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RESEARCH ARTICLE

Negative Ion In-Source Decay Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry for Sequencing Acidic Peptides

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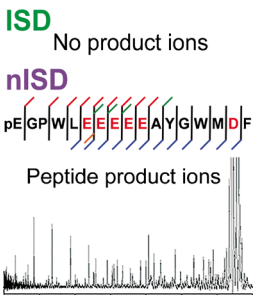

Abstract. Matrix-assisted laser desorption/ionization (MALDI) in-source decay was studied in the negative ion mode on deprotonated peptides to determine its usefulness for obtaining extensive sequence information for acidic peptides. Eight biological acidic peptides, ranging in size from 11 to 33 residues, were studied by negative ion mode ISD (nISD). The matrices 2,5-dihydroxybenzoic acid, 2-aminobenzoic acid, 2-aminobenzamide, 1,5-diaminonaphthalene, 5-amino-1-naphthol, 3-aminoquinoline, and 9-aminoacridine were used with each peptide. Optimal fragmentation was produced with 1,5-diaminonphthalene (DAN), and extensive sequence informative fragmentation was observed for every peptide except hirudin(54–65). Cleavage at the N–Cα bond of the peptide backbone, producing c′ and z′ ions, was dominant for all peptides. Cleavage of the N–Cα bond N-terminal to proline residues was not observed. The formation of c and z ions is also found in electron transfer dissociation (ETD), electron capture dissociation (ECD), and positive ion mode ISD, which are considered to be radical-driven techniques. Oxidized insulin chain A, which has four highly acidic oxidized cysteine residues, had less extensive fragmentation. This peptide also exhibited the only charged localized fragmentation, with more pronounced product ion formation adjacent to the highly acidic residues. In addition, spectra were obtained by positive ion mode ISD for each protonated peptide; more sequence informative fragmentation was observed via nISD for all peptides. Three of the peptides studied had no product ion formation in ISD, but extensive sequence informative fragmentation was found in their nISD spectra. The results of this study indicate that nISD can be used to readily obtain sequence information for acidic peptides.
Keywords: Negative ion mode, In-source decay, nISD, Deprotonated acidic peptides, MALDI/TOF

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Introduction

Matrix-assisted laser desorption/ionization (MALDI) has several benefits that are useful for biomolecule analysis, such as higher tolerance for contaminants and impurities and the small amount of sample consumed in the ionization process [1]. One important feature that makes MALDI useful for analysis and sequencing of peptides is that only singly charged ions are typically produced, making spectral interpretation relatively straightforward. Analytes can form protonated, $[M+H]^+$, or deprotonated, $[M-H]^-$, species in MALDI. However, the majority of MALDI studies on peptides have only employed the positive ion mode [1–23]. Extensive studies that have employed the negative ion mode for peptide sequencing have involved post-source decay (PSD) [24–27] or MALDI with collision-induced dissociation (CID) [28, 29].

In-source decay (ISD), where the ions formed by MALDI dissociate in the source before being pulsed into a mass analyzer (normally time-of-flight, TOF) has been a successful technique for peptide sequencing. The focus of ISD studies has primarily been sequencing protonated peptides. However, many of the peptides that have important functions in the human body are highly acidic. For example, gastrin (a stomach peptide) and hirudin (a peptide involved in blood coagulation) contain a large number of acidic residues [30, 31]. Acidic peptides can be difficult or even impossible to observe using positive ion mode techniques because they often do not readily produce protonated ions [25, 32]. However, acidic peptides

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have been shown to readily form deprotonated ions using negative ion mode techniques [24–27, 32, 33].

Negative ion mode ISD (nISD) has been demonstrated by several groups. De Pauw and coworkers [34] published a study on the rational selection of a MALDI matrix for ISD; peptide signal intensity produced by three matrices was compared in positive and negative ion mode. De Pauw and coworkers [35] later proposed that specific sites within a peptide are capable of providing the negative charge and producing observable product anions in nISD. In addition, they stated that nISD product ions containing acidic residues are generally more intense than product ions that do not contain acidic residues. De Pauw and coworkers [36] also considered 2-aminobenzoic acid and 2-aminobenzamide as potential ISD matrices and tested these matrices for nISD with the peptide [Glu^{1}]-fibrinopeptide b. Asakawa and coworkers [37] stated that nISD provides useful complementary sequence information to positive ion mode ISD. This group also published a study on 5-nitrosalicylic acid as a MALDI matrix for ISD, and tested this matrix for nISD with a tetraphosphopeptide [38]. It was determined that 5-nitrosaylicylic acid can be used with ISD and nISD to determine the sites of post-translational modifications within a peptide. Most recently, Takayama and coworkers [29] performed MALDI ISD and nISD experiments on whole proteins followed by positive or negative ion CID of the ISD product ions to provide information on the secondary protein structures. In addition, Takayama and coworkers [39] performed MALDI ISD and nISD on four whole proteins and identified amino acid residues that served as protonation or deprotonation sites in the positive and negative ion modes, respectively.

The present study is the first to focus primarily on the applications of nISD as a useful technique for sequencing acidic peptides that do not readily protonate and are therefore difficult or impossible to sequence using positive ion methods.

Experimental

All experiments were performed on a Bruker Ultraflex MALDI/TOF MS (Billerica, MA, USA) equipped with a 337 nm nitrogen laser. In nISD experiments, ions were accelerated from the source using a voltage of −20 kV. For ISD experiments, the accelerating voltage was 25 kV. To improve signal intensity, 1000 laser shots were acquired per spectrum at a frequency of 25 Hz. During the nISD and ISD experiments, laser fluence was at 20%–30% above the ionization threshold for each peptide. Delayed extraction time was optimized at 300 ns for nISD experiments and 180 ns for ISD experiments. Reflectron TOF was employed with all experiments due to its increased resolution over linear TOF.

The peptides studied contain at least four acidic residues and less than three basic residues and were: GLP-2 (rat), (Tyr)-C-peptide (dog), gastrin I(1–17) amide (human), hirudin(54–65), [Glu^{1}]-endo Glu^{7a}-fibrinopeptide b, oxidized insulin chain A (bovine), fibrinopeptide b, and ACTH(22–39). Peptides were purchased from VWR (Radnor, PA, USA), except for oxidized insulin chain A and ACTH(22–39), obtained from Sigma-Aldrich (St. Louis, MO, USA), and [Glu^{1}]-endo Glu^{7a}-fibrinopeptide b, obtained from Anaspec EGT (Fremont, CA, USA). The matrices used were dihydroxybenzoic acid (DHB), 2-aminobenzoic acid (2-AA), 2-aminobenzamide (2-AB), 1,5-diaminonaphthalene (DAN), 5-amino-1-naphthol (5A1N), 3-aminoquinoline (3-AQ), and 9-aminoacridine (9-AA). Matrices were purchased from VWR, except for 5A1N that was purchased from Sigma-Aldrich.

Final matrix solution concentration was 10 nmol/μL (10^{-2} M) in 50:50 acetonitrile:water (ACN:H_2O) (v/v). To help with pre-ionization, additives were added to the matrix solutions at 1.0% (v/v). Solution additives were ammonium hydroxide (NH_4OH) for deprotonation and formic acid (FA) for protonation. The final peptide concentration was 10 pmol/μL (10^{-5} M) for the solution applied to the sample plate. A stainless steel target plate was employed with all samples. The final concentration ratio for each sample spot was analyte:matrix 1:1000.

Results and Discussion

Matrix Optimization

All nISD experiments were performed initially with DHB, which is a well established matrix for peptide ionization [14, 33, 40–42], in order to obtain information about the peptide precursor ions. Supplemental Figures 1–7 show the precursor spectra of the peptides in this study. The dominant precursor ion for each peptide was $[M - H]^-$. Adduct ions from addition of sodium, $[M - H + Na]^-$, or potassium, $[M - H + K]^-$, were observed for some peptides but were at very low intensities compared to $[M - H]^-$. Minimal adduct formation in negative ion mode MALDI is an advantage over positive ion mode MALDI and has been previously observed by our group [33]. Obtaining precursor spectra in nISD (and ISD) is important because this technique does not allow for selection of a precursor ion prior to tandem mass spectrometry (MS/MS). Therefore, it is possible for product ions to be produced by something other than the $[M - H]^-$ precursor. To examine precursor ion formation, DHB was used because it transfers the least amount of energy to the peptide, in comparison to the other matrices in this study, and is also a very common MALDI matrix [34, 35, 42]. The laser fluence was set at slightly above the ionization threshold for each peptide. Thus, ionization with minimal fragmentation was produced, allowing for observation of all possible precursor ions for each peptide.

Previous studies by De Pauw and coworkers showed that the matrices 2-AA, 2-AB, [36] and DAN [23, 34] are very useful for obtaining peptide fragmentation via ISD. In our nISD experiments, these matrices were found to provide sequence informative fragmentation, with DAN producing the most sequence coverage for all peptide samples except hirudin(54–65). In an attempt to improve product ion intensity and increase the range of fragmentation, the matrices 5A1N, 3-AQ, and 9-AA were studied because they are similar to DAN with their ring structures and side chain amide groups. However, none of the

nISD spectra of any of the peptide samples showed increased signal intensity or more extensive fragmentation with these matrices beyond what is observed when using DAN. As an example, Figure 1 shows nISD spectra for the peptide gastrin I(1–17) amide with the matrices DHB and DAN, and Supplemental Figure 8 shows the nISD spectra of this peptide with all of the other matrices except 9-AA. (9-AA would evaporate from the target plate almost immediately after sample spotting, before insertion of the target into the source, when using the NH_4OH additive).

The established peptide sequencing nomenclature from Roepstorff and Fohlman [43], as modified by Biemann [44], is used to identify peptide precursor and product ions. In the nISD spectra of all peptides studied, the dominant product ion series are c′ and z′ ions, where the prime symbol indicates the addition of a hydrogen to the product ion (i.e., c′ is $[c+H]^-$). These product ions are produced from cleavage of the N–Cα bond of the peptide backbone.

Optimized nISD Spectra

Figure 2 shows the nISD spectrum of (Tyr^0)-C-peptide (dog) with DAN as the matrix. The ions observed are c_n', where n = 5–15, 17–20, 22, 24, 25, and 30, and z_n', where n = 8, 10, 13–15, 17, 19, 22, 23, 25, and 27–29. Note that the ion pairs c_{13}'/z_{17}' and c_{15}'/z_{19}' are isobaric, which has been reported with this peptide previously by De Pauw and coworkers [23]. No cleavage corresponding to c or z ions is observed at the proline residues (residues 17 and 24 from the N-terminus). Since proline forms a ring structure that includes the N–Cα bond of the peptide backbone, cleavage of this bond only opens the ring; breaking two bonds in the proline residue is required in

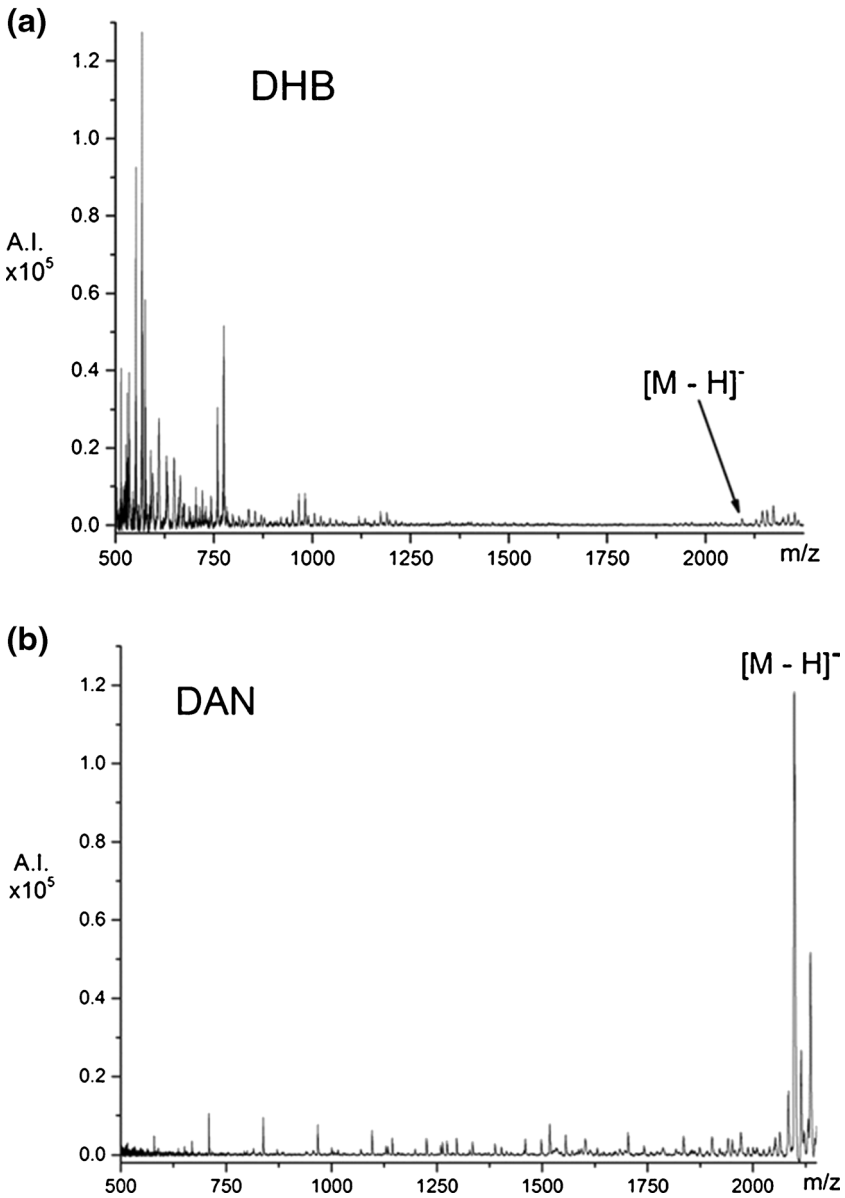

Figure 1. nISD spectra of gastrin I(1–17) amide with the matrices (**a**) DHB and (**b**) DAN

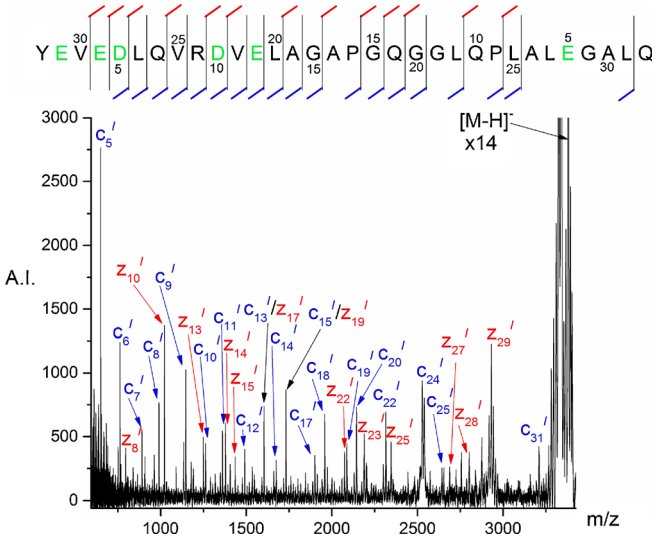

Figure 2. nISD spectrum of (Tyr^0)-C-peptide with DAN as the matrix. Acidic residues are highlighted in green

order to see cleavage at that site. Thus, no N–Cα bond cleavage at proline was observed in the nISD spectra of any peptide studied. The absence of cleavage at proline residues is observed with radical-driven dissociation techniques (ECD, ETD, ISD) in situations where c and z ion formation is common [23].

Figure 3 shows the nISD spectrum of the peptide GLP-2 with DAN as the matrix. The c_n' ions are n = 6–24, 30, and 32, and z_n' ions are n =6, 8, 10, 12–15, 18–20, 23–28, 30, and 31. Some b_n ions are seen where n = 8–11, 15, 19, and 20. De Pauw and coworkers [34] have also observed low intensity b ions in nISD. The c-series are dominant in the lower *m/z* region of this peptide. However, at increasing *m/z*, c ions decrease in intensity and only the z ions are observed. At the higher *m/z* region beginning from the N-terminus the last acidic residue is n = 21, and the next acidic residue does not appear in the sequence until n = 33. From the C-terminus, z ions are observed at low intensities. There does not appear to be a trend correlating z ion intensity with proximity to an acidic residue. In contrast, the c ions are most intense when cleavage is located at the C-terminal side of an aspartic acid (D) residue or a glutamic acid (E) residue. One item to note is that the most intense product ion, c_{15}', is located C-terminal to an aspartic acid residue and N-terminal to an asparagine (N) residue. Previous studies by Wysocki and coworkers have discussed enhanced cleavage C-terminal to aspartic acid residues via positive ion mode CID [45, 46], which could help explain the enhanced cleavage observed in the present study.

The nISD spectrum of [Glu^1]-endo-Glu^{7a}-fibrinopeptide b is shown in Figure 4 with DAN as the matrix. Complete sequence coverage is observed for this peptide. The c_n' ions are observed from n = 6 to n = 14, and the z_n' ions are observed from n = 5 to n = 13. A possible w_n ion is observed at n = 14, which corresponds to a loss of the H side chain from the glycine residue at the N-terminal side of the z_{14} product ion. The c_{12}' and w_{15} (precursor ion minus the side chain of the N-terminal glutamic acid residue) contain all of the acidic residues in the peptide and are the most intense product ions in the spectrum. Neutral losses are observed for z_{10}', c_8', c_9', and c_{12}' that correspond either to elimination of water or the carboxylic acid groups from glutamic acid residues. An intense product ion is $[M - H - COO - 72]^-$; 72 Da may correspond to $C_2H_6N_3$, which is cleavage from the side chain from the C-terminal arginine residue. Side chain losses from arginine have been previously observed by Coon and coworkers via positive ion mode ETD [47], and the loss of 72 Da, specifically, has been observed in positive ion mode CID by Laskin and coworkers [48].

The nISD spectrum of fibrinopeptide b with DAN is shown in Figure 5. N-terminal sequence coverage begins at c_5' and goes to c_{13}', except for c_{11}', which is difficult to observe because of an intense neighboring ion. C-terminal sequence coverage begins at z_5' and goes to z_{13}', except for z_{12}'. A y ion is also observed at y_{13}. A potential internal ion observed at *m/z* 1256 corresponds to simultaneous cleavage at z_{13} and c_{13}, resulting in the loss of pyroglutamate from the N-terminus

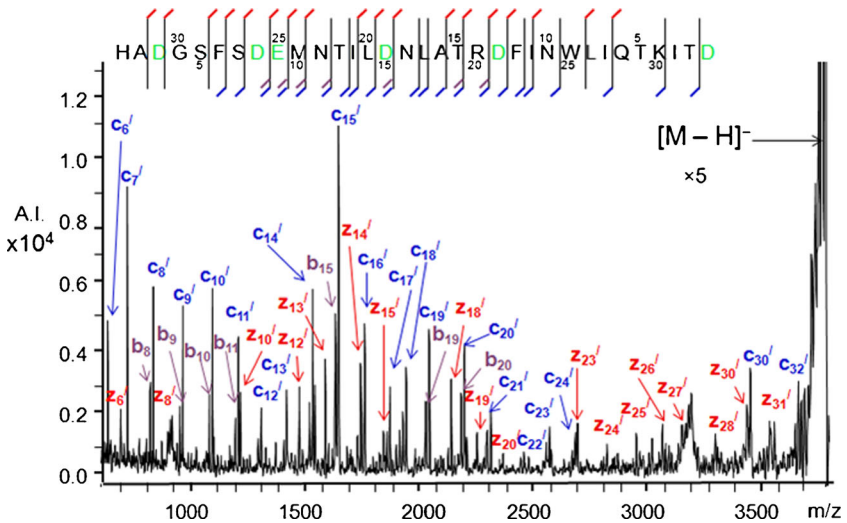

Figure 3. nISD spectrum of GLP-2 with DAN as the matrix. Acidic residues are highlighted in green

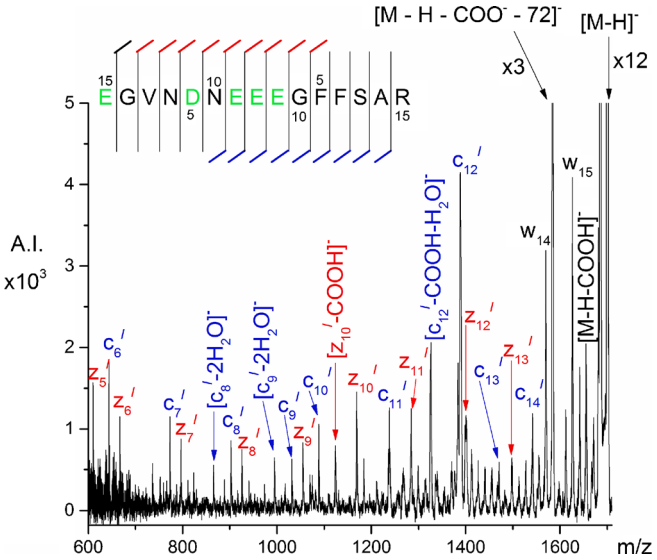

Figure 4. nISD spectrum of [Glu[1]]-endo-Glu[7a] fibrinopeptide b with DAN as the matrix. Acidic residues are highlighted in green

and an arginine residue from the C-terminus. The most abundant product ions are the c ions, which are most intense near the acidic residues. In contrast, the z ions are all at relatively the same intensity throughout the peptide. The most intense product ion is c_5', which is C-terminal to an aspartic acid residue and N-terminal to an asparagine residue. This is analogous to c_{15}' in the spectrum of GLP2. [Glu[1]]-endo-Glu[7a] fibrinopeptide b also has the residues of aspartic acid and asparagine positioned in the same manner, but the c ion between these two residues falls below the lower *m/z* range of the spectrum (Figure 4).

The peptides that did not produce extensive sequence informative fragmentation via nISD are oxidized (ox.) insulin chain a, ACTH(22–39), and hirudin(54–65). For nISD of ox. insulin chain A, shown in Supplemental Figure 9, fragmentation is sequestered around the oxidized cysteine residues. Cysteine residues in insulin chain A are oxidized when insulin chain A is cleaved from insulin chain B, causing the disulfide bonds to break and form a highly acidic cysteic acid group in place of the thiol group. The main product ions observed were c_n' ions where n = 6–15 and 19. The only z_n' ions observed were n = 11, 13, and 16. A few b_n ions (n = 6, 7, 18, and 20) and x_n ions (n = 16 and 18) were observed. The majority of these ions were potassium adducts (i.e. $[c_n' + K]^-$). Loss of the cysteic acid was observed in several of the product ions as well. Owing to the high acidity of cysteic acid [49], charge sequestration around these residues may occur and limit fragmentation, as previously observed in negative ion mode CID by our group [32] as well as Gaskell and coworkers [50] and Russell and coworkers [51]. The most intense product ions observed in the nISD spectrum of ox. insulin chain A correspond to the products $[z_{16} - SO_3H]^-$ and $[x_{16} - SO_3H]^-$, which are product ions that contain all of the oxidized cysteine residues in the peptide. The product ion $[x_{18} - SO_3H]^-$ also has greater intensity than most of the other product ions. However, this nISD spectrum is still considerably more informative than the positive ISD spectrum, where even the precursor ion is not observed (see Supplemental Figure 16).

Although the spectrum of ACTH(22–39) does not show extensive sequence-informative fragmentation in comparison to the previously discussed peptides, some informative fragmentation is observed in the middle section of the peptide, as shown in Supplemental Figure 10. The c_n' ion series is observed at n = 7–13 and 16. In contrast to the other peptides, several y ions (n = 6 and 8–10) are prevalent and involve fragmentation with the charge remaining at the C-terminus. The y_n ions observed are n = 6 and 8–10. The only z ion is z_{10}'. A possible internal ion is observed representing cleavage at c_{15} and z_{17} at *m/z* 1462, which are C-terminal to a valine residue and a proline residue, respectively. ACTH(22–39)

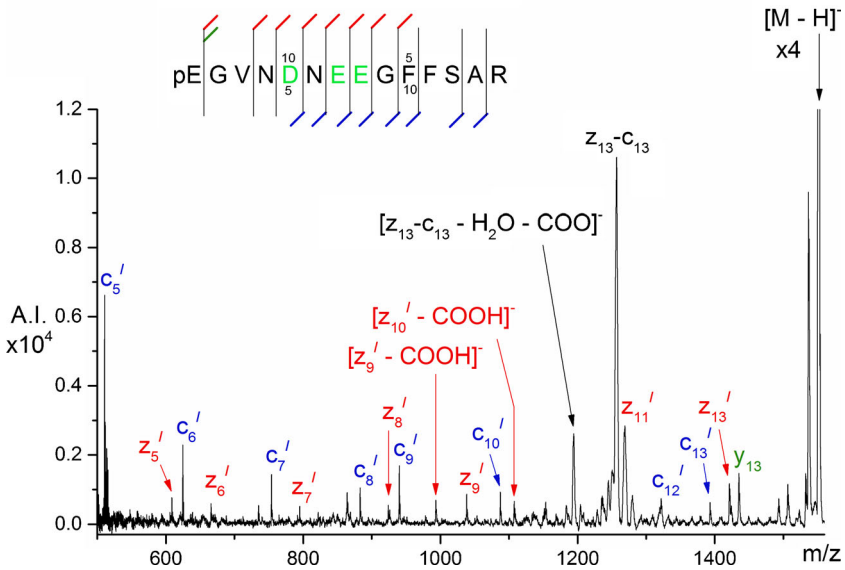

Figure 5. nISD spectrum of fibrinopeptide b with DAN as the matrix. Acidic residues are highlighted in green

contains two proline residues at positions 3 and 15 from the N-terminus. No product ions occur in the spectrum N-terminal to these residues, which is observed for (Tyr^0)-C-peptide and gastrin I(1–17) amide. The most intense product ions are c_7' and c_{12}', which are both located C-terminal to glutamic acid residues.

Hirudin(54–65) is the smallest peptide in this study, with only 12 residues. DHB was found to be the optimal nISD matrix for this peptide rather than DAN. When this peptide was analyzed with DAN (Supplemental Figure 19), only the precursor ion $[M - 2H]^-$, the precursor minus a neutral loss $[M - H - OH]^-$, and the product ions c_{11} and z_{11} were observed (corresponding to loss of the C-terminal residue and N-terminal residue, respectively), and the low *m/z* region of the spectrum contained significant chemical noise peaks. When using DHB (Supplemental Figure 11), product ions were observed in the nISD spectrum corresponding to cleavage adjacent to glutamic acid residues with what appears to be the adduction of matrix fragments. Adduction of matrix fragments during MALDI is atypical, so this assignment is speculative. Other nISD product ions are c_n' (n = 4 and 8), z_n' (n = 4, 5, and 9), and a possible y_4 ion. The lack of extensive observable fragmentation for this peptide may be due to the small number of residues in the peptide. For example, a previous study by De Pauw and coworkers showed that the amount of positive ion ISD fragmentation decreased as the peptide size decreased; this trend was observed with each matrix in the study [34]. In addition, DHB generally produces less nISD fragmentation than DAN [34, 35, 42], as we have observed. The cause of the lack of fragmentation (i.e., peptide size and/or matrix choice) when studying hirudin(54–65) with DAN, in comparison to DHB, is unclear at this time. However, the spectrum of hirudin(54–65) with DHB still contains more sequence information than what is obtainable with positive ISD (Supplemental Figure 19).

Fragmentation Pathways

The observed product ions in nISD indicate that the technique is likely a radical-driven dissociation. Cleavage at the N–Cα bond, as observed in nISD, is also found for radical-driven dissociation techniques such as electron capture dissociation (ECD) [52–54] and electron transfer dissociation (ETD) [55, 56] as well as positive ion mode ISD, which is considered a radical-driven dissociation [19, 35, 57]. Other ion types are observed depending on the peptide. nISD product ions are not reported below *m/z* 500–600 due to interference from matrix ions and considerable signal overlap of the isotopic distributions of peaks in that region.

There is some disparity in the literature regarding the exact nature of the c- and z- ions produced via ISD and nISD. De Pauw and coworkers [23, 34, 37, 42] have reported that these ions are c′ and z′ for both ISD and nISD, which is in agreement with the present study. In contrast, Takayama and coworkers [14, 29] reported that these ions are c and z′ in both ISD and nISD. However, in a different study, Takayama and coworkers [58] agreed that the ions are c′ and z′ in ISD and did not comment on the nature of the ions in nISD. This discrepancy is likely the result of limited resolution observed in nISD/TOF, which results from the kinetic energy spread found in nISD product ions. This kinetic energy spread occurs because of the excess energy required for the ISD process, ultimately causing peak broadening since TOF analysis is based on ion velocity and kinetic energy. In the present study, to increase *m/z* accuracy, calibration of the instrument was performed before every nISD experiment with a mixture of peptides with established masses and varying in size. In addition, the calibrant mixture was spotted with DHB, and the laser fluence was set at the minimum threshold to achieve ionization for all peptides in the mixture, to minimize fragmentation.

Comparison of nISD and ISD

While most of the acidic peptides in this study produce fragmentation in the positive ion mode by ISD, more product ions were observed in nISD. Both modes show no evidence of enhanced cleavage at the more highly acidic or basic sites, with the exception of enhanced cleavage adjacent to oxidized cysteine residues in nISD. Also, for these acidic peptides, the intensity of the product ions was greater for negative ions than in the positive ion mode (see Supplemental Figures 12–18 for optimized ISD spectra). In fact, for the highly acidic peptides gastrin I(1–17), ox. insulin chain A, and hirudin(54–65), little to no precursor was observed in the positive ion mode spectra, and no ISD fragmentation was observed. Previously, our group [33] published a study on negative ion mode production in MALDI. The acidic peptide gastrin I(1–14) had much lower precursor ion intensity in the positive ion mode compared with the negative ion mode. Also, no adduct ions were observed with gastrin I(1–14) in the negative ion mode, but $[M+Na]^+$ and $[M+K]^+$ were at nearly the same signal intensity as $[M+H]^+$ in positive ion MALDI. The results are very similar to what is observed in the present study in terms of observed precursor ions and signal intensity.

Figure 6 shows the negative and positive ion mode ISD spectra of gastrin I(1–17) amide with DAN as the matrix. In Figure 6a, extensive cleavage is observed via nISD. Cleavage occurs at nearly every residue, but no c or z ions formed corresponding to cleavage at the proline residue near the N-terminus. N-terminal cleavage begins at c_n' where n = 6 and goes to n = 16. For C-terminal product ions, cleavage begins at z_n' where n = 8 and goes to n = 16, with the exception of n = 15, which is at the proline residue. Some y_n ions are also observed where n = 7 and 9–11. The most intense product ions are

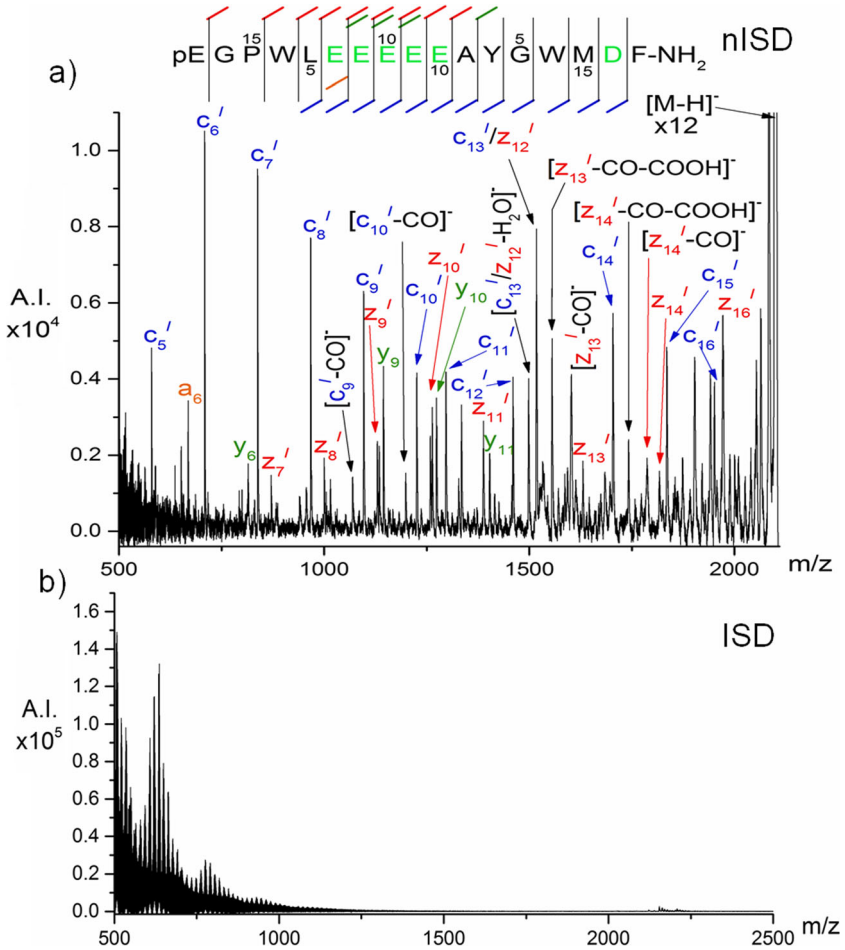

Figure 6. (**a**) nISD spectrum of gastrin I(1–17) amide and (**b**) ISD spectrum of gastrin I(1–17) amide. Acidic residues are highlighted in green. The matrix used to induce fragmentation in both spectra is DAN

adjacent to the acidic residues in the peptide; for example, in comparison to the negative ion mode (Figure 6a), the positive ISD spectrum of gastrin I(1–17) amide in Figure 6b shows very little precursor ion, $[M+H]^+$, and no ISD product ions. The intense peaks at the lower *m/z* region of the spectrum in Figure 6b are the result of matrix ion interference.

Conclusions

The usefulness of nISD for sequencing peptides that will readily deprotonate was demonstrated using several acidic peptides. Extensive fragmentation and sequence coverage was observed for all but ox. insulin A, which has highly acidic oxidized cysteine residues, and hirudin(54–65), which is the shortest peptide in the study. The optimal MALDI matrix for nISD of acidic peptides was found to be DAN, and the dominant nISD product ions produced are c′ and z′ ions. While extensive cleavage occurs for most of the peptides, enhanced cleavage is observed near the acidic residues, specifically for c′ ions. No significant trends were found for the z′ ions except that they typically involve cleavage near an acidic residue. Overall, the results from this study indicate that nISD can be successfully used to obtain extensive sequence informative fragmentation for acidic peptides that readily deprotonate and form negative ions. In comparison to positive ion mode ISD, nISD produces a greater amount of sequence informative fragmentation for acidic peptides.

Acknowledgments

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