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3. Experimental

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For the hydrolysis, this salt is stirred in 100 ml of AcOEt and 50 ml of 2N HCl. After extraction with AcOEt, the org. layer is washed with H_2O (2 × 100 ml), dried ($MgSO_4$), and evaporated. Recrystallization from Et_2O/pentane (30/100 ml) and drying (0.2 mbar/100°) yield 3.40–4.03 g (27–32% overall from *rac*-**5b/6b**) colourless **5b**. M.p. = 161°. $[\alpha]_{589}^{20}$ = –67.1; ee > 99.9% (determined by GC analysis on a permethylated β-cyclodextrine phase after methylation by CH_2N_2 (reaction time 10 min, r.t.) and acetylation to the arylacetate-methylester).

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Chimia 45 (1991) 123–126

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Martin Schär*, K. Olaf Börnsen, Ernst Gassmann, and **H. Michael Widmer**

Abstract. The potential of matrix-assisted laser desorption and ionization mass spectrometry (LDI-MS) is demonstrated by monitoring and analyzing the digestion of (human) pTH (1–34), a synthetic peptide with carboxypeptidases Y and B. All occurring ion signals in the mass spectra could be identified as degraded peptides. By calculating the mass differences between successive degraded peptides, it was possible to identify the released amino acids and to determine 8 amino acids of the C-terminus of the original peptide. For a single MS measurement, only 2 pmol of substrate was needed. Time-course analysis of the cleavage of the first amino acid residue gave insight into the kinetics involved. These measurements strongly support the hope that quantitative information about concentrations can be extracted from LDI-MS.

1. Introduction

The determination of the amino-acid sequence in peptides and proteins is a major requirement in biomedical and biochemical research. With the stepwise *Edman* degradation of the N-terminus, this information can be obtained. This method is usually slow, it needs pure samples and can be impaired by chemical contaminations. Further N-terminal blocking groups can be a severe obstacle for the degradation process. With the development of new mass spectrometric and ionization methods as fast-atom-bombardment mass spectrometry (FAB-MS) [1], plasma-desorption mass spectrometry (PD-MS) [2][3], electrospray mass spectrometry (ES-MS) [4–6], and recently matrix-assisted laser desorption and ionization mass spectrometry (LDI-MS) [7–11], novel analytical tools have become available. They offer interesting opportunities for peptide

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Abstract. The potential of matrix-assisted laser desorption and ionization mass spectrometry (LDI-MS) is demonstrated by monitoring and analyzing the digestion of (human) pTH (1–34), a synthetic peptide with carboxypeptidases Y and B. All occurring ion signals in the mass spectra could be identified as degraded peptides. By calculating the mass differences between successive degraded peptides, it was possible to identify the released amino acids and to determine 8 amino acids of the C-terminus of the original peptide. For a single MS measurement, only 2 pmol of substrate was needed. Time-course analysis of the cleavage of the first amino acid residue gave insight into the kinetics involved. These measurements strongly support the hope that quantitative information about concentrations can be extracted from LDI-MS.

1. Introduction

The determination of the amino-acid sequence in peptides and proteins is a major requirement in biomedical and biochemical research. With the stepwise *Edman* degradation of the N-terminus, this information can be obtained. This method is usually slow, it needs pure samples and can be impaired by chemical contaminations. Further N-terminal blocking groups can be a severe obstacle for the degradation process. With the development of new mass spectrometric and ionization methods as fast-atom-bombardment mass spectrometry (FAB-MS) [1], plasma-desorption mass spectrometry (PD-MS) [2][3], electrospray mass spectrometry (ES-MS) [4–6], and recently matrix-assisted laser desorption and ionization mass spectrometry (LDI-MS) [7–11], novel analytical tools have become available. They offer interesting opportunities for peptide

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Central Analytical Department
Ciba-Geigy Ltd.
CH-4002 Basel

analysis as sequencing and identification of post-translational modifications.

Due to the low selectivity of LDI-MS measurements, the high sensitivity, a dynamic range of at least 1:100, the complete absence of fragments in the mass spectra and the negligible amount of doubly or higher charged particles, this mass spectrometric method is especially well suited for the measurement of complex protein and peptide mixtures as they occur in enzymatic digestions.

The analysis of a carboxypeptidase digestion is done by the mass spectrometric identification of the degraded peptides. Instead of determining chemically the released amino acids, the mass differences between successive degraded peptides are determined, and based on these residual masses the released amino acids can be identified. As long as the accuracy of the residual mass determination is better than 1 Da, it is theoretically possible to identify and distinguish unambiguously all primary amino acids except Leu and Ile (same mass) and Gln and Lys (mass difference 0.04 Da). Gln and Lys must be chemically derivatized by acetylation of the primary amino group to be distinguished by mass spectrometric methods.

With LDI-MS, a typical accuracy for the mass determination of ±0.05% can be achieved. Thus, the absolute error for a peptide with mass > 2000 Da would be larger than 1 Da. For the identification of the released amino acids, the difference of the masses is important. If the truncated peptides were measured in the same measurement the determined mass should have at least the same systematical error which is cancelled out in the calculation of the released masses. Nevertheless the statistical errors have to be added for the calculation of the residual mass which usually increases the error and, therefore, leads to a greater uncertainty of the amino-acid determination. If more than one measurement is needed for the sequence determination the time-to-mass conversion has to be performed in a consistent way, *i.e.* using the same peptide signals as standards for all calibrations. However, the accuracy of the mass determination defines the limit for the accuracy of the sequence determination of peptides.

Since the work by *Hayashi et al.* [12] and *Kuhn et al.*[13], carboxypepdidases have been often used in the analysis of carboxy-terminal amino-acid sequences in polypeptide substrates. Carboxypeptidase Y from yeast has a broad specifity and can cleave most primary amino acids including Pro except His, Arg or Lys as C-terminal residues for which the cleavage is slow [14]. It offers a useful tool for structural studies of proteins and peptides.

The peptide used as a test substance for monitoring enzymatic digestion was (human) pTH (1–34), a synthetic linear polypeptide consisting of 34 amino acids without Cys. It has a molecular mass of 4117.8 Da, and the amino-acid sequence is:

Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Phe.

The goal of this paper is merely to show the applicability of LDI-MS for the analysis and monitoring of a C-terminal digestion of a known peptide with carboxypeptidases and to demonstrate how quickly and efficiently data on the amino-acid sequence can be obtained with this mass spectrometric method.

2. Experimental

2.1. *Digestion*

Chemicals. Carboxypeptidase Y from yeast and B from porcine pancreas were obtained from *Carlsberg Biotechnology Inc.*, Denmark.

2.9 mg of the lyophilized substance containing 19.3% carboxypeptidase Y was diluted in 14 ml of deionized water and stored in portions at –20°.

Carboxypeptidase B was taken from an aqueous soln. (0.7 mg/0.5 ml) and was further diluted in deionized water to a concentration of $7 \cdot 10^{-7}$ M and stored at –20°.

1 mg of the lyophilized peptide (human) pTH (1–34) from *Bachem AG*, CH–4416 Bubendorf, was dissolved in 6.1 ml of 50 mM NaAc buffer, pH 5.8 and stored at –20°. For a soln. of pH 7.4 1 mg of the peptide was dissolved in 6.1 ml of 50 mM Tris (Tris(hydroxymethyl)aminomethane).

Digestion Procedure. 25 µl of the enzyme (16 pmol) and 12.5 µl of substrate (500 pmol) were mixed and incubated at 20° or 37°, respectively. After 1, 2, 4, 8, 15, 30, 60, and 120 min, aliquots of 2 µl were removed from the sample from the soln. and the digestion terminated by the addition of 4 µl of 0.1 M sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, see matrix preparation). These samples were frozen at –20°. For the analysis by LDI-MS, 0.5 µl of this soln. was used. For the LDI-MS measurement, the mixture could be directly added to the probe tip without further preparation.

2.2. *Mass Spectrometry with LDI-MS*

Principle. The method has been extensively described in several publications [7–11]. The key idea behind LDI-MS is to mix the analyte molecule with a light-sensitive small molecule. The large molar excess of these molecules leads to a statistical distribution of the analyte molecules in the matrix. With a short UV-laser pulse, energy is deposited in the matrix resulting in an explosive ejection of the top molecular layers of the solid matrix. Some of analyte molecules which are embedded in the matrix are desorbed together with the ejected matrix molecules and are either negatively or positively charged. They can then be easily analyzed in a time-of-flight mass spectrometer.

Matrix Preparation. The peptide in a 10^{-5} M aq. soln. is mixed with 0.1 M of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) from *Fluka*, Buchs, Switzerland and dissolved in 50% MeCN and 50% of a 90% aq. EtOH soln. with a ratio of *ca.* 1:1. Typically 0.5 µl of this mixture was added to the gold covered metallic probe tip. The solvents were either rapidly pumped off or evaporated at r.t. The rod with the probe tip was inserted into the vacuum system of a time-of-flight mass spectrometer.

Apparatus. The instrument is a 1.7-m linear time-of-flight mass spectrometer. It was built at *Ciba-Geigy* and has been described in detail [10]. Some details of the original system have been modified: the repeller plate is now on a constant potential of ±25 kV. This electric field of the 2-stage ion optic accelerates the produced ions which are then mass analyzed in the time-of-flight mass spectrometer. Further the Nd:YAG/Dye laser used in previous works [10][11] has been replaced by a N_2 laser with a wavelength of 337 nm, 2–6 µJ output energy and a pulse length of 3 nsec (*Laser Science Instruments, Inc.*, Cambridge, MA, USA). The pulsed laser beam is focussed with a quartz lens to an area of 300 × 600 µm on the solid matrix. The ion were detected with a tandem microchannel plate detector held at –3 kV and a transient recorder with a time resolution of 10 ns. The whole system was controlled by a PC *via* IEEE-Bus. New improved software for peak detection and calibration was used to increase the accuracy of the mass determination.

To enhance the signal-to-noise ratio, several single shots were summed for a mass spectrum. All spectra shown in this paper are original measurements without data treatment like smoothing or background subtraction. All measurements were made in the negative-ion mode where the ions were detected as deprotonated species.

Time-to-Mass Conversion. The conversion was done by using an internal standard of known mass as the photo-products of sinapinic acid in the lower mass range or a peptide of known mass. The peptide was added to the existing matrix on the probe tip. With this procedures, an accuracy of the molecular mass determination of typically ±0.05% was obtained. The calibration was also performed with strong ion signals identified as degraded peptides (see *Table*).

3. Results and Discussion

The abbreviations M–1, M–2... are standing for the original peptide which has lost 1, 2... amino acids. The notation (11–33) refers to the loss of one amino acid at the C-terminus and ten amino acids at the N-terminus.

Fig. 1 shows an example of a digestion of pTH (1–34) with carboxypeptidase Y. The sample was measured after 60 min digestion time at 37°. For the measurement, 0.5 µl (2 pmol) substance was used. It is important to point out that the sum of all amounts of the truncated peptides give 2 pmol. The concentration of the single degraded peptides in solution are only a fraction thereof. The lower limit of the sensitivity of LDI-MS measurements lies clearly in the fmol range.

The original peptide of mass 4117.8 Da could not be detected and has been completely digested. A series of ion signals of high intensity can be detected between 3000 and 4000 Da. In the lower mass range, several peaks appear and can be identified as either C-terminally degraded peptides or as peptide fragments which are truncated from both the C- and the N-terminus. The increase of the background towards the lower masses is due to the strong ion signals of the sinapinic acid. The unwanted low-mass matrix molecule and photo-adduct ions can be drawn out by an electric field which is applied perpendicular to the flight direction while these ions are flying by. The small peaks with masses M + 61 accompanying the intense ion signals above 3000 Da origin from photo-adducts of the sinapinic acid. Although these photo-adducts cannot be completely avoided, they can be clearly distinguished from the actual peptide signals and represent no problem for the analysis of LDI-MS measurements.

The intense ion signals can be identified as the truncated peptides which have lost up to eight amino acids of the C-terminus. The identification of the cleaved residues leads to the identification of the C-terminal se-

Table. *Sequence Determination of pTH (1–34) with Carboxypeptidase Digestion* (amino acids are abbreviated by their one-letter code). Digestion conditions: enzyme Y, molar ratio [E]:[S]=1:30, T:37°, pH:5.8, digestion time: 60 min.

Peptide	Sequence	M_r [Da] calc.	M_r [Da] exp.	Δ [Da]
	1 10 20			
M	SVSEIQLMHNLGKHLNSMER	4117.80	–	–
	21 30 34			
	VEWLRKKLQDVHNF			
M–1	...NLGKHLNSMERVEWLRKKLQDVHN	3970.62	3970.62	0[a])
M–2	...NLGKHLNSMERVEWLRKKLQDVH	3856.51	3856.71	–0.20
M–3	...NLGKHLNSMERVEWLRKKLQDV	3719.37	3720.42	–1.05[b])
M–4	...NLGKHLNSMERVEWLRKKLQD	3620.24	3620.24	0[a])
M–5	...NLGKHLNSMERVEWLRKKLQ	3505.15	3506.25	–1.10
M–6	...NLGKHLNSMERVEWLRKKL	3377.02	3377.74	–0.72
M–7	...NLGKHLNSMERVEWLRKK	3263.86	3265.04	–1.18
M–8	...NLGKHLNSMERVEWLRK	3135.68	3137.00	–1.32
M–13	...NLGKHLNSMERV	2422.82	2422.41	0.41
M–17	...NLGKHLNS	1907.19	1907.90	–0.71
M–19	...NLGKHL	1706.01	1706.90	–0.89
M–20	...NLGKH	1592.85	1593.20	–0.35

[a]) Ion signals used for mass calibration.
[b]) Peptide M–3 was detected with different digestion conditions. See *Fig. 1b*.

quence: -Lys-Leu-Gln-Asp-Val-His-Asn-Phe with the already mentioned ambiguity of the assignment of Leu/Ile and Gln/Lys.

Protein M–5 is very low in intensity and an ion signal with reasonable signal-to-noise ratio was only detected between 8 and 15 min digestion time (not shown). This points out the necessity of a time-course digestion in order to detect all degraded peptides which can be digested with the chosen enzyme.

However, it was not possible to detect the ion signal of peptide M–3 with an enzymatic digestion with carboxypeptidase Y even with a molar enzyme to substrate ratio of less than 1:100 and T = 20°. It could be detected with a mixture of carboxypeptidase Y and carboxypeptidase B. This enzyme has a different specificity. It cleaves only the basic amino acids.The inset *b*) in *Fig. 1* was measured with a mixture of carboxypeptidase Y and B at a pH of 7.4. The hydrolysis rates for this enzyme mixture under the chosen digestion conditions were different from the rates of measurement shown in *Fig. 1a* and allowed to detect the ion of the truncated peptide M–3. This is a general problem of the enzymatic digestion to detect peptides with high hydrolysis rates which follow peptides with low rates and high concentrations. This is the case for the peptides M–5 and M–3. Carboxypeptidase Y releases Asn slowly while the cleavage of His is fast. The concentration of M–1 remains therefore high and the peptides M–2 and especially M–3 are significantly lower in concentration and the detectable ion signals are thus very weak. Asp is also only slowly released and the ion signal of M–4 is, therefore, rather intense. The slow release of Asp again is, together with the fast release of Gln, responsible for the low ion signal of the M–5 peptide. The series of intense signals ends with the peptide M–7. The C-terminal sequence -Arg-Lys-Lys of M–7 can only be very slowly digested by carboxypeptidase Y even under the chosen high molar enzyme: substrate ratio of 1:30. Although the basicity the tripeptide Arg-Lys-Lys almost prevents further digestion, the peptides M–13, M–17, M–19, and M–20 can be detected. The low ion signal of petide M–13 is followed by an only partially resolved second ion signal whose molecular mass is 13.4 Da higher. This ion signal cannot be easily assigned.

Because of the strong ions signals from the sinapinic acid matrix, no further truncated peptides in the mass region below 1500 Da could be identified. With a different matrix molecule as 2,5-dihydroxybenzoic acid it is possible to measure analyte molecule ions down to 600 Da without matrix signal interference. For this work, sinapinic acid was chosen as matrix molecule because of its low sublimation rate, high peptide yield and acceptable small amounts of photoadducts.

The gap between the peptides M–7 and M–17 is due to the very low concentration of these peptides caused by the slow cleavage of the basic tripeptide Arg-Lys-Lys. With adjusted digestion conditions and the use of a different enzyme it is possible to detect degraded peptides below M–8 (data shown elsewhere [15]). The three ion signals originating from the peptide fragments 11–27 (Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys), 12–30 (Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp), and 12–33 (Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn) may stem from an endoproteinase impurity. Because of the high concentration of M–1 (1–33), M–7 (1–27), and M–4 (1–30), the ions of the doubly truncated peptides have still enough intensity to be detected.

The *Table* summarizes the results of the measurement shown in *Fig. 1*. Only C-terminally degraded peptides are listed. Note

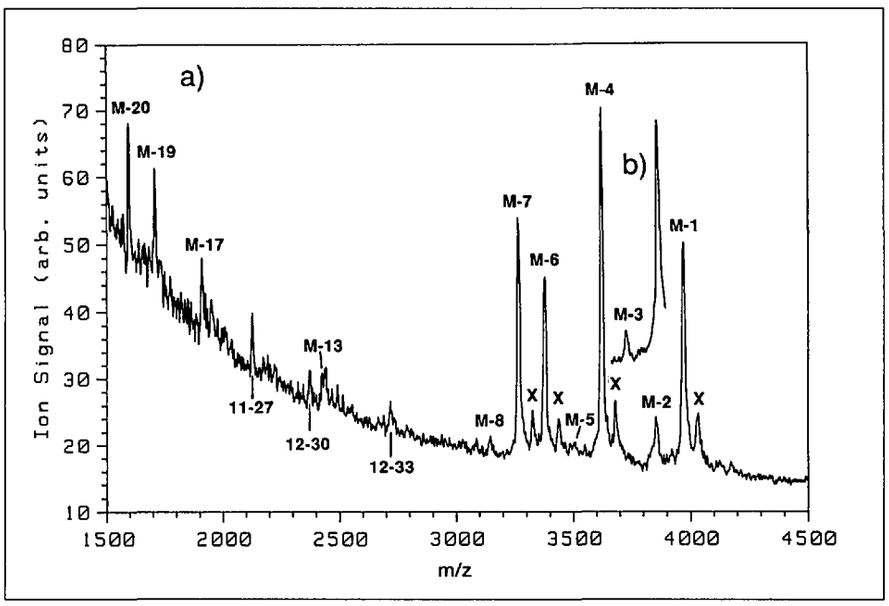

Fig. 1. a) *Negative-ion LDI-MS of pTH (1-34) digested with carboxypeptidase Y after 1 h.* 2 pmol of substrate was used. 2 µl of the substrate soln. was mixed with 4µl of 0.1 M sinapinic acid mixture. Conditions of the digestion: molar ratio [E]:[S] = 1:30, *T* = 37°, 50 mM of sodium acetate buffer, pH = 5.8. 20 shots were summed. No background subtraction was performed. b) *Negative-ion LDI-MS of pTH (1–34) digested with with carboxypeptidases Y and B in 50 mM Tris, pH 7.4 at 37°.* Digestion time was 120 min. Molar ratio [E_Y]:[S]=1:60, CPB [E_B]:[S]=1:60. 30 single shots were summed for this spectrum. No background subtraction. X: Adduct from sinapinic acid.

CHIMIA 45 (1991) Nr. 4 (April)

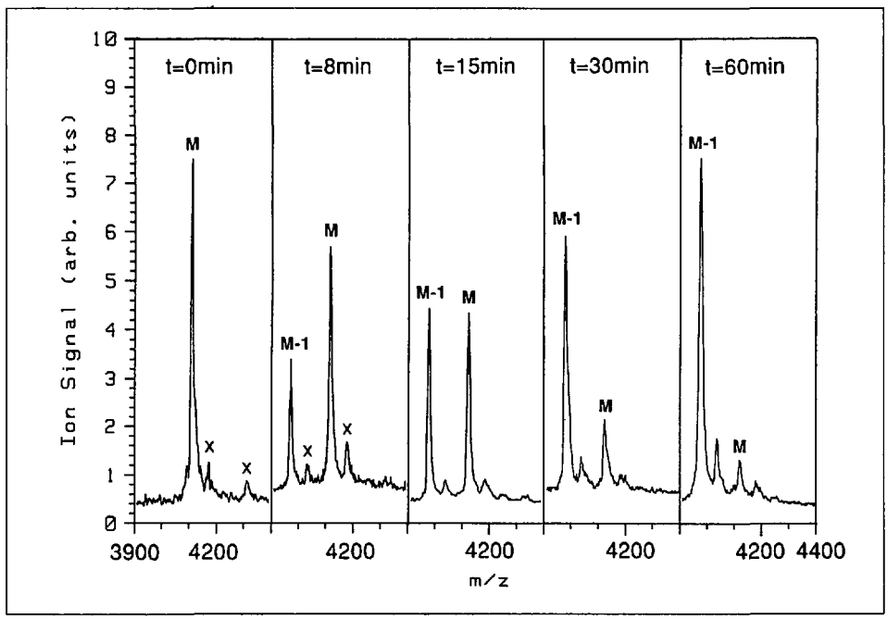

Fig. 2. *Negative-ion LDI-MS of a digestion of pTH (1–34) with carboxypeptidases Y and B in 50 mM Tris, pH 7.4 at 20°.* Molar ratio [E_{CPY}]:[S] = 1:60, CPB [E_{CPB}]:[S] = 1:60. 20 single shots were summed for each spectrum. No background subtraction. x: Adduct from sinapinic acid. Occurring in all spectra but denoted only in the spectra taken at t = 0 and t = 8 min.

that the error in mass determination is generally better than the typical value of ±0.05%. Although some Δ values are above 1 Da it is still possible to identify the released amino acids and to reveal the first eight amino acids from the C-terminal of the original peptide [15] within the limitations given for mass spectrometric analysis.

Fig. 2 shows 5 LDI-MS measurements in the mass range from 3900 to 4400 Da of a time-course analysis of an enzymatic digestion of pTH (1–34) where aliquots of 2 µl were withdrawn prior to the adding of enzyme, t = 0 min, after 8, 15 min, 30, and 60, stopped with 4 µl 0.1M sinapinic acid and analyzed by LDI-MS. The digestion was carried out with a mixture of carboxypeptidases Y and B at 20° and pH 7.4. This digestion conditions were used to decrease the cleavage rate for Phe, the first amino acid at the C-terminus and to monitor the release with respect to digestion time. The digestion with carboxypeptidase Y at pH 5.8 of the aromatic Phe was too rapid to be monitored. Carboxypeptidase Y is responsible for the cleavage of Phe and the decrease in the hydrolysis rate of the -Asn-Phe might be due to the lower activity of carboxypeptidase Y at the higher pH value.

The measurements show the original peptide of mass 4117.8 Da and the truncated peptide M–1 of mass 3970.62 Da which has lost Phe. Again the mass at M+61 Da which origins from an attached sinapinic acid residue can be detected in all measurements. A further photo-adduct at M+206 Da can be detected in the spectrum taken at t = 0 min. The production of this adduct can be controlled by the laser power and concentration of the petide in solution but it cannot be completely suppressed. The measurements in *Fig.2* give insight into the kinetics of the enzymatic cleavage of this particular peptide bond. The smooth change of the M and M–1 ion signal intensities as a function of time is, furthermore, a strong hint that the ion intensities of an LDI-MS measurements could be used to obtain at least semi-quantitative information about analyte concentrations. The reproducibility of the solid matrix for the LDI-MS measurement is crucial for any quantification. The crystallization process of the matrix has, therefore, to be controlled and carried out under the same conditions. Nevertheless, it is known that very strong ion signals are often underrepresented and that molecules with different tertiary structures give ion signal intensities which do not represent the actual concentration ratio in the matrix. Quantification of LDI-MS is still a difficult task and further investigations are under way in our laboratory.

4. Conclusion

In this paper, it was shown that LDI-MS is a useful tool for monitoring and analyzing peptide digestions. With the mass spectrometric information it is possible to identify the released amino acids for rapid identification and confirmation of the C-terminal amino-acid sequence of peptides in very short time and almost no chemical expenditure.

The mass spectrometric method is completely unimpaired by amino-acid contaminations of the sample solution. Therefore, the ratio of substrate/enzyme can be chosen in a wide range allowing rapid digestions. The conditions of a digestion can be adjusted almost 'On Line' with the information obtained from LDI-MS measurements. The time needed for a LDI-MS measurement (including sample preparation, measurements, and calibration) takes only a few min. The sensitivity of the method is high: for a complete digestion 500 pmol substrate was used. This amount is mainly dependent on the number of aliquots which must be withdrawn during a digestion. A single LDI-MS measurement can be performed with as little as 2 pmol total amount of peptide.

A necessary prerequisite for mass spectrometric amino-acid determination is that the error of the mass differences between measured degraded peptides must be below 1 Da. This allows order to distinguish all 20 primary amino acids except Leu/Ile and Gln/Lys which cannot be identified by mass spectrometric means alone.

The problems occurring during the analysis of a digestion with LDI-MS are of chemical or biochemical nature and not of the mass spectrometric method. Problems as significant differences in the hydrolysis rates of different peptide bonds or amino-acid sequences which cannot be degraded can be solved with the use of different enzymes and adjusted digestion conditions. Together with the results obtained with LDI-MS it is possible to solve sequencing problems faster and more reliable than it was possible with standard methods like the N-terminal *Edman* degradation.

LDI-MS measurements are straight-forward. Our instrument can be operated and the results interpreted by any untrained person in every biochemistry laboratory. Thus, the main effort can be concentrated on the chemical problems.

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