Protein and Polymer Analyses up to *m/z* 100 000 by Laser Ionization Time-of-flight Mass Spectrometry

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Hitherto, ^{252}Cf plasma desorption mass spectrometry (PDMS) has been used to study peptides and proteins in the molecular weight range from 1 kDa to 35 kDa.[1,2] Fast atom bombardment mass spectrometry (FABMS) and secondary ion mass spectrometry (SIMS) have been applied to the analyses of proteins and polymers molecules.[3,4] On the other hand, in the area of laser desorption time-of-flight (TOF) mass spectrometry (MS), though there have been many papers on analyses of organic compounds, the molecular weight of these compounds has been relatively low.[5]

For the purpose of investigating the mass spectrometry of high-mass molecular organic compounds, we developed a laser ionization TOF mass spectrometer. To assess the utility of this spectrometer for high masses, we evaluated and tried the mass spectrometer on various organic compounds.[6,7] This spectrometer was able to obtain useful spectra up to *m/z* 100 000. Typical spectra of proteins and polymers with molecular weights up to 25 kDa are shown in this paper.

EXPERIMENTAL

Apparatus

The construction of the laser ionization TOF mass spectrometer (Shimadzu LAMS-50K) is shown in Fig. 1. A nitrogen laser (wavelength: 337 nm, pulse width: about 15 ns, pulse energy: 4 mJ max.) was used for sample ionization. The sample, mounted on the sample holder, was capable of small movements in the plane perpendicular to the ion axis. The magnification image of the sample surface could be observed on the TV monitor. These features provided reliable analysis of the selected area. The ion acceleration voltage of 5 kV was applied to the sample holder.

Generally, TOF-MS has the following characteristics: very high transmission; measurement times of less than a few hundreds of μs; unlimited mass range, low mass resolution.

A new "gradient electric field type ion reflector"[8] for a TOF mass spectrometer has been developed in order to improve mass spectral resolution by time focusing. The TOF mass separation system was designed to select the "reflection type" (above-mentioned) or the "linear type" (without reflecting electric field). For ion detection, a micro channel plate (MCP) was used, which was equipped with an ion to electron converter in order to improve the sensitivity of detection for high-mass ions.

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Two TOF systems were constructed. The first system utilized a digital wave memory and accumulation circuits. This system could accumulate the spectrum data of 8 K words within 1 ms. In the first place, a "one shot" TOF spectrum was stored into the wave memory, in the subsequent accumulating circuits the spectrum was accumulated in sequence. The second system utilized a constant fraction discriminator (CFD) and a multi-stop time-to-digital converter (TDC). The time intervals between "start" and "stop" pulses were measured with a time resolution of 1 ns. In these experiments, the first system (AD method) was only used.

The measuring circuit was controlled with a microprocessor [Intel 80286, Intel, CA, USA], and the TOF spectrum obtained with those measurement systems could be provided with various processes, e.g., smoothing, background subtraction, peak detection and mass number calibration.

Sample preparation

We investigated the method of sample preparation to increase the yield of high-mass ions and to prolong the production time. As a result, it was found that the yield of ions and the sustained production time largely depended on the method of sample preparation and that the "ultra fine metal plus liquid matrix method", described below, was extremely effective.

A sample was made into a solution having a concentration of about 10 μg/10 μL using distilled water as the solvent. About 10 μL of this sample solution was dripped onto the sample holder. On the other hand, an ultra fine metal powder ((UFP), in these experiments, we used cobalt powder of about 300 Å diameter purchased from Vacuum Metallurgical Co. in Japan) and glycerol were dissolved with organic solvents, e.g., ethanol and acetone. About 10 μL of this solution was also dripped onto the sample holder. A mixture of the two solutions was then vacuum dried for a short time to remove volatile compounds of the solution. The sample holder, with sample, was then introduced into the mass spectrometer to start analysis. This sample preparation method was simple with preparation times of less than a few minutes.

Data acquisition

Several one-shot spectra were accumulated from a single sample introduction to produce the final spectrum, giving total data acquisition times of 2 or 4 min; in some cases two or more significant spectra were

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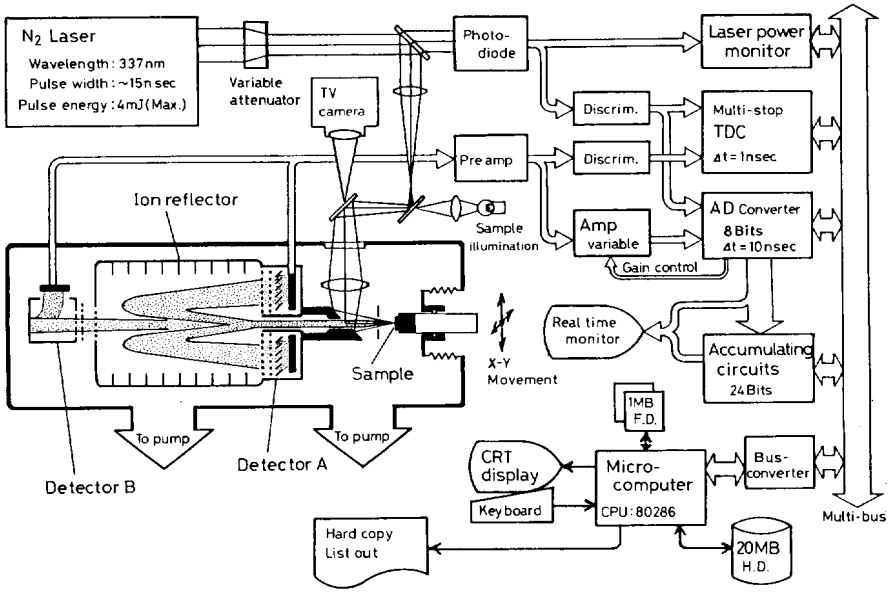

Figure 1. Construction of the laser ionization TOF mass spectrometer.

obtained from a single sample introduction. The final spectrum data were smoothed and sometimes background was subtracted and the position of the peak tops determined. Mass number calibrations were carried out by using cationized molecular ion peaks ($[M+Na]^+$) of poly(ethylene glycol) ((PEG); mixture of PEG 200, 400, 600, 1000, 1500 and 2000) as measures of isotopically averaged mass (chemical mass).

RESULTS AND DISCUSSION

This mass spectrometer was successfully applied to the detection of high-mass molecular ions, e.g., lysozyme, mol. wt 14306 Da (Fig. 2); chymotrypsinogen, mol. wt 25717 Da (Fig. 3); poly(propylene glycol) (PPG), average mol. wt 4 kDa (Fig. 4); and PEG20K, average mol. wt ca 20 kDa (Fig. 5).

It was impossible to detect high-mass molecular ions from these samples without using the "ultra fine metal plus liquid matrix method" for sample preparation.

UFP has the following features in comparison with bulk: high photo-absorption, low heat capacity, and extremely large surface area per unit volume. "Rapid heating" of the sample was used as a volatility enhancement technique of organic compounds[9] and it was achieved by irradiating a pulsed laser on the sample surface. We suppose that UFP in the sample enhances the speed of sample heating, by laser irradiation, due to the above mentioned features. Consequently, molecular ions are formed more easily. By the addition of glycerol to the sample, molecules of the sample are replenished to the laser beam irradiation position and it is considered that the ion formation is allowed to continue for a long time.

Figure 2 shows the singly- and many of the doubly-charged cluster ions of lysozyme. Quasi-molecular ions (may be mostly in the form of $[M+Cation]^+$) of lysozyme were also clearly found.[10] This mass spectrometer could detect $[n\times M+Cation]^+$ and $[n\times M+2\times Cation]^{2+}$ ($n=1$–7) of lysozyme. As far as we know, ions of $m/z \geq 100000$ are the highest organic ion yet observed by laser ionization/desorption mass spectrometry.

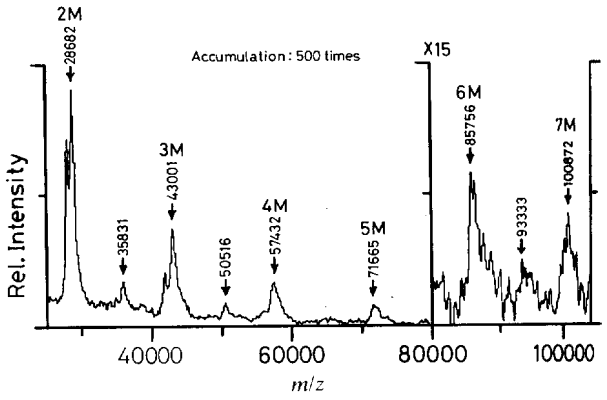

Figure 2. Laser ionization mass spectrum of lysozyme from chicken egg white, mol. wt 14306 Da.

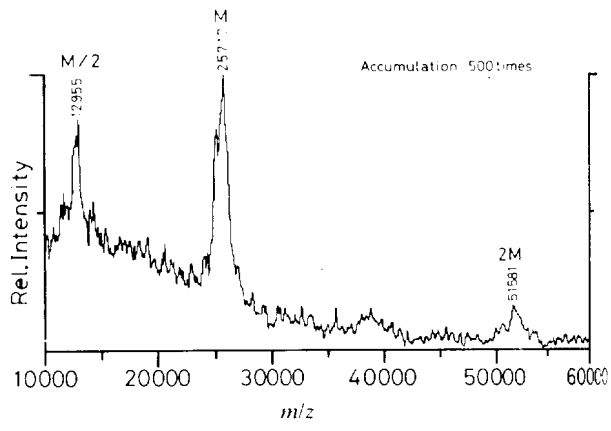

Figure 3. Laser ionization mass spectrum of chymotrypsinogen, mol wt 25717 Da.

Figure 3 shows the singly- and doubly-charged quasi-molecular ions and the cluster ion of chymotrypsinogen. This spectrometer was also capable of detecting the quasi-molecular ion of carboxypeptidase-A (mol. wt 34472 Da),[10] bovine insulin (mol. wt 5733 Da),[6] cytochrome-C (mol. wt 12384 Da)[7] and so on.

The widths of all the peaks associated with the molecule (over 1000 Da wide in most cases) were broader than those expected from the distribution of isotopes in the molecule. The origin of these observed broad peaks is uncertain. Impurities in the sample, together with adduct ions $[M+Na]^+$, $[M+K]^+$ and $[M+H]^+$, as well as metastable peaks arising from these ions, are all possible contributors.

Figure 4 shows cationized molecular ion peaks $[M+Na]^+$ of PPG having the nonlinear structure. The intensity of fragment peaks was so weak compared to that of the molecular ions that it was easy to calculate the average molecular weight from this spectrum. Figure 5 shows the molecular ion region spectrum of PEG20K. Each peak for the different polymerization (n) is not separated. It is supposed, however, that the shape of this spectrum shows the distribution envelope of PEG20K molecules.

We observed the spectrum about 10 times for each sample above. Those peak tops, taken in all cases to be the cationized molecular ion $[M+Na]^+$, allowed the molecular weight to be determined within ±1%.

In conclusion, analytically useful spectra from proteins and polymers with molecular weights as high as 34 kDa were obtained by using the laser ionization TOF mass spectrometer. The time required to obtain such spectra was very short compared with PDMS and comparable to liquid SIMS.[3]

The methods commonly used at present for molecular weight determination of proteins and polymers are sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE), gel permeation chromatography (GPC) and viscosity method. These methods normally allow a molecular weight determination precision of ca±10%. The precision may be influenced by other properties of the molecule such as conformational state and hydrophobicity. In addition, with these latter techniques, the measurement times are considerably longer. In comparison with these methods, the precision of this laser ionization TOF mass spectrometer was at least one order of magnitude higher and its measurement time was one or two orders shorter.

We developed the method of sample preparation ("ultra fine metal plus liquid matrix method") to be able to form high-mass molecular ions up to at least 34 kDa for laser ionization mass spectrometry. Theoretically, TOF-MS has no limitation of mass range and this spectrometer was capable of detecting organic ions up to m/z 100 000. Further improvements on this method of sample preparation should be promising, enabling the detection of high-mass molecular ions over 34 kDa.

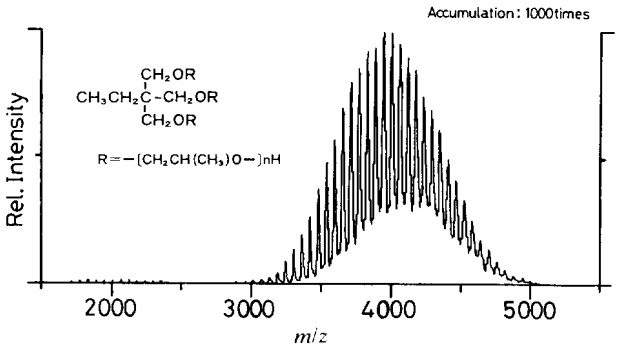

Figure 4. Laser ionization mass spectrum of poly(propylene glycol), average mol. wt 4 kDa (PPG4K).

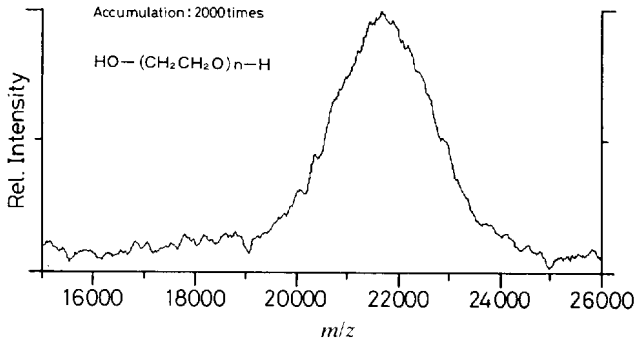

Figure 5. Laser ionization mass spectrum of poly(ethylene glycol), average mol. wt ca 20 kDa (PEG20K).

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