Factors Affecting the Ultraviolet Laser Desorption of Proteins

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The production of high-mass quasimolecular ions from proteins by matrix-assisted ultraviolet laser desorption is described. A simple time-of-flight system using a Q-switched frequency-quadrupled Nd-YAG laser to desorb protein molecules is shown to have a mass range of up to 116 000 u by the observation of intact, singly charged quasimolecular ions from 700 fmol of β-galactosidase subunit (mol.wt = 116 336 Da). Both positive- and negative-ion spectra of proteins are shown. Four new matrix materials, with properties as good as or better than nicotinic acid, are described. A mass resolution of approximately 500 (full width at half maximum definition) is demonstrated for proteins with mol.wt < 20 000 Da. Product species, formed by fast photochemical reactions in the matrix, are observed to form adduct ions with protein molecules. These adduct ions are a significant cause of the observed broadness of protein quasimolecular ion peaks. The practical physical considerations in detection of large-mass quasimolecular ions from laser desorption, such as detector overloading, are discussed.

Lasers, of a variety of wavelengths and pulse durations, have been used to produce gas-phase neutral molecules and ions from solids for over twenty years. Laser desorption (LD) of ions has been studied in depth by many groups and extensively reviewed.[1,2] In the past year, two new methods of producing pseudomolecular ions using LD have been published. Both of these new techniques use ultraviolet (UV) pulsed lasers to desorb the molecules, and time-of-flight mass measurement. Also, both use a matrix to absorb the UV light and to convert electronic excitation into vibrational/translational energy, expelling the sample molecule, intact and ionized, into the gas phase.

The essential difference between the two techniques is the type of matrix. Tanaka *et al.*[3] report using a mixture of analyte, glycerol and finely divided metal powder (to absorb the UV) and irradiating the resultant liquid slurry with a pulsed nitrogen laser. Karas and Hillenkamp[4] report using a dried mixture of the sample and nicotinic acid (a strong UV absorber at the fourth harmonic frequency of a Nd-YAG laser). Both techniques show similar results, i.e., the production of copious numbers of pseudomolecular ions with $m/z > 20\,000$ from sample molecules.

In the work described below, our laboratory took as its model the technique of Hillenkamp and Karas. Here we report the successful reproduction of the technique, using different experimental conditions and apparatus. Both positive and negative ions of proteins were observed. A new finding is that with a linear time-of-flight (TOF) instrument, mass resolutions of $m/\Delta m \cong 500$ (full width at half maximum (FWHM)) have been observed for desorbed proteins, a substantially higher resolution than has been reported previously for this technique. Using this improved resolution, adduct ions caused by addition of part of the matrix ring structure to the protein molecule have been observed. We also report a survey of matrix materials, and the demonstration of several new matrices that perform as well as (or better than) nicotinic acid. Several key experimental considerations and their effects on resolution and signal intensity are discussed, including: (i) detector gains under high current flux conditions; (ii) field effects in the ionization of matrix molecules; and (iii) laser intensity and beam homogeneity. The observation of an intact pseudomolecular ion of *Escherichia coli* β-galactosidase subunit ($m/z \cong 116\,000$) from a small sample (700 fmol) demonstrates the mass range of the technique.

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EXPERIMENTAL

The time-of-flight mass spectrometer used was a simple linear instrument with a two meter flight path. The sample was loaded onto a 2 mm diameter stainless-steel probe tip-face that, when inserted into the mass spectrometer, was aligned to produce a homogeneous acceleration field (see Fig. 1). Ions produced were accelerated in two stages. Total accelerations of up to (±) 20 kV were used. Low-mass ions could be suppressed selectively by using pulsed deflection plates set close to the source end of the flight tube. The detector was a chevron-type tandem microchannel plate array, with the front plate of the assembly at approximately −2 kV. The typical operating pressure

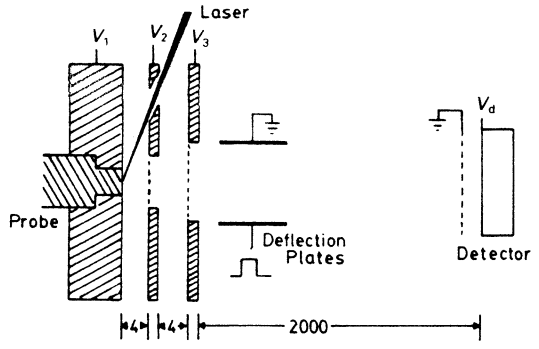

Figure 1. A schematic diagram of the linear TOF mass spectrometer used. Dimensions are shown in mm, and the drawing is not to scale. V_1 and V_2 are varied to set the accelerating potential, and V_3 is held at ground. V_d (ca −2 kV) sets the gain of the detector.

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for the mass spectrometer was 10 μPa, which was reached 2 min after sample introduction.

The laser used was a HY-400 Nd-YAG laser (Lumonics Inc., Kanata (Ottawa), Ontario, Canada), with a 4th harmonic generation/selection option. The laser was tuned and operated at conditions that produced maximum temporal and energy stability. Typically, the laser was operated with an output pulse width of 10 ns and an energy of 15 mJ of UV per pulse. To improve the spatial homogeneity of the beam, the amplifier rod was removed from the laser. The output of the laser was attenuated with a 935-5 variable attenuator (Newport Corp., Fountain Valley, California, USA) followed by a cuvette (containing an acetonitrile + acetone (250:1) solution), aperture stopped with a 1 mm diameter pinhole and focused onto the sample using a 12 in focal length fused-silica lens. The incident angle of the laser beam, with respect to the normal of the probe's sample surface was 70°. The spot illuminated on the probe was therefore not circular, but a stripe of approximate dimensions 100 × 300 μm (measured by burn marks on paper). The start time for the data system (i.e., the time the laser actually fired) was determined using a beam splitter and a P5-01 fast pyroelectric detector (Molectron Detector Inc., Cambell, California, USA). The laser was operated in the Q switched mode, internally triggering at 5 Hz, using the Pockels cell Q-switch to divide that frequency to a 2.5 Hz output.

The data system for recording the spectra produced was a combination of a TR8828D transient recorder and a 6010 CAMAC crate controller (both manufactured by Lecroy, Chestnut Ridge, New York, USA). The transient recorder had a selectable time resolution of 5–20 ns. Spectra were accumulated using custom software that permitted the accumulation of up to 256 laser shots in 131 000 channels, with the capability of running at up to 3 Hz. The data were read from the CAMAC crate using a Proteus IBM AT compatible computer. During the operation of the spectrometer, the spectra (shot-to-shot) could be readily observed on a 2465A 350 MHz oscilloscope (Tektronix, Inc., Beaverton, Oregon, USA).

RESULTS AND DISCUSSIONS

The first result was that the technique of Karas and Hillenkamp[4] could be reproduced using a different laser optical system and type of TOF mass spectrometer. Intense pseudomolecular signals from proteins were produced using a nicotinic acid matrix, with a sample of the order of 1 pmol of protein on the probe tip. Similar results were observed for both positive and negative ions (e.g., insulin, Fig. 2). The linear TOF system used could be switched from positive to negative ions easily and both modes used to look at a single sample. Singly, and often, doubly charged pseudomolecular ions were observed in both positive and negative modes. Ions characteristic of dimers, trimers, etc. were also observed for many proteins although the intensity of these ions varied, depending on the protein, its concentration in the matrix and the matrix used. No intense fragment ions were observed in the positive mode for any of the proteins examined. The negative-ion-mode spectrum of insulin showed significant signals due to the A- and B-chains.

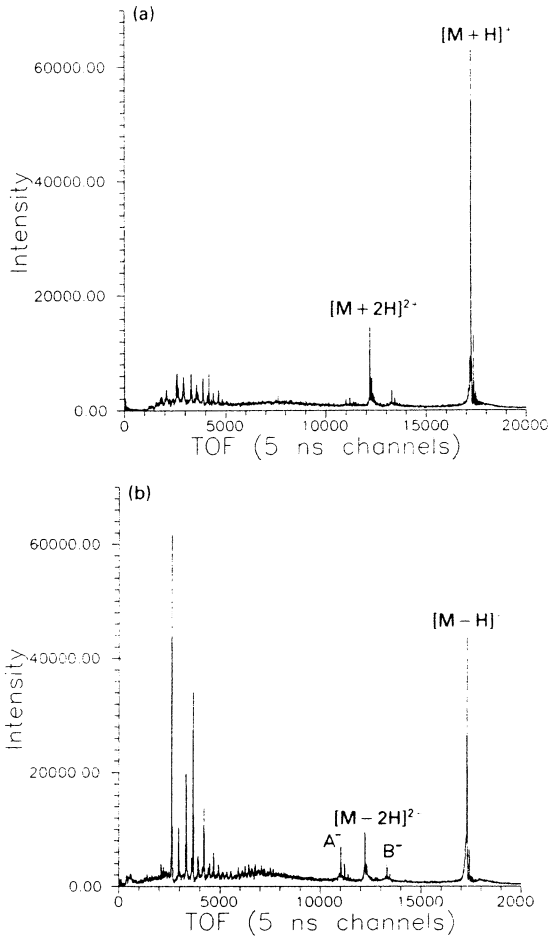

Figure 2. (a) The positive-ion LD TOF mass spectrum of bovine insulin from a 2-pyrazinecarboxylic acid matrix (measured $m/z = 5735$). Conditions: $V_1 = +15$ kV; $V_2 = +12.5$ kV; 100 laser shots summed; total protein loaded = 3 pmol. (b) The negative-ion LD TOF mass spectrum of bovine insulin from a 2-pyrazinecarboxylic acid matrix (measured $m/z = 5733$). Conditions: $V_1 = -15$ kV; $V_2 = -12.5$ kV; 100 laser shots summed; total protein loaded = 3 pmol. Ions of mass < 500 were strongly suppressed by pulsed deflection plates.

Power-density thresholds for the observation of protein pseudomolecular ions from matrices of ca 2×10^7 W/cm^2 have been reported previously.[4,5] The power-density thresholds determined in our instrument are substantially lower, between 5×10^5 and 10^6 W/cm^2. These power density values were obtained by calculations based on the measured dimensions of the illuminated spot and measurements of the laser power after attenuation, assuming a uniform beam. The output power of the laser used was high enough that this value could also be checked by direct illumination of the probe by the 5 mm diameter unfocused laser beam by the removal of the lens. Pseudomolecular ions of insulin were observed at a power density of 7×10^5 W/cm^2 using the unfocused beam. A possible explanation for the discrepancy between the presently and the previously reported result is the difference in the area illuminated on the sample. Our illuminated area is >600 times larger than that in the LD microprobe instrument used for the previous measurement. Therefore, if the ion production scales linearly with the surface area illuminated, a larger beam area should increase the sensitivity of detection of ion production, in our case by a factor of several orders of magnitude.

The angle of incidence of the laser beam in the present instrument (70°) is much larger than that in the instrument of Karas *et al.*[4,5] The existence of small (< 1 mm diameter) "hot spots" in the unfocused laser beam, producing small regions of high power density on the sample, is another possible explanation for the discrepancy in the two results. The effect of laser power density on mass resolution is discussed below.

Sample preparation was optimized for the production of homogeneous samples, in order to produce similar signals from the entire face of the probe tip. The standard conditions that evolved were: dissolving less than 0.2 g/L of the sample in a 5–10 g/L solution of matrix in water (or 1:1, water + ethanol) and depositing 1.0 μL of the solution on the probe tip. All of the protein and peptide samples tried so far have dissolved sufficiently in the matrix solution to produce ions. The solution was then gently dried with a hot-air gun. This simple method of sample preparation was used for all the matrices shown in Table 1 (see below). Mixing of solutions of the neat sample and matrix on the probe tip were tried, but the reproducibility of this method was not always satisfactory.

The sensitivity of the technique is very high for proteins. With a typical sample loading of 0.1–20 pmol of analyte on the probe tip (3 mm^2) good signals were observed. For most peptides, the optimum signal was produced with a sample coverage of < 2 pmol/mm^2 on the probe. The laser focus used in these studies was a stripe of dimensions 0.1 × 0.3 mm (0.03 mm^2); therefore as little as 3% of the sample on the probe was used for one measurement. At very high protein concentrations, i.e., 10^{-4} M, the signal intensity decreased, noise increased and the pseudomolecular ion peak widened. This behaviour suggests that there should be a 10^3–10^5 molar excess of matrix for optimum detection.

After demonstrating successfully the desorption of proteins from nicotinic acid, a limited survey of other possible matrix materials was undertaken, using insulin as a test analyte. Samples were prepared as described above (0.1 g/L of insulin in a 5–10 g/L solution of the matrix) and 0.5 μL of the solution deposited on the probe. The results are given in Table 1, where (s) indicates a strong and (w) a weak pseudomolecular ion signal. Matrices were selected for their: (i) capacity to absorb strongly at $\lambda = 266$ nm; (ii) solubility in aqueous solutions (for mixture with proteins); and (iii) low boiling/sublimation points. The five compounds that produced strong signals all sublimed easily. The UV absorption of the matrix alone was not a sufficient criterion for selecting a useful matrix, as was demonstrated by the almost complete loss in signal intensity upon changing the matrix from nicotinic acid (3-pyridinecarboxylic acid) to isonicotinic acid (4-pyridinecarboxylic acid), which both have very similar UV absorption spectra. The physical properties of the matrix were also very important. Materials that either melted readily or did not sublime, produced either weak signals or none at all. It is not necessary for the matrix to contain a carboxylic acid moeity, as evidenced by the strong signals observed from thymine matrices. The presence of nitrogen in the chromophore is also not necessary, as vanillic acid (3-methoxy-4-hydroxybenzoic acid) is an excellent matrix material. The cyclic π-electronic structure of the chromophore was also not necessary, as thiourea matrices produced strong signals. Table 2 lists the peptides and proteins observed up to the present time. The numbers in brackets are the molecular weights either of the intact molecule or of protein subunits, using the sequences compiled in the Dayhoff Protein Sequence Data Base[6] and the currently accepted IUPAC values for the isotopically averaged atomic masses. The ribonuclease A sample used did not yield abundant ions either from nicotinic or 2-pyrazinecarboxylic acids as matrices, but produced strong signals from thymine, vanillic acid or thiourea matrices.

One experimental factor that has proved to be important is the behaviour of the detector under high current-flux conditions. A microchannel plate array has a very high gain under low current conditions, but can easily become paralyzed by large currents. Each channel in a microchannel plate, once triggered, takes several ms to recover. Therefore, each channel can only detect a single ion from a given laser desorption event (one ion per pulse). If a large number of low-mass ions is produced by the laser pulse, both the gain and sensitivity of the detector are drastically decreased by the time that high-mass ions arrive.

The drop in detector gain with high low-mass ion fluxes is of substantial practical importance. It was discovered that for nicotinic acid and 2-pyrazinecarboxylic acid, the laser intensity threshold for the production of low-mass ions was dependent on the accelerating electric field strength at the probe tip. At fields of higher than 10^6 V/m, it was impossible to produce high-mass pseudomolecular ions without

Table 1. Results of a survey of matrices

Useful matrices[a]	No observable signal
3-Pyridinecarboxylic acid (s)	3-Pyridinesulfonic acid
2-Pyrazinecarboxylic acid (s)	*P*-Toluenesulfonic acid
Thymine (s)	4-Pyridineethanesulfonic acid
3-Methoxy,4-hydroxybenzoic acid (s)	2,3-Pyridinedicarboxylic acid
Thiourea (s)	2,4-Pyridinedicarboxylic acid
Uracil (w)	2,5-Pyridinedicarboxylic acid
Purine (w)	3,4-Pyridinedicarboxylic acid
2-Pyridinecarboxylic acid (w)	Guanidine hydrochloride
4-Pyridinecarboxylic acid (w)	Urea
3,4-Dimethoxybenzoic acid (w)	

[a] (s) = a strong pseudomolecular ion signal; (w) = a weak pseudomolecular ion signal.

Table 2. Proteins and peptides observed by LD TOF-MS

Ala_4Val (401.5)[a]	Bovine ribonuclease A (13 682)
Leucine enkephalin (555.6)	Human milk lysozyme (14 693)
Methionine enkephalin (573.7)	Horse heart apomyoglobin (16 951)
$Ala_{10}Val$ (827.9)	Bovine trypsin (23 311)
Gramicidine S (1141.5)	Porcine pepsin (34 504; 34 688)
Substance P (1347.7)	Yeast alcohol dehydrogenase (YAD1-36 740; YAD2-36 766)
Human beta-endorphin (3465.0)	Bovine albumin (66 267)
Bovine insulin B-chain (ox) (3495.5)	Rabbit phosphorylase B (97 093)
Bovine insulin (5733.5)	*E. coli* β-galactosidase (116 336)
Porcine proinsulin (9082)	
Human apolipoprotein $CIII_1$ (9421)	

[a] Mol. wts shown in brackets.

producing large numbers of low-mass ions. If the acceleration field was kept below this threshold value however, protein pseudomolecular ions were emitted without high fluxes of low-mass ions. The discovery of this effect greatly improved our sensitivity to high-mass ions. In order to use such low fields in the vicinity of the sample and still accelerate the ions produced from the surface to a high enough velocity for them to be detectable, it was necessary to use a two-stage acceleration region, as described above. Using this configuration and a total acceleration of 22 kV, a positive-ion spectrum of *E. coli* β-galactosidase (subunit mol.wt ≅ 116 000) showing singly, doubly and triply charged pseudomolecular ions was obtained (Fig. 3). The mechanism for the enhanced production of low-mass ions at higher fields is not currently known, but the effect is very reproducible. In the negative-ion mode, the addition of a magnetic field to deflect electrons from the detector, thereby decreasing the total number of particles striking the detector, was also helpful.

The practical mass resolution of the instrument was $m/\Delta m \cong 700$ (FWHM definition) measured using the cation of the dye Brilliant Green ($m/z = 385.26$) as a representative low-mass ion. The resolution obtained for small proteins (< 20 000) was $m/\Delta m = 500$ (FWHM). The dominant factor determining the mass resolution of the instrument was the laser power density. The resolution of a pseudomolecular ion could be increased by an order of magnitude by decreasing the laser intensity to near the production threshold of ca 10^6 W/cm^2. Conversely, laser power densities much higher than threshold dramatically decrease the mass resolution of the spectra obtained. Increasing the homogeneity of the laser's spatial profile, by the addition of a stop into the optical system and removing the laser's amplifier rod, also increased the resolution obtained.

Mass calibrations could be obtained by examining the mass spectra of mixtures of analytes. Because of the instrumental time-resolution effects, it was not practicable to use low-mass ions (e.g., sodium and potassium cations) to calibrate the spectrum in the high-mass region. Up to 5 different peptides over the mass range of 500–6000 were mixed and applied to the probe tip, and the spectrum that resulted was analyzed in detail to obtain an internally consistent calibration for all the species observed. This procedure demonstrated that the most intense signal observed for the proteins examined was from the $[M+H]^+$ ion. Low-mass peptides (< 2000) had significant signals from alkali cationized ions, but these peaks were either small or absent for larger molecules. Other calibrations were performed using a low-mass protein (insulin) and the prompt photon peak, using the photon peak as a "time-zero" marker.

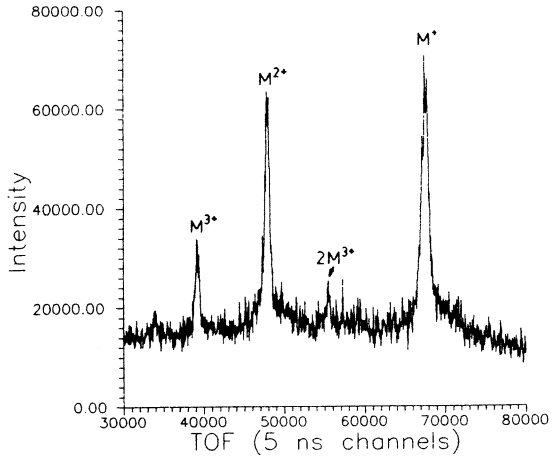

Figure 3. The high-mass region of the positive ion LD TOF mass spectrum of *E. coli* β-galactosidase from a 2-pyrazinecarboxylic acid matrix ($m/z = 116\,000$, determined from M^+ and M^{2+} ions). Conditions: $V_1 = +20$ kV; $V_2 = +17.5$ kV; 200 laser shots summed; total protein loaded = 0.7 pmol.

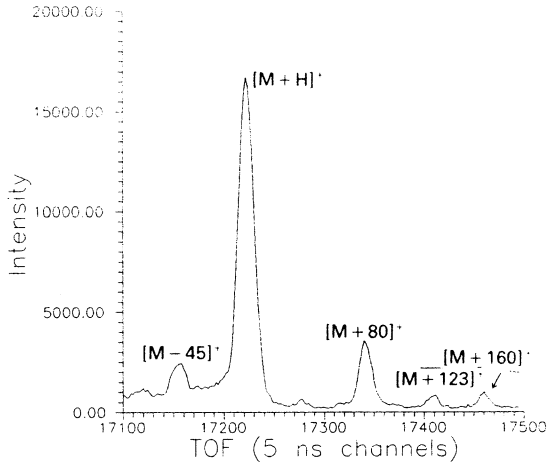

Figure 4. The pseudomolecular-ion region of a positive-ion LD TOF mass spectrum of bovine insulin from a 2-pyrazinecarboxylic acid matrix. Conditions: $V_1 = +15$ kV, $V_2 = +12.5$ kV; 100 laser shots summed; total protein loading = 4 pmol.

Figure 4 shows the insulin pseudomolecular ion region (sample desorbed from a 2-pyrazinecarboxylic acid matrix) with a mass resolution of 500 (FWHM), sufficient to resolve several ions in the vicinity of the $[M+H]^+$ species. The most intense ion usually observed is the protonated adduct ion $[M+H]^+$ (determined to a mass of ±1 by the procedure explained above), but substantial intensity is also seen in the $[M-45]^+$ ion (typical of peptides in most types of desorption) and an $[M+80]^+$ adduct ion. The $[M+80]^+$ ion can be assigned to the addition of 2-pyrazinecarboxylic acid to the insulin molecule with the loss of a carboxylic acid group and the addition of a proton. Similar adducts have been seen previously in the low-mass spectra of nicotinic acid as an ion with $m/z = 2035$. This assignment is supported by the discovery of a similar adduct ion in spectra observed from vanillic acid, but shifted to higher mass because of the hydroxyl and methoxyl groups on the ring. The formation of adduct ions of this type is strongly dependent on the type of matrix used. Matrices of 2-pyrazinecarboxylic acid produce intense adducts of this type compared with nicotinic acid, while thymine does not seem to form ring-addition adducts at all. This lack of adduct formation can be attributed to the lack of a carboxylic acid moiety on thymine, which may be necessary for the adduct-formation reaction. Negative-ion mass spectra of proteins also contain adduct ions.

The demonstration of adduct formation has implications for the ultimate mass accuracy of the technique

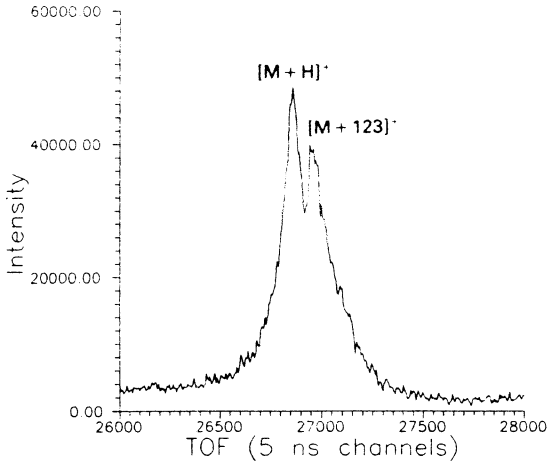

Figure 5. The pseudomolecular-ion region of a positive-ion LD TOF mass spectrum of bovine ribonuclease A, from a vanillic acid matrix (measured $m/z = 13\,684$ and $13\,691$, in separate measurements). Conditions: $V_1 = +15$ kV; $V_2 = +12.5$ kV; 200 laser shots summed; total protein loaded: 2 pmol.

when observing high-mass ions. Figure 5 shows the pseudomolecular-ion region of the mass spectrum of ribonuclease A (vanillic acid matrix). Clearly, without sufficient mass resolution to resolve the higher-mass adduct ion, the molecular weight determined by the centroid of the superposition of the two signals would be too high. The separation of the matrix-dependent adduct ions from the protonated pseudomolecular ion is aided by using a matrix of vanillic acid, because of its higher molecular weight.

SUMMARY

In this paper, the desorption of intact high-mass protein pseudomolecular ions has been demonstrated, using the solid-phase, matrix-assisted desorption technique. All of the proteins so far examined have produced such ions, up to mol.wt 116 000 Da. Three new matrices that produce good quality spectra are reported. Negative high-mass pseudomolecular ions produced by this type of desorption are demonstrated for the first time. The resolution of the method has been improved by approximately one order of magnitude to $m/\Delta m \cong 500$ (FWHM). A detailed examination of the pseudomolecular-ion region of the spectra obtained, demonstrated the existence of adduct ions formed by the addition of portions of the matrix molecules.

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