Cinnamic Acid Derivatives as Matrices for Ultraviolet Laser Desorption Mass Spectrometry of Proteins

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The paper reports the discovery of three new matrices for the matrix-assisted laser desorption of proteins. These new matrices (sinapinic, ferulic and caffeic acids) are cinnamic acid derivatives that have several practical advantages over the nicotinic acid matrices previously used. These materials form much less intense photochemically generated adduct peaks in the protein quasimolecular ion signal and the adduct peaks that are present are easier to resolve. These matrices produce intense protonated-molecule ions from all of the proteins (over 50) so far examined. These new matrices are also very stable in a vacuum, allowing for their convenient use in very high vacuum applications (e.g., Fourier transform ion cyclotron resonance mass spectrometry).

For the past nine months, our laboratory has been vigorously pursuing the application of the matrix-assisted laser desorption (LD) technique[1] to protein molecular weight determination. This technique allows the mass spectral analysis of minute amounts of proteins by a very soft desorption and ionization of large numbers of protein molecules in a single laser shot. By isolating protein molecules in a relatively volatile matrix substance (nicotinic acid) the mixture can be simultaneously put into the gas phase and ionized by the use of an appropriate high energy ultraviolet laser pulse (266 nm light from a Nd-YAG laser). Intact protein subunits have been observed with molecular weights $>100\,000$ and non-covalently bound subunit clusters have been observed of masses $>200\,000$.[1-5]

Our laboratory has been examining a variety of compound classes, with the goal of finding new (and hopefully better) matrices for use with proteins in LD mass spectrometry. In an earlier publication,[3] several matrices other than nicotinic acid were described. Pyrazine 2-carboxylic acid and vanillic acid, in particular, were found to have some desirable properties. In this communication we will expand on the subject of matrices, particularly on the formation of photochemically generated adducts of matrix molecules to the protein molecules being studied. These adducts complicate the signals obtained from the ionized, intact molecule and pose a serious problem for the determination of accurate molecular weights in those cases where the adductions cannot be resolved from the parent ions. This adduction problem only becomes readily apparent when the mass resolution of the instrument becomes high enough to distinguish their presence, i.e., an $m/\Delta m > 100$ (Full width at half maximum) at molecular weight 6000.

In the course of a survey of possible matrix materials, a new class of matrix compounds has been discovered that greatly reduces the problem of photochemically generated adducts, leading to an effective gain in mass resolution for proteins. These matrices are all derivatives of cinnamic acid. The most effective of these derivatives are 3-methoxy-4-hydroxycinnamic acid (ferulic acid), 3, 4-dihydroxycinnamic acid (caffeic acid) and 3, 5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid). Each of these new matrices has a much lower tendency to produce photochemically generated adducts than matrices with simple conjugated ring chromophores. Also, because of the higher molecular weights of these matrix molecules, any adduct formed is easier to resolve.

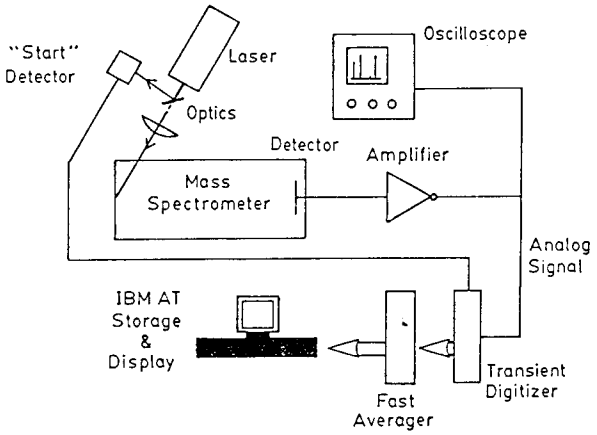

Figure 1. A schematic diagram of the Rockefeller laser-desorption mass spectrometer and data system.

EXPERIMENTAL

Instrumental

The mass spectrometer used was built at Rockefeller University using a commercially available laser (HY 400, Lumonics Inc., Kanata, Ontario, Canada) and transient recorder (TR8828D, Lecroy, Chestnut Ridge, NY, USA). The details of the instrument have been described.[3] Figure 1 shows the schematic plan of the instrument and data system. The portion of Fig. 1 designated "Optics" contains a beam splitter, a variable attentuator and a twelve-inch focal-length lens. The differences between the current arrangement and that

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described in Ref. 3 are: (i) the cuvette beam attenuator has been removed; (ii) the aperture stop near the focusing lens has been removed; (iii) a mode-selection aperture (2.0 mm diameter) has been placed in the oscillator cavity of the laser, mounted on the oscillator shutter assembly; and (iv) the pyroelectric detector used to generate the "start" pulse has been replaced by a phototransistor.

Sample Preparation

After substantial experimentation with matrices and solvents, a matrix solution concentration of 10 g/L was found to be the most generally applicable. Therefore, as a standard procedure to test the usefulness of a matrix, a 10 g/L solution was used. Nicotinic acid and pyrazine 2-carboxylic acid (Aldrich Chemical Co., Milwaukee, WI, USA) solutions were prepared with neat water (distilled-in-glass grade, Burdick & Jackson Laboratories, Muskegan, MI, USA). Vanillic, ferulic, caffeic and sinapinic acids (Aldrich Chemical Co.) were dissolved in a 50% mixture of ethanol and water. The protein to be examined was first dissolved in 0.1% trifluoroacetic acid and then added to the matrix solution, so that the final protein concentration of the mixture was less than 0.1 g/L. Other methods of mixing the protein and the matrix solution were tried, but the direct mixture of the two solutions was found to be the most satisfactory for general use. Typically 0.5 μL of matrix/protein solution was then applied to a metal probe tip and dried, either by ambient air or by a stream of forced room temperature air. Initially, heated forced air was used to dry the solution rapidly, but that method was found to give variable results for some of the matrices, particularly the cinnamic acid derivatives. Drying samples in vacuum was also found to be a suitable technique that had the advantage of speeding up the drying procedure. Lyophylization was also used, although results of this method were quite variable.

It should be noted that solutions of cinnamic acid derivatives are light sensitive and should be stored in the dark. Typically, solutions of these chemicals were made freshly every morning and stored in the dark between usages.

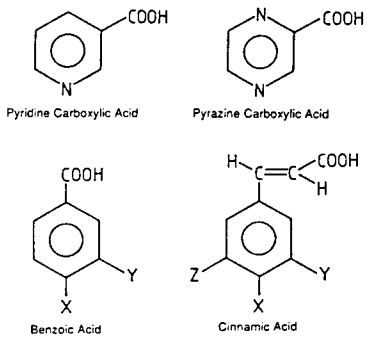
Spectra

In order to obtain the high mass-resolution ($m/\Delta m = 300$–500) in the spectra shown, the most important operational parameter was the careful regulation of the laser intensity. The laser irradiance per shot was kept as low as possible, i.e., close to the threshold of protein-ion production. Spectra were obtained by adding together the transient records of 50 to 200 individual shots. To obtain the maximum output power stability, the laser was operated with a continuous triggering frequency of 5Hz and on every second pumping lamp flash, the oscillator was Q-switched with the internal Pockels cell, resulting in an output frequency of 2.5 Hz. Therefore, 200 shots could be accumulated in 80 s. Typical laser irradiances used were approximately 1 MW/cm^2 at the sample. The acceleration voltage (V_{acc}) listed with each spectrum refers to the initial acceleration in the ion source.[3] A further 2 kV is added at the detector by post-acceleration.

The spectra shown below have time-of-flight (TOF) as the abscissa, in units of 5 ns (called bins), and intensity as the ordinate, in arbitrary units, dependent on the number of individual shots summed and the amplifier gain. These plots are the raw data as collected from the transient recorder, with the indicated number of shots directly summed. No background subtraction has been done.

RESULTS AND DISCUSSION

The four general classes of matrices that have been found to work most satisfactorily for a range of proteins are shown schematically below. The symbols X, Y and



Z represent either hydroxy or methoxy groups. The only useful pyridine carboxylic acid derivative of the many examined is nicotinic acid. Similarly, the only useful pyrazine carboxylic acid derivative is pyrazine 2-carboxylic acid, a structural analogue of nicotinic acid. The benzoic acid derivative that proves to be useful is 4-hydroxy-3-methoxybenzoic acid (vanillic acid). Dihydroxy- and dimethoxybenzoic acid derivatives show very limited utility as matrices, and polyhydroxyphenols are also of limited usefulness. The cinnamic acid derivatives with substitution at the 3, 4 and 5 positions are excellent matrix materials, producing very strong, stable signals of either positive (protonated) or negative (deprotonated) molecular ions. The further advantages of these cinnamic acid derivatives are discussed below.

Figure 2 compares the insulin molecular-ion signals obtained from nicotinic and vanillic acids. Clearly, the extent and the separation of the higher mass satellite peaks (i.e., photochemically produced adducts) is enhanced by using the heavier vanillic acid. The masses of these satellite peaks identify them as being caused by the addition of the matrix molecule less COOH to the intact protein. Thus, for nicotinic acid (mol. wt = 123), the first adduct peak is at $[M+78]^+$ (where M is the protein) and for vanillic acid mol. wt = 168) at $[M+123]^+$. Nicotinic acid is known to be resistant to the loss of CO_2 relative to its isomers.[6] Presumably, the absorption of one or more photons is sufficient to drive a reaction resulting in the loss of COOH. While it is not clear from the mass spectra alone by what reaction pathway these products are formed, it is clear that some reaction is occurring. Possible mechanisms are: (i) the production of a highly excited matrix molecule that reacts directly with the protein with the loss of COOH; or (ii) the production of an excited matrix molecule that loses COOH, forming a product that, in turn, reacts with the protein.

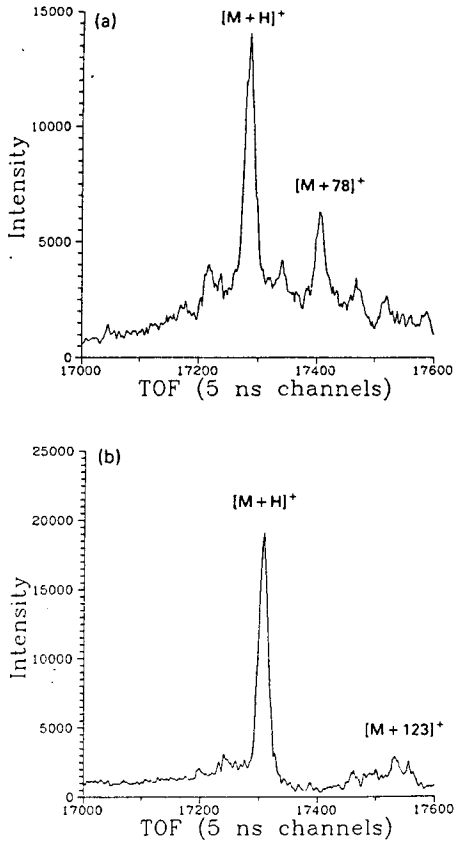


Figure 2. The molecule-ion region of the positive-ion LD time-of-flight mass spectra of bovine insulin (mol. wt = 5733.5), comparing photochemically generated adducts produced with (a) a nicotinic acid matrix and (b) a vanillic acid matrix. Conditions: V_{acc}, 15 kV; 100 laser shots summed; total protein loaded, 4 pmol; displayed bin size, 10 ns.

The ratio of adduct signal intensity to protonated-molecule ion signal intensity increases with the mass of the protein. Figure 3(a) shows, for comparison with Fig. 2, the extent of adduction found in human milk lysozyme (mol. wt = 14,693) using vanillic acid. This type of result, found to be quite general for proteins, made it important to find a class of matrices that does not produce such extensive adduction.

One such class of matrices is the cinnamic acid derivatives referred to above. Figure 3(b) shows the molecular-ion signals obtained from human milk lysozyme using a sinapinic acid matrix. The decreasd intensity of the photochemically generated adduct peaks and the separation obtained between the molecular ion and the adduct peaks has clearly improved the quality of the information available. The clear resolution of the adduct from the $[M+H]^+$ ion allows for much better accuracy in determining the centroid (ca 100 ppm.), and thus the molecular weight of the protein. Because of the improved signals obtained from these matrices, all of the work being carried out in our laboratory now uses sinapinic acid as the matrix of choice.

The type of adduct species observed using cinnamic acid derivatives is also different from those described above. The mass difference between the $[M+H]^+$ species and the adduct indicates that the adduct is formed by the loss of water rather than the carboxylic acid group. The photochemical and pyrolytic loss of CO_2 from cinnamic acid are well documented reactions.[7] The product of this loss is a neutral molecule and therefore may not be as reactive a species as the products of the much higher energy nicotinic acid reaction. The site and mechanism of the dehydration reaction causing the low intensity adduct peaks is not known. The amount of adduction increases with increasing protein molecular weight but at a much slower rate than in the case of the matrix less COOH adducts from nicotinic acid.

A practical advantage to using matrices such as ferulic, caffeic and sinapinic acid is their vacuum stability. Both nicotinic acid and pyrazine 2-carboxylic acid are rather volatile materials that will readily vacuum sublime at room temperature. Using our typical matrix/sample loading of 5 μg on a probe tip, signals were obtained from either nicotinic or pyrazine 2-carboxylic acid for only 20 min after its introduction into the vacuum system. The best signals were produced for less than 10 min after entering the vacuum. Examination of the probe tip under a microscope after 20 min in the vacuum system showed that the matrix had largely sublimed. The heavier, less volatile cinnamic acid derivatives do not readily sublime at room temperature. Samples prepared with these matrices remain stable in the vacuum for more than 10 h at pressures of 2×10^{-8} Torr, with no significant decrease in signal intensity or quality. The relatively low vapor pressure of these materials may make them the matrices of choice for applications requiring high vacuum, such as Fourier transform-ion cyclotron resonance mass spectrometry.

The results of examining a large number of different proteins (over 50) with ferulic, caffeic and sinapinic

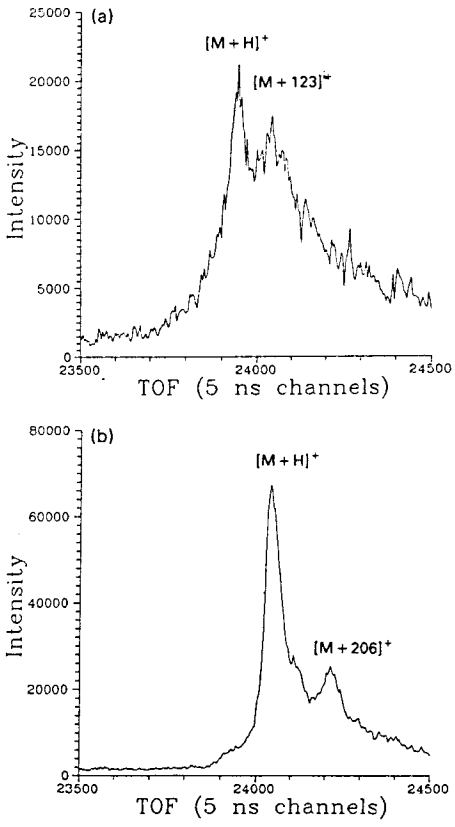


Figure 3. The molecule-ion region of the positive-ion LD time-of-flight mass spectra of human milk lysozyme (mol. wt = 14693, comparing the photochemically generated adducts produced with (a) a vanillic acid matrix and (b) a sinapinic acid matrix. Conditions: V_{acc}, 20 kV; 100 laser shots summed; total protein loaded, 2 pmol, displayed bin size, 20 ns.

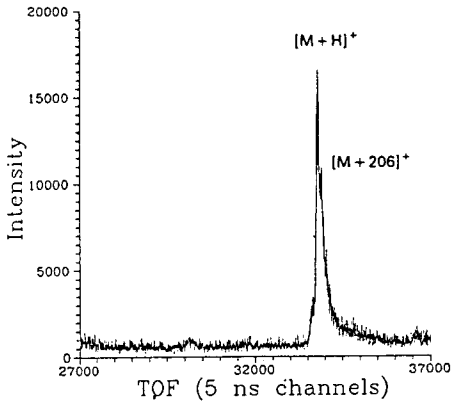


Figure 4. The mass spectrum of bovine erythrocyte carbonic anhydrase II {mol. wt = 29 020 (calc.), mol. wt = 29 019 ± 3 (measured)} using a sinapinic acid matrix. The region of the spectrum shown corresponds to a mass range of 18 000–35 000. Conditions: V_{acc}, 20 kV; 200 shots summed; total protein loaded 2 pmol; displayed bin size, 20 ns.

acid have demonstrated that these matrices can be used for most types of proteins. Fibrous proteins, such as rabbit-muscle actin and tropomyosin, respond as well as globular enzymes, such as horse erythrocyte carbonic anhydrase II (Fig. 4) and *E. coli* β-galactosidase. Every protein that has been examined has produced a signal corresponding to the individual subunits of the protein. In general, the most intense signals in the spectrum are the intact subunit with one, two or three charges in both positive and negative spectra. Higher mass cluster ions ($[2M+H]^+$, $[3M+H]^+$, etc.) are usually of significantly lower intensity.

Acknowledgement

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