

A Practical Ion Trap Mass Spectrometer for the Analysis of Peptides by Matrix-Assisted Laser Desorption/Ionization

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The present paper describes the performance of a newly configured matrix-assisted laser desorption/ionization quadrupole ion trap mass spectrometer (MALDI-ITMS), designed for biological applications that require the determination of the primary structures of proteins, e.g., the rapid identification of proteins and the elucidation of posttranslational modifications. The strategy used for solving problems of this type involves enzymatic digestion of the protein, followed by MALDI ion trap mass spectrometric analysis of the components of the resulting complex mixture of peptide ions. The new instrument is demonstrated to be a highly practical tool for analyzing proteins. In particular, mixtures containing as many as 30 peptide components can be rapidly and sensitively analyzed without prior chromatographic separation of the components. Informative tandem mass spectra can be obtained from the peptide components with m/z values up to 3500. A single subpicomole sample loading of a complex peptide mixture is more than sufficient for a complete set of experiments that includes both low- and high-resolution molecular mass determinations as well as a complete MS/MS study of the various components present in the sample. Extensive use is made of improved methods for trapping, isolating, fragmenting, and detecting ions in the ITMS (details are to be provided in two future papers).

The quadrupole ion trap mass spectrometer (ITMS) is a potentially powerful tool for the analysis of biopolymers.¹ The capacity of the ion trap for high-sensitivity multistage tandem mass spectrometry (MSⁿ)^{2,3} is particularly appealing for biological applications that require the determination of the primary structures of proteins, e.g., the identification of proteins and the elucidation of posttranslational modifications. An attractive strategy for solving problems of this type involves enzymatic digestion of the protein, followed by mass spectrometric peptide mapping and tandem mass spectrometric analysis of individual components of the resulting complex mixture of peptide ions. This strategy, which does not require prior chromatographic separation of the peptides, is potentially fast and highly sensitive.

At present, the most efficient methods for ionizing peptides are electrospray ionization (ESI)⁴ and matrix-assisted laser desorption/ionization (MALDI).⁵ MALDI has a number of properties that make it particularly attractive as an ion source for ion trap mass spectrometry of complex peptide mixtures that are produced by enzymatic digestion of proteins. First, the pulsed nature of the MALDI source matches well with the pulsed operation of the ITMS.^{6–9} Second, the low sample consumption per laser pulse allows interrogation of a single sample (containing ≤ 1 pmol of each component) by many thousands of laser pulses. These features allow large numbers of experiments to be performed on a single low-abundance sample of a mixture of peptides, and they also enable judicious tuning of experimental parameters. The capabilities described above are advantageous for biological applications in which the proteins of interest are in low abundance. Additional properties of MALDI that make it well suited for the analysis of protein digests include its proclivity for producing abundant yields of ions from the components of complex mixtures of peptides, the easily interpretable spectra obtained from mixtures of peptides (as a result of the dominance of singly protonated species), and the tolerance of MALDI for salts, buffers, and other common biochemical additives and impurities.¹⁰

Notwithstanding the advantages outlined above, the match between the MALDI ion source and the ITMS has proved challenging. This difficulty is reflected by the current absence of a commercial MALDI-ITMS that can be used for biological applications with ease comparable to that of MALDI in conjunction with the time-of-flight mass analyzer⁵ or ESI in combination with the quadrupole analyzer.⁴ For the coupling to be successful, it is necessary to solve several problems. Crucial among these is the question of how to trap peptide ions efficiently. The wide kinetic energy distributions of ions produced by MALDI¹¹ render these ions difficult to trap without large loss. In addition, the desorbed

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peptide ions are internally excited¹² and are prone to undergo fragmentation prior to, during, and after trapping. Once the ions of interest are trapped, it is necessary to eject them efficiently for detection and mass measurement. Unfortunately, using conventional resonant ejection with ac amplitudes greater than $7 V_{p-p}$ at $q < 0.25$ (the values conventionally used),⁶⁻⁹ the mass resolution has been observed to deteriorate sharply for peptide ions with $m/z > 2500$, making it difficult to measure higher masses accurately. This is a severe limitation for the measurement of singly charged proteolytic fragments produced by MALDI. Additional challenges relate to efficient isolation and effective fragmentation of ions for MSⁿ analysis.

An intrinsic limitation of the ITMS is the restricted dynamic range imposed by the maximum number of ions that the trap can hold (estimated to be in the range 10^5 – 10^7) before space-charge effects degrade the analyzer performance.¹³ MALDI generates copious matrix ions in addition to the peptide ions of interest. If these matrix ions are not discriminated against, they will flood the trap and seriously degrade its performance. There are several ways to “clean” these ions from the ITMS during or after trapping.¹⁴ However, the most straightforward and efficient method of discrimination is simply to prevent them from entering the trap in the first place. Such a strategy requires an “external ion source” wherein ions are produced external to the trap. During transport to the trap, the low-mass matrix ions separate in space and time from the higher mass peptide ions. Appropriate adjustment of the amplitude of the radio frequency (rf) trapping field as a function of time should allow for complete discrimination against the matrix ions and efficient trapping of peptide ions.

In two upcoming papers, we address in detail ion trapping,¹⁵ ion detection,¹⁵ mass isolation,¹⁶ and ion activation.¹⁶ These papers describe means for trapping MALDI peptide ions with ~40% efficiency, isolating selected ion species with >95% efficiency, inducing extensive collision-induced dissociation of selected ions with m/z up to 3500, and measuring ions with $m/z > 2500$ with improved resolution and mass accuracy. The achievement of these design goals has paved the way to a practical, sensitive ITMS that can be used to solve challenging and important biological problems. In the present paper, we describe the design and performance of this MALDI-ITMS instrument.

EXPERIMENTAL SECTION

Mass Spectrometer. Figure 1 shows a schematic representation of the MALDI-ITMS that we assembled at The Rockefeller University. Ions are generated external to the ion trap, whereupon they are accelerated, focused with an electrostatic lens, and injected axially into the trap. To optimize trapping, the timing of the laser pulse is adjusted with respect to the time that the rf field is turned on so that ions of interest penetrate the entrance endcap when the rf field has low amplitude, collide with and are translationally cooled by He buffer gas in the trap, and approach the exit endcap when the rf field has ramped to higher amplitude—a

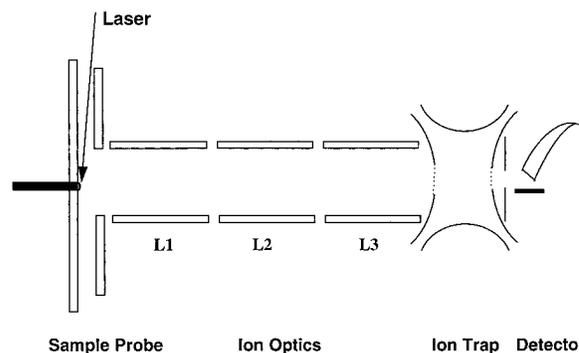


Figure 1. Schematic of the matrix-assisted laser desorption/ionization ion trap mass spectrometer. The objects from left to right are the sample probe plate, source ion extraction plate, three tube lenses (L1, L2, and L3), ion trap, detector ion extraction plate, and channeltron detector fitted with a conversion dynode. The distance between the sample probe and the source ion extraction plate is 1.27 cm. Both the diameter and the length of the tube lenses are 2.57 cm. The source ion extraction plate, tube lenses, and entrance endcap are each separated by a distance of 0.32 cm. The detector ion extraction plate is ~0.5 cm from the exit endcap. The sample probe is biased at 25 V with respect to ground, while both the extraction plate and tube lens L2 are biased at -150 V, and tube lenses L1 and L3 are grounded. The detector ion extraction plate and conversion dynode are biased at -3 and -10 kV, respectively. The front face of the channeltron is operated at a potential between -1.3 and -1.7 kV.

strategy that has been termed dynamic trapping.^{17,18} We use a variation of dynamic trapping that yields particularly high efficiencies (up to 40%).¹⁵ Our variation of dynamic trapping, which we call matched dynamic trapping, involves ramping the rf amplitude to a high level (V_1) and then dropping the rf amplitude over an appropriate time period to a lower level (V_2).¹⁵ The trapped ions are then analyzed via an instability rf ramp incorporating resonant ejection for mass range extension.¹⁹

Matrix-assisted laser desorption of peptides is effected by pulses of light (355 nm wavelength, 10 ns pulse duration) from a frequency-tripled Nd-YAG laser (Lumonics HY400, Kinata, Canada). The laser beam is focused with a lens (focal length, 20 cm) to a small elliptical spot on the sample probe tip (tip diameter, 2 mm), illuminating ~1% of the area of the sample. The beam passes through a fused silica window on the vacuum chamber and strikes the sample probe at an angle of 75° to the surface normal. The energy of the laser pulse incident on the sample is controlled by an adjustable light attenuator (Newport Model 935-5).

Ion acceleration and transport are accomplished with a five-element lens (Figure 1) that was designed with the assistance of the SIMION ray tracing program.²⁰ The kinetic energy of ions injected into the trap can be varied in the range 5–100 eV. Minimization of velocity components perpendicular to the ion optical axis (which is collinear with the “z-axis” of the ion trap) is important for effective trapping of ions. The ion beam was, therefore, collimated but not strongly focused into the trap. The lens was designed to ensure that the ion beam passes through

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the entrance apertures on the trap endcap (see below), irrespective of the point of origin of ions on the 2 mm diameter probe.

The mass spectrometer vacuum chamber was constructed in-house and incorporated a Finnigan ITMS electronics kit (Finnigan MAT, San Jose, CA). The standard commercial ITMS electronics was modified by inclusion of a scan rate reduction board (Ionstore, Oak Ridge, TN) for improved mass resolution and an rf amplifier with a gain of 3.5 (Ionstore, Oak Ridge, TN) to boost the amplitude of the supplemental ac field. Ions are injected axially into the trap through an endcap. To improve the efficiency of ion transfer into the ion trap, we replaced the standard entrance endcap (containing a single 1 mm diameter hole) with a standard Finnigan ITMS detector endcap (containing seven holes). Spaces between the ion trap electrodes are sealed with Teflon spacers to increase the efficiency for differential pumping of the detector region. The pressure in the detector region is monitored with an ion gauge mounted on the main vacuum chamber. Helium gas was introduced into the trap through a needle valve. Although the pressure inside the trap was not directly measured, it was inferred to be in the millitorr range from a calculation of the conductance of the trap enclosure and the known pumping speed of the two turbomolecular ion pumps (Varian Model 9699008, Palo Alto, CA). The base pressure in the ion source and detector regions (in the absence of helium gas) was measured with an ionization gauge (calibrated with N_2) at 1×10^{-8} Torr, and the working pressure was maintained between 5×10^{-6} and 9×10^{-6} Torr (not corrected for gas type). Ions were detected with a channeltron detector (Everlast CDEM Model 1305, Detector Technology, Inc., Sturbridge, MA), operated with voltages in the range -1.3 to -1.7 kV (gain 10^4 – 10^5). Detection efficiency was improved through the use of a -10 kV conversion dynode.

Data Acquisition. The ion trap was controlled using the commercial Finnigan scanning electronics. The analog signal from the Finnigan ITMS preamplifier is amplified and fed into a LeCroy 9450 digital oscilloscope (LeCroy Corp., Chestnut Ridge, NY). An intensity versus time spectrum was acquired for each laser pulse and transferred through a GPIB interface to an IBM-compatible computer. Data acquisition and transfer were accomplished with a commercial software package (TOFWARE, Ilys Software, Pittsburgh, PA). Spectra were acquired by summing the digitized transients from 10 to 1000 laser pulses at 4 Hz. The number of data points per scan was chosen to ensure that every resolved peak is composed of no fewer than eight data points. This data system has the useful property that the oscilloscope can be triggered at an arbitrary time subsequent to the laser desorption event. Thus, we can monitor the fate of ions during the whole scan process of trapping, cooling, isolation, resonant excitation, and resonant ejection.

A typical scan function used for molecular mass measurements of peptides is shown in Figure 2a. The scan consists of a sequence of trapping, cooling, and detection. The laser is fired after a delay with respect to the turning on of the rf trapping field. The delay is adjusted to maximize the trapping of peptide ions while minimizing the trapping of low-mass matrix ions.¹⁵ Subsequent to trapping ($\sim 200 \mu\text{s}$ after firing the laser), ions are collisionally cooled for a period of ~ 15 ms. Finally, the trapped ions are detected and their masses (strictly mass-to-charge ratios (m/z), but here $z = 1$) determined by resonant ejection at $q_z = 0.0906$ with a supplemental ac amplitude of $3.1 V_{p-p}$ for masses up to 6000 Da (effective scan rate, 41 kDa/s). Above $m/z = 6000$, resonant ejection is accomplished at $q_z = 0.0604$.

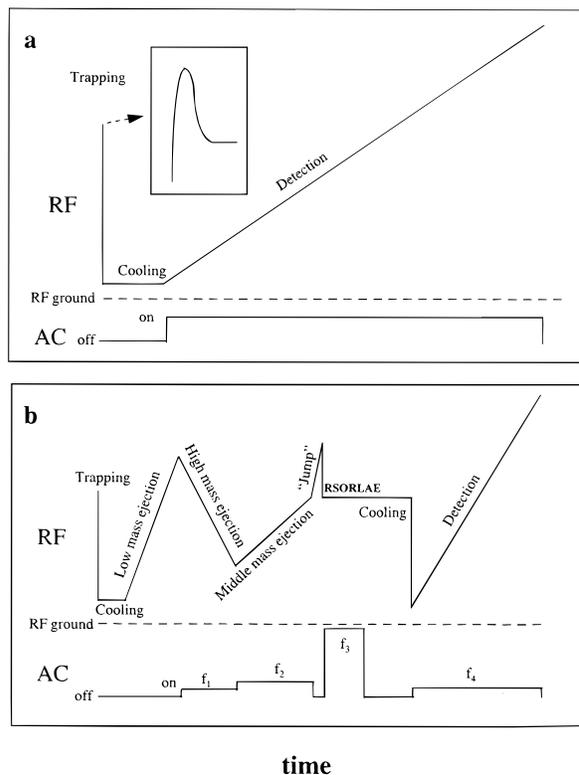


Figure 2. Radio frequency scan functions for (a) MS and (b) MS/MS experiments. For a definition of "jump" and RSORLAE, see text. The insert in (a) shows a detail of the trapping waveform.

A typical scan function used for MS/MS measurements is shown in Figure 2b. Following trapping and cooling, isolation of an ion of a given mass is carried out in three scan segments. Low-mass ions (usually <600 Da) are ejected by an up-ramp of 10 ms duration. This is followed by a down-ramp of 30 ms duration with a mass extension ratio of 20 at $1.4 V_{p-p}$ to eject high-mass ions. Subsequent to high-mass ejection, the intermediate-mass ions are ejected by a ramp of 60 ms duration with a mass extension ratio of 10 at $1.4 V_{p-p}$. These three scan segments are adjusted so that ions of interest can be isolated with a resolution of 3–5 Da. We selected this particular sequence of mass isolation scan segments to avoid sharp discontinuities in the rf amplitude as a function of time, because we have found that sudden jumps (which give rise to sudden changes in the trapping volume for a given ion species) can produce significant loss of ions from the trap.¹⁶ Just prior to excitation, the rf amplitude is ramped upward (10 ms risetime "jump") and then abruptly dropped to the rf amplitude used for excitation. The excitation is performed with large ac amplitudes at a frequency that is red-shifted by $\sim 5\%$ from the secular frequency of the isolated ions. This new excitation scheme, which we term red-shifted off-resonance large-amplitude excitation (RSORLAE),¹⁶ produces effective dissociation of peptides up to $m/z = 3500$ (see Results). The amplitude and duration of the ac excitation are adjusted to produce the required degree of fragmentation. Typical values employed are $21 V_{p-p}$ and 30 ms. After the ac excitation is turned off, the rf amplitude is maintained at the same level for a period of 30 ms to allow for decomposition of the parent ion species and cooling of the product ions. The subsequent ion detection scan segment is identical to that used for the simple m/z measurement. All the experiments described here were performed at an injection energy of 25 eV and an einzel lens focusing potential of -150 V.

Sample Preparation. Kemptide, bradykinin, [Glu¹]fibrinopeptide B, *N*-acetyl- β -endorphin, bovine insulin, and bovine ubiquitin were obtained from Sigma Chemical Co. (St. Louis, MO). Synthetic N-terminal EGF-like domain of the human coagulation factor X was kindly supplied by Dr. Yan Yang (Hunter College, City University of New York). The peptide ladder was synthesized in the laboratory of Dr. Stephen Kent (Scripps Research Institute, La Jolla, CA). RecA was a gift from Dr. Kenji Adzuma (The Rockefeller University). All the present experiments were performed with a matrix of 2,5-dihydroxybenzoic acid (DHB) obtained from Aldrich Chemical Co. (Milwaukee, WI). Of 40 different laser desorption matrices that we tested, DHB proved to be the best (data not shown), i.e., the ion response was high and the fragmentation of injected ions prior to excitation was moderate. Saturated stock solutions of DHB matrix were prepared in 1:1 (v/v) acetonitrile/H₂O. The working matrix solutions were prepared by diluting the stock matrix solutions with 2–3 volumes of 1:1 (v/v) acetonitrile/H₂O. Solutions of peptide were prepared in 0.1% TFA/H₂O. Endoproteinase Glu-C was sequencing grade (Boehringer Mannheim, Indianapolis, IN); the digestions were carried out as suggested in the manufacturer's literature. Peptide samples were prepared for mass spectrometric measurement by mixing, directly on the probe, equal volumes (0.5–1 μ L) of the peptide (or digest) and the working matrix solutions. The mixture was dried at ambient temperature and inserted into the spectrometer for mass determination. A single sample loading, containing subpicomole quantities of each peptide, can be irradiated by many thousands of individual laser pulses without depleting all of the sample. We have found that such a single sample loading is more than sufficient for a complete set of experiments that includes both low- and high-resolution molecular mass determinations as well a complete MS/MS study of the various components present in the sample.

RESULTS AND DISCUSSION

Mass Range, Resolution, and General Features of the MALDI-ITMS Mass Spectra. The performance of the MALDI-ITMS instrument was evaluated through a series of experiments on more than 2000 peptides. Figure 3 illustrates representative mass spectra obtained over the mass range 0.7–8.5 kDa, showing the regions of the mass spectra encompassing the protonated peptides. For masses less than 6 kDa, ions were detected using a mass extension ratio of 10 at 35 371 Hz ($q_z = 0.0906$) with an ac amplitude of 3.1 V_{p-p} . For masses greater than 6 kDa, ions were detected at a mass extension ratio of 15. Figure 3a shows the mass spectrum of kemptide ($MM_{\text{calc}} = 771.9$ Da, $MM_{\text{measd}} = 771.5$ Da, where MM is the isotopically averaged molecular mass) obtained by adding the spectra from 50 laser shots. The total sample loading of peptide on the probe was 100 fmol. Matched dynamic trapping¹⁵ was used (see Experimental Section) with $V_1 = 350$ Da and $V_2 = 70$ Da (rf level specified by the Finnigan ITMS control software). The spectrum is dominated by a peak corresponding to protonated kemptide, designated $(M + H)^+$. The resolution is ~ 250 fwhm. A low-intensity peak is observed 17–18 Da below the $(M + H)^+$ peak, which likely arises through the loss of NH₃ and/or H₂O from the protonated peptide. Analogous NH₃/H₂O loss peaks are observed in the majority of the MALDI-ITMS spectra of peptides that we have obtained. A small $(M + Na)^+$ peak is also detected.

Figure 3b shows the spectrum of *N*-acetyl- β -endorphin ($MM_{\text{calc}} = 3064.5$ Da, $MM_{\text{measd}} = 3064.7$ Da). Again, the $(M + H)^+$ species

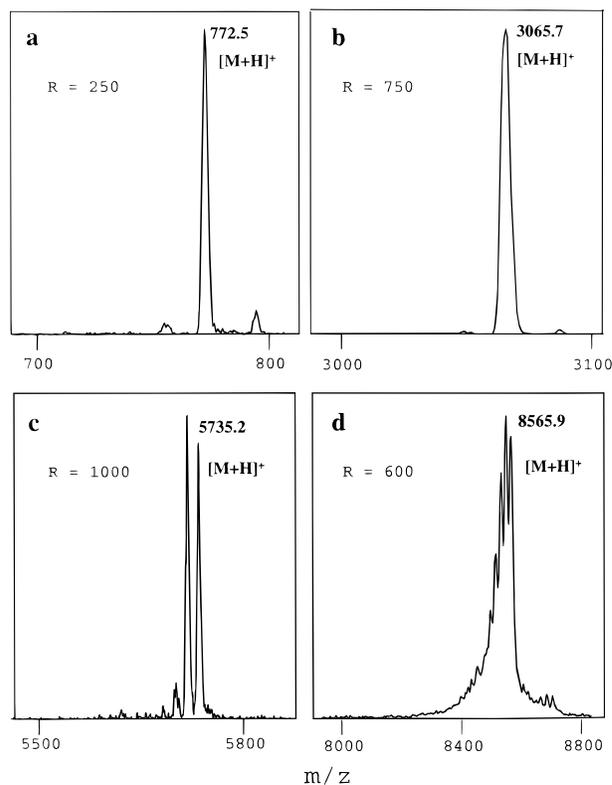


Figure 3. MALDI ion trap mass spectra of (a) kemptide ($V_1 = 350$ Da, $V_2 = 70$ Da, 100 fmol loading, 50 laser shots), (b) *N*-acetyl- β -endorphin ($V_1 = 470$ Da, $V_2 = 220$ Da, 100 fmol loading, 50 laser shots), (c) bovine insulin ($V_1 = 600$ Da, $V_2 = 300$ Da, 1 pmol loading, 100 laser shots), and (d) bovine ubiquitin ($V_1 = 650$ Da, $V_2 = 550$ Da, 2 pmol loading, 100 laser shots). "R" designates the fwhm resolution.

dominates the mass spectrum (resolution = 750 fwhm), and the low-intensity peaks are analogous to those seen for kemptide. Figure 3c shows a spectrum of bovine insulin ($MM_{\text{calc}} = 5734.6$ Da, $MM_{\text{measd}} = 5734.2$ Da). The improved resolution (1000 fwhm at $m/z = 5735$) of the current measurements compared with previous determinations of insulin by MALDI-ITMS^{1, 6–9} reveals a resolved cluster of peaks in the region of the protonated molecule which has not previously been resolved by ITMS. The improved resolution is a result of our improved resonant ejection conditions.¹⁵ In contrast to the spectra obtained from kemptide and β -endorphin, the base peak is no longer the protonated intact molecule, $(M + H)^+$, but a fragmentation product, $(M + H - 17)^+$, that likely arises through the elimination of NH₃. Also evident are peaks that arise from further consecutive losses of NH₃/H₂O. Similar losses have been observed in Fourier transform ion cyclotron resonance mass spectrometry using a MALDI source.²¹ The products of consecutive loss of NH₃/H₂O are also evident in the spectrum (Figure 3d) of bovine ubiquitin ($MM_{\text{calc}} = 8564.9$ Da, $MM_{\text{measd}} = 8564.9$ Da), but the resolution of 600 fwhm is only sufficient to partially resolve these products.

For accurate mass assignments, it is crucial that we are able to resolve NH₃/H₂O losses. Under normal (rapid scan, low resolution) operating conditions, the resolution of the instrument is sufficient to partially resolve consecutive losses of 17–18 Da from singly protonated ubiquitin ($MM_{\text{calc}} = 8564.9$ Da), allowing

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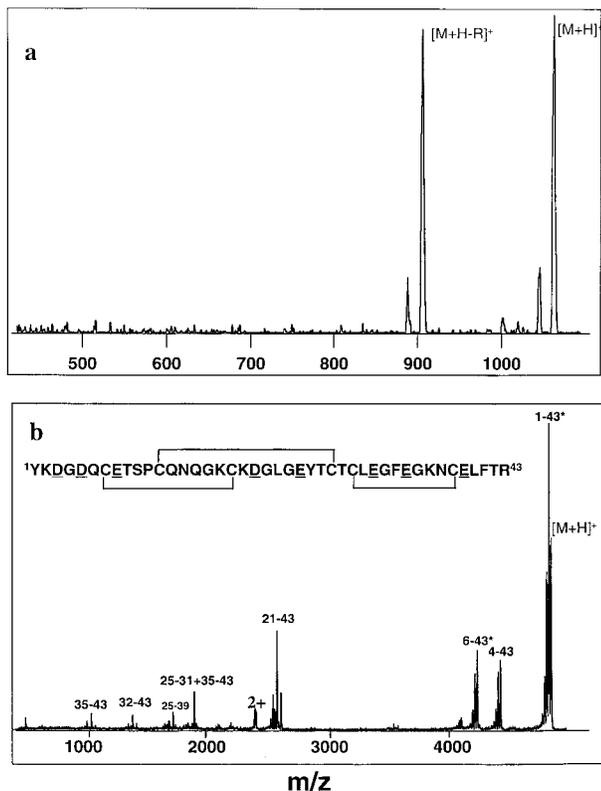


Figure 4. MALDI ion trap mass spectra illustrating facile decomposition reactions that take place during injection of protonated peptides into the ion trap. (a) Bradykinin ($V_1 = 450$ Da, $V_2 = 90$ Da, 100 fmol loading, 20 laser shots); one dominant fragment is observed, corresponding to the loss of the C-terminal Arg (R) residue.²⁶ (b) Synthetic N-terminal EGF-like domain of human coagulation factor X ($V_1 = 550$ Da, $V_2 = 400$ Da, 0.5 pmol loading, 100 laser shots); the fragments all arise from cleavages at the C-terminal of Asp/Glu residues and at the disulfide bonds. The sequence is given together with the disulfide linkages; the numbers above the peaks designate the residues of the assigned fragments. An asterisk indicates the loss of $\text{NH}_3/\text{H}_2\text{O}$.

accurate mass assignments only up to 8.5 kDa. It should be pointed out that the resolution is dependent on the mass extension ratio. Smaller mass extension ratios give better resolutions (data not shown).

Protein ions produced by MALDI with masses higher than that shown in Figure 3 can be readily trapped, but the quality of the spectra are inferior to those obtained with the time-of-flight analyzer.⁵ Thus, for example, we obtain an intense signal from bovine cytochrome *c* at a m/z value in the region of the intact protein, but the resolution at $m/z \approx 12\,000$ (140 fwhm) is insufficient to resolve the $\text{NH}_3/\text{H}_2\text{O}$ losses, rendering accurate mass assignment impossible. Thus, for the present instrument, the range for accurate mass measurement is limited to $m/z \leq 8500$.

Fragmentation upon Injection of Peptide Ions into the Ion Trap. Even in the absence of resonant excitation, we observed fragmentation upon injection of single-component peptide ions into the trap. The pattern and extent of this fragmentation was dependent on the composition and mass of the peptide and was frequently found to exhibit a high degree of selectivity. For example, the spectrum of bradykinin evidenced a single dominant fragmentation pathway that occurs through the facile loss of a terminal arginine residue from the protonated peptide²⁶ (Figure

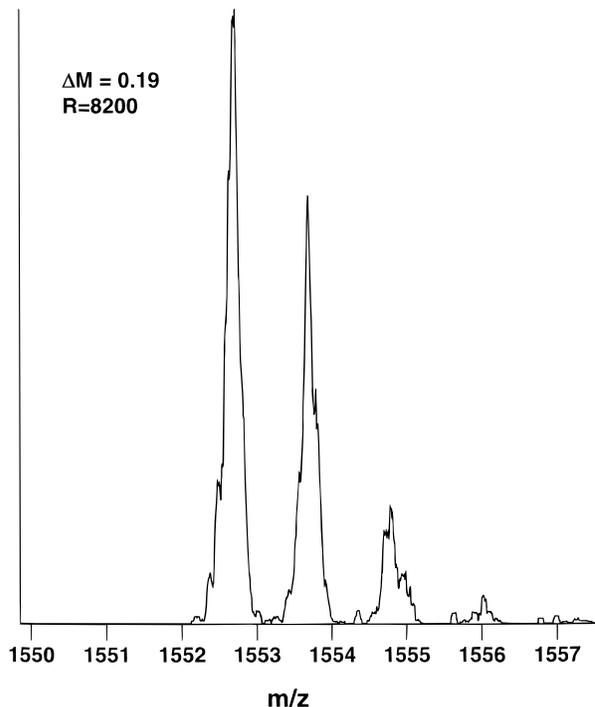


Figure 5. High-resolution spectrum of the $[\text{Glu}^1]\text{fibrinopeptide B}$ in the region of the $(M + H - 17)^+$ ion species. The spectrum was obtained with a 20-fold reduction in the scan rate and a mass extension ratio of 3 (ac modulation frequency, 119 936 Hz; ac amplitude, 6 V_{p-p}).

4a). Other fragments were detected in the spectrum, but their intensity is more than 20-fold weaker.

We have shown previously that peptide ions tend to fragment preferentially at the C-termini of acidic residues (Asp and Glu)²² as well as across disulfide linkages (Qin, J.; Chait, B. T. Presented at the Ninth Symposium of The Protein Society, Boston, MA, July 8–12, 1995). Thus, for example, the majority of the fragmentation seen in the spectrum of the protonated N-terminal EGF-like domain in human coagulation factor X occurs at the acidic residues and/or at the disulfide linkages (Figure 4b). Fragmentation upon injection was observed to occur most readily for peptide ions with masses less than 1200 Da or for those that contain acidic residues or interchain disulfide linkages. Conversely, relatively little fragmentation was observed upon injection of peptides with masses greater than 1500 Da that do not contain these labile bonds.

The above observations concerning fragmentation upon injection were made with single-component samples. An interesting but as yet unexplained finding is that considerably less fragmentation was observed when complex peptide mixtures were injected into the trap, irrespective of the amino acid composition of the components.

High Resolution at Slow Scan Rates. Relatively high resolutions can be obtained by reducing the scan rate and increasing the value of q_z for resonant ejection.²³ Figure 5 shows a high-resolution spectrum of $[\text{Glu}^1]\text{fibrinopeptide B}$ in the region of the intense $(M + H - 17)^+$ ion species. The spectrum was obtained with a 20-fold reduction in the scan rate and a mass extension ratio of 3 at 119 936 Hz with a modulation amplitude of

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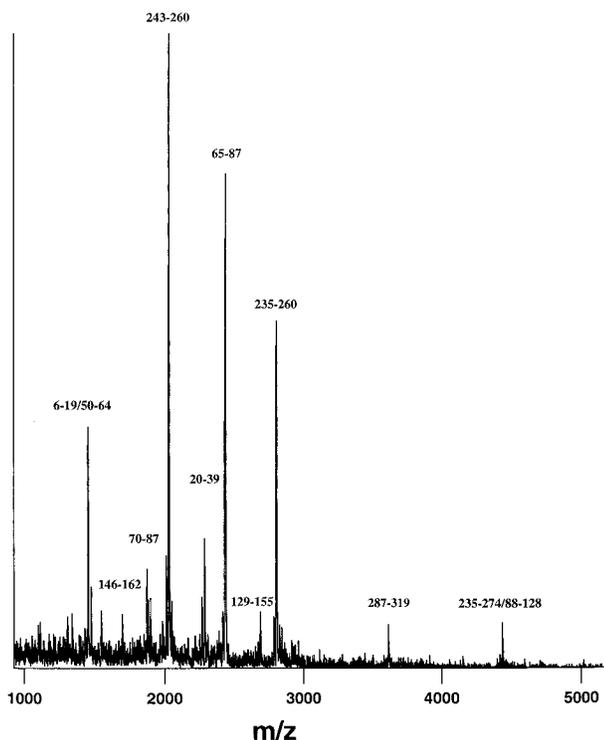


Figure 6. MALDI ion trap mass spectrum of the peptide mixture produced by endoproteinase Glu-C digestion of *E. coli* RecA protein (38 kDa). A ~ 0.5 pmol aliquot of the digested protein was loaded into the mass spectrometer, and the spectrum was acquired from 50 laser shots. Assignment of the peak labeled 6–19/50–64 as a mixture of two peptides was confirmed by a MS/MS experiment (data not shown).

$6 V_{p-p}$ (resulting in an effective scan rate reduction of 6.67.) The peak width is 0.19 Da, yielding a resolution of 8200 (fwhm). Unfortunately, the utility of high resolution in our ITMS is compromised by several practical limitations. First, we observe a large diminution in the ion signal-to-noise ratio, arising, in part, from the dilution effect caused by spreading the peak into its isotopic components. Fragmentation of the peptide ions may also contribute to the signal reduction because fragmentation is enhanced by the use of high values of q_z (i.e., large ac amplitudes) for ejection and by the long period of off-resonance excitation experienced by the ions when the scan rate is reduced. Our observation that high resolutions can only be obtained at high values of q_z is probably related to the mechanism for ion ejection. Differentiation of two species of almost the same mass requires that their absorption profiles for resonant ejection do not overlap. If the absorption profile has a constant bandwidth (Δf), then it should be easier to resolve the profiles at higher resonant frequencies (f), since the resolution is proportional to $f/\Delta f$. This requirement for high ejection frequencies ($q_z \geq 0.3$) also imposes an upper limit (~ 2000 Da) on the mass for which high resolutions can be obtained. Finally, calibration of high-resolution spectra is not yet a straightforward process, despite recent advances.²⁴

Mixture Analysis. The utility of MALDI-ITMS for mixture analysis was evaluated by measuring more than 100 different peptide mixtures. The ability to directly analyze mixtures without prior separation of the components can eliminate the need for chromatography and can potentially provide large gains in analysis

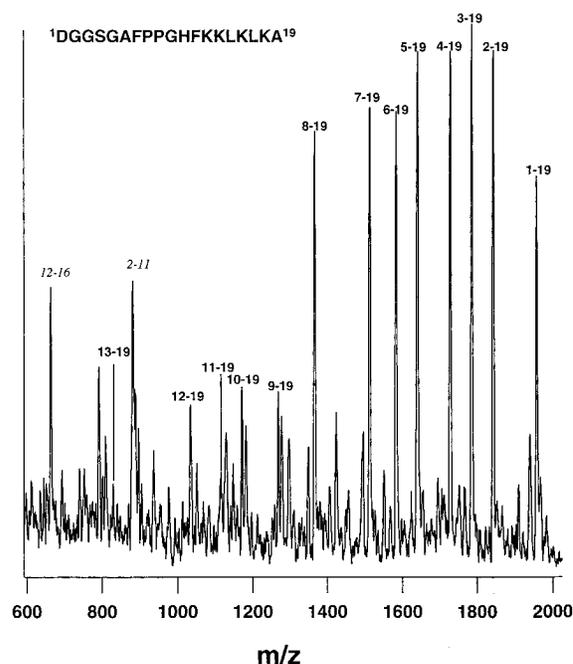


Figure 7. MALDI ion trap mass spectrum of a synthetic peptide ladder mixture containing 13 components using a loading of 5 fmol of each component. The ladder consists of the following peptides: 1–19, 2–19, 3–19, ..., 13–19 of the sequence D¹GGSGAFPPGHFKLKLKA.¹⁹ All 13 peptides in the mixture are detected, along with several fragments, which arise from fragmentation in the ion trap.

speed and sensitivity. Figure 6 illustrates this utility with a spectrum obtained from a proteolytic digest of *Escherichia coli* RecA protein (354 amino acid residues; $MM_{\text{calc}} = 38\,102$ Da). The digestion was carried out using endoproteinase Glu-C, under conditions where cleavage occurs primarily C-terminal to Glu residues. Inspection of Figure 6 shows the presence of at least 10 major cleavage products, which were identified from their measured masses (as indicated). This spectrum, together with similar analyses on a large collection of other mixtures (data not shown), demonstrated that complex mixtures of peptides can be routinely analyzed in our MALDI ion trap mass spectrometer with high sensitivity and speed.

Mass Calibration and Mass Accuracy. Mass calibration (internal or external) is straightforward and fast. For internal calibration, peptides of known mass are mixed with the sample of interest, yielding mass accuracies as high as 50 ppm. For external calibration, a calibration file is generated from a spectrum of calibrant peptides in the absence of the sample of interest. Such an external calibration file can be used reliably for weeks. We find that a three-point linear regression external calibration covering the mass range of the analytes of interest gives mass accuracies better than 200 ppm. For example, 12 separate measurements of the molecular mass of renin substrate tetradecapeptide DRVYIHPFLLVYS ($MM_{\text{calc}} = 1759.04$ Da) yielded the following experimental values using an external calibration: 1759.12, 1759.14, 1759.14, 1759.18, 1759.12, 1759.19, 1759.02, 1758.96, 1759.03, 1758.96, 1759.10, and 1759.17 Da. The mean MM calculated from these measurements is 1759.09 Da, and the standard deviation of a single measurement is 0.08 Da.

In the present instrumental configuration, large variations in ion intensity (up to a factor of 20) did not lead to significant shifts in the measured masses of peptides with m/z values between 1000

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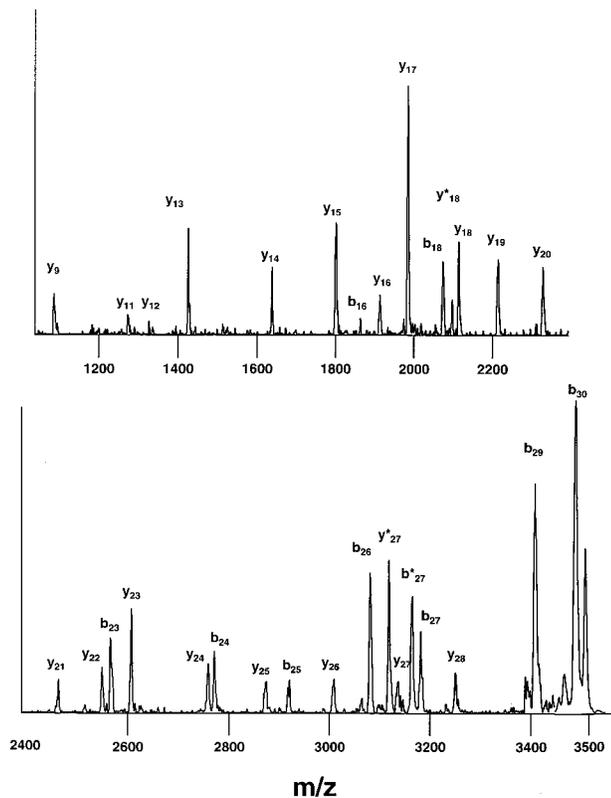


Figure 8. Tandem mass spectrum of oxidized insulin B-chain ($M_{\text{calc}} = 3495.9$ Da). The precursor ion was isolated and then excited by RSORLAE at an ac frequency of 50 617 Hz, amplitude of 21 V_{p-p} for 30 ms, rf setting = 518 m/z units. The product ions were detected at a 10 \times mass extension ratio with an amplitude of 1.4 V_{p-p} . An asterisk indicates the loss of $\text{NH}_3/\text{H}_2\text{O}$.

and 8500; i.e., shifts, if present, are smaller than 200 ppm. It appears that damaging space-charge effects are minimized by the use of a fast scan rate (41 kDa/s), together with improved resonant ejection detection at low q_z .¹⁵ By contrast, when the scan rate was reduced by a factor of 3, we observed significant peak broadening and mass shifts, indicating the presence of space-charge effects under these slower scan conditions.

Sensitivity. No attempt was made to determine the absolute detection limits. Rather, we took a practical approach to determine the amount of loading on the mass spectrometer probe that resulted in spectra of good quality. Satisfactory spectra can be obtained with a loading of 5 fmol for most of the peptides that are commonly used for characterizing mass spectrometer performance (e.g., substance P, bombesin, and renin substrate). Similar sensitivity can be achieved for mixture analysis. Figure 7 shows the spectrum of a synthetic peptide ladder mixture containing 13 components using a loading of 5 fmol of each component. All 13 peptide components were detected, along with five fragmentation products, which arise from fragmentation upon injection of ions into the trap. We found that the presently described MALDI ion trap mass spectrometer has a sensitivity comparable with that of our previously described MALDI linear time-of-flight instrument.¹⁰

MS/MS Experiments. The capacity to perform multiple-stage mass spectrometry is one of the most appealing features of the ITMS. We have found it feasible to carry out such analysis on ions produced by MALDI through the development of efficient procedures to isolate and dissociate ions.¹⁶ High sensitivity and high information content were achieved through the development of a new excitation scheme that deposits high levels of internal

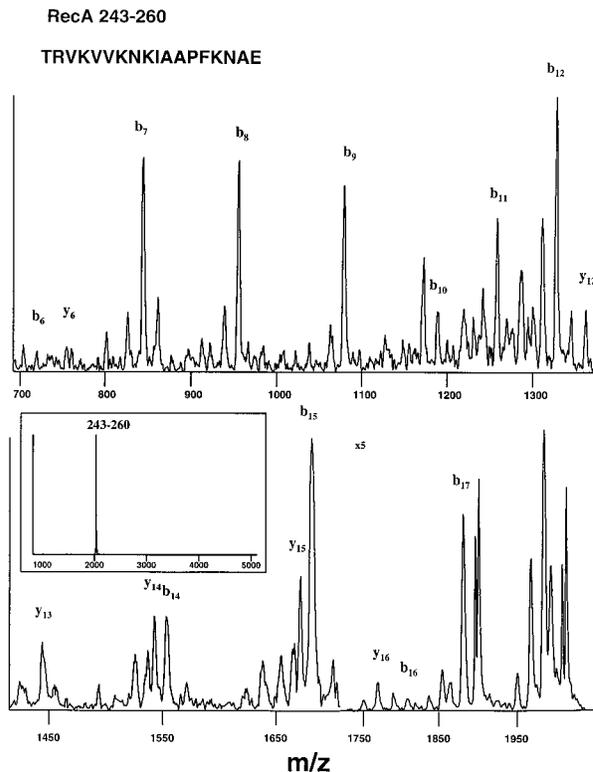


Figure 9. MS/MS of a product (RecA 243–260) of the endoproteinase Glu-C digestion of *E. coli* RecA (see Figure 6). The CID spectrum of RecA 243–260 was obtained by RSORLAE of the mass-isolated precursor (see inset) at an ac frequency of 59 124 Hz, amplitude of 20 V_{p-p} for 30 ms, rf setting = 354 m/z units. The spectrum is the average of 500 laser shots.

energy into peptide ions, without incurring ion loss. This scheme, which involves excitation of the ions of interest at a frequency red-shifted from the secular frequency by $\sim 5\%$ with a large excitation amplitude (21 V_{p-p}), was found to be effective in fragmenting ions with m/z values up to 3500. Excitation and dissociation are sustained over an extremely long time window, allowing for the observation of dissociation channels with relatively slow rate constants. Figure 8 shows a MS/MS spectrum of bovine insulin B chain (oxidized) using a 1 pmol loading on the probe. A large proportion of the amino acid sequence can be readily deduced from the mass spectrum, which is dominated by b and y series ions. The sequence information present in Figure 8 is comparable with that obtained by Cooks and co-workers using liquid SIMS ionization,¹ a method that leads to considerably greater input of internal energy into peptides than does MALDI.

Such MS/MS experiments are of great potential use for the analysis of individual components of complex mixtures, obviating the necessity for chromatographic separations. Figure 9 shows the MS/MS analysis of one of the components (residues 243–260) of the endoproteinase Glu-C digest of *E. coli* RecA protein (see Figure 6). Fragmentation is extensive and is dominated by sequence-defining b- and y-type ions. Sixteen out of the 18 amino acid residues of RecA 243–260 can be deduced from the b_n and y_n ion series. The experiments shown in Figures 6 and 9 were performed with a single 0.5 pmol loading of the sample. The same 0.5 pmol loading was used to perform additional MS/MS experiments on the other three intense V8 fragments with $m/z < 3000$. In each case, we obtained results similar to those shown in Figure 9. In more than 20 such analyses of different mixtures, we have

not yet experienced complete depletion of sample on the probe; a single loading was always sufficient for all the experiments that we wished to do. Extensive evaluation of the MS/MS capacity of MALDI-ITMS from $m/z = 600$ to 3500 was carried out on more than 100 peptides using subpicomole loadings of sample. The systematics of the CID spectra obtained with our new excitation scheme will be detailed elsewhere.²⁵

CONCLUSIONS

We have demonstrated an effective coupling of MALDI and ion trap mass spectrometry that takes advantage of the excellent properties of the two techniques. The described instrument is a useful tool for a host of challenging biological applications that can be addressed by the analysis of peptides mixtures where the masses of the components are less than 8.5 kDa. A particularly attractive feature is the ability to perform high-sensitivity tandem mass spectrometry on the components of complex mixtures of peptides produced by MALDI. The instrument has already proved very useful for protein characterization—particularly for site-

specific identifications of posttranslational modifications (e.g., phosphorylation, glycosylation, and disulfide mapping).²⁷ We are currently applying MALDI ion trap mass spectrometry to the identification and characterization of gel-separated proteins.²⁷

ACKNOWLEDGMENT

This work was supported in part by grants from the NIH (RR00862 and GM38724). The excellent technical assistance of Mr. Herbert Cohen is gratefully acknowledged. We thank Dr. Nathan Yates and Professor Richard Yost for supplying the "ICMS" control software; Dr. Hans Reiser, Dr. Randall Julian, Jr., and Professor Graham Cooks for supplying the simulation program, "TTSIM"; and Drs. Douglas Goeringer and Scott McLuckey for informative discussions. J.Q. would like to thank Mr. Yingming Zhao, Mr. Steven Cohen, and Dr. Rong Wang for teaching him techniques in biochemistry.

Received for review November 29, 1995. Accepted February 26, 1996.[⊗]

AC9511612

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[⊗] Abstract published in *Advance ACS Abstracts*, April 1, 1996.