Peptide Analysis to the Atomole Level Using a Curved-Field Reflectron MALDI-TOF Mass Spectrometer†

Amina S. Woods,[a] D. P. Little,[a] Timothy Cornish,[a] R. J. Cotter[a] and Daniel P. Little[b]

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Post-source decay (PSD) mass spectra of several peptides were obtained on a time-of-flight mass spectrometer with a curved-field reflectron to determine their amino acid sequences using very small quantities of sample. Peptides included angiotensin II, modified angiotensin II, and the tryptic fragments from somatostatin. Samples were prepared on the mass spectrometer probe using a piezo-electric micropipetting apparatus that enabled deposition of nanoliter volumes. The curved-field reflectron then enables recording of PSD mass spectra of mass-selected precursors without the need for stepping the reflectron voltage to bring different mass regions in focus. Using this instrument, sequence-specific ions could be observed with as little as a few atomoles of peptide.

Introduction

The peptides and proteins required for biological research are generally available in only very small quantities, which are further reduced by the purification requirements of the analytical techniques used to determine their amino acid sequences or other structural features. While automated Edman degradation[1),2)] can be utilized for peptides that are easily fractionated from other interfering peptides, are not N-terminally blocked and are available in at least the 2–3 picomole level, there is increasing interest in mass spectral methods for the analysis of peptides down to the femtomole range or below. The relatively recent application of mass spectrometry to such analyses is largely the result of the introduction of two ionization techniques: *electrospray ionization* (ESI)[3),4)] and *matrix-assisted laser desorption/ionization* (MALDI).[5),6)] ESI has been most often used with quadrupole mass spectrometers, with the triple-quadrupole providing a tandem arrangement that can be used for mass selecting, fragmenting and sequencing individual peptides from a mixture. MALDI, because it utilizes a pulsed laser to form the ions, is generally incorporated into a *time-of-flight* (TOF)[7),8)] mass spectrometer. Time-of-flight mass spectrometers have an inherent sensitivity advantage that arises from their ability to simultaneously record ions of all masses from each laser pulse. Thus, the combination of MALDI-TOF has found considerable use in the determination of the molecular weights of very small quantities of proteins and peptides, in the femtomolar or subfemtomolar region. In addition, strategies that employ endo- and exopeptidases prior to mass analysis can be utilized to extend these highly sensitive molecular weight measurements to enzymatic peptide mapping[9)] and amino acid sequencing.[10)]

It is also possible to use the MALDI-TOF to mass select, fragment and sequence peptides directly, when the time-of-flight mass analyzer includes a *reflectron.* Reflectrons were originally developed to improve mass resolution by insuring that ions with the same mass but different kinetic energies are time focused at the detector.[11),12)] However, these same reflectrons will cause ions formed by fragmentation in the drift region between the ion source and the reflectron to arrive at the detector at different times, enabling the recording of mass spectra of the product ions from each precursor ion that is mass-selected in the drift region by an electronic gating system. Fragmentation in the drift region is generally known as post-source decay (PSD)[13)] and includes both metastable (unimolecular) decomposition and fragmentation induced by collisions when inert gases are admitted to raise the background pressure. PSD mass spectra can be used to record amino acid sequence-specific ions from a single peptide in the presence of other peptides, in order (for example) to obtain the sequences of all peptides in a tryptic digest.[14)] At the same time, the major problem with using the reflectron in this way is that product ions of different masses cannot be simultaneously focused, and it becomes necessary to record PSD spectra for each precursor at several different reflectron voltages.[15)] The resultant loss of this important multichannel recording advantage when recording product ions has been addressed previously by this laboratory with the introduction of the *curved-field* reflectron,[16)] which enables the recording of amino sequence mass spectra for each peptide without stepping the reflectron voltage. More than convenient, this provides a very distinct advantage in improving the sensitivity for sequencing small quantities of peptides.

In this report we utilize a curved-field reflectron MALDI-TOF mass spectrometer to obtain amino acid sequences by post-source decay from as little as 10 atomoles of peptides. Critical to the preparation of such samples is the use of a piezo-electric microdrop pipettor capabale of depositing nanoliter volumes of peptide and matrix solutions on the sample probe.

† This paper is dedicated to the memory of Professor Makoto Suzuki.

[a] *Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine (Baltimore, MD 21205, U.S.A.)*

[b] *Sequenom, Inc., (San Diego, CA 92121, U.S.A.)*

Materials and Methods

The peptides: *Angiotensin II* (DRVYIHPF), *Angiotensin II-modified* (DRVYVHPF), and *Somatostatin-28* (SANSNPAMAPRERKAGCKNFFYKTFTSC, with a disulfide bridge between Cys_{17} and Cys_{28}) were obtained from Sigma (St. Louis, MO), and have calculated protonated molecular ion masses: $MH^+ = 1047.2$, 1033.2, and 3149.6, respectively. Peptides were dissolved in solutions of 20% acetonitrile, 80% water, and 0.1% TFA. The angiotensin solutions (100 pmol/microliter) were diluted 200 fold in a saturated solution of α-cyano-4-hydroxycinnamic acid, obtained from Aldrich (Madison, WI), in 50% ethanol.

Enzymatic digestion of somatostatin was carried out using trypsin (modified sequencing grade) obtained from Boehringer Mannheim (Mannheim, Germany). The enzyme was dissolved in 20 μl of 1 mM HCL for a final concentration of 1.25 $\mu g/\mu l$. 50 μl of somatostatin solution (1,000 pmol) was reacted with 48 μl of 25 mM NH_4HCO_3 at pH 8.4 and 2 μl trypsin solution. The mixture was vortexed and incubated at room temperature for 3 hours, and the solution diluted 200 fold in matrix solution.

The final peptide solutions were deposited on the sample slide of a Kratos (Manchester, England) Kompact MALDI IV time-of-flight mass spectrometer, using the Autodrop Sytem (Microdrop, Norderstedt, Germany). The mass spectrometer is equiped with a 337 nm pulsed N_2 laser for MALDI ionization, and a curved-field reflectron for obtaining PSD spectra. The instrument was operated in the positive ion mode with 20 kV extraction voltage.

Theory of Operation

In a simple time-of-flight mass spectrometer ions are extracted from the source region to the same final kinetic energy: $eV = mv^2/2$, where m is the mass of the ion, e is its charge, and V is the accelerating or extraction voltage. In effect, this means that ions of different mass have different velocities, so that their masses can be determined by the time need to traverse a fixed drift length D: $t = (m/2eV)^{1/2}D$. Because ion velocity is determined during extraction from the source, fargmentation that occurs within the drift region does not alter the flight time, *i.e.*, a post-source decay product ion will appear at the detector at the same time as its precursor ion. For methods involving molecular ion measurments (tryptic mapping, ladder sequencing) this is an advantage, since the sensitivity is not reduced even when extensive fragmentation occurs.

Although post-source product ions have the same velocities as their precursors in the drift region, they do not have the same kinetic energies. Rather, for a product ion $KE = (m_f/m)eV$ where m and m_f are the masses of the precursor and product ions, respectively, and eV is the kinetic energy of the precursor ion. Therefore, the flight times of product ions formed in the drift region can be distinguished in a reflectron instrument, since product ions will penetrate the reflectron less deeply and for a shorter time period. In particular, for a single-stage reflectron, the flight time of a product ion is:

$$t = \left[\frac{m}{2eV}\right]^{1/2}\left[D + 4\frac{m_f}{m}d\right]$$

where D is now the total drift length traveled (before and after the reflectron) and d is the average penetration depth into the reflectron for a precursor ion. Thus, as illustrated in Fig. 1a, fragment ions formed in the drift region prior to entering the reflectron lead their precursor ions as they strike the detector. However, as also illustrated in Fig. 1a, product ions are not as well focused as the precursor ion; in particular, focusing decreases with decreasing mass. The single-stage reflectron is in fact a first order kinetic energy focusing device, with optimal focusing occuring when the flight times in the drift and reflectron regions are nearly the same, *i.e.*, when $D = 4d$. For the lower mass product ions this can be achieved by lower the reflectron voltage. Thus, the most common method for obtaining PSD mass spectra on instruments with a single-stage reflectron is to record the mass range in several segments.

The single-stage reflectron uses a single retarding electrical field generally constructed using a series of lens elements connected resistively to provide a linearly increasing voltage from the entrance to the back of the reflectron. Higher order kinetic energy focusing can be provided by dual-stage reflectrons, which utilize two constant retarding electrical fields, but such reflectrons still require the acquisition of product ion mass spectral data at several reflectron voltages. Quadratic reflectrons have also been described[17] in which flight times in the reflectron would be completely independent of ion energy, but are not able to accomodate a drift region in which post-source fragmentation is carried out. Thus, as described by our laboratory earlier,[16] we

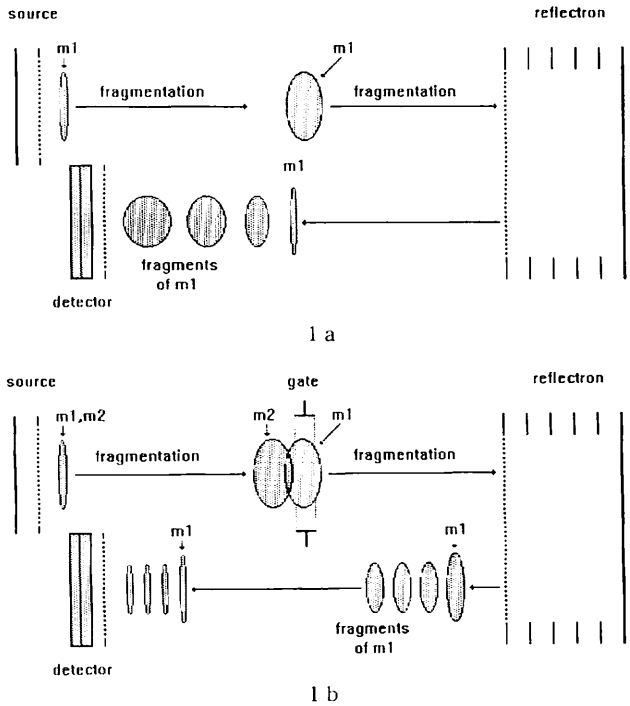

Fig. 1. Illustration of the time dispersion and focusing of precursor and product ions using (a) a single-stage reflectron and (b) a curved-field reflectron.

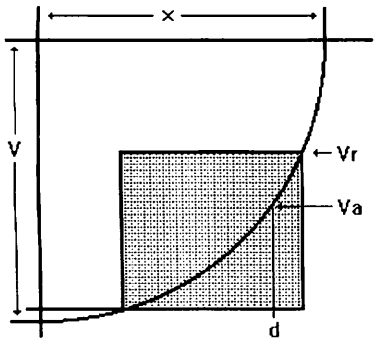

Fig. 2. Diagram of the voltage vs depth on the curved-field reflectron. V_r is the voltage at the back of the reflectron, V_a is the accelerating voltage and x is the distance from the entrance to the reflectron.

developed a reflectron in which the voltages placed on the retarding lenses follow the equation for the arc-of-a-circle (Fig. 2). This *curved-field reflectron* provides kinetic energy focusing of sufficiently high bandwidth that product ions remain in approximately constant focus over the entire mass range, as illustrated in Fig. 1 b. Also illustrated in Fig. 1b is the gating system used to mass select the precursor ion. Because precursor ions and their products have the same velocity, gating can be accomplished before or after fragmentation has occured, but provides better mass selection when located at some distance into the drift length.

Results and Discussion

Using conventional pipetting techniques, our attempts to analyze samples containing less than 1 fmol/

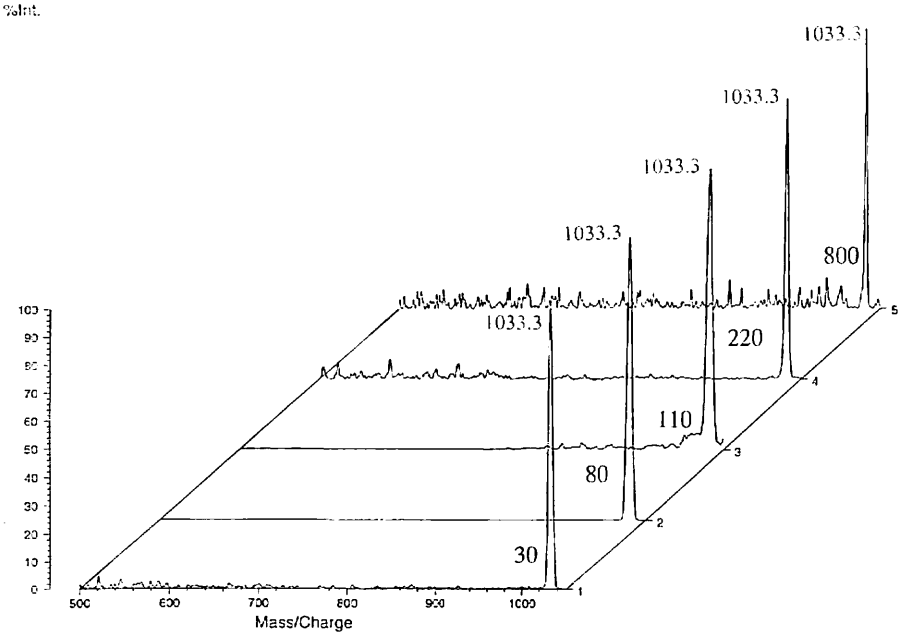

Fig. 3. Mass spectra of 30, 80, 110, 220, and 800 atomole samples of modified angiotensin II, recorded in the reflectron mode.

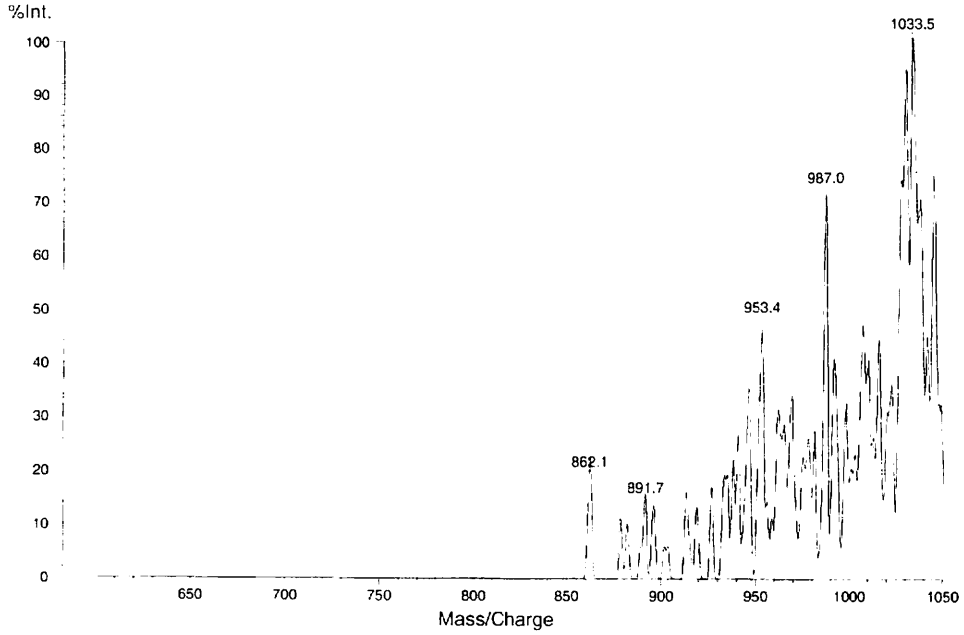

Fig. 4. Mass spectrum of 3 atomole sample of modified angiotensin II, recorded in the linear mode.

μl have produced mass spectra with poor signal/noise. However, using the microdrop pipetor we were able to deposit samples and acquire mass spectra of modified angiotensin II from 3 atomoles to 800 atomoles. Figure 3 shows the mass spectra aquired in the reflectron mode from the 30, 80, 110, 220, and 800 atomole sample spots deposited on a 30 well sample slide. Signal/noise is excellent throughout this sample size range; it was mediocre for the sample spot containing 3 atomoles although the MH^+ ion at 1033.5 is easily distinguished in Fig. 4.

Figure 5 shows the post-source decay mass spectra of 3000, 300, and 0.3 fmol modified Angiotensin II samples deposited on the sample probe. The same fragment ions are observed in all of the PSD mass spectra, although the signal/noise is reduced at the lower sample sizes. Figure 6 shows the PSD mass spectra for the MH^- ion of Angiotensin II from 3000, 300, and 0.3 fmol samples, while Fig. 7 gives the assignment of the sequence ions for these spectra. All of the PSD mass spectra were acquired at a single reflectron voltage.

Figure 8 is the mass spectrum of 7.5 fmol of the somatostatin tryptic digest deposited with the microdrop pipetor. Several of these tryptic fragments contain the disulfide bond between Cys_{17} and Cys_{28}. In Fig. 9, spectrum 3 is the digest, while spectra 1 and 2 are the PSD mass spectra of the tryptic fragments NFFWK (residues 19–23; $MH^-=741.9$) and SANSNPAMAPR (residues 1–11; $MH^-=116.2$), respectively.

Conclusions

Successful mass analysis of very small quantities of peptides and other biological molecules requires the

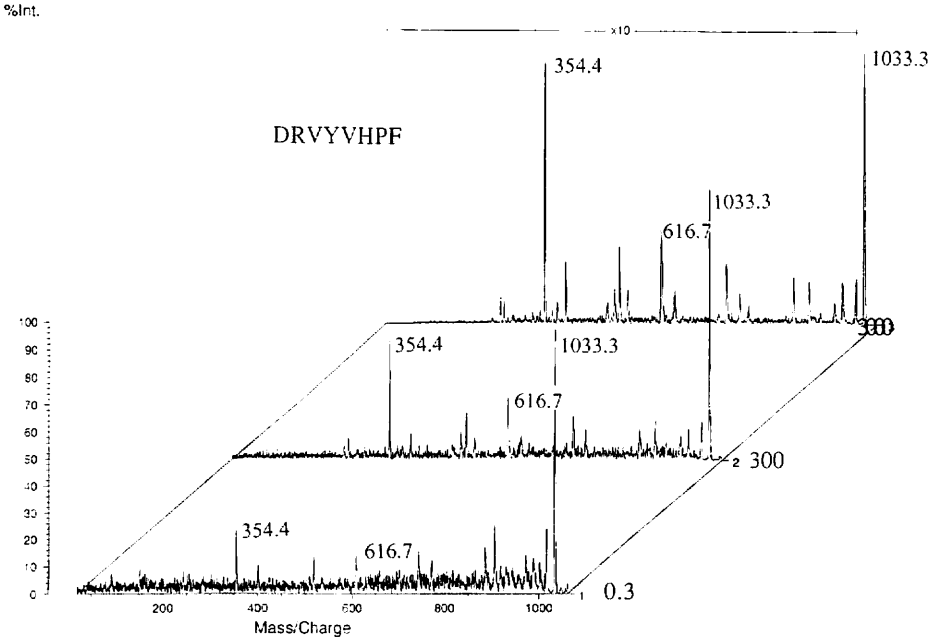

Fig. 5. PSD mass spectrum of 0.3, 300, and 3000 femtomoles of modified angiotensin II deposited on the sample slide.

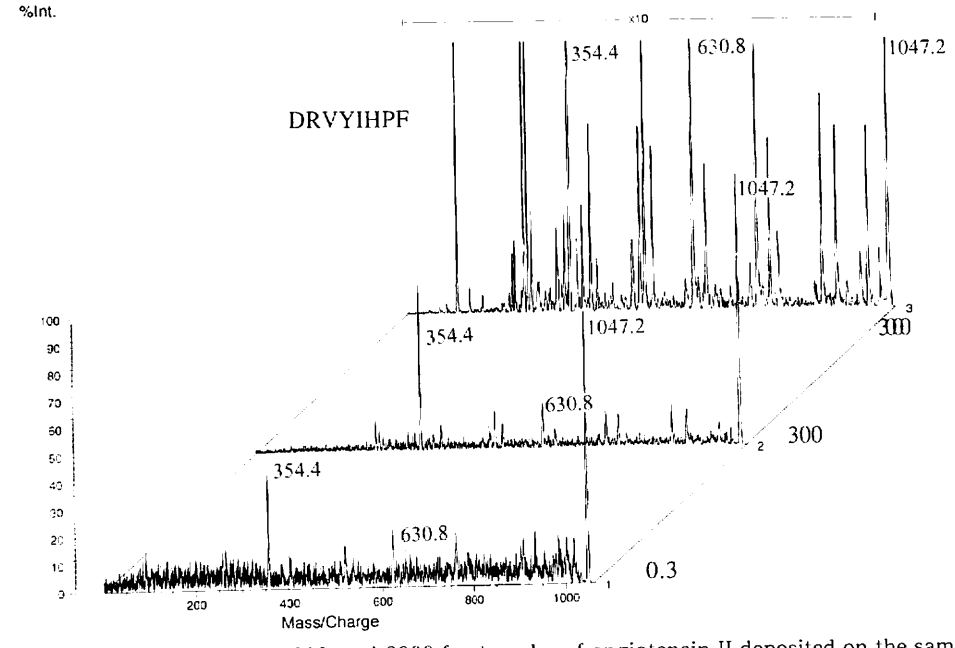

Fig. 6. PSD mass spectrum of 0.3, 300, and 3000 femtomoles of angiotensin II deposited on the sample slide.

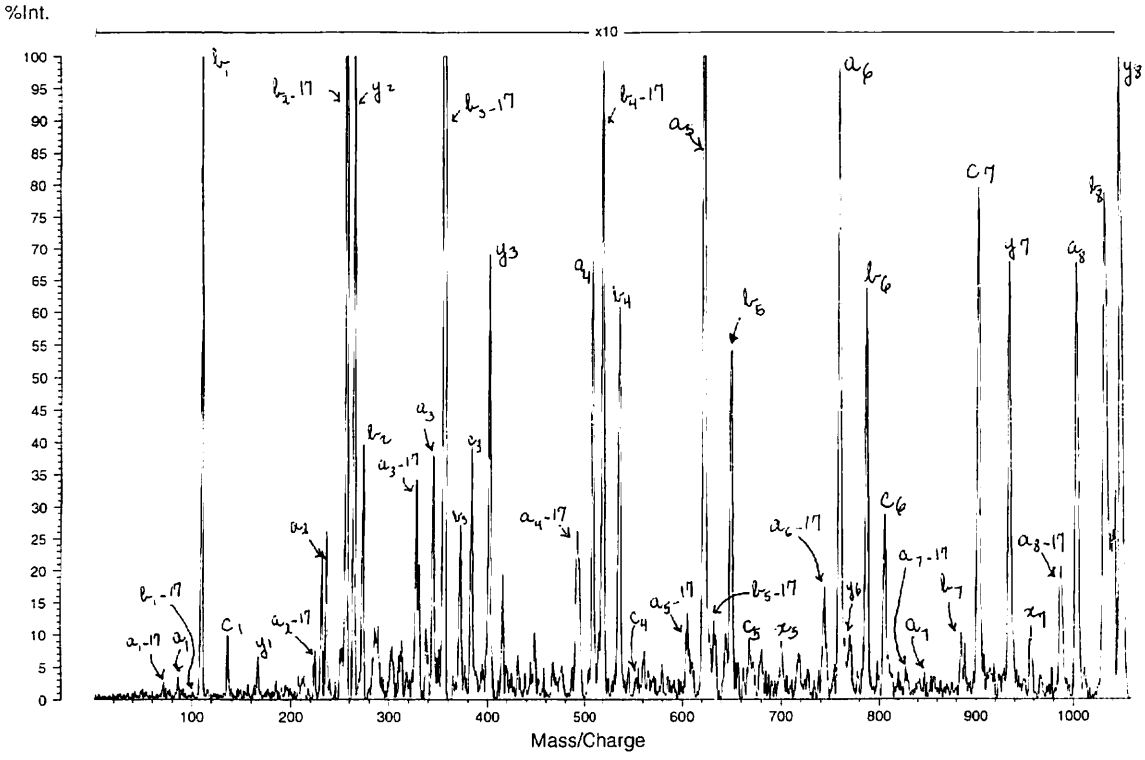

Fig. 7. Assignment of fragment ions obtained in the PSD mass spectra of angiotensin II.

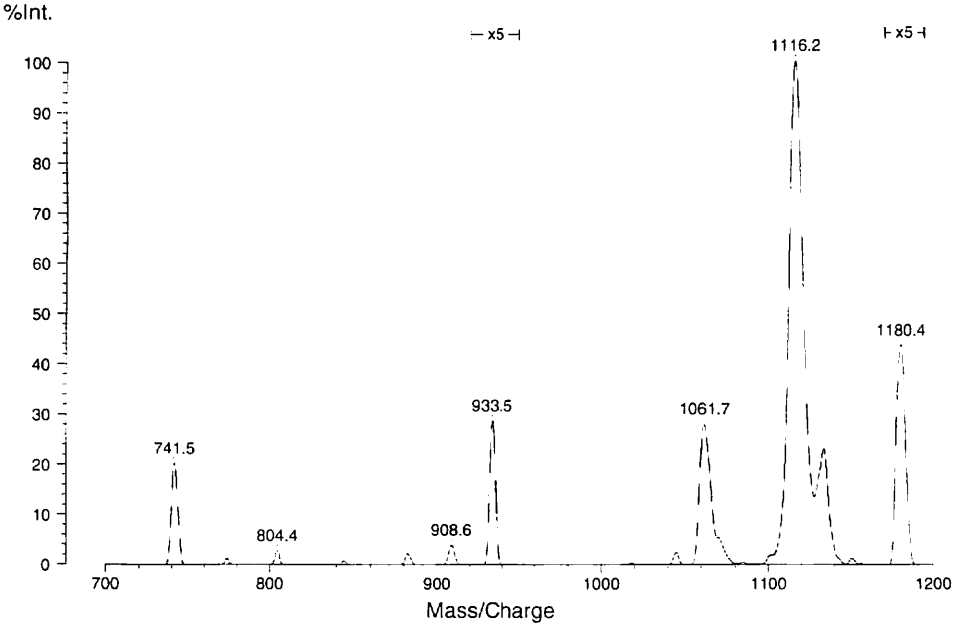

Fig. 8. Mass spectrum of the tryptic digest of somatostatin 28, with 7.5 femtomoles deposited. The peaks correspond to the MH^+ ions for residues 19–23 at 741.5, residues 1–11 at 1116.2, and the disulfide linked residues 15–18 and 24–28 at 933.5, residues 14–18 and 24–28 at 1061.7, and residues 14–19 and 24–28 at 1180.4.

maximum utilization of the sample. The microdrop pipeting device used in this work enables deposition of samples in nanoliter volumes as smaller, more concentrated spots that improve the ion signal from each laser shot. Concommitent with these smaller spots, and no less critical, is the ability to obtain the maximum data from each laser shot. Thus, the ability to record the entire mass range in PSD mass spectra, without stepping the reflectron voltage, maximizes the data utilized in acquiring mass spectra, and becomes particularly important when it is necessary to obtain sequence data from several peptides in the same digest. In this report, the curved-field reflectron has been effective in the mass analysis of as little as 3 atomoles of peptide and the amino acid sequencing of 0.3 fmol.

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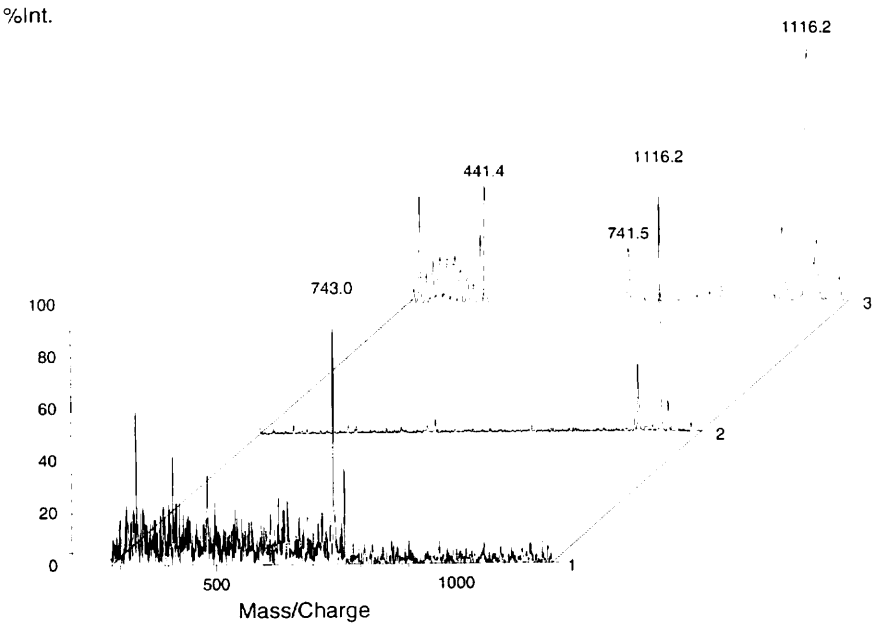

Fig. 9. Mass spectra of (1) PSD of fragment NFFWK, residues 19–23, (2) PSD of fragment SASNSNPAMARP, residues 1–11, and (3) peptide fragments from the tryptic digest of somatostatin 28.

the sale of products by Kratos (Manchester, England) related to researh described in this paper. In addition, RJC serves as a consultant to Kratos Analytical. The terms of these arrangements have been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies.

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