

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