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Delayed Extraction Time-of-flight MALDI Mass Spectrometry of Proteins above 25 000 Da

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Matrix-assisted laser desorption/ionization (MALDI) with delayed extraction (DE) has been optimized for mass analysis of high-mass proteins and glycoproteins with masses above 25 000 Da. Under optimized experimental conditions, i.e. using a weak extraction field strength and a long delay time, a steep drop in mass resolution above 30 000 Da is no longer observed and an improvement of more than a factor of 10 is obtained compared with the non-DE case, at least up to 66 kDa in a 1.2 m time-of-flight mass analyzer. On this level of resolution the factors limiting further improvements become apparent, i.e. adduct ion formation between matrix and analyte, but also cationization and further non-matrix-related adducts, as well as prompt fragmentation. Moreover, heterogeneity of the sample is often the reason for the detection of broad signals for larger proteins. Within these limitations, DHBs (a 9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) gave by far the best results. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

The major problem with time-of-flight (TOF) instruments for the analysis of MALD ions, i.e. the relatively poor mass resolution, has recently been overcome by the implementation of delayed extraction,[1–4] a method based on the idea of 'time lag energy focusing' originally described in 1955 by Wiley and McLaren.[5] With this technique, ions are generated with the extraction field switched off, i.e. in zero or vanishing electric field, and only extracted after a predetermined and optimized delay time. Within the delay time, ions will spread out into the extraction gap owing to their initial velocities, thus spreading out in space and acquiring different total kinetic energies, when accelerated by the delayed extraction. In a two-stage acceleration ion source, with an adequate set-up of the first and second acceleration potential gaps, it becomes possible to compensate for the initial ion velocity distribution to a large extent. Substantial improvement in mass resolution compared with static ion extraction has been reported. A resolution $M/\Delta M$ (FWHM) of 12 500 for insulin, with the isotopes resolved nearly to the baseline, has been shown in a 6.4 m reflectron mass spectrometer.[4,6] Even in a small (1.2 m) linear mass analyzer a mass resolution of 4000 for peptides can be obtained.[4] For ions up to the 10 kDa range, limitations in mass resolution are determined instead by the time resolution limits of the detector and digitizer systems, especially in small instruments.

However, larger proteins exhibit an apparent drop in resolution. For cytochrome C resolution of 2000[7] and 1025[3] and for carbonic anhydrase a value of 840[4] have been reported. All authors state that an enhancement in mass resolution can only be obtained up to masses of 30 000 Da. Their straightforward explanation for the failure of the DE method in the higher-mass range is the peak broadening due to prompt (in a linear TOF) and prompt and metastable (in a reflection TOF) fragmentation as well as adduct ion formation. In the present work we demonstrate that in a small linear TOF mass analyzer (1.2 m) considerably better results can be achieved for proteins over the whole mass range up to 66 kDa by applying 'soft' delayed extraction conditions and 'soft' matrices.

EXPERIMENTAL

Mass spectrometry

All experiments have been carried out with a MALDI TOF mass spectrometer (Voyager-RP-DE, PerSeptive Biosystems, Framingham, MA, USA) in the liner positive ion mode. The total acceleration voltage was 25 kV; the voltage on the first grid and the delay time between ion production and extraction were adapted to the mass of the analyte. Between 20 and 30 single scans were accumulated for each spectrum.

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Computer simulations of protein signals, including their isotopic pattern folded with an instrumental resolution, were performed using IsoPro 2.1 software (M. W. Senko, National High Magnetic Field Laboratory, Tallahassee, FL, USA). It is stressed that signals shown in the paper are all accumulated from a larger number of shots without directed selection of spectra and are usually registered considerably above threshold irradiance. They have all been processed in the instrumental software (GRAMS/386) by at least five-point, but usually 19-point (for larger proteins), Sawiztky–Golay smoothing; thereby, accidentally occurring non-significant very-high-resolution values are not taken into consideration.

Materials

Matrix materials were used without further purification. Sinapinic acid (Aldrich) was dissolved at a concentration of 20 g l^{-1} in a mixture of 1:1 acetonitrile/0.1% aqueous TFA. DHBs, a 9:1 mixture of 2,5-dihydroxybenzoic acid (Fluka) and 2-hydroxy-5-methoxybenzoic acid (Aldrich), was used at 20 g l^{-1} in 1:2 acetonitrile/0.1% aqueous TFA. Proteins–insulin (human; Sigma), myoglobin (horse; Serva), carbonic anhydrase (Boehringer Mannheim), ovalbumin (Sigma), α-amylase (bacillus species; Sigma) and bovine serum albumin (Sigma)—were used without further purification. Samples were dissolved in 1:1 acetonitrile/0.1% aqueous TFA to a final concentration of 10^{-5} M; 0.5 μl of sample and 1 μl of matrix were mixed together on the sample plate and dried in a stream of air.

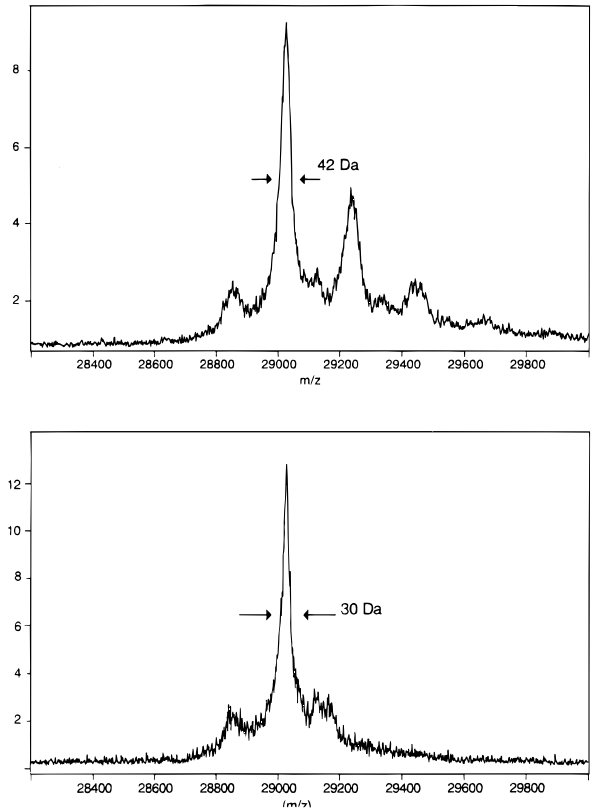

Figure 1. DE-MALDI mass spectra of carbonic anhydrase using hard extraction conditions (900 V mm^{-1}): A, matrix sinapinic acid, delay time 300 ns, 53 spectra averaged, FWHM resolution 700; B, matrix DHBs, delay time 250 ns, 30 spectra averaged, FWHM resolution 960.

RESULTS AND DISCUSSION

For the following work, which focuses on proteins in the mass range above 25 kDa, the 'classical' protein matrices sinapinic acid and DHBs were used. Carbonic anhydrase (CAH, MW 29 024.6 Da) was the first case inspected (Fig. 1). The voltage of the first grid was set to 91% of the total acceleration voltage (corresponding to an electric field strength of about 900 V mm^{-1} in the first gap) and the delay time was adapted to yield the highest mass resolution possible. As expected owing to the measured and reported initial velocity differences of different matrices,[8] the optimized delay time for CAH with sinapinic acid is higher than with DHBs (300 *vs.* 250 ns). Nevertheless, even under optimized delay time the mass resolution determined from the peak width for carbonic anhydrase with sinapinic acid is only about 700 (Fig. 1A) compared with 960 with DHBs (Fig. 1B).

As reported in a previous paper,[9] (metastable) fragmentation is promoted even under DE conditions as a function of the initial extraction voltage and can only be reduced by substantial lowering of the electric field; this indicates that collisional excitation is still not negligible. On one hand, linear TOF analysis would not be essentially affected by metastable fragmentations—at least as long as small neutrals are cleaved off—but collisional impediment of the acceleration will directly result in peak broadening. Starting from these considerations, long delay times inherently connected to lower extraction field strengths were inspected as well as the role of the matrix. On the other hand, delayed extraction will be strongly affected by matrix-dependent fast metastable fragmentation.[10,11]

In general, the drop in resolution apparently occurring once the 10 kDa limit has been passed should not come as a surprise, because the FWHM resolution required to at least partially resolve the isotopic masses of a 17 kDa protein would be 20 000 or more, or roughly 17 000 to obtain the isotopic envelope (peak width of 8 Da). In other words, two signals at mass 1000 and 1001 Da of equal height are only separated by a 92% valley for truly Gaussian peak shapes (and therefore not resolved in practice) at a mass resolution of 1000 if the FWHM (50% height) resolution definition is applied. If the mass resolution is determined from the FWHM, as typically done in the high-mass range, one implicitly assumes a monoisotopic signal. Therefore measured peak widths can only be used as a measure for the instrumental resolution as long as they are considerably larger than the natural width of the isotopic distribution. For the intermediate range, when signal widths are approaching these widths, deconvolution from the known isotopic pattern is needed in order to determine significant values. In the following we will use the terms 'FWHM resolution' and 'deconvoluted resolution' to differentiate between the two methods.

In a first step the protein standard myoglobin was reinspected. For the apomyoglobin signal a minimum peak width of 9.6 Da is obtained under optimized con-

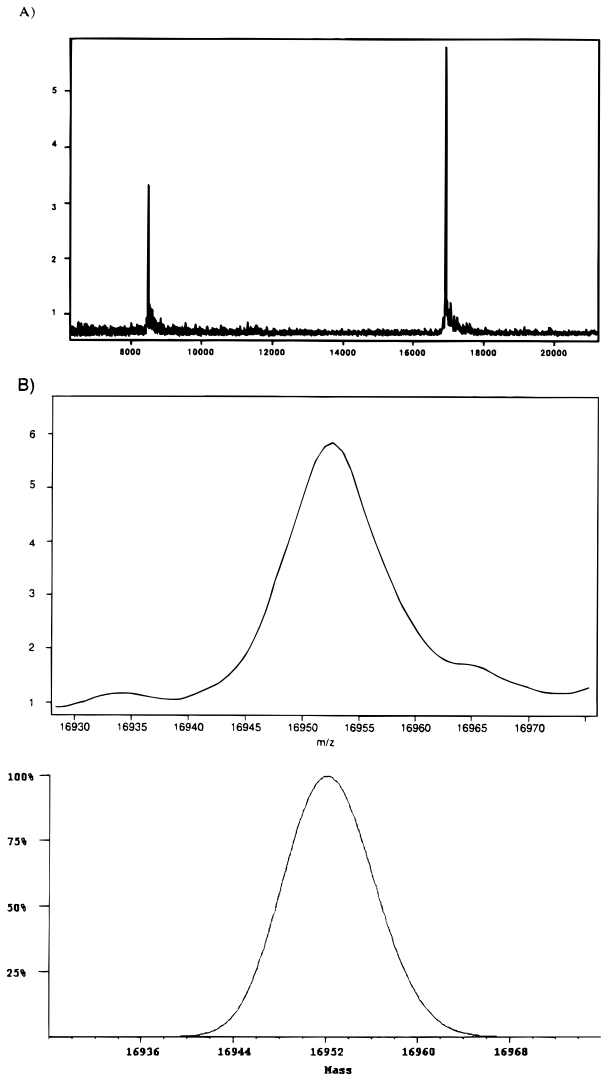

Figure 2. DE-MALDI mass spectrum of apomyoglobin, matrix DHBs, delay time 550 ns, grid voltage 94.5%, 35 spectra averaged: A, overview; B, singly charged molecular ion peak from A showing a width at half-maximum of 9.6 Da; C, computer simulation of apomyoglobin with a resolution of 3500.

ditions. Figure 2A shows the molecular ion range and Fig. 2B only the singly charged molecular ion region. A decrease in extraction field strength to 500 V mm^{-1} (corresponding to 1375 V for the first extraction gap, i.e. 94.5% of the total acceleration voltage) has to be accounted for by a higher delay time, in this case 550 ns. By measuring the FWHM, a resolution of 1765 is calculated. Computer simulations, including the isotopic pattern, however, reveal that this peak width is only obtainable with an instrumental resolution of 3500 (Fig. 2C).

Using the above optimization approach, CAH was reinspected (Fig. 3). While no improvement was observed for the sinapinic acid matrix (Fig. 3A), 'soft' extraction and a delay time of 800 ns using DHBs yielded a considerable improvement, i.e. a peak width of 21 Da, corresponding to an FWHM resolution of nearly 1400 (Fig. 3B). Taking the natural isotopic distribution into account, a slightly higher deconvoluted resolution of 1600 is calculated. For these investigations, spectra with high signal intensities were chosen,

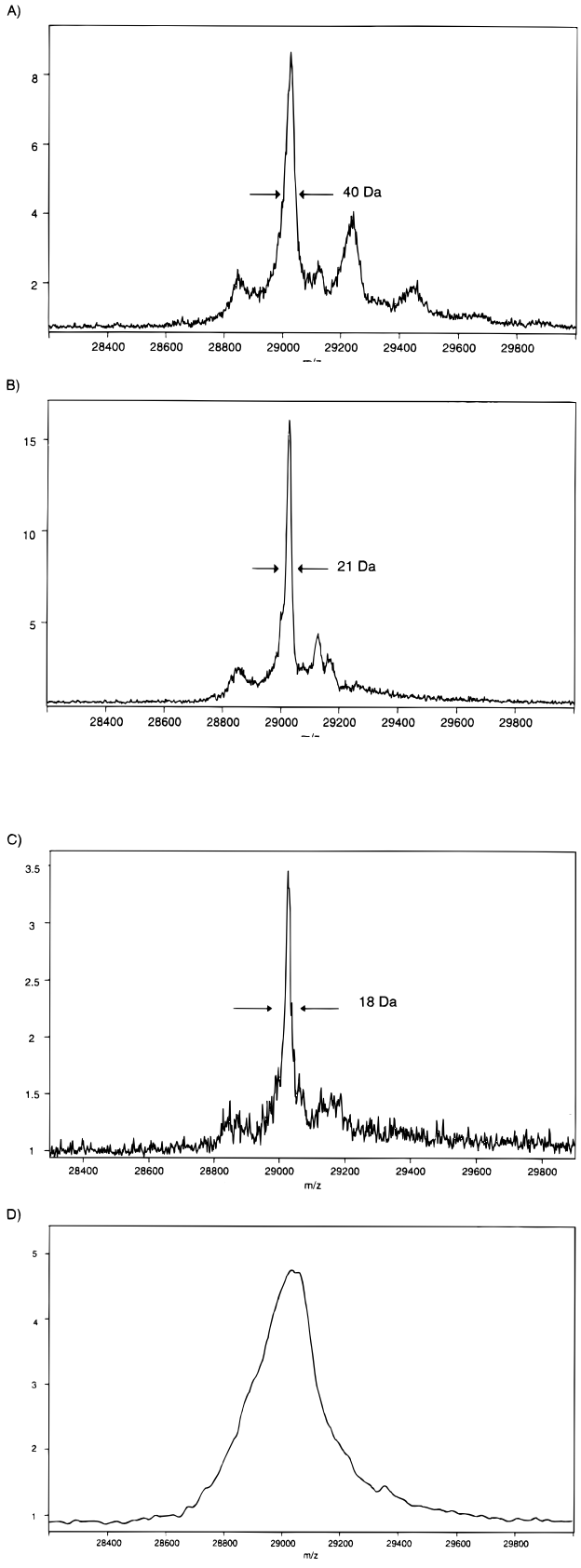

Figure 3. DE-MALDI mass spectra of carbonic anhydrase using soft extraction conditions (530 V mm^{-1}) and a delay time of 800 ns: A, matrix sinapinic acid, 55 spectra averaged, FWHM resolution 725; B, matrix DHBs, 40 spectra averaged, FWHM resolution 1400; C, matrix DHBs, laser irradiance a factor of two lower than in B, 39 spectra averaged, FWHM resolution 1600; D, matrix DHBs, without DE, 86 spectra averaged, FWHM resolution 125.

but still better resolution is obtained from signals registered near threshold. The spectrum of carbonic anhydrase in Fig. 3C is taken at low laser irradiance and the singly charged molecular ion signal has a peak width of 18 Da, corresponding to an FWHM resolution of 1600 or a deconvoluted resolution of about 2000. For comparison, the peak without application of DE (Fig. 3D) now has a mass resolution of only about 125. There is no drop in signal intensity or in S/N ratio by application of long delay times compared with short delay times; this is valid also for higher masses shown later.

The unsymmetrical peak shape clearly visible in the extension of the CAH spectrum with sinapinic acid (from Fig. 1A) shown in Fig. 4 reveals the major reason for the enhanced peak width: prompt, comparatively fast metastable fragmentations resulting in losses of one or two NH_3 or H_2O or other small neutrals such as CO_2. This agrees with earlier observations on the matrix dependence of metastable fragmentation and proves that also with respect to prompt, comparatively fast metastable fragmentation, DHBs is less critical. In addition, a long delay time and low acceleration field strength reduce flight time dispersion as a result of impeding collisions. Consequently, further investigations with increasing mass of the proteins were restricted to the DHBs matrix.

It is clear from the DHBs spectra that the next limitation with increasing mass is adduct ion formation. To investigate the origin and character of adduct and fragment ions, a spectrum of insulin with DHBs was recorded (Fig. 5). Some signals are clearly attributable, such as adduct ions at [M + 138 Da] and [M + 154 Da], as well as cationized species and loss of 44. Others cannot be rationalized in a straightforward way. With respect to the mechanism of matrix adduct ion formation, it is noteworthy that there is no simple explanation for this process, such as cluster formation between analyte ions and neutral matrix molecules or fragments. Whereas both $[M + 154]^{\bullet +}$ and $[M + 138]^{\bullet +}$ are odd-electron ions, $[M + 111]^+$ can be rationalized to be the adduct between MH^+ and a neutral decarboxylation product, i.e. 1,4-dihydroxybenzene, which does not show up in the ion spectrum. This points to complex photochemical processes occurring in the plume.

With increasing mass, peaks get broader as expected, but when using DHBs and long delay times, the degradation in mass resolution is much less steep than reported

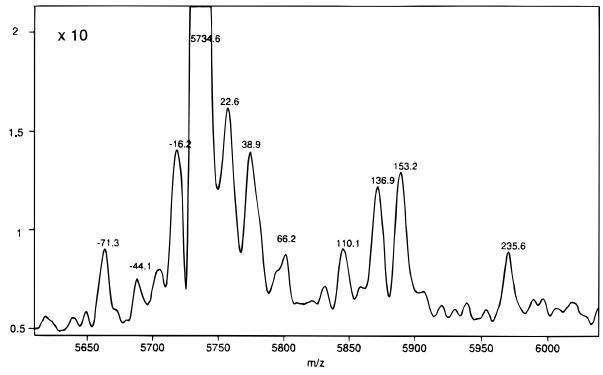

Figure 5. DE-MALDI mass spectrum of insulin showing fragment and adduct ions in molecular ion region. Ion signal intensities are blown up by a factor of 10.

before. Figure 6 shows the spectrum of α-amylase (from *Bacillus amyloliquefaciens*), which, besides sharp peaks of the protonated molecule and the adduct ion $[M + 138]^{\bullet +}$, exhibits increasing contributions of adduct ions which cannot be resolved from each other. The relatively broad peak of the matrix adduct signal compared with the molecular ion, as well as the fact that the valley between the protonated and the adduct ion is less pronounced than might be expected from the needle-like shape, indicates that there are several other adducts in between. One possible contribution could come from sulfuric or phosphoric acid adducts, as e.g. reported in ESI[12] measurements; indeed, for some samples an improvement was found after barium acetate precipitation (data not shown). Sodium- and potassium-attached molecular ions could also contribute, but a mass resolution of 10 000 would be necessary to at least partially resolve them at this mass. The protein mass determined from six measurements on different spots of the sample and by external calibration with CAH monomer and dimer amounts to 54 850 ± 9 Da, which is in good agreement with the mass 54 851 Da reported in the literature.[13] A mass resolution of 880 can be calculated from the peak width of 62 Da. Again, computer simulations reveal that the deconvoluted mass resolution manifested in this experiment is somewhat larger than that determined from the peak width. It should be mentioned here that the optimized values for delay time and first-gap voltage yield optimized mass resolution in a limited *m/z* window. For mass cali-

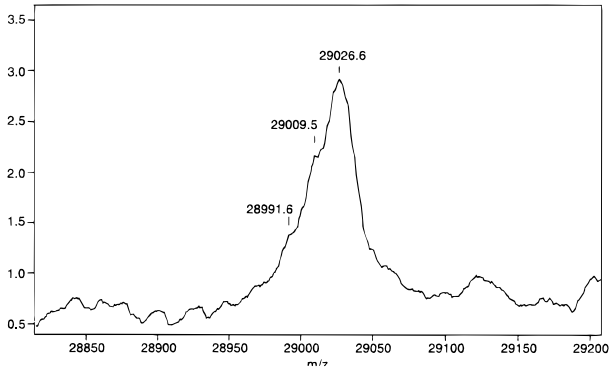

Figure 4. DE-MALDI mass spectrum of molecular ion region of carbonic anhydrase with sinapinic acid as matrix, conditions as in Fig. 3, 45 spectra averaged.

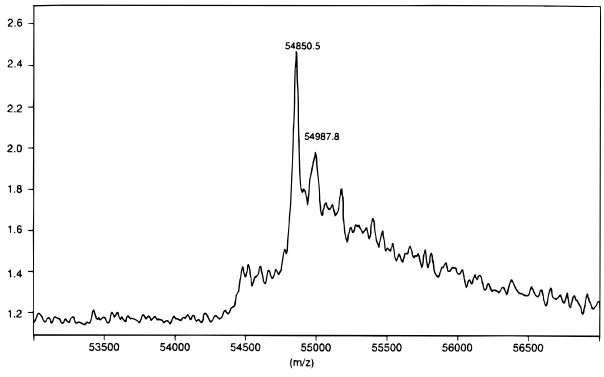

Figure 6. DE-MALDI mass spectrum of α-amylase, matrix DHBs, delay time 1050 ns, grid voltage 93.5%, 58 spectra averaged, FWHM resolution 880.

bration using standards with large differences in m/z values, a compromise between delay time and grid voltage has to be chosen. In the case of α-amylase a delay time of 800 ns and a first grid voltage of 93.5% of the acceleration voltage was chosen. Although the mass resolution for all masses of interest was not optimal under these conditions, the adducts could be clearly resolved from the molecular ion peak and the peak shape was sufficient to obtain good mass accuracy.

It should be stressed here that it is not the numerical value of resolution which is relevant but the fact that a distinct and high level of resolution is required for determination of protein heterogeneity. Moreover, the mass determination accuracy is strongly affected by MALDI signal spreads due to fragmentations, impurity adducts such as cations and matrix adducts. Also, inherently, the instrumental resolution requirements steadily increase with increasing mass. Even though DHBs is doubtless the actual matrix of choice, adduct ion formation, which unfortunately grows in intensity with the mass of the analyte, forms a major drawback, especially when samples are naturally heterogeneous. This can be seen in the case of bovine serum albumin (BSA). Figure 7A shows the MALDI spectrum, revealing two additional signals which cannot be explained by adduct ion formation alone. It is well known that BSA preparations, depending on the supplier, vary in purity and can contain greater or lesser quantities of protein modifications. In a recently published ESI mass spectrum of BSA[14] the sample was from the same supplier as the one used in this study) a second strong signal at $[MH + 107]^+$ was identified. Even though the instrumental resolution is not significantly lower in the DE-MALDI case, the second component is not resolvable in the MALDI mass spectrum because it mixes with the 138 matrix adduct ion to give a composite signal at $[MH + 127]^+$. This also means that BSA can only be used as a calibrant for the high-mass range if the true BSA molecular ion signal is at least partially resolved from adducts or modified components as obtained with the experimental parameters described above. For comparison, the MALDI spectrum of BSA without delayed extraction is shown in Fig. 7B. Reliable mass determination may nevertheless be achievable also in the higher-mass range for homogeneous samples, even for non-resolved adduct ions, if one assumes that both calibrant and sample exhibit the same average shift to higher masses by adduct ions. The problem that adduct ions deteriorate mass separation also occurs for chicken egg ovalbumin, where only part of the glycan heterogeneity is resolved in the MALDI mass spectrum (Fig. 8), but the majority is obscured by matrix adduct ions, thus resulting (unfortunately) in considerably inferior practical resolution, as e.g. shown for ESI in Ref. 15.

One additional feature that is obvious in all the mass spectra we have shown, especially at increasing mass, is the broad unresolved peak component at lower mass. Moreover, it appears especially for amylase and BSA that the signals of the intact proteins are sitting on a broad hill extending over nearly the whole mass range which the non-DE signal would cover (see Fig. 7). It is suggested that this arises from the formation of larger fragments produced before and during acceleration, as well as by decay of larger adduct ions again within the early acceleration phase. The latter could be regarded as a not fully declustered/desolvated molecular ion. Even though this broad hill gives some signal deterioration, it does not appear to be a limiting factor for the mass determinations undertaken.

The precision achieved in the DE mode is considerably better than with continuous extraction and amounts to ± 50 ppm for successive measurements from one laser spot and to ± 200 ppm over the complete crystalline rim of the DHBs preparation; presumably the latter is due to the different heights of the DHBs rim. This precision is obtained over the complete useful laser irradiance range from threshold to irradiance values when the molecular ion intensities start to decrease. Mass calibration was performed either externally from a second spot or internally using the masses

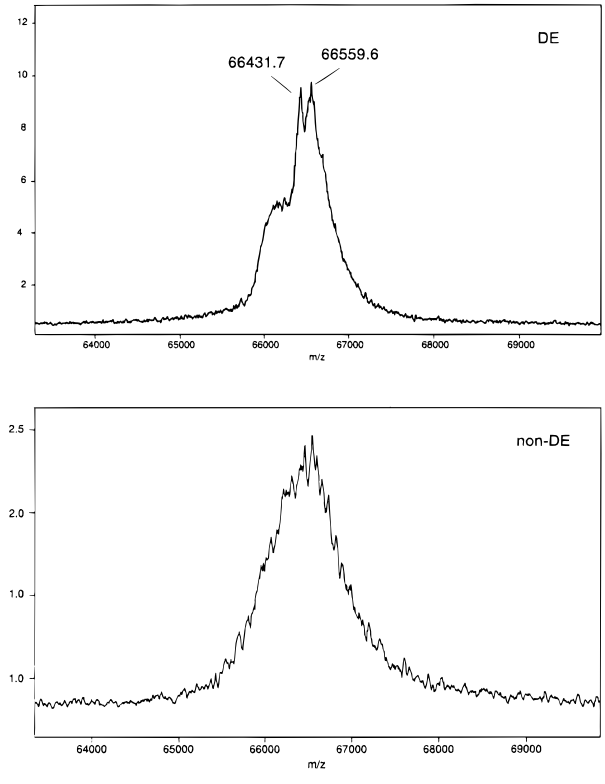

Figure 7. DE-MALDI mass spectra of bovine serum albumin, matrix DHBs: A, delay time 950 ns, grid voltage 92.5%, 54 spectra averaged; B, without DE, 71 spectra averaged.

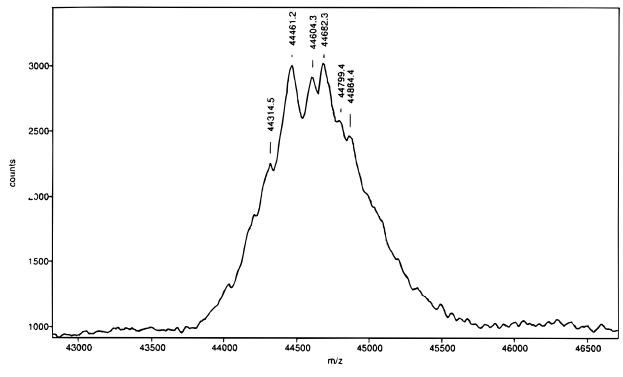

Figure 8. DE-MALDI mass spectrum of ovalbumin, matrix DHBs, delay time 700 ns, grid voltage 92%, 93 spectra averaged.

of the monomer and dimer of a calibrant. The mass determination accuracy even for a two-point external calibration for masses greater than 25 000 Da reaches the 100 ppm level. For internal calibration a mass accuracy and precision of less than 0.5 Da for carbonic anhydrase was obtained using the monomer and dimer ions of apomyoglobin. In this case the delay time and grid voltage optimized for carbonic anhydrase were used, resulting in a decreased mass resolution of 700 for the monomer of apomyoglobin, which is, however, still sufficiently high for high mass accuracy.

The reported data prove that the mass resolution in DE-MALDI-TOF is indeed not dropping steeply in the mass range above 20 kDa if extraction conditions and the matrix are optimized. Rather, a gradual slow decrease in resolution is found, as expected theoretically from the increase in energy spread of the analyte ions for constant initial average velocity and velocity spread. Numerical simulations using the simplest assumptions (normal emission of ions into the acceleration region from a flat surface without any collisions, with an initial average velocity of 500 m s^{-1} and a spread of ± 250 m s^{-1}) yield a theoretical resolution for the instrumental configuration used which is only roughly a factor of two better than that actually measured (see Fig. 9). In addition, these numerical calculations deliver some theoretical limitations for practical optimization. When the mass resolution is calculated and plotted as a function of the voltage in the first gap, a steep drop is exhibited for first-gap voltages that are too small. This drop is mass-dependent and occurs earlier for larger masses, which agrees with the experimental finding that shorter delay times and thus slightly larger first-gap voltages are required e.g. for BSA in comparison with amylase. This effect an be rationalized by the increasing contribution of the initial energy and energy spread of larger ions.

The impression of a steep drop in mass resolution is caused by the broad signals observed, which result mainly from fragmentation, cationization and matrix-related or other adducts. Whereas fragmentation appears to be no severe problem for a DHBs matrix and a considerable improvement is obtainable under optimized DE conditions, i.e. relatively long delay times and related low acceleration fields, matrix adducts still form a major drawback. Moreover, the results substantiate the belief that calibration requires carefully chosen calibrants to avoid severe faults because of natural sample heterogeneity (see BSA). Even though DHBs forms the best compromise in matrix properties actually known, further progress in matrix choice and preparation protocols is needed to fully use the advantages of time-of-flight measurements using delayed extraction.

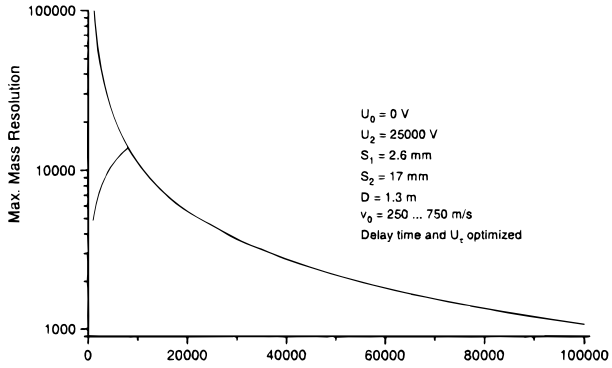

Figure 9. Numerical simulation of maximum mass resolution as a function of mass (delay time and extraction voltages optimized) for instrumental configuration and starting conditions given on graph. The second decay line refers to the case where detector and digitizing limitations of 2 ns become decisive.

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