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Charge Derivatization of Peptides for Subsequent Analysis by Matrix-Assisted Laser Desorption/ Ionization Mass Spectrometry

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John Allison

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CHARGE DERIVATIZATION OF PEPTIDES FOR SUBSEQUENT ANALYSIS BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY

By

Rui Lu

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ABSTRACT

CHARGE DERIVATIZATION OF PEPTIDES FOR SUBSEQUENT ANALYSIS BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY

By

Rui Lu

Our research goals are to analyze polypeptides using 2D-polyacrylamide gel electrophoresis with matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. After a mixture of polypeptides is separated, analytes are frequently digested and electroblotted, resulting in complex mixtures in specific locations on a membrane. If the membrane can be prepared for direct MALDI analysis, all components of the mixture are usually not detected, certainly not detected with equal response. Charge derivatization, attaching a fixed positive charge to the N-terminus of peptides, can increase the number of components detected and can provide useful MS/MS spectra. We are investigating formation of charged-derivatives of peptides on membranes. Our approach is to use liquid phase or gas phase reagents to react with peptides on membranes to form charged-derivatives. With gas phase reagents, without solvents, spots on membranes or gels will not spread. The gas phase reagents react with single peptides, and mixtures of up to ten peptides on a polyvinylidene difluoride membrane (PVDF). The trimethylamine acetyl derivatives formed have increased response in MALDI. This charge derivation method can be used to detect peptides for which a peak is not formed, or peptides whose concentrations are too low to be accurately detected by MALDI.

Dedicated to my family

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CI	Chemical ionization
CID	Collision-induced dissociation
CIF	Charge-induced fragmentation
CRF	Charge-remote fragmentation
C-terminus	Carboxyl-terminus of peptides
DCC	dicyclohexylcarbodiimide
DMAA	dimethylalkylammonium
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
ESI	Electrospray ionization
FAB	Fast-atom bombardment
HPLC	High-performance liquid chromatography
KE	Kinetic energy
L	Linker ($-CH_2CO-$)
⊕-L-M	Trimethylamine acetyl charged-derivative of the molecule
⊕′-L-M	Dimethylamine acetyl charged-derivative of the molecule
m/z	Mass-to-charge ratio value
MALDI-MS	Matrix-assisted laser desorption/ionization mass spectrometry
MES	2-(N-morpholino)ethane sulfonic acid
$[M+H]^+$	Protonated molecule
MS/MS	Tandem mass spectrometry
NHS	N-hydroxysuccinimide

Sulfo-NHS	N-hydroxysulfosuccinimide
NMPA	N-methylpyridinium acetyl
N-terminus	Amino-terminus of peptides
PE	Polyethylene
PSD	Post-source decay
PU	Polyurethane
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TMAA	Trimethylamino acetyl
TMPB	trimethylpyridiniumbutyl
$TMPP^{+}$-Ac	Tris-(2,4,6-trimethoxyphenyl)phosphonium acetyl
TOF	Time-of-flight

CHAPTER ONE. INTRODUCTION

In the early days of mass spectrometry analysis, mass spectrometry was principally used to determine the exact masses and relative abundances of the elements and their isotopes [1-2]. In the 1940s and 1950s, mass spectrometry analysis was directed to organic compound identification. The available ionization methods mainly electron ionization (EI), chemical ionization (CI) [3] and field ionization (FI) [4], require thermal evaporation of the analyte, thereby limiting mass spectrometry to the analysis of low molecular weight and thermally stable compounds. In the last two decades, the characterization of peptides, proteins and other biomolecules encountered in biotechnology launched the application of mass spectrometry to large and often thermally liable bioorganic molecules. The extension of mass spectrometry for the identification and structural determination of large biomolecules became possible with the development of various desorption ionization methods [5-8], which are capable of producing ions directly from the condensed phase.

Pulses of laser light have been employed in an ion source to produce intact gas phase peptide ions from solid samples [7]. However, the mass spectral quality depended critically on the specific physical properties of the biological compound (e.g. photoabsorption, volatility) under study [9-10]. This situation is changed dramatically with the development of matrix-assisted laser desorption/ionization (MALDI) in 1988 by Karas and Hillenkamp [11].

MALDI-MS provides the means to volatilize peptides and proteins readily and to make the condition for volatilization largely independent of the physical properties of the sample molecules. In this technique, the samples are prepared by cocrystallization of the analyte with a small molecular weight, UV absorbing organic matrix, on a sample probe target. Ionization is triggered by a UV laser. Mass analysis is done by a time-of-flight (TOF) instrument.

MALDI-MS TOF is versatile and effective for the analysis of peptides and proteins because of the properties and capabilities of the technique. Chapter two will discuss the instrumentation of a MALDI-MS TOF experiment, introducing the reader to MALDI operating principles, sample preparation, functionality of the reflectron, and post-source decay techniques for peptide structural analysis.

In protein analysis, after a mixture of polypeptides is separated, analytes are frequently digested and electroblotted, resulting in complex mixtures in specific locations on a membrane. If the membrane can be prepared for direct MALDI analysis, all components of the mixture are usually not detected, certainly not detected with equal response. Charge derivatization, attaching a fixed positive charge to the N-terminus of peptides, can increase the number of components detected and can provide useful MS/MS spectra [12-14]. Chapter three will evaluate the charge derivatization methods that have been explored.

Although charged-derivatives offer attractive advantages in the mass spectrometry analysis of proteins, additional work is still required in this field. The goal of our approach is to develop a method, which improves the sensitivity of peptide analysis by MALDI, and provides structure information of peptides by MALDI-PSD, which is easy and fast enough to be used in routine analytical work and which does not cause mass spectral interference by non-volatile solvents or reagents. In chapters four and five, we will summarize the results of research regarding charge derivatization methods and their usefulness in MALDI-MS analysis.

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CHAPTER TWO. INTRODUCTION TO MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT (MALDI-TOF) MASS SPECTROMETRY

Introduction

Before the introduction of matrix-assisted laser desorption/ionization (MALDI) [1-2] and electrospray ionization (ESI) [3-5] techniques, mass spectrometric analysis of organic compounds was limited to small, volatile sample molecules. With the extension of mass spectrometry to large nonvolatile and often thermally fragile biomolecules, research during the 1970s and 1980s has been directed to the development of ionization methods capable of ionizing such molecules directly from the solid or liquid state. This work led to the development of MALDI and ESI techniques [6]. With these two new techniques, today, routine mass spectrometric analyses of proteins in the interesting mass range between 10 and 100kDa with only picomolar amounts of sample have become possible. Due to the advantages and limitations of these two techniques, they both contribute to establish mass spectrometry as a tool in fields like molecular biology and biomedicine.

There are several features that make MALDI an attractive analytical technique for biomolecules. First of all, MALDI can be used to identify molecules in a broad mass range. The accessible mass range for MALDI has been extended to more than 300,000 Daltons [7]. MALDI has been successfully applied to peptides, proteins, olignucleotides [8] and oligosaccharides [9]. Secondly, MALDI spectra have high resolution. A mass

accuracy of 0.01% for proteins up to 30,000 Daltons has been reported [7]. MALDI has an outstanding sensitivity. Typically no more than 1 picomole of analyte is the total amount of sample required and used for analysis. The absolute sample consumption for recording the mass spectra can be estimated to be in the low attomole (10^{-18} mole) range [10], thus virtually the whole sample can be recovered after analysis. The ability to examine complex mixtures and tolerance to contaminations are other reasons of more and more interest in MALDI. Although singly charged molecular ions dominate the mass spectra, MALDI has the ability to elucidate structural information of the molecules under investigation, by analyzing fragment ions [11-12].

Operation principles

Many types of lasers have been used to generate ions for mass spectrometric analysis [13-16]. Two main types of lasers employed are CO_2 lasers emitting light at 10.6μm in the infrared (IR), and N_2 ultraviolet (UV) lasers at 337nm. In order to reduce thermal decomposition of labile biomolecules, the use of short pulse lasers (typically 1-100 nanoseconds) is essential. Photon beams generated by pulsed lasers have good spatial resolution, focused down to spots of submicrometer diameters by suitable microscopic objectives [17]. Direct laser- desorption of intact biomolecules without a matrix seems to be limited to molecular masses of about 1000 Daltons [14,18-20]. Too high of an irradiance results in extensive fragmentation, with increasing and finally prominent formation of unspecific low mass ions. These limitations promoted a search for highly UV- absorbing organic compounds to be used as a matrix.

The matrix can serve several functions to enhance desorption of large molecules. The matrix is present in large excess relative to the analyte (typically 1000:1). In the matrix, which is like a solvent for analyte molecules, analyte molecules are solvated, and separated from each other. Since the analyte molecules are separated from each other, the aggregation, which prevents molecular ion formation, is limited. As matrix desorption is usually performed from solid material, the ability of a matrix to form a "solid solution" of analyte molecules is essential. The matrix absorbs laser energy and transfers it into excitation energy of the solid system. Top molecular layers of the sample then will experience a rapid phase change. Beavis and Chait proposed that, as a result of the volume excitation, a dense gas is formed, which expands supersonically into the vacuum [21]. The matrix also plays an active role in the ionization process of analyte molecules [22,23]. The analyte ionization is assumed to take place via matrix/analyte collisions and charge transfer within the gas phase plume [24-26].

Ions formed by pulsed laser desorption will be analyzed by a time-of-flight (TOF) mass analyzer. An illustration of a linear MALDI-TOF is shown in Figure 2.1.

After ionization, there are two major steps in a time-of-flight analysis: acceleration and the flight time measurement itself. The TOF mass analyzer is conceptually the simplest of the mass analyzers in common use. The measurement is based on the fact that if ions of different masses leave the ion source simultaneously, they will take different times to reach the detector [27]. The flight time of an ion with mass, m, formed in ion source with kinetic energy, KE, a distance from the source to the detector, d, is calculated as follows:

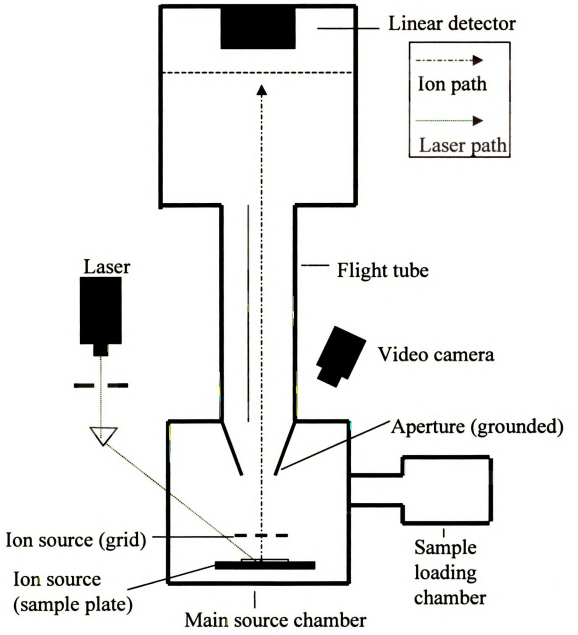


Figure. 2.1. MALDI-TOF mass spectrometer (linear mode).

$$KE = 1/2mv^2$$

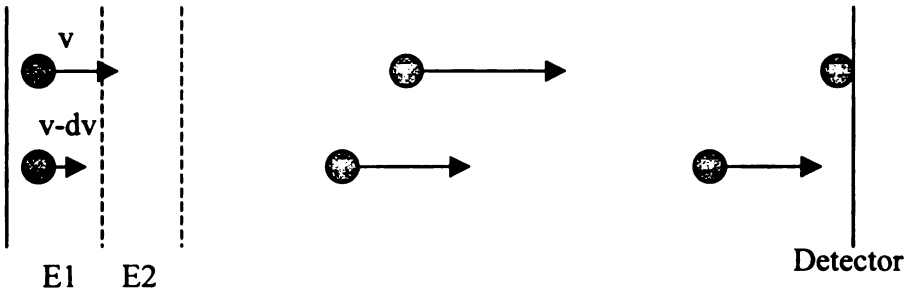
$$v = (2KE/m)^{1/2}$$

$$t = d/v = d(m/(2KE))^{1/2} \quad (1)$$

A TOF mass analyzer is therefore used with sources like those that use pulsed-lasers, and requires the use of a collector with very good time resolution. Compared with other types of mass spectrometers, TOF has several advantages besides its simplicity and robustness [28]. The whole mass spectrum is obtained in every single cycle of the measurement without the need to scan any voltages or currents. Furthermore, because of the few ion optical elements through which the ions must pass, the transmission from the point of the ionization to the detector is usually higher than in other mass spectrometers. However, there is a severe problem associated with this technique - low resolution. This problem is created by the initial velocity of the ions in the ion source and the duration of ionization. As shown in Figure 2.2(a), and Figure 2.2(b), initial ion velocities [29] and the duration of ionization are sources of broadening of the flight time distribution. In addition, when desorption occurs in a strong electric field, energy is presumably lost by collisions within the neutral plume, resulting in further mass-dependent energy dispersion [30].

The introduction of reflectrons and delayed-extraction led to the impressive renaissance of TOF mass spectrometry. In 1973, Mamyrin suggested a compensation of the flight time differences of ions with an electric deceleration field and reversing their flight direction before detecting them [31]. Figure 2.3 presents a diagram of a reflectron TOF mass spectrometer. The reflectron is also called an ion mirror. Ions with high energies penetrate deeper into the reflectron field, and spend more time there than low energy

a) Initial ion velocity v



b) Ion formation time t

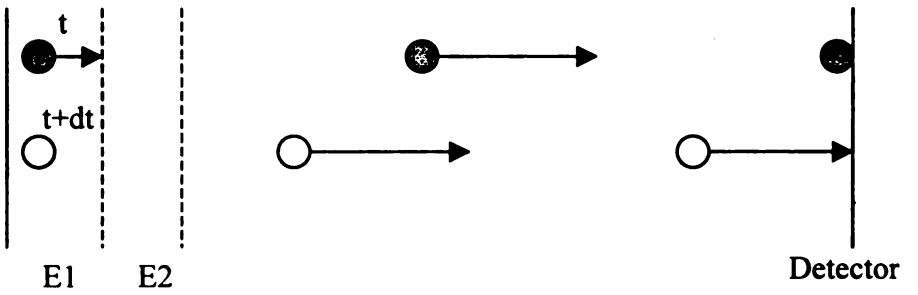


Figure. 2.2(a). An illustration of the initial velocity distribution of the ions in the ion source causing the distribution on detector. 2.2(b). An illustration of ions with the same m/z but formed at different times in the ion source, arriving at the detector at different times.

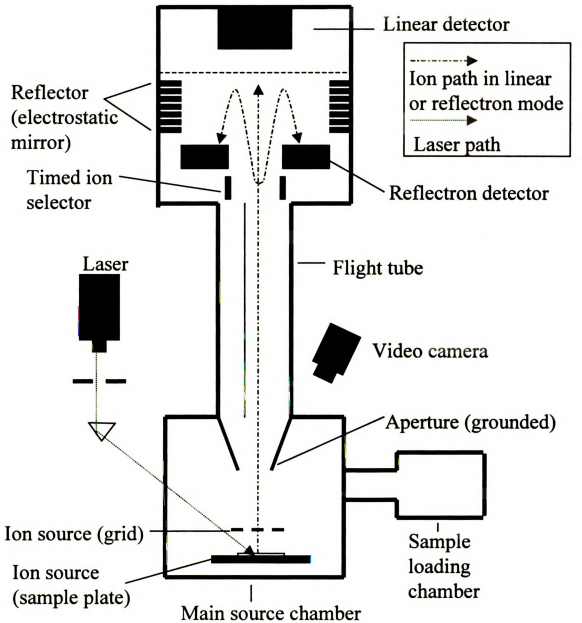


Figure. 2.3. Diagram of a MALDI-TOF mass spectrometer with a reflector.

ions. The retarding field of the reflector is adjusted to compensate for the different flight times of the ions in the drift regions. Ions with same mass will hit the detector at the same time. The resolution of MALDI-MS spectra has been improved from hundreds to more than 10,000 for biomolecule analytes [32-34].

More recently, the development of delayed-extraction dramatically improves the resolution achievable in MALDI-TOF mass spectrometry. In the conventional MALDI-TOF instrument, ions are generated by the laser beam near the surface of the sample plate and are continuously extracted by a dc potential. In delayed-extraction, a short time delay (less than 300 nanoseconds) is applied between laser desorption/ionization and ion acceleration events [35]. During the delay time, the ions which have the greater initial kinetic energy will travel further into the ion source than those with lower kinetic energy. When the extraction field is turned on, the ions having the lower kinetic energy will traverse a longer distance in the electric field, thereby acquiring more kinetic energy in a compensatory effort to give all ions of the same m/z the same flight time. In principle, delayed-extraction allows ions of the same mass to arrive at the detector at the same time, giving rise to a sharp peak or greater resolution. Some improvement in MALDI spectral quality with delayed-extraction may also be explained by dissipation of the very high local pressure (tens of atmospheres) in the laser plume following the laser pulse [29, 30].

Sample preparation

Proper sample preparation is critical for successful analysis by MALDI-MS [36, 37]. A saturated matrix solution, usually in water and acetonitrile, at a concentration of 5-100 mg/μL depending on the solubility of the matrix, is used. The analyte is prepared in a solvent, which is miscible with the matrix solution, at the concentration of about 10-1pmol/μL. The matrix and analyte solutions are mixed to achieve a final matrix:analyte molar ratio in the range of 100:1 to 10000:1. An aliquot of 1 to 2 μL of the mixture will then be deposited on a metal sample plate. During the drying process, the matrix co-crystallizes with the analyte from the solution.

The nature of matrix-analyte interactions in the mixture is not well understood. Studies show that small molecules such as amino acids and peptides will be embedded in the matrix crystal lattice while larger molecules are distributed preferentially on the crystal surface [38]. Matrix crystal formation and size varies depending on the choice of matrix and solvent. In general, it has been observed that a decrease in crystal morphology quality is attributed to a lower quality MALDI- MS spectrum [39]. High salt concentrations in the mixture may induce the decreasing quality of the crystal formation. HPLC or simply washing with a drop of water may need to be carried out to desalt the sample [40]. Alternatively, solution dilution, which can reduce the interference from a salt, may also lead to improved spectra.

The sample can be introduced into a MALDI spectrometer on various support surfaces. The mainly used surface is metal. Other than that, inert polymeric supports such as membranes have also been employed for picomole sample handling in protein mass spectrometric analysis [41-43]. There is considerable interest now in the direct analysis by MALDI-MS of biological samples that have been adsorbed onto a transfer membrane because of implications in coupling gel electrophoresis with MALDI-MS. After proteins are separated in a gel, they are electroblotted quantitatively onto a membrane; their relative positions are not changed. Protein digestions often are applied to proteins in a gel before electroblotting. Membranes with separated proteins or peptide spots will then be washed with distilled water to remove contaminations before adding matrix and introduction to the MALDI mass spectrometer. Various membranes, such as polyvinylidene difuoride (PVDF) membrane [44, 45], polyurethane (PU) membrane [46], polyethylene (PE) membrane [42] and nitrocellulose [43,47] have been reported to be suitable for analyzing picomole levels of a protein that had been immobilized on the membrane.

Characteristics of MALDI-MS spectra

Peaks representing the intact protonated molecules in the positive ion mode dominate MALDI-MS spectra. Na^+, K^+ and matrix adduct peaks are also observed. Multiply charged ions are observed but at relatively lower abundance. Peaks corresponding to fragmentation are rarely observed. The lack of fragmentation makes this technique ideal for mixture analysis [48-50]. Under proper laser irradiance most of the signals in the lower mass range can be assigned to matrix ions. The mass resolution of the ion peaks

can be estimated by $m/\Delta m$ (Δm is the width at the half-height of the peak). Besides the spread of initial ion energies in the ionization process, unresolved adduct ion peaks on the high mass side and peaks due to cleavage of small groups on the low mass side, are also believed to contribute to low resolution for some peaks. Depending on the attained signal-to-noise ratio, the precision of mass determination for proteins is typically in the range of 10-50 mass units at 10,000 Daltons and 100-500 mass units for a protein of 100,000 Daltons in mass.

Post-source decay

The MALDI technique is powerful in determining molecular masses of large biomolecules. However, in order to identify and characterize the biomolecules, structural information is also desired. In linear MALDI-MS spectra, relatively little or no fragmentation was observed. Studies using reflectron MALDI-TOF mass spectrometers revealed that MALDI ions undergo post-source fragmentation [51-52], but the fragments are not separated from the protonated molecules by a linear TOF mass analyzer, because they have approximately the same velocity. All fragment ions from post-source decay are detected at the end of the field free region, as an increase in the peak width of the intact protonated molecule. A fragment ion has a kinetic energy KE_f as in (2):

$$KE_f = (M_f/M_p)KE_p \qquad (2)$$

In equation (2), M_f and M_p are masses of fragment and precursor ions respectively, and KE_p is the kinetic energy of the precursor ion.

Since these fragment ions and precursor ions have different kinetic energies, a reflector can separate them. As mentioned earlier in this chapter, in a reflectron-TOF instrument, at the end of the flight tube, an ion mirror is placed, whose electric field forces the ion in the reverse direction of their initial movement. As illustrated in Figure 2.4, by scanning the reflector voltage, starting at the optimum value for precursor ions down to zero, a particular ion of a certain mass with a certain KE will be focused onto the detector. The entire mass range of PSD fragment ions for a particular precursor ion can be detected by lowering the reflector potential in several intervals. Typically the ratio of the mirror voltage to the acceleration voltage is lowered in steps, in a way that each ratio is about 75% of the preceding ratio. The observable fragment ion mass is proportional to the mirror ratio times the precursor ion mass, as shown in equation (3):

$$M_f = (KE_f/KE_p)\, M_p = \text{mirror ratio} \times M_p \qquad (3)$$

Basically, the reflectron is tuned to sequentially focus the lower energy fragments and a series of related spectra are obtained. A program then combines those spectra into a single spectrum showing both the fragment ions and their precursor ion.

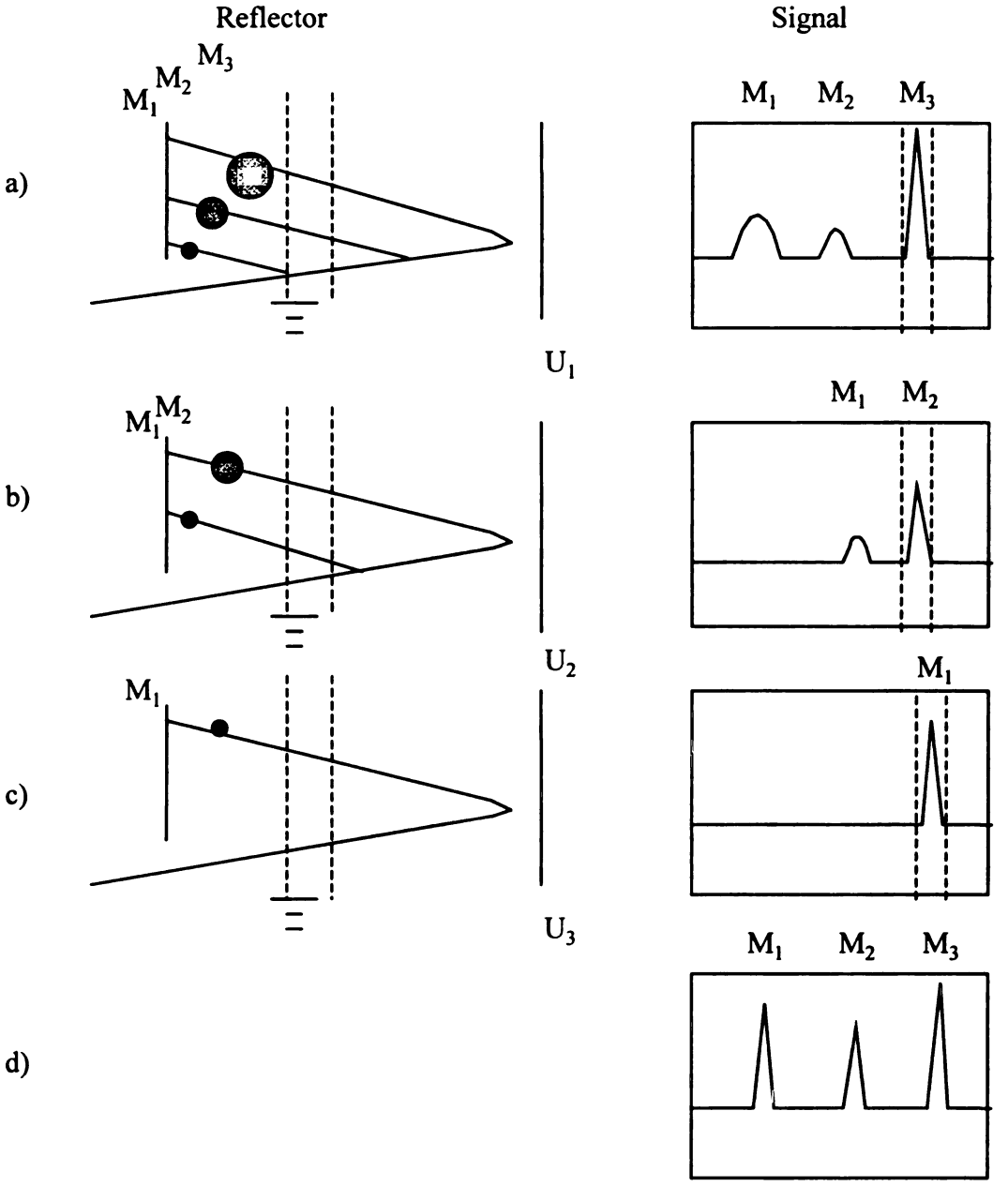


Figure. 2.4. An illustration of a PSD spectrum formed by relectron MALDI-TOF. At high reflector voltage (u_1), the precursor ions are focused on the detector; at lower reflector voltage (u_2, u_3), fragment ions are focused on the detector.

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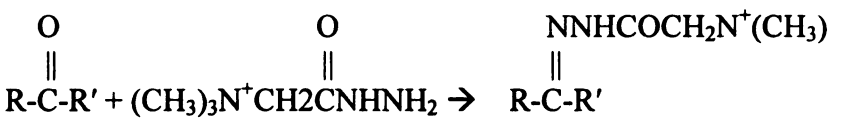
CHAPTER THREE. CHARGE DERIVATIZATION OF PEPTIDES

Introduction

Chemical derivatization has played an important role in mass spectrometry from the earliest practice of the technique [1]. In the early years of mass spectrometric analysis, the primary ionization techniques were electron ionization (EI) and chemical ionization (CI). These mass analysis techniques have a common disadvantage. The sample analyzed must have enough volatility in order to be analyzed. The major impetus for chemical derivatization's early use was to confer the necessary volatility to sample compounds. In the 1970s and 1980s, the development of desorption/ionization techniques, e.g. field desorption [2], plasma desorption [3], and fast atom bombardment (FAB) [4], enabled the analysis of underivatized peptides by mass spectrometry. The advent of FAB ionization has led to the use of chemical derivatization for detectability enhancement by the intentional introduction of a charged group into the molecule. In 1985, a study reported that by applying Girard's reagent T in the derivatization of the ketosteroid androsterone, which is undetectable under normal FAB, the quaternary ammonium derivative as shown in (1), gave an intense peak in the mass spectrum indicating the present of the compound [5].



Girard's reagent T quaternary ammonium derivative (1)

Later on, several types of derivatives were developed to lower the detection limits of peptide analysis. It is assumed that derivatives with a fixed charge group increase ionization efficiencies of the analytes during analysis by desorption/ionization techniques.

Other than the molecular weight, which is important information in identifying molecules, structural information from fragments is also desired. The utility of FAB with tandem mass spectrometry, MS/MS, with fragmentation methods such as collision-induced dissociation (CID), produces a variety of structurally significant fragment ions [6-8]. However, the many types of fragment ions that arise during desorption/ionization and MS/MS [8] can complicate peptide mass spectra. Peptides undergo cleavage of a single peptide bond with charge retention at either the amino-terminal (N-terminal) or the carboxyl-terminal (C-terminal) fragment in the CID experiment. N-terminal fragment and C-terminal fragment ions are a_n, b_n, c_n and x_n, y_n, as shown in Figure 3.1. Fragmentation at a peptide bond, followed by further cleavage of the side chain of the amino acid at that peptide bond forms ions of types d_n, v_n and w_n (Figure 3.1). Internal fragment ions formed by fragmentation at two distant peptide bonds are also observed.

The structure of peptide fragment ions and the mechanisms that produce them are represented by the nomenclature recommended by Johnson et al. [9]. The mechanism shows that the fragmentation is charge-induced [7]. Alternative a_n [10], c_n [11] and y_n [7] structures are also supposed for peptides formed by the charge-remote fragmentation

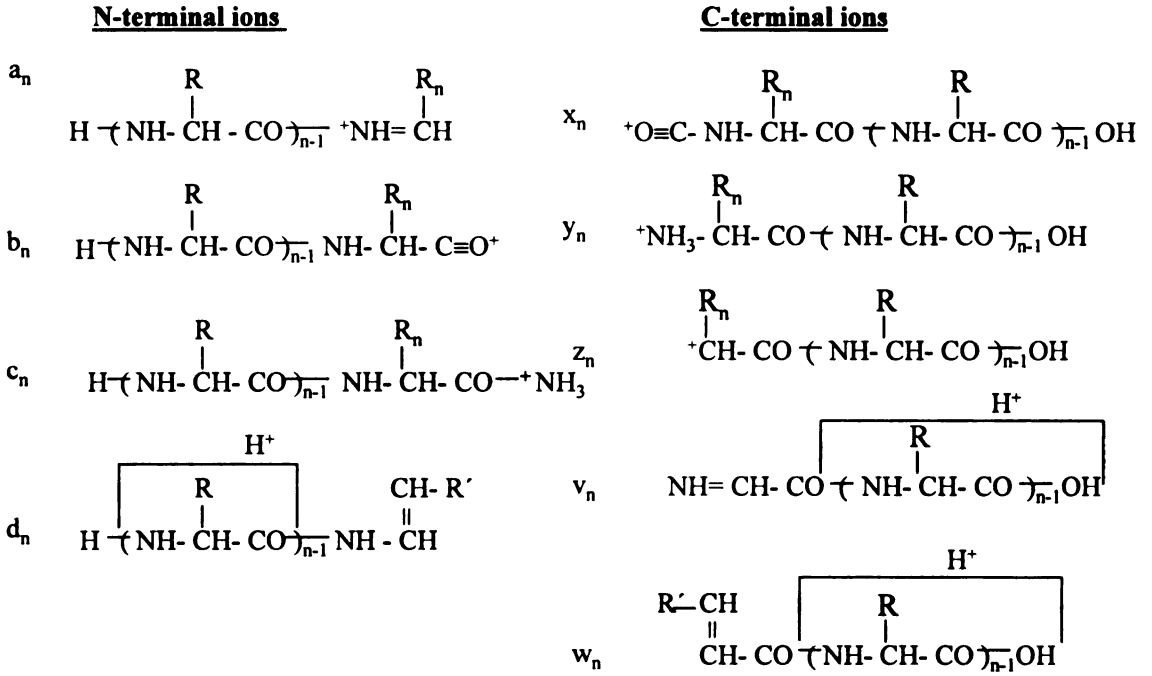


Figure. 3.1. Structure of N-terminal and C-terminal peptide fragment ions.

mechanism, as shown in Figure 3.2. An alternative b_n structure [12, 13], a more stable structure than that originally proposed for b_n ions, is presented in Figure 3.2.

Charge-remote fragmentation (CRF) [14, 15] can be distinguished from charge-induced fragmentation (CIF). The principle of these two reaction types is shown in Figure 3.3. In CIF, a mobile proton is located close to a certain bond, lowering activation energies for fragmentation. As a result the charge is moved to one or the other part of the fragmented peptide forming a detectable fragment ion [13,16,17]. If a mobile proton is lacking, fragmentation reactions by the CIF mechanism are suppressed since the activation energy is too high. In the case of CRF, no mobile proton is needed for fragmentation, but the internal energy of the peptide is high enough to overcome the activation energy [11, 13, 16, 17]. It is assumed that in low-energy CID, the CIF mechanism is the predominant mechanism [18, 19], while CRF was observed frequently in high-energy CID [15, 20].

The charge localization influences the type of fragmentation [21]. Peptides devoid of basic centers (acylated N-terminus, no basic amino acids) result in simple spectra dominated by b_n and y_n ions. These ions are formed via the CIF mechanism [7]. One of the amide nitrogens is initially protonated. This is rationalized by the assumption that the protonating hydrogen is attached randomly to any of the equally basic amide nitrogen atoms of the peptide backbone. This triggers cleavage to both the b_n ion and release of a neutral C-terminal peptide fragment or fragmentation to form a y_n ion by transfer of a hydrogen atom from the N-terminal moiety [22].

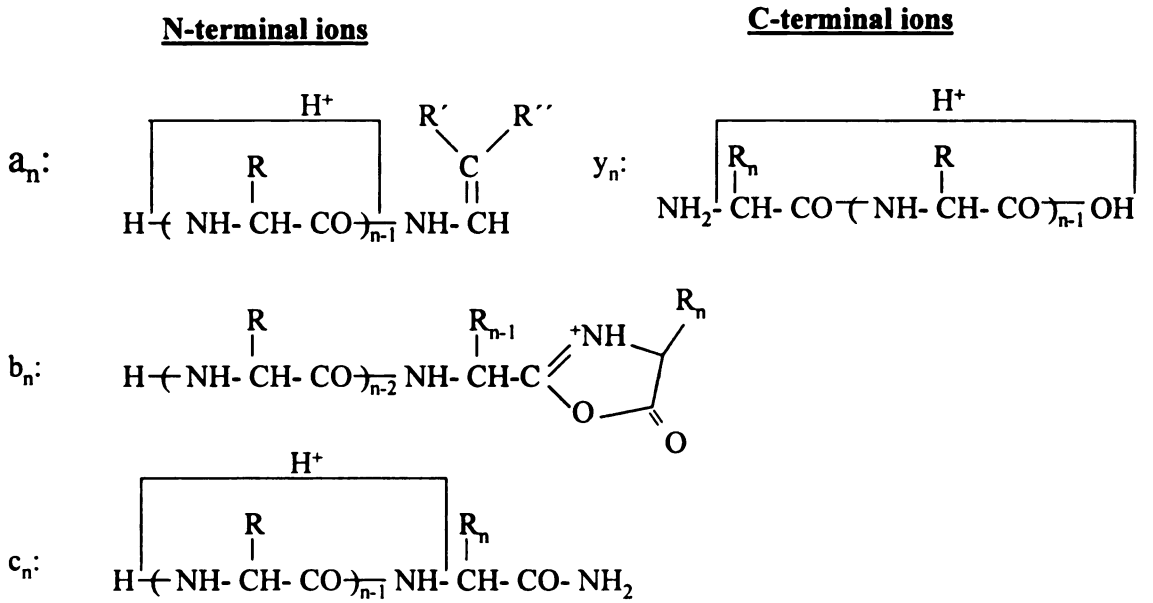


Figure. 3.2. Alternative structure of a_n, b_n, c_n and y_n ions.

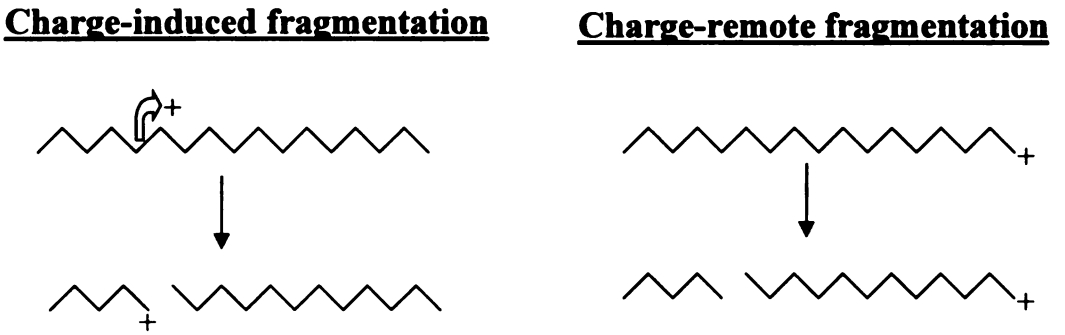


Figure. 3.3. Charge-induced fragmentation (CIF) and charge-remote fragmentation (CRF) mechanisms.

In the presence of a basic amino acid such as arginine (Arg), or a less basic amino acid such as histidine (His) and lysine (Lys), the appearance of the CID spectra changed dramatically. This effect is attributed to the tendency of the proton to be preferentially attached to the basic amino acid, in which case charge-remote fragmentation occurs [23]. This process competes with the protonation of one of the less basic amide nitrogen atoms, as discussed above, and produces fragments by a mixture of charge-induced and charge-remote fragmentation mechanisms [7]. If the basic amino acid is present at the C-terminus, mainly y_n, v_n, w_n and b_n ions are observed. Conversely, when a basic amino acid is at or near the N-terminus of the peptide, a_n, b_n and d_n ions are formed.

Derivatization can be used in conjunction with tandem mass spectrometry to obtain simplified mass spectra of peptides. A charged group stabilized on either the N-terminus or the C-terminus of the peptide can facilitate interpretation of the mass spectra. It is assumed from the observed fragmentation pattern of the peptides in MALDI-PSD spectra, that charged peptide derivatives form fragments through a charge-remote fragmentation mechanism [34]. As a result, only certain types of ions are observed. Proposed structures of the fragment ions of charge-derivatized peptides are shown in Figure 3.4 (taken from reference [50]). Because the protonated peptides and charged peptide derivatives differ in mass depending on the type of derivatization, an asterisk is added to denote the presence of the charged moiety.

Charge derivatization limits the types of fragmentation of peptides. Derivatization at the N-terminus causes N-terminal charge remote fragment ions to be observed; derivatization

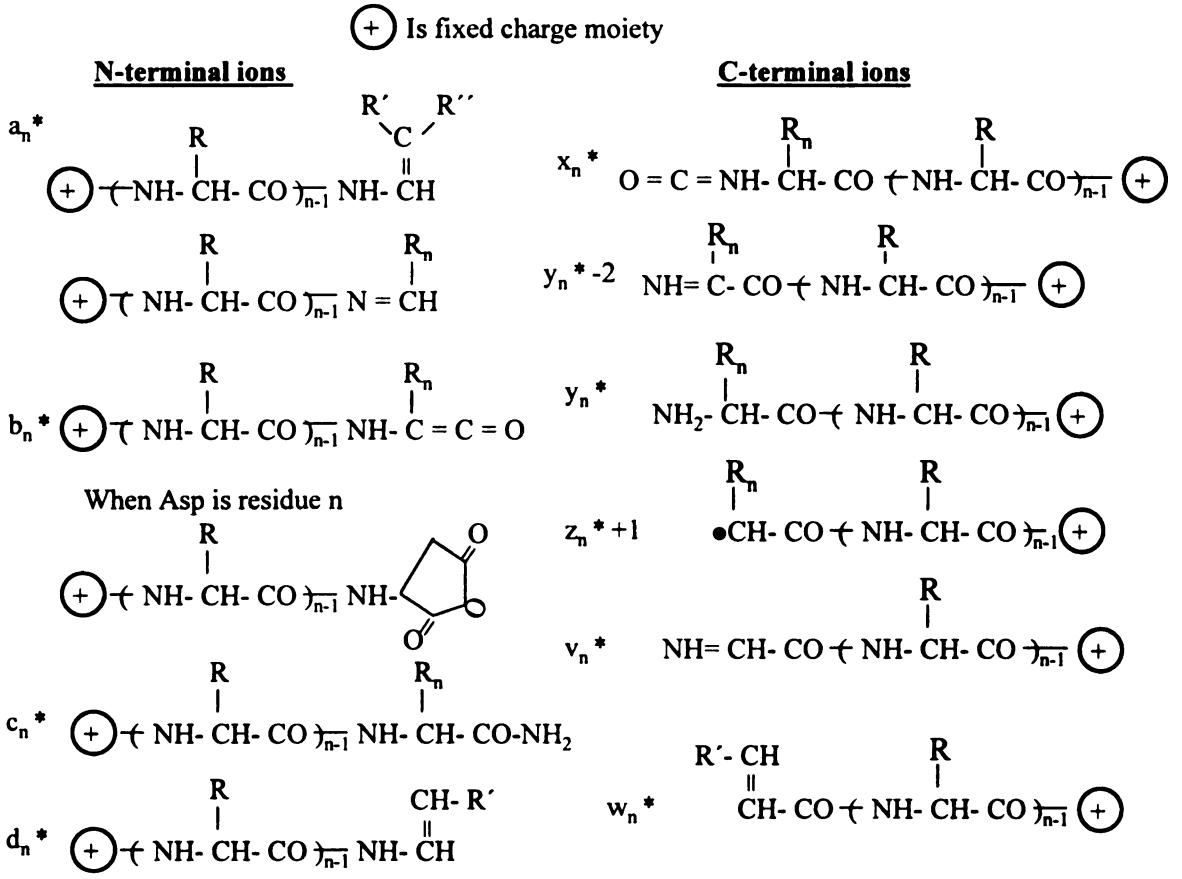


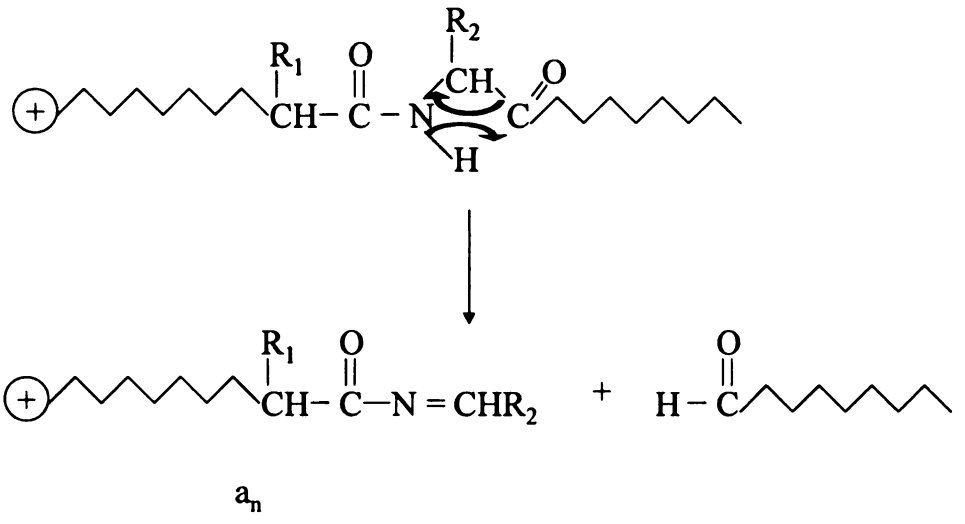
Figure. 3.4. Structures of the fragment ions of charge-derivatized peptides.

at the C-terminus causes C-terminal fragments to be observed. A study using high energy CID showed that CID mass spectra of N-terminal charged-derivatives produce primarily a_n*, and d_n* and a few b_n* and c_n* ions[24], while C-terminal charged-derivatives give primarily y_n*-2, y_n*, v_n* and w_n* ions [25-26].

The diversity of the fragmentation of an N-terminal charged-derivative results in various sources of the hydrogen shift. When amide hydrogen atoms shift, a_n* ions are formed as illustrated in Scheme 3.1.

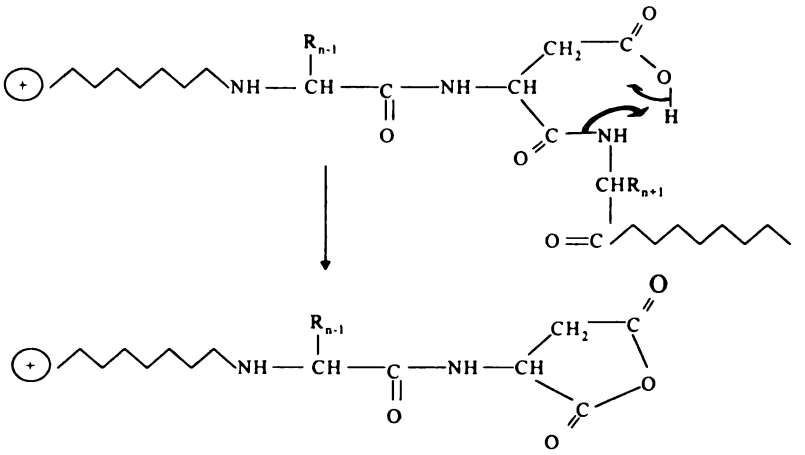
The shift of a hydrogen atom from a side chain produces ions with a cyclic termination. For example, when the side chain is aspartic acid, the terminal hydrogen on aspartic acid side chain is labile. This hydrogen atom is at a position that facilitates formation of three different fragmentation intermediates. Through these intermediates, low energy paths for dissociation are possible. The mechanism of the side chain hydrogen shifting produces b_n*, c_{n-1}* and d_n* ions as shown in Scheme 3.2.

Recently, MALDI-TOF mass analysis has received more and more attention in its potential for biomolecule analysis. Charge derivatizations have been applied to the analysis of peptides by MALDI [27-35]. In linear MALDI-TOF, only a peak representing the C^+ cation for such charged-derivatives was observed. However, the ions do fragment following their generation and acceleration. Mass spectra representing these fragment ions can be acquired in reflectron TOF experiments, and are referred to as post-source decay (PSD) spectra [36-37]. As in high-energy collision-induced dissociation, the data



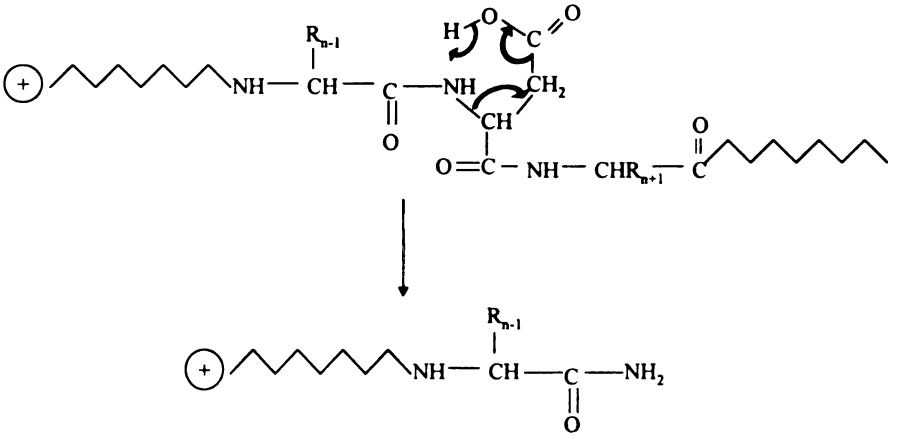
Scheme 3.1. Mechanism of amide hydrogen shift forming the a_n ions.

a).



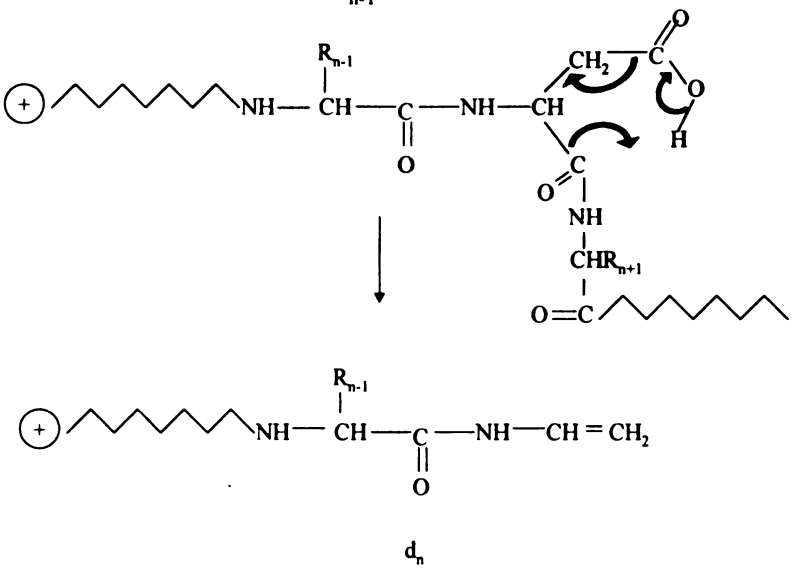
b_n

b).



c_{n-1}

c).



d_n

Scheme 3.2. (a). Formation mechanism of b_n ions; (b). formation mechanism of c_{n-1} ions; (c). formation mechanism of d_n ions, when the side chain is aspartic acid.

obtained by MALDI-TOF illustrated that the charge-remote fragmentation mechanism is the primary fragmentation mechanism in MALDI-TOF PSD [34]. The PSD spectra of peptides are complicated with mixed types of fragment ions. It is charge derivatization that makes PSD an effective technique for obtaining structural information, through interpreting simplified PSD spectra.

In summary, the major advantages of charged-derivatives are that they simplify the fragmentation of peptides. This is important for peptides with a completely unknown sequence. Also, they improve the detectability of the peptide analyte in the mass spectrometer.

There exists a diversity of charged-derivatives in that both positive and negative charge derivatives have been studied [38, 39]. A charged group can be attached to the N-terminus or the C-terminus, or to the side chain of the peptides [7, 40, 41]. Also attachment of a charged group to both the N-terminus and side chain of the peptides, or both the C-terminus and side chain of the peptides, has been observed [7, 40, 41].

N-terminal charged-derivatives

Several N-terminal charge derivatization approaches have been developed since 1984. The structures of those charged groups in the derivatives are shown in Figure 3.5.

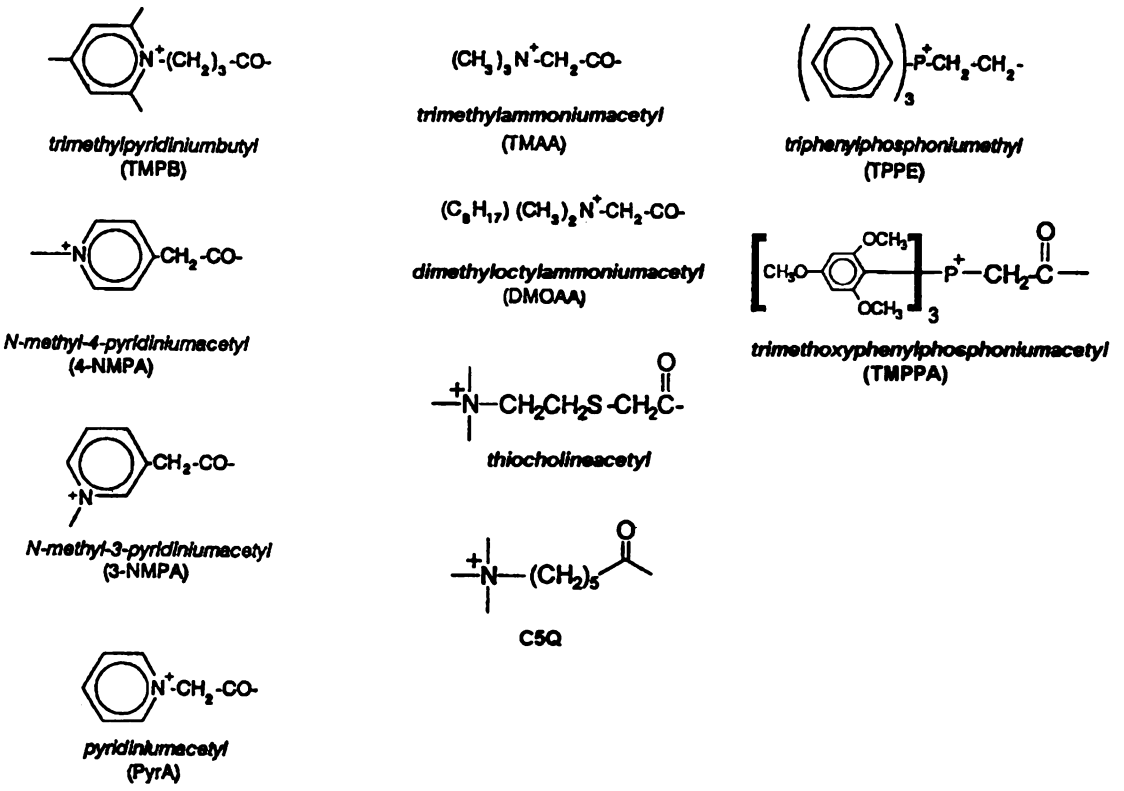


Figure. 3.5. Structures of charged groups in N-terminal derivatization.

The earliest N-terminal charge derivatization on peptides for sequence mass spectrometric analysis was performed in 1984 by Kidwell et al. [42, 43]. The peptides were derivatized by reaction with methyliodide, as presented in (2):

$$NH_2\text{-Pep-COOH} \xrightarrow{CH_3I} (CH_3)_3N^+\text{-Pep-COOH} \qquad (2)$$

This derivatization approach had low yield, so another approach [42, 43] involving successive derivatization of the peptide N-terminus with chloroacetyl chloride followed by reaction with trimethylamine to give a trimethylamino acetyl derivative was proposed.

Other amines have also been studied, as shown in (3).

$$NH_2\text{-Pep-COOH} \xrightarrow{i,\ ii} (CH_3)_3N^+\text{-}CH_2CO\text{-NH-Pep-COOH} \qquad (3)$$

Reagents: i= Hal-CH_2CO-R_1 ii=R_2–$\underset{\displaystyle R_3}{\overset{|}{N}}$–$R_4$

a) Hal = Cl, R_1 = Cl, R_2=R_3=R_4=C_2H_5
b) Hal = Cl, R_1 = Cl, R_2=R_3=R_4=CH_3
c) Hal = I, R_1 = $OC(O)CH_2I$, R_2=R_3=CH_3, R_4= CH_3, C_6H_{13} or C_8H_{17}

These derivatives were analyzed by FAB. The derivatives had detection limits in the low picomole range.

In 1989, iodoacetic anhydride followed by thiocholine iodide was selected to attach a quaternary ammonium group to a peptide N-terminus [44]. By controlling pH at each step, selective derivatization of the N-terminus can be achieved. The pH control allows

selective derivatization of the N-terminus because of the differences between the pK_a of the N-terminus and those of the basic amino acid side chains. However, protection of cysteines was required to prevent derivatization of the side chain [45]. The reaction was completed after several hours and needed HPLC to purify the product before they could be analyzed in the mass spectrometer. Analysis by FAB followed by CID-MS/MS gave typical N-terminal charge-remote fragment ions, but the spectrum was complicated by the loss of trimethylamine from the derivatives.

In 1990, Vath and Biemann proposed a gas phase N-terminal charge derivatization by devising a technique for peptides deposited on a capillary tube as solid phase derivatized with first gas phase chloroacetyl chloride, then with gas phase trimethylamine [46]. The reaction vessel is shown in Figure 3.6.

This two step reaction attached a trimethylamino acetyl moiety to the N-terminus of the peptide. It took three hours to complete, and could be carried out at a subnanomole level. The CID spectra of these derivatives are simplified compared with spectra of underivatized peptides. The products did not need to be purified since excess reagents had been evacuated. However, it is not possible to eliminate derivatization on a side chain by controlling pH in the gas phase reaction.

Three years later, a similar reaction scheme for attaching a dimethylalkylammonium (DMAA) group was developed [47]. Appropriate nucleophiles can be trimethylamine, dimethyloctylamine or pyridine. The derivatization involved reaction of at least 100

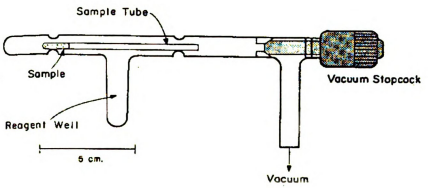
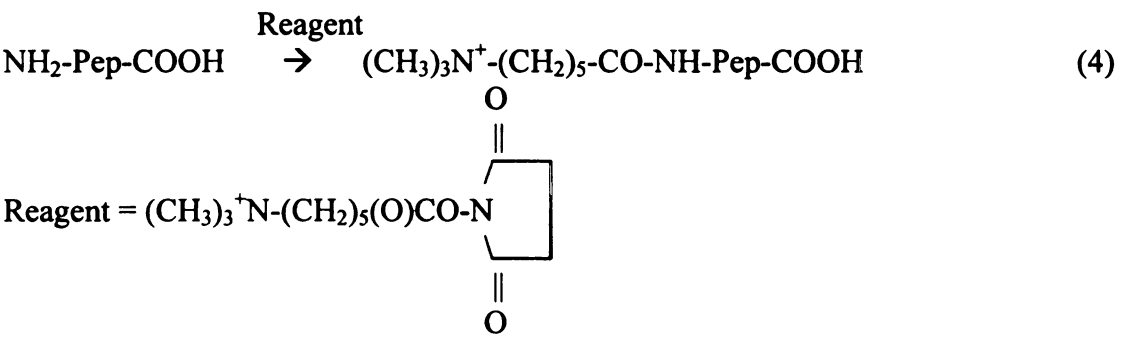


Figure 3.6. Picture of a gas phase N-terminal charge derivatization reaction vessel. (taken from reference [46])

picomoles of peptides with iodoacetic anhydride followed by reaction with dimethylalkylamine. Control of pH can be used to achieve 70-90% selectivity for the N-terminus over the side chain of the peptides. The procedure involved a total of two hours of reaction time, and gave a yield of 60-80%. Excess reagents and nonvolatile buffers must be removed by HPLC. Increased signal intensity was observed in mass spectra of derivatives. This is presumably the result of increased surface activity. However, trimethyl ammonium derivatives gave a slight decrease in signal intensity compared to that for the underivatized peptides. In CID-MS/MS spectra, a small loss of the alkyl group from the dimethylalkylammonium functionality was observed.

In 1994, Bartlet-Jones et al. developed another derivatization approach, which is called "C5Q" [27], as shown in (4).



(4)

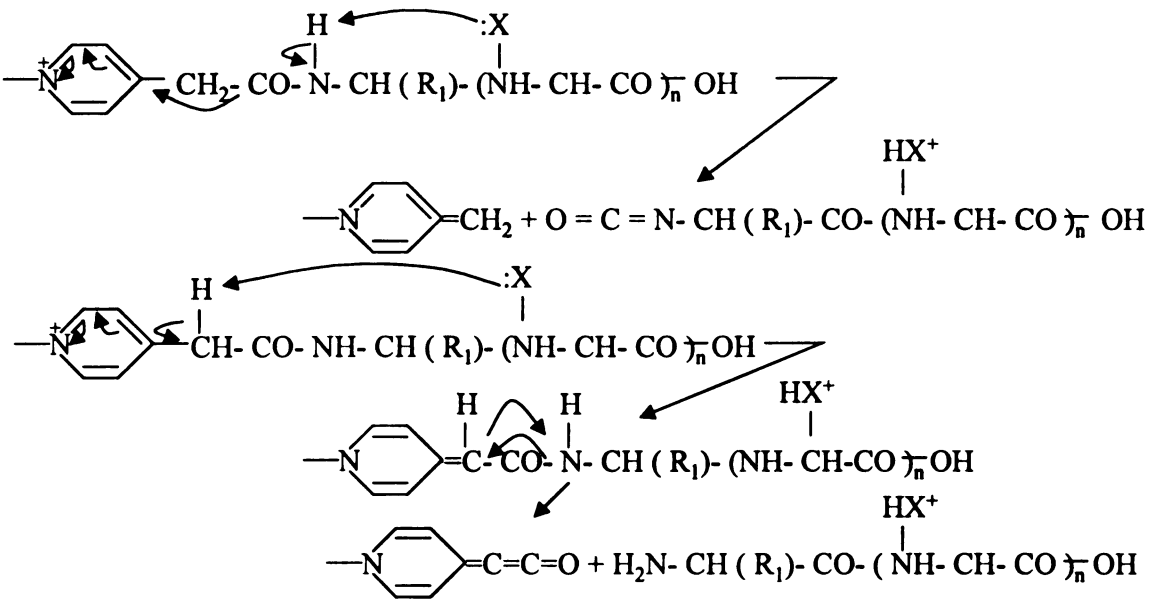
The reaction can be performed sequentially in little more than four hours with quantities of 5-10 pmol for the complete procedure. HPLC was required to remove excess reagents. When the peptide was charge derivatized, and basic side chains were modified, only N-terminal fragment ions were produced in MALDI-PSD spectra [32,35] primarily a_n* and b_n* ions. The b_n* ions are formed by a hydrogen shift from the beta position. The b_n*

ions of charge derivatized peptides are usually present in spectra when very high laser power was used for desorption [32]. In another study of the "C5Q" charged-derivative, by using MALDI-PSD, several a_n* and b_n* fragment ion peaks were observed, but the most abundant fragments were resulting from a combination of backbone cleavage and the loss of trimethylamine from the derivative, e.g. a_n*-59 and b_n*-59 ions.

N-methyl-4-pyridinium acetyl (4-NMPA), N-methyl-3-pyridinium acetyl (3-NMPA) and trimethylpyridiniumbutyl (TMPB) derivatives have also been studied. However, the reaction took place in a relatively long period of time and the yield of the reaction was low [24]. In CID-MS/MS spectra, the 4-NMPA derivatives produce ions due to fragmentation of derivative-peptide bonds. It is believed that these losses are produced when arginine on the side chain extracts a proton, which eliminates the derivative moiety as shown in Scheme 3.3.

The spectra of 3-NMPA derivatives show that elimination of the derivative is less favored for the 3-NMPA derivatives than for 4-NMPA. An explanation proposed is that the pyridinium nitrogen is para to the acetyl linkage in the 4-NMPA and meta in the 3-NMPA. The former is able to transfer charge via resonance to the peptide, which leads to the leaving of the derivative moiety as a neutral molecule. In TMPB derivative MS/MS spectra, loss of trimethylpyridine has been observed which complicates the spectra.

N-terminal charge derivatization using quaternary phosphonium groups have also been explored. Vinyl triphenylphosphonium bromide [40] and 2-bromoethyl

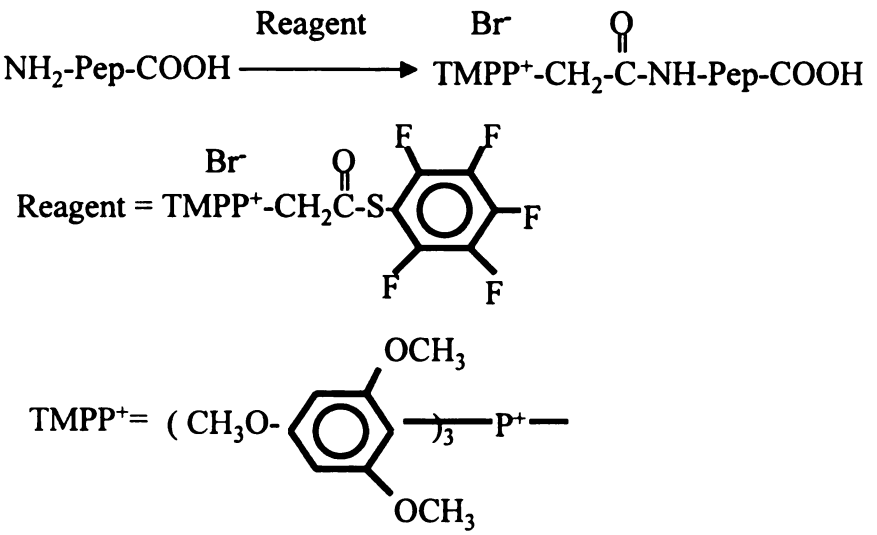


Scheme 3.3. Formation mechanism of fragment ions from 4-NMPA derivatives by loss of charged moiety.

triphenylphosphonium bromide [25] have been used to attach a triphenylphosphonium positive charged moiety on peptides. The derivatization reactions have greater than 75% yield but with some difficulties in reproducibility. pH control was used to prevent reaction with basic side chains, but it required removal of the buffer salts prior to mass spectrometric analysis. In CID-MS/MS spectra, 2-bromoethyl triphenylphosphonium bromide derivatives produced abundant charge-remote fragments arising from the derivatized terminus. But in the study of vinyl triphenylphosphonium bromide derivatives, the spectra are dominated by ions that are solely produced from the derivative moiety and thus contain no sequence information. The peptide fragment ions were of rather low relative abundance in the spectra [24].

Huang et al. introduced a derivatization procedure that attached a tris(2,4,6-trimethoxyphenyl) phosphonium acetyl ($TMPP^+$-Ac) group to the peptide N-terminus [29-30]. The reaction is shown in Scheme 3.4.

A nearly quantitative modification can be achieved with 5-10 fold molar ratio of the reagents and base over the analytes. The reaction took 15 minutes, and 10-picomole of peptide can be derivatized. Selective N-terminal derivatization was achieved by pH control. No HPLC was necessary before mass spectrometric analysis of the products. But $TMPP^+$-Ac derivatization can be adversely influenced by the presence of certain amino acids located at the terminus. For example, when the N-terminus bears an arginine in the chain, the derivatization reaction efficiency is reduced by enhanced rate of hydrolysis of the reagent [29].



Scheme 3.4. Mechanism of $TMPP^+$-Ac charge derivatization.

The $TMPP^+$-Ac derivative has been used to direct the fragmentation of peptides during analysis by MALDI-PSD [29,30,32]. In the spectra, a_n* ions are dominating, a few b_n*, c_n* and d_n* ions are also observed. When an aspartic acid residue is present at the n^{th} residue, b_n*, d_n* and c_{n-1}* ions are more abundant than a_n* ions. This is due to the hydrogen shift from the aspartic acid side chain forming three different intermediates, through which low energy paths of fragmentation take place. This reagent has been used to derivatize peptides located in an electrophoretic gel or membrane [35]. The modified peptides were then analyzed by MALDI and MALDI-PSD directly from the gel or the membrane surface.

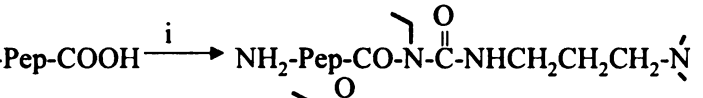
C-terminal charged-derivatives

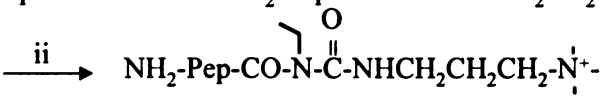
C-terminal charge derivatization has been explored. Several approaches are shown in Scheme 3.5.

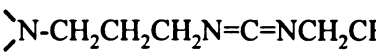
Coupling of the peptide C-terminus to a charged pyridinium salt with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) was used to generate charged-derivatives [43]. Alternatively, after EDC reacted with the peptide C-terminus, by adding methyliodide, the terminal nitrogen of EDC was quaternized, to form a charged-derivative. However, these reagents also derivatize the unprotected acidic side chains of the peptide. The reaction shown in Scheme 3.5(a) and 3.5(c) are selective C-terminal derivatization procedures [48, 49]. These two procedures are very time consuming and required large samples. A C-terminal triphenyl phosphonium derivatization approach was

a. $NH_2\text{-Pep-CO-OCH}_3 \xrightarrow{i,\ ii} NH_2\text{-Pep-CO-NH-N}^+\text{-}$

Reagents: i=carboxypeptidase, $NH_2\text{-N(CH}_3)_2$, ii=CH_3I

b. $NH_2\text{-Pep-COOH} \xrightarrow{i}$ 

$\xrightarrow{ii}$ 

Reagents: i=  , ii = CH_3I

c. $NH_2\text{-Pep-COOH} \xrightarrow{i,\ ii} CH_3CO\text{-NH-Pep-CO-OCH}_2CH_2\text{-N(CH}_3)_3$

$\xrightarrow{CH_3I} CH_3CO\text{-NH-Pep-CO-OCH}_2CH_2\text{-}^+N(CH_3)_3$

Reagents: i = Ac_2O, ii = $HOCH_2CH_2N(CH_3)_2$

d. $NH_2\text{-Pep-COOH} \xrightarrow[DCC]{NH_2CH_2CH_2\text{-}P^+\text{-}(C_6H_5)_3} NH_2\text{-Pep-CO-NHCH}_2CH_2\text{-}P^+\text{-}(C_6H_5)_3$

e. $NH_2\text{-Pep-COOH} \xrightarrow[EDC]{NH_2CH_2CH_2\text{-}^+N(C_6H_5)_3} NH_2\text{-Pep-CO-NHCH}_2CH_2\text{-}^+N(C_6H_5)_3$

Scheme 3.5. Mechanisms of some C-terminal charge derivatizations.

reported in 1991 [25]. The peptide was mixed with 2-aminoethyltriphenyl phosphonium bromide and dicyclohexylcarbodiimide (DCC) at pH 5 (Scheme 3.5.d). The derivatization products can be analyzed without purification, and increased signal was observed in MALDI spectra of the derivative [30]. However, for all C-terminal charge derivatization reactions, there is a potential to derivatize acidic side chains.

C-terminal charge derivatization and N-terminal charge derivatization have both been explored. However, under certain pH control, specific N-terminal derivatization can be accomplished without modifying the side chains of the peptide [24]. In contrast, it is difficult to prepare C-terminal derivatives without simultaneous alkylation of the carboxyl group of the side chains. Also, N-terminal charged-derivatives produce fewer types of fragment ions than C-terminal charged-derivatives making the spectra of N-terminal charged-derivatives simpler than those of C-terminal charged-derivatives. In response to all these advantages of N-terminal charged-derivatives, more efforts were put into N-terminal charge derivatization.

Research goal

A useful N-terminal charge derivatization must have several attributes. First of all, the derivatives produce a series of charge-remote fragments upon analysis by MALDI. The fragment ions must be dominated by a_n* and d_n* ions. The derivatized peptides should not lose the charged moiety as a neutral fragment to an appreciable extent because fragment ions that lack the derivative moiety would complicate the interpretation of the

spectrum. Secondly, derivatization should not adversely affect ionization efficiency of the peptide. The derivative should have a lower detection limit in the mass spectrometer than the original peptide. Third, the reaction should be fast and with high yield of the derivative desired, be specific to the N-terminus without unwanted derivatization of peptide side chains. The ideal charge derivatization also should not require purification prior to mass spectrometric analysis.

Although charged-derivatives offer attractive advantages in the mass spectrometry analysis of proteins, additional work is still required in this field. The goal of our approach is to develop a method, which improves the sensitivity of peptide analysis by MALDI, and provides structure information of peptides by MALDI-PSD, which is easy and fast enough to be used in routine analytical work and which does not cause mass spectral interference by non-volatile solvents or reagents.

We put our effort on peptide N-terminal quaternary amino group derivatization. It is assumed that since a quaternary amino group is smaller than a quaternary phosphonium group, the attachment of a quaternary amino group will be less hindered. Charged quaternary amino groups may direct peptide fragmentation more efficiently through secondary structure effects.

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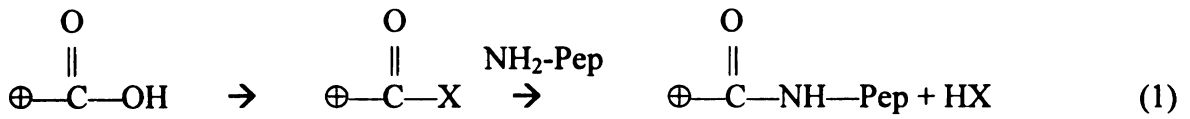
CHAPTER FOUR. LIQUID PHASE CHARGE DERIVATIZATION

Derivatization of peptides with betaine hydrochloride and dicyclohexyl carbodiimide

Introduction

Betaine hydrochloride with a quaternary amino group has been chosen as the derivatizing reagent. Formation of an amide bond between betaine hydrochloride and the N-terminus of peptides is an endothermic reaction [1]. Carboxylic acids do react with amines at elevated temperatures and amides can be produced this way. The temperatures, however, at which such transformations occur at reasonable rate, far exceed the limits considered safe for complex peptides. Therefore, in order to form an amide bond, one of the groups, the carboxylic group of betaine hydrochloride or the N-terminus of the peptides, must be activated.

Activation of the N-terminus of peptides is a challenging problem. Electron-releasing substituents should enhance the nucleophilicity of the nitrogen atom, but the substitution will also decrease the rate of acylation because of the bulkiness of the substituent. The activation of the carboxyl group remains the underlying principle of the derivatization methods in use [2], as shown in (1).

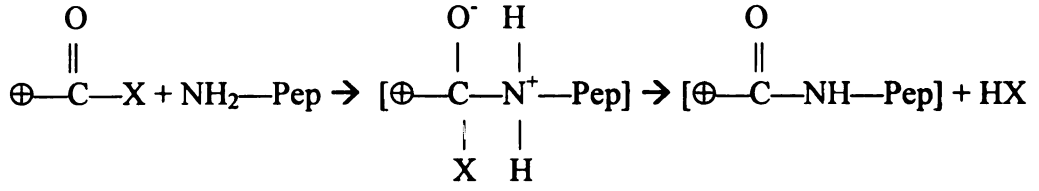


(1)

X = Halide or carboxylate group

The nucelophillic species undergoes addition at the carboxyl group, followed by elimination of the halide or carboxylate group. Acyl halides and anhydrides are reactive acylating reagents because of a combination of the inductive effect of the halogen or oxygen substituent on the reactivity of the carboxyl group and the ease with which the tetrahedral intermediate can expel such relatively good leaving groups [1, 2].

In (1), "X" is an electron-withdrawing group, which renders the carbon atom of the carboxyl group sufficiently electrophillic to facilitate the nucelophillic attack by the amino group. The tetrahedral intermediate thus formed is stabilized by the elimination of X^- as shown in (2):

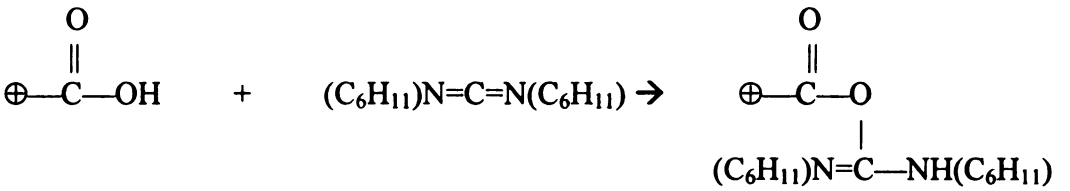


tetrahedral intermediate (2)

Therefore, an appropriate activation method should be found. An attractive approach to the formation of the charged-derivative is the use of "coupling reagents". These are compounds used in peptide synthesis. In this case, we can use them as coupling reagents that connect the peptides' N-terminus (NH_2—Pep) with betaine hydrochloride through an

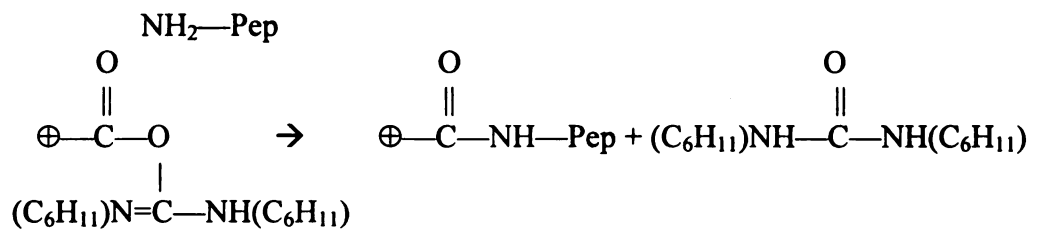
amide bond. One of the most successful coupling reagents used is 1,3-dicyclohexylcarbodiimide (DCC) [3].

DCC will first react with betaine hydrochloride to form an intermediate as shown in (3):



Betaine hydrochloride DCC intermediate (3)

Then into the reaction mixture is added the peptides whose N-terminus will attach to the carboxyl group of betaine hydrochloride, releasing N, N'-dicyclohexylurea as shown in (4):



intermediate charged-derivative dicyclohexylurea (4)

The micro-scale derivatization using DCC and betaine hydrochloride with peptides was demonstrated.

Experimental

1. Materials

Betaine hydrochloride ($(CH_3)_3N(Cl)CH_2CO_2H$, FW 153.61) [4] , 1,3-dicyclohexyl carbodiimide ($(C_6H_{11})N=C=N(C_6H_{11})$, DCC, FW 206.33, 1.0 M solution in dichloromethane) [5] were obtained from Aldrich (Milwaukee, WI).

Lys-Arg-Thr-Leu-Arg-Arg (FW 829.0), methionine enkephalin-Arg-Phe (FW 877.0), Tyr-adrenocorticotropic hormone fragment 4-9 (FW 1068.2), allatostatin II (FW 1067.2), and bradykinin (FW 1067.2) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

α-cyano-4-hydroxycinnamic acid [6] obtained from Aldrich Chemical Co. has been recrystallized and used as the MALDI matrix.

2. Instrument

MALDI-MS experiments were carried out on the PerSeptive Biosystems Voyager Elite time-of-flight mass spectrometer equipped with a model VSL-337ND nitrogen laser (Laser Science, Newton, MA) (337nm, 3-nsec pulse length) and a dual microchannel plate detector (Galileo, Sturbridge, MA). The instrument has delayed extraction

capabilities, and can be operated in either linear or reflectron mode. The acceleration voltage in the ion source used was 20kV. Data were acquired with the data system provided and based on a transient recorder with 2 ns resolution.

Time-to-mass conversion was achieved by either external or internal calibration using matrix (m/z 172.2) and a standard of bradykinin (m/z 1067.2).

All experiments were performed using α-cyano-4-hydroxycinnamic acid as matrix.

3. Sample preparation

Peptides were prepared by dissolving the peptides in 1:1 (v/v) solution of acetonitrile/water at a concentration of 1nmol/μL.

Betaine was first prepared as 4.5nmol/μL aqueous solution. Then 1μL of the betaine solution was air dried in a plastic vial. Dichloromethane was added in, while keeping the concentration of betaine constant at 4.5nmol/μL.

DCC was diluted to 4.5nmol/μL using dichloromethane.

A saturated matrix solution was prepared in a 1:1 (v/v) solution of acetonitrile/water and mixed in equal volumes with peptides or derivatization products. The mixture was allowed to air dry before being introduced to the mass spectrometer.

4. Charge derivatization

Pipette the peptide solution (4μL of 1nmol/μL in 1:1 (v/v) acetonitrile/water) into a plastic vial and let it air dry.

To a solution of the betaine hydrochloride (4μL of 4.5nmol/μL in dichloromethane) was added DCC (4μL of 4.5nmol/μL in dichloromethane). Then 1μL of the betaine and DCC solution was pipetted into a plastic vial. After standing at room temperature for 30 minutes, the mixture was added into the vial with solid peptide. The mixture was vortexed for 1 minute. The vial was sealed and set in room temperature. The reaction was stopped after 2 hours, by adding 1:1 (v/v) acetonitrile/water into the reaction mixture, diluting the mixture to a solution at the concentration of 5pmol/μL. Then 1μL of this diluted reaction solution was mixed with equal volume of matrix.

Results and Discussion

In the MALDI-MS spectra shown below, we use 'L' as linker between the charged moiety and the peptide N-terminus, '⊕' as the charged moiety and a two-letter symbolic name for each peptide.

The yield of the reaction is relatively low. For a particular peptide, allatostatin II (m/z 1067.2), the charged-derivative peak (m/z 1167.4) height in the spectra (Figure 1) is 1/3 of the original peptide protonated peak (m/z 1068.4).

Not only the desired derivatives (⊕—CO—NH—Pep) are formed for the peptides, but also the coupling products of molecules of peptides themselves are formed (5):

$$NH_2\text{-}Pep_1\text{-}COOH + NH_2\text{-}Pep_2\text{-}COOH \rightarrow NH_2\text{-}Pep_1\text{-}CO\text{-}NH\text{-}Pep_2\text{-}COOH + H_2O \qquad (5)$$

As shown in Figure 4.1, the peaks at m/z 2116.8, m/z 2216.8 for allatostatin II (Al, FW 1067.2) are two peptide molecules coupled and the charged-derivative of the coupled molecules respectively. Compared with the MALDI-MS spectrum of allatostatin II before the derivatization, the intensity of the protonated peptide peak decreased. The intensity of the peptide protonated peak in the spectrum taken after derivatization is only 1/10 of the intensity of the peptide protonated peak in the spectrum taken before the reaction (Figure 4.1,4.2). This suggested that 90% of the peptide molecules have been transformed to a derivative, coupled molecules or coupled-derivative molecules.

In a mixture of peptides, the coupling can take place between molecules of one peptide themselves, and also can take place between molecules of one species with the molecules of the other species. These phenomena are shown in Figure 4.3 methionine enkephalin (Me, FW 877.0) coupled with Tyr-adrenocorticotropic hormone fragment 4-9 (Ty, FW

1068.2). In the MALDI-MS spectrum, the peak of the protonated product is at m/z 1928.5 (877.0 + 1068.2 –18= 1927.2). The cross-coupled product was derivatized by betaine

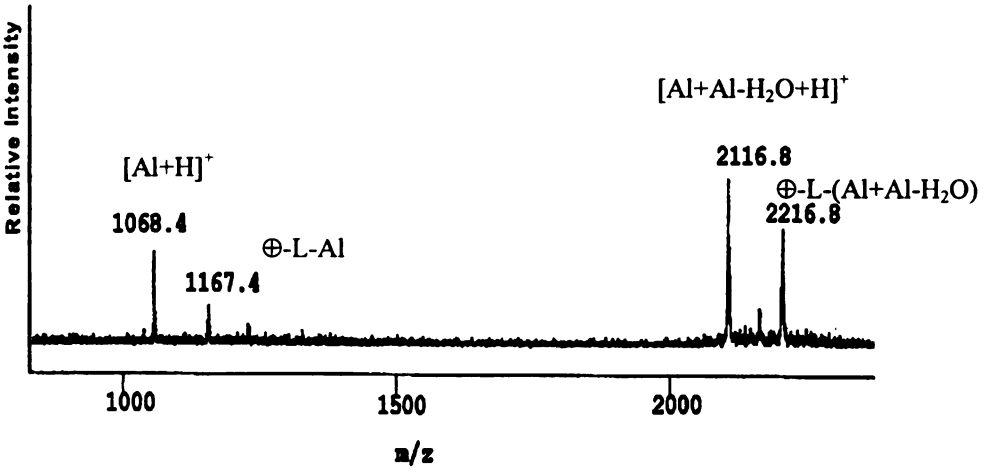


Figure. 4.1.MALDI-MS spectrum of trimethylamino acetyl charged-derivative ⊕-L-Al (m/z 1167.4) of allatostatin II (Al, FW 1068.4), peptide coupling reaction product $[Al+Al-H_2O+H]^+$ (m/z 2116.8) and its trimethylamino acetyl charged-derivative ⊕-L-$(Al+Al-H_2O)$ (m/z 2216.8).

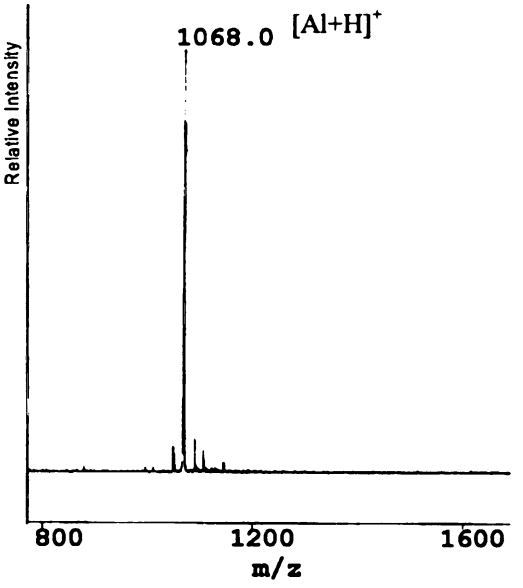


Figure. 4.2. MALDI-MS spectrum of allatostatin II protonated peak $[Al+H]^+$ at m/z 1068.0.

hydrochloride and DCC, formed the trimethylamino acetyl derivative represented by a peak 100 m/z units higher (m/z 2027.5). The coupling between the molecules of the same species Tyr-adrenocorticotropic hormone fragment 4-9 (FW 1068.2) is also present. The coupling product and its trimethylamino acetyl derivative are at m/z 2119.8 and m/z 2218.8.

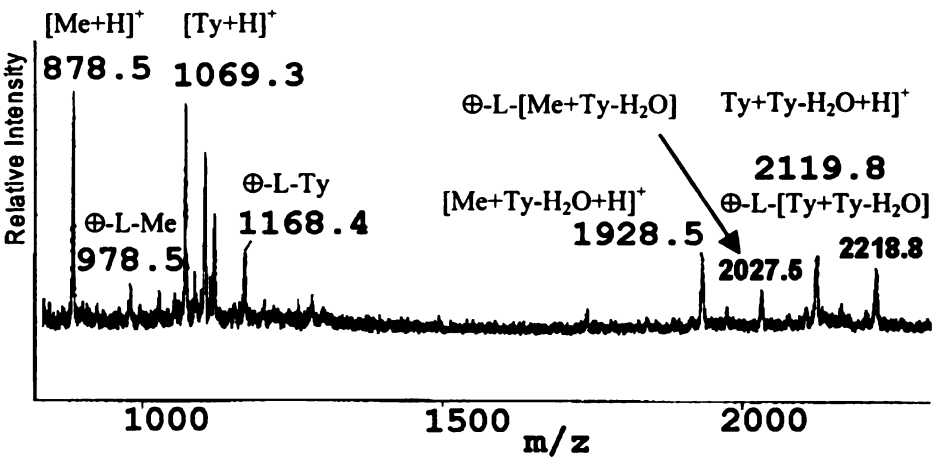


Figure. 4.3. MALDI-MS spectrum of a two-peptide mixture: methionine enkephalin (Me, FW 877.0) and Tyr-adrenocorticotropic hormone fragment 4-9 (Ty, FW 1068.2), protonated peaks $[Me+H]^+$ (m/z 878.5) and $[Ty+H]^+$ (m/z 1069.3), their derivative peaks at m/z 978.5 and 1168.4 respectively. In the m/z 2000 range, there are peaks related to the coupling between peptide molecules.

The MALDI-MS spectrum of the mixture of betaine hydrochloride and DCC is shown in Figure 4.4. We can locate the peak for protonated DCC at m/z 207.9. At 18 m/z units higher, is a peak representing the product of DCC hydrolysis. The yield of the intermediate of this two-step derivatization reaction, produced by betaine hydrochloride and DCC reaction, is approximately 40% of the total of the original reagent. The peak represent the intermediate is at m/z 325.0.

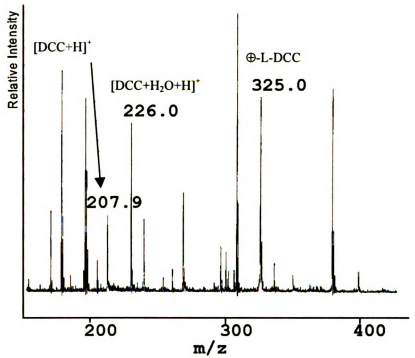


Figure. 4.4. MALDI-MS spectrum of the mixture of betaine hydrochloride and DCC.

There are several shortcomings of the DCC method. First of all, DCC has very low solubility in water. The reaction must taken place in an organic solvent. However, peptides and betaine hydrochloride are hydrophilic compounds. They usually are prepared as aqueous solution. The differences in the hydrophilic and hydrophobic properties of the reagents tend to complicate the experimental procedure, and may cause the low product yield.

Second, the N, N′-dicyclohexylurea by-product, while indeed insoluble in most organic solvents, is not entirely insoluble, therefore it frequently contaminates the product of coupling. Contamination is one of the causes of the suppression of signals.

A more disturbing side reaction is the intramolecular rearrangement of the intermediate. The attack on the activated carboxyl group by the nearby nucelophillic N results in an O→ N shift, yielding an N-acylurea derivative as by-product. This by-product is undesirable not only because they represent a loss of the carboxyl component from betaine, but also because they introduced another contamination to the product of peptide derivatization.

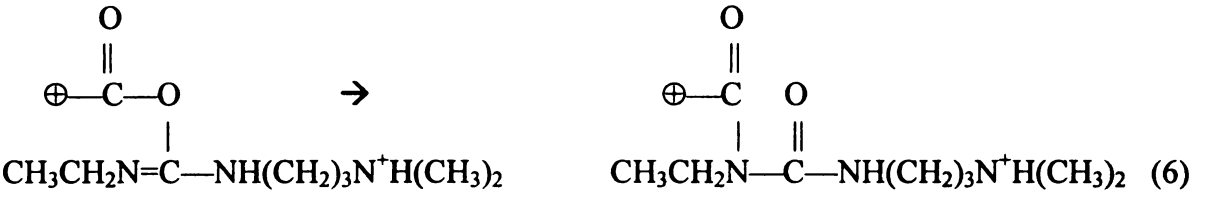
Obviously, some amount of the "coupling reagent", DCC, has not reacted with betaine hydrochloride, but reacted as a coupling reagent between two peptide molecules. The formation of coupled peptides and cross-coupled peptides tends to complicate the MALDI-MS spectrum of the final derivatization product. This may cause difficulties when we try to identify peptides in peptide mixtures by the MALDI-MS spectrum.

Derivatization of peptides with betaine hydrochloride, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide and N-hydroxysuccinimide

Introduction

The DCC coupling reaction has several shortcomings, such as low solubility of DCC in water, contamination induced by the by-products of the reaction, and the side reaction forming N-acylurea. A remedy for this imperfection could be in the use of water-soluble carbodiimides [7, 8]. 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC, FW 191.71), an ionic carbodiimide ($CH_3CH_2N{=}C{=}N(CH_2)_3N^+H(CH_3)_2\ Cl^-$), is one of the water soluble carbodiimides. The experimental procedure could be simplified and the yield of the reaction should be improved [9] by using reagents with similar hydrophobic/hydrophilic properties. The by-product of the EDC coupling reaction can be removed by a drop of water applied on the dried sample spot, on a sample plate, before it is loaded into a MALDI mass spectrometer.

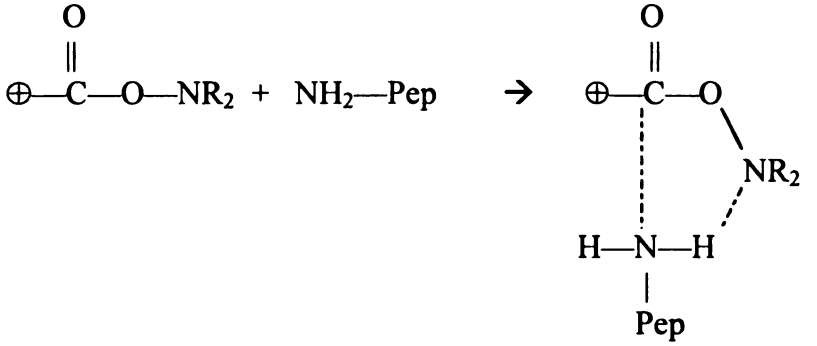
However, the amine-reactive intermediate formed by EDC and betaine hydrochloride, an O-acyl isourea, is unstable in aqueous solutions. The similar side reaction as in the case of DCC, renders rearrangement of the carboxyl and releasing of an N-substituted urea as shown in reaction (6).



(6)

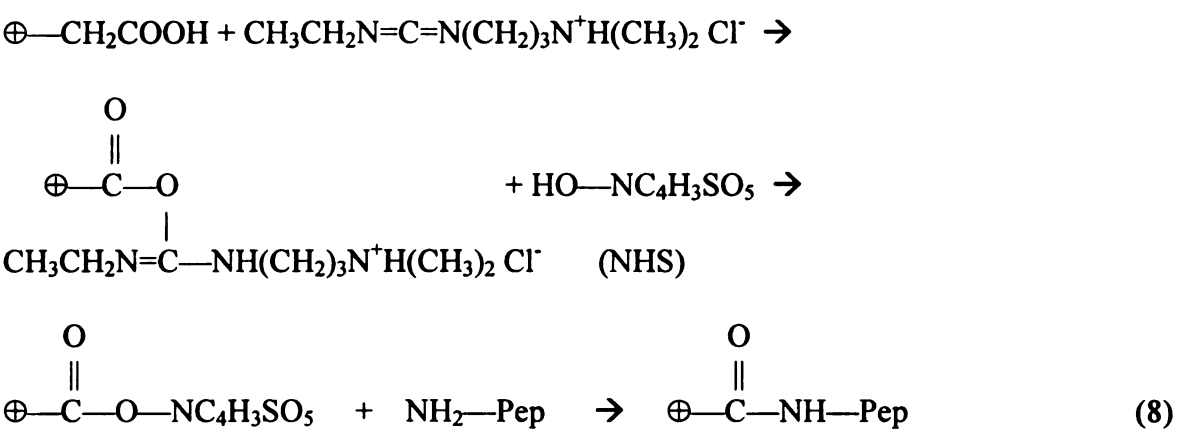
We need to find a way to activate the carboxyl group of betaine hydrochloride. The intermediate of the reaction should be able to react with amines readily, and it must be stable and not rearrange or decompose into other species.

Active esters [10, 11] have the properties that we want. They have activated carboxyl groups and they are stable. One of the active esters is N-hydroxysuccinimide (NHS) [12-14] ester. So, N-hydroxysuccinimide and its water-soluble analog sulfo-NHS are chosen to modify carboxyl group on betaine to form an amine active ester. This is accomplished by mixing the NHS with betaine hydrochloride molecules and a dehydrating agent such as EDC. The reactivity of O-acyl hydroxyamines is usually explained with their anhydride character, and with anchimeric assistance [15, 16] provided by the nitrogen atom adjacent to the ester oxygen as shown in (7):



(7)

The chemical reaction in the derivatization process is shown as follows:



(8)

Experimental

1. Materials

Betaine hydrochloride ($(CH_3)_3N(Cl)CH_2CO_2H$, FW 153.61) and β-mercaptoethanol ($HSCH_2CH_2OH$, FW 78.13) were obtained from Aldrich Chemical Co. (Milwaukee, WI). (1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC, FW 191.71), 1-hydroxyl-2,5-dioxo-3-pyrrolidine sulfonic acid monosodium (Sulfo-NHS, FW 217.13) and 2-[N-morpholino] ethane sulfonic acid (MES) were purchased from Pierce (Rockford, IL).

Allatostatin II (FW 1067.2) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

α-cyano-4-hydroxycinnamic acid, obtained from Aldrich Chemical Co., has been recrystallized and used as the MALDI matrix.

2. Sample preparation

Peptides were prepared by dissolving the peptides in a 1:1 (v/v) solution of acetonitrile/water at a concentration of 1nmol/μL. 4.5μL of the peptide solution was air dried on the bottom of a plastic vial.

Betaine, EDC, and Sulfo-NHS were dissolved separately in MES buffer at pH 6, to 1nmol/μL, 2.1nmol/μL, and 5.1nmol/μL respectively.

A saturated matrix solution was prepared in a 1:1 (v/v) solution of acetonitrile/water and mixed in equal volumes with solution of peptides or derivatization products. The mixture was allowed to air dry on a sample plate before being introduced to the mass spectrometer.

3. Charge derivatization

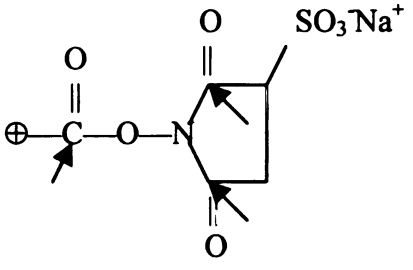
First, 4.5μL EDC solution was added into 4μL of betaine solution. Into this mixture was added 4μL of the sulfo-NHS solution. The reaction took place in room temperature for 15 minutes. Then 0.5μL β-mercaptoethanol was added to quench the EDC. Then the mixture was added into the vial with air-dried 4.5nmol peptide on the bottom of the vial. The

mixture was vortexed immediately, and then was set in an ultrasonic water bath for 2 hours. The reaction was stopped after 2 hours, by adding 1:1 (v/v) acetonitrile/water into the reaction mixture, diluting the mixture to a solution at the concentration of 5pmol/μL. Then 1μL of this diluted reaction solution was mixed with equal volume of matrix.

Result and Discussion

The yield of this EDC and sulfo-NHS derivatization method is low, as shown in Figure 4.5. The peak at m/z 1184.9 corresponds to the hydrolyzed derivative of Allatostatin II (m/z 1067.2).

There exists a certain ambiguity in the reaction of N-hydroxysuccinimide esters. The strained five membered ring is fairly sensitive to nucelophiles which can open it [17]. There are three carboxyl groups in the intermediate active ester as present in Scheme 4.1:



Scheme 4.1. Structure of the intermediate in the active ester reaction.

However, the open ring product is not observed in the MALDI-MS spectrum. (Figure 4.5)

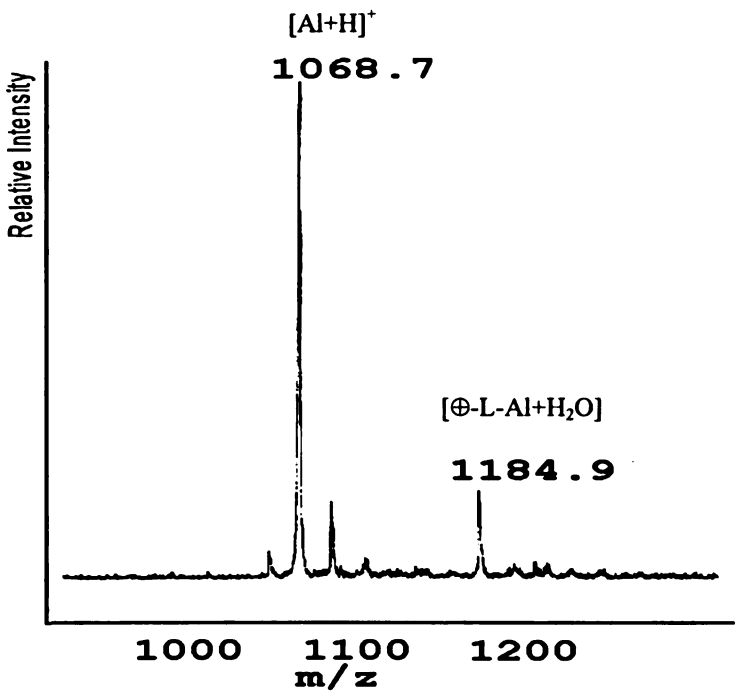


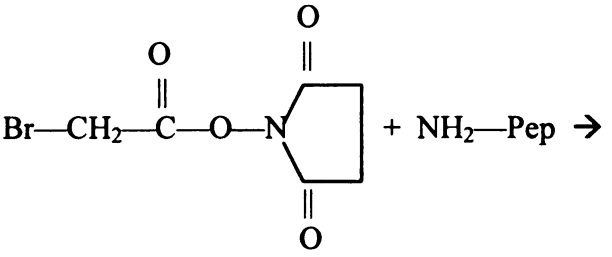
Figure. 4.5. MALDI-MS spectrum of hydrolyzed derivative [⊕-L-Al+H2O] (m/z 1184.9) of allatostatin II.

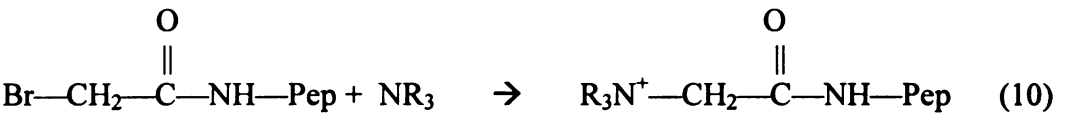
Besides other drawback of EDC, there is the potential that the positive charge on EDC may hinder the formation of an O-urea intermediate with the positively charged betaine molecule.

Derivatization of peptides with trimethylamine and bromoacetic acid N-hydroxysuccinimide ester

Introduction

Bromoacetic acid N-hydroxysuccinimide ester is commercially available. This is a heterobifunctional active ester with two potential leaving groups: $HO\text{-}NC_4H_4O_2$ and Br^-. It allows bromoactylation of the N-terminus of peptides, followed by coupling to other compounds, such as NR_3 (10).





In this way, we introduce a positively-charged amine group onto the N-terminus of the peptides.

Experimental

1. Materials

Bromoacetic acid N-hydroxysuccinimide ester (FW 236.0) was purchased from Sigma Chemical Co. (St. Louis, MO). Trimethylamine ($(CH_3)_3N$, FW 59.11) in 25% (wt.) water solution was obtained from Aldrich Chemical Co. (Milwaukee, WI). Poly(vinylidene difluoride) (PVDF) membrane, pore size 0.45 μm, was bought from Millipore Co. (Bedford, MA). 2-[N-morpholino] ethane sulforic acid (MES) was purchased from Pierce (Rockford, IL).

Tyr-Ala-Gly-Phe-Leu-Arg (FW 725.8), des-Asp-angiotensin (FW 1181.4), Lys-Arg-Thr-Leu-Arg-Arg (FW 829.0), methionine enkephalin-Arg-Phe (FW 877.0), Tyr-adrenocorticotropic hormone fragment 4-9 (FW 1068.2), and allatostatin II (FW 1067.2), were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

α-cyano-4-hydroxycinnamic acid, obtained from Aldrich Chemical Co., has been recrystallized and used as the MALDI matrix.

2. Sample preparation

Peptides were prepared by dissolving the peptides in 1:1 (v/v) solution of acetonitrile/water at a concentration of 0.1nmol/μL. Then a 1μL aliquot of peptide was added into 12μL 0.3M MES at pH 6.

Trimethylamine was diluted with water to 5 μmol/μL.

Bromoacetic acid N-hydroxysuccinimide ester was prepared as 0.01M solution in dry THF.

A saturated matrix solution was prepared in a 1:1 (v/v) solution of acetonitrile/water and mixed in equal volumes with peptides or derivatization products. The mixture was allowed to air dry on a sample plate before being introduced into the mass spectrometer.

3. Charge derivatization

(1). The peptide buffer solution was cooled to 0°C, then added in 5μL solution of bromoacetic acid N-hydroxysuccinimide ester (0.01M in THF) solution. The mixture was immediately vortexed for 1 minute, then cooled to 0°C for 5 minutes. After 5 minutes, the temperature of the solution was elevated to ambient temperature and at that temperature for 2 to 3 minutes. Then 5 μL trimethylamine (5μmol/μL in water) was added to the mixture. The final mixture was vortexed and heated up to 37°C for 20

minutes. The mixture was vortexed every 10 minutes to ensure mixing. After 20 minutes, the mixture was cooled down to ambient temperature and the solution was diluted to 5pmol/μL with 1:1 (v/v) acetonitrile/water.

(2). This reaction was also conducted on a PVDF membrane. First, 2μL, 20pmol/μL peptide solution in 1:1 (v/v) acetonitrile/water, was dropped on a piece of membrane. Into this droplet was added 1μL of 1nmol/μL bromoacetic acid N-hydroxysuccinimide ester in THF. The mixture droplet was air dried on the membrane. Then 2μL of 5μmol/μL trimethylamine aqueous was dropped on the same place of the dried peptide bromoacetic acid N-hydroxysuccinimide ester mixture. The droplet was dried in air after 30 minutes.

Results and Discussion

Relatively high yield was obtained for this derivatization method. As shown in the MALDI-MS spectrum for allatostatin II (m/z 1067.2), the derivative peak is the most intense one in the spectrum. The intensity of the protonated peptide peak is only 1/6 of the intensity of the derivative's peak (Figure 4.6).

However, at shorter reaction times, such as 10 minutes, the reaction is not complete. In Figure 4.7, we located the bromoactyl peptide peak of allatostatin II (m/z 1067.2), $[Br^{79}—CH_2—CO—NH—Pep+H]^+$ at 1189.5

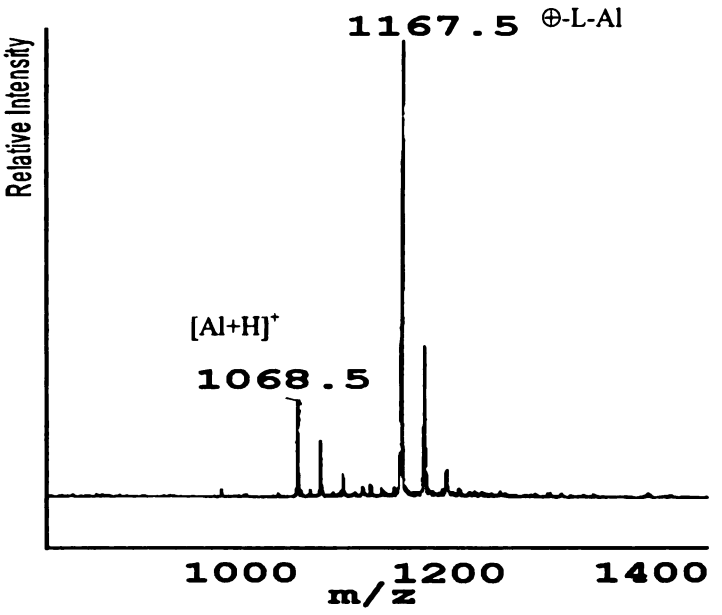


Figure. 4.6. MALDI-MS spectrum of trimethylamine actyl derivative of allatostatin II ⊕-L-Al (m/z 1167.5), and the peak of protonated allatostatin II $[Al+H]^+$(m/z 1068.5).

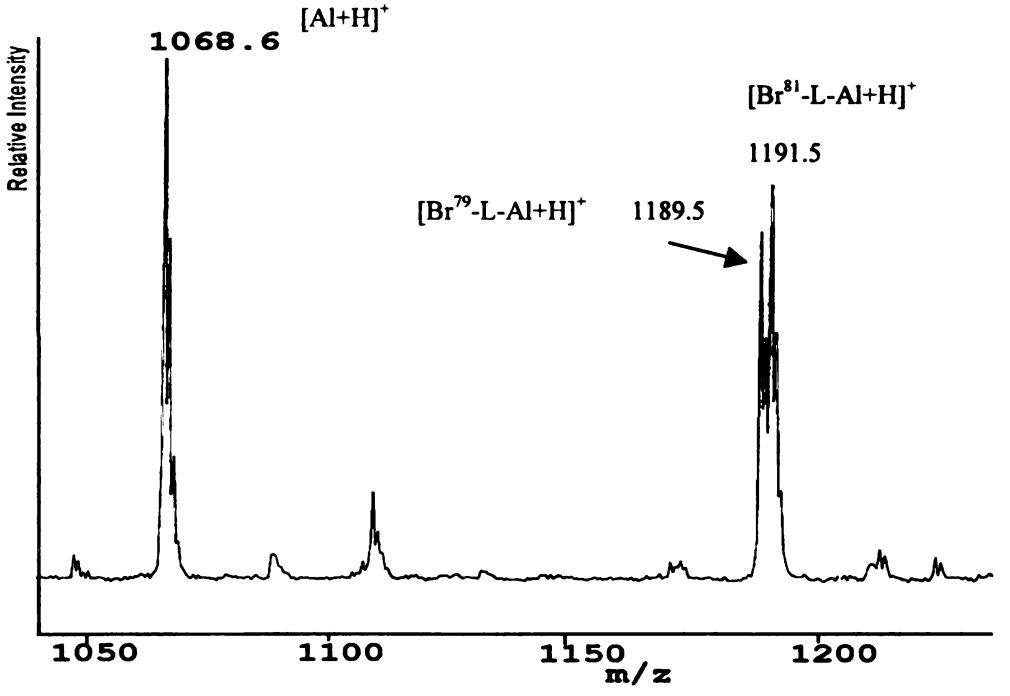


Figure. 4.7. MALDI-MS spectrum of protonated bromoactyl allatostatin II $[Br^{79}$-L-Al+H$]^+$ (m/z1189.5) and its isotopic peak $[Br^{81}$-L-Al+H$]^+$ at m/z 1191.5.

Mixture of peptides can be derivatized as presented in Figure 4.8, methionine enkephalin-Arg-Phe (Me, FW 877.0), Tyr-adrenocorticotropic hormone fragment 4-9 (Ty, FW 1068.2), and their derivative peaks at 977.7 and 1168.8 respectively, were observed in the spectrum.

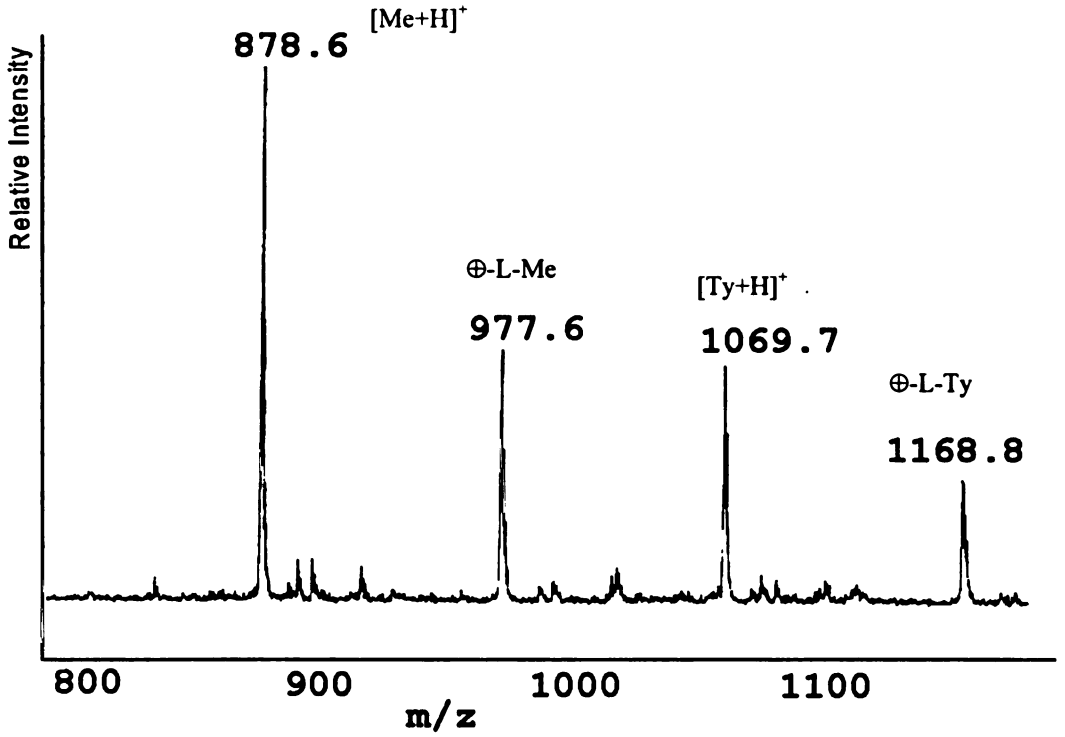


Figure. 4.8. MALDI-MS spectrum of the derivatives of methionine enkephalin-Arg-Phe (Me, FW 877.0), Tyr-adrenocorticotropic hormone fragment 4-9 (Ty, FW 1068.2), at m/z 977.6 (⊕-L-Me) and m/z 1168.8 (⊕-L-Ty) respectively.

The derivatization of mixture peptides on a membrane is also successful (Figure 4.9). Tyr-Ala-Gly-Phe-Leu-Arg (TA, FW 725.8), methionine enkephalin-Arg-Phe (Me, FW 877.0), Tyr-adrenocorticotropic hormone fragment 4-9 (Ty, FW 1068.2) and des-Asp-angiotensin (AA, FW 1181.4) were components in the mixture on the membrane. After the derivatization process as described above, the corresponding derivatives for each

peptide can be detected on the membrane by MALDI-MS. However, the derivatization efficiencies for these peptides are different. In the best case, for Tyr-adrenocorticotropic hormone fragment 4-9 (Ty, FW 1068.2), the derivative's yield is 50%, estimated from the peak height ratio between the protonated peptide peak and the derivative peak. For Tyr-Ala-Gly-Phe-Leu-Arg (TA, FW 725.8) and des-Asp-angiotensin (AA, FW 1181.4), 30% yield was estimated.

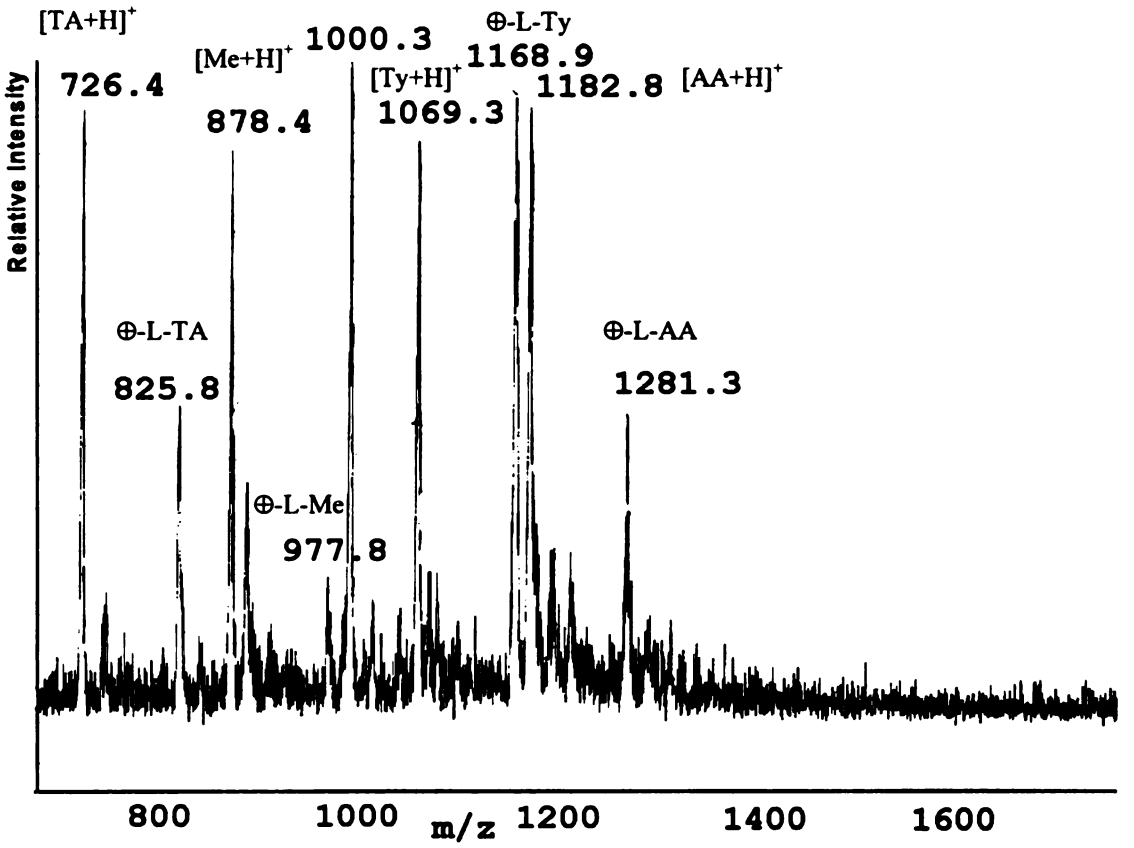


Figure. 4.9. MALDI-MS spectrum of charged-derivatives of peptide mixture on membrane.

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CHAPTER FIVE. GAS PHASE CHARGE DERIVATIZATION

Derivatization of peptides with chloroacetyl chloride and trimethylamine on a membrane

Introduction

The spectral simplicity and apparent lack of fragmentation of ionized proteins characterize MALDI-MS spectra. This suggests that MALDI could be applied to the analysis of mixtures [1]. Peptide and protein mixtures are important analytical targets. However, when a mixture of proteins is encountered, the resulting MALDI spectra frequently show fewer peaks than the number of components in the mixture. In response to this suppression effect, a separation technique should be used. Protein separation and purification by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [2] is a fast and above all an extremely sensitive and almost quantitative method. By use of high-resolution 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE), complex protein mixtures can be separated. The 1 or 2 dimensional separations, based on characteristics of analyte, such as molecular weight, isoelectric point, mobility, yield a spatial array of separated polypeptides.

However, the interfacing of the two analytical methods is not straightforward. A gel is not like a metal surface, which is a conducting surface for a time-of-flight measurement [2]. While success in time-of-flight measurements directly from a gel surface has been

reported [3], there still exist many problems of on-gel detection. The obvious problem is the fact that gels become fragile when dehydrated. They are moisture sensitive, and can crack and disintegrate in the vacuum conditions of the mass spectrometer [2]. They form uneven surfaces, which cause a spread of flight times in MALDI-TOF. Also, detergents, critical to separations, notably SDS, can severely interfere with crystal growth and MALDI analysis [4-6].

Another way to interface gel electrophoresis to MALDI is via membranes. It is increasingly common for separated proteins to be transferred onto more robust polymer membranes by the application of an orthogonal electric field [2]. When electroblotting is carried out correctly, the proteins are transferred quantitatively and the relative position of each is retained. Proteins are transferred free of buffers, SDS, and other contaminations. MALDI-MS analysis of proteins, electroblotted onto polymer membranes after SDS-PAGE separation has been reported [7-9]. The proteins or peptides are desorbed directly from the blot membranes after matrix application. Several commercially available membranes, such as polyvinylidene difuoride (PVDF) membrane [10-11], polyurethane (PU) membrane [12], and polyethylene (PE) membrane [13] have been adopted as sample supports in MALDI-MS. On these chemically inert membranes, proteins are present in a highly purified state, in comparison to classical techniques, essentially free of buffers, salts, detergents or contaminations by other proteins.

Several laboratories have had success in desorbing peptides and proteins directly from PVDF [11, 14, 15]. It is the membrane that has been used in this research. Our goal in

this project is to combine charge derivatization chemistry, which has been successfully demonstrated for enhanced MALDI analysis of peptides, with PAGE and membranes. After a mixture of polypeptides is separated, analytes are digested and electroblotted, resulting in complex mixtures in specific locations on a membrane. If the membrane can be prepared for direct MALDI analysis, all components of the mixture are usually not detected, certainly not detected with equal responses. Charge derivatization, attaching a fixed positive charge to the N-terminus of the peptides, can increase the number of the components detected and can provide useful MS/MS spectra. We are investigating formation of charged-derivatives of peptides on membranes. Our approach is to use gas phase reagents to react with peptides on membranes to form charged-derivatives.

Because of the high sensitivity of the mass spectrometer, only a small amount of the derivatives needs to be introduced into the instrument, usually by using a small aliquot if the derivatization is carried out by conventional solution chemistry. However, when solvents are added to a membrane, the separated spots of proteins or peptides can spread. Damage of the separation is undesired. Other advantage of gas phase reactions over liquid phase reactions is that excess reagents can be removed by evacuation of the reaction vessel. We have therefore developed this two step derivatization of picomole quantities without transfer of the sample or exposing it to bulk solvent or reagents. After the derivatization, the entire product on a membrane was transferred onto a sample probe.

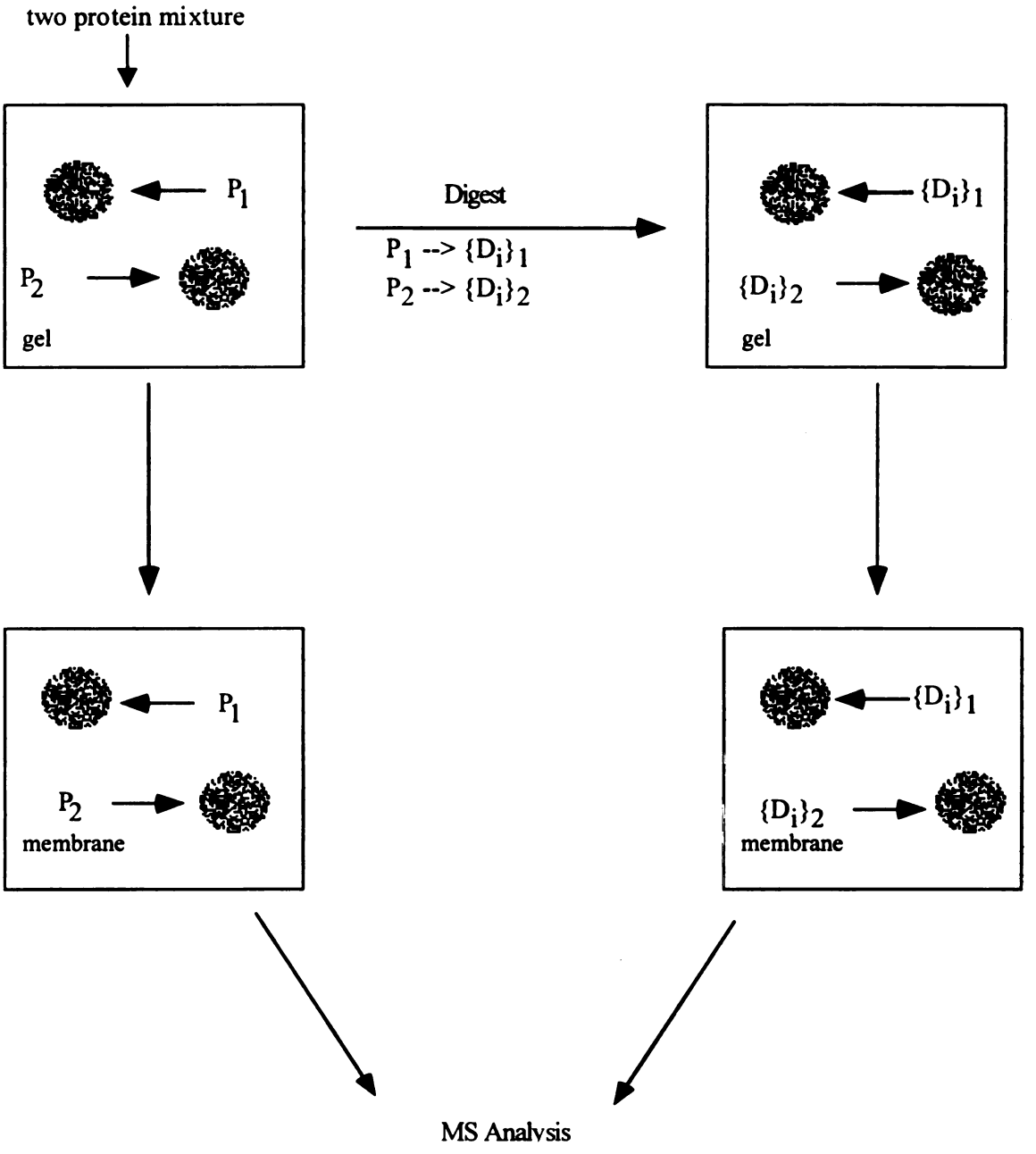
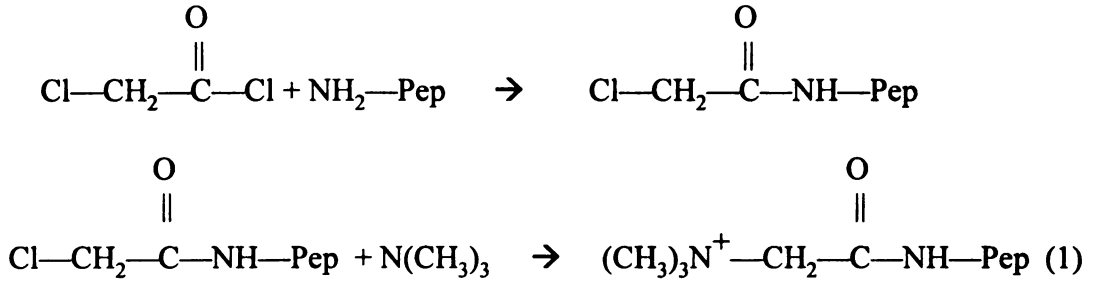


Figure. 5.1. Illustration of protein separation on gel followed by electroblotting onto a membrane; separated proteins digested on gel and then electroblotted onto a membrane.

The only limitations are the requirements that the reagents have an appreciable vapor pressure at the working temperature. The reagents we used are chloroacetyl chloride and trimethylamine. They both have relatively high vapor pressures at room temperature. Chloroacetyl chloride is a bifunctional compound, used as linker between the peptide's N-terminus and a trimethylamine moiety (1).



Experimental

1. Materials

Chloroacetyl chloride ($ClCH_2COCl$, FW 112.94) and trimethylamine (FW 59.11, 25wt.% solution in water) were obtained from Aldrich (Milwaukee, WI). Poly(vinylidene difluoride) (PVDF) membrane, pore size 0.45 μm, was bought from Millipore Co. (Bedford, MA).

Tyr-Ala-Gly-Phe-Leu-Arg (FW 725.8), des-Asp-angiotensin (FW 1181.4), Lys-Arg-Thr-Leu-Arg-Arg (FW 829.0), methionine enkephalin-Arg-Phe (FW 877.0), Tyr-adrenocorticotropic hormone fragment 4-9 (FW 1068.2), neoromedin U-8 (FW 1111.3) and allatostatin II (FW 1067.2), were purchased from Sigma Chemical Co. (St. Louis,

MO) and used without further purification.

α-cyano-4-hydroxycinnamic acid, obtained from Aldrich Chemical Co., has been recrystallized and used as the MALDI matrix.

2. Instrument

MALDI-MS experiments were carried out on the PerSeptive Biosystems Voyager Elite time-of-flight mass spectrometer equipped with a model VSL-337ND nitrogen laser (Laser Science, Newton, MA) (337nm, 3-nsec pulse length) and a dual microchannel plate detector (Galileo, Sturbridge, MA). The instrument has delayed extraction capabilities, and can be operated in either linear or reflectron mode. The acceleration voltage in the ion source used was 20kV. Data were acquired with the data system provided and based on a transient recorder with 2 ns resolution.

Time-to-mass conversion was achieved by either external or internal calibration using matrix (m/z 172.2) and a standard of bradykinin (m/z 1067.2).

All experiments were performed using α-cyano-4-hydroxycinnamic acid as the MALDI matrix.

3. Sample preparation

Peptides were prepared by dissolving the peptides in 1:1 (v/v) solution of acetonitrile/water at a certain concentration. Then 1μL of the peptide solution was dropped onto the membrane surface and air-dried.

The PVDF membrane was washed with 1:1 (v/v) acetonitrile/water then methanol and dried in air before use.

A saturated α-cyano-4-hydroxycinnamic acid matrix solution, 1μL, prepared in a 1:1 (v/v) solution of acetonitrile/water was dropped on the membrane surface with peptides or derivatization products. The mixture was allowed to air dry before being introduced into the mass spectrometer.

4. Charge derivatization

Membrane with peptides dropped and dried on the surface was put in a reaction vessel, which has 10 mL of the proper reagent in a vertical sidearm (Figure 5.2).

Picture of the glass reaction vessel

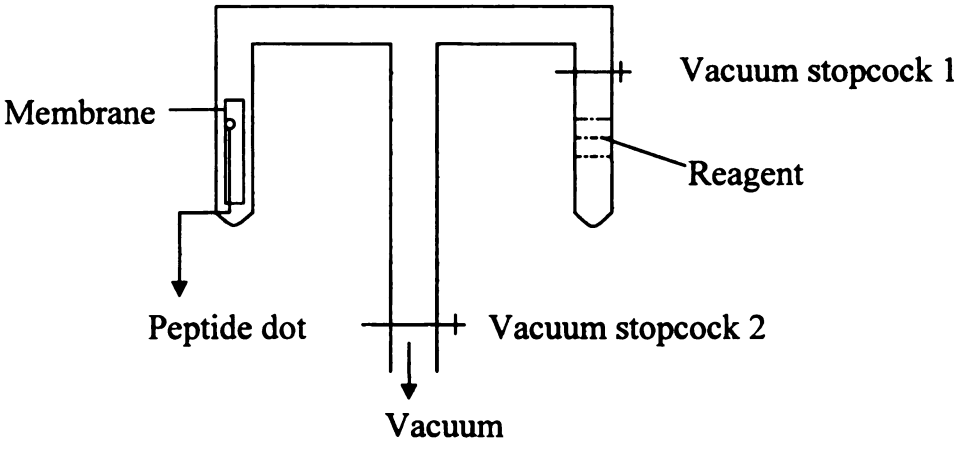


Figure. 5.2. A glass reaction vessel with two side arms which containing membrane and reagents, is connected to a vacuum pump.

At the beginning, the reagent in the right side arm was chloroacetyl chloride, and the vacuum stopcock 1 was closed. The vessel was evacuated to remove the air, then sealed. Vacuum stopcock 1 was then opened to let the reagent vapor fill the reaction vessel at the room temperature. After the reaction between the peptides and chloroacetyl chloride on the membrane has taken place for a period of time, the vacuum stopcock 1 was closed. Another reagent, trimethylamine was loaded and before opening vacuum stopcock 1, the vessel was evacuated to remove the excess chloroacetyl chloride reagent vapor from the vessel. When the vacuum stopcock 1 opened again, the peptides were exposed to trimethylamine vapor, which was heated up to 45°C for a period of time, to finish the derivatization. Onto the derivatized peptide dots on membrane, 1μL of α-cyano-4-hydroxycinnamic acid matrix solution was added. After drying in the air, the membrane was introduced to the MALDI-MS on a sample plate.

Another reaction vessel we employed is a plastic mold, into which a reservoir with reagent can be attached. The membrane with air-dried peptides' dots can be fitted in the mold and it is the only place through which the reagent vapor can go, as shown in Figure 5.3.

In these experiments, with the glass vessel or with the mold, only the second reagent, trimethylamine, was warmed to 45°C. All other parts of the reaction vessels and the reaction with chloroacetyl chloride were kept at room temperature.

Another reaction vessel employed

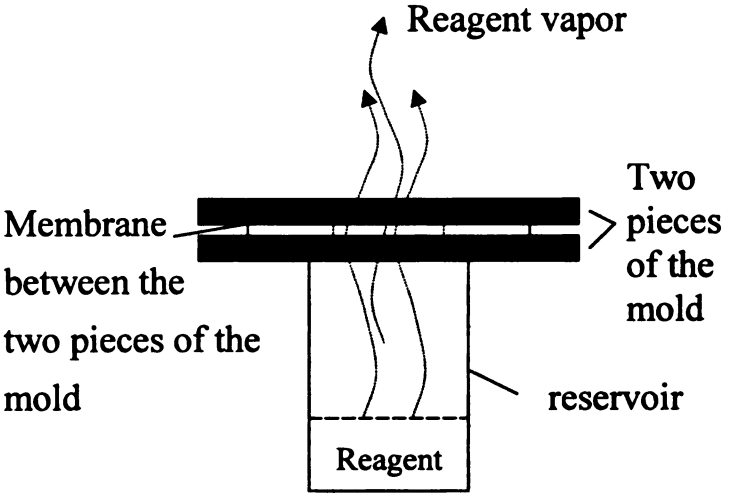


Figure. 5.3. Another vessel for the reaction, the membrane was fixed on a mold and connected with a reagent container. The reagent molecules collided with the peptide molecules stabilized on the membrane when the reagent vapor diffused out.

Results and Discussion

Peptides in mixtures can be detected directly from a membrane. The resulting spectrum has good resolution and signal-to-noise ratio, as shown in Figure 5.4.

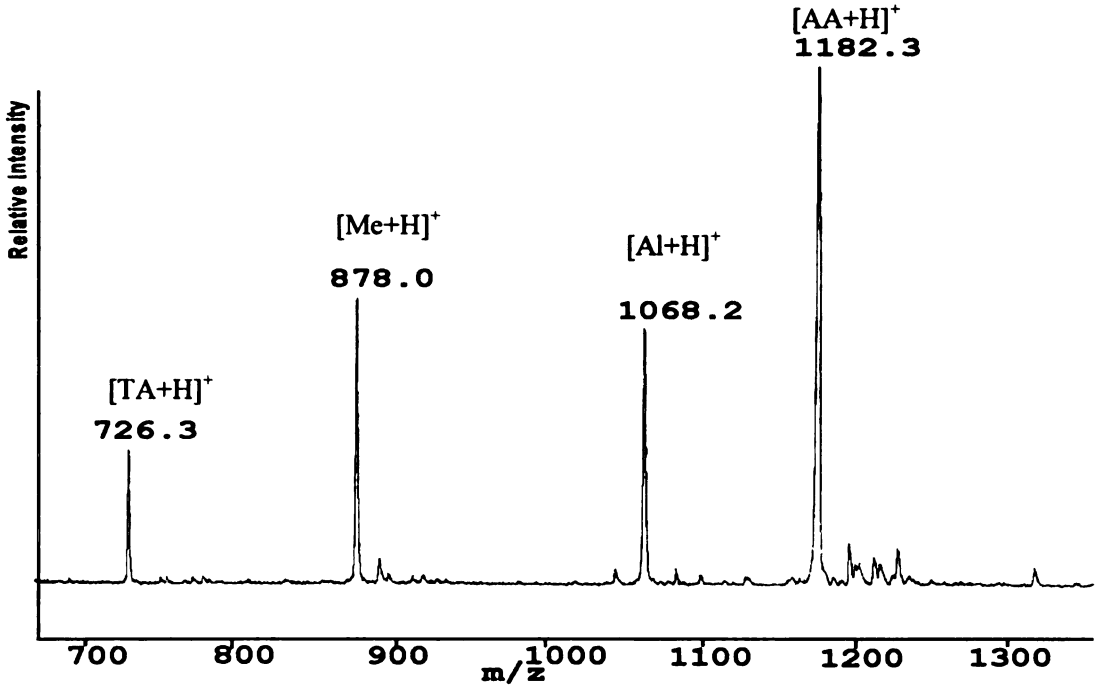


Figure. 5.4. MALDI-MS spectrum of a four peptide mixture of Tyr-Ala-Gly-Phe-Leu-Arg (TA, FW 725.8), methionine enkephalin-Arg-Phe (Me, FW 877.0), allatostatin II (Al, FW 1067.2), and des-Asp-angiotensin (AA, FW 1181.4), deposited on PVDF. Peaks at m/z 726.3, 878.0, 1068.2, and 1182.3 represent the four protonated peptide species respectively.

After the peptides were exposed to the first reagent vapor, chloroacetyl chloride vapor, for 10 minutes, the membrane was taken out from the reaction vessel and the product of the first step of the derivatization was detected on the membrane. The spectrum of three component peptide mixture after the first step derivatization is presented in Figure 5.5.

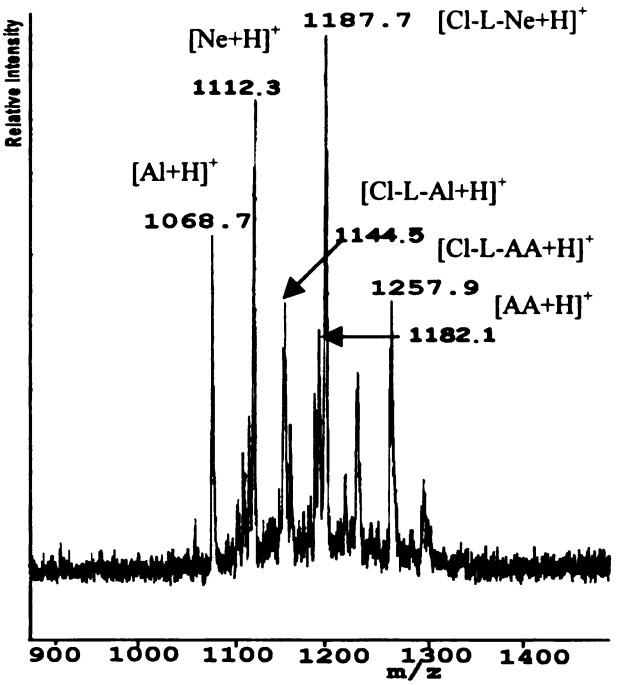


Figure. 5.5. MALDI-MS spectrum of chloroacetyl derivatives of allatostatin II (Al, FW 1067.2), neoromedin U-8 (Ne, FW 1111.3), and des-Asp-angiotensin (AA, FW 1181.4). The m/z values shift up by 76 for each peptide.

However, the resolution and signal-to-noise ratio of those peaks decreased dramatically compared with those peaks detected on membrane without going through the reaction.

As presented in Figure 5.6, the two step derivatization was complete. The peptides were first reacted with the chloroacetyl chloride vapor for 2 hours. The chloroacetyl derivatives of this step were then reacted with trimethylamine in the gas phase, for 2 hours, to yield trimethylamino acetyl derivatives. The peaks of protonated peptides as shown in Figure 5.4, are virtually disappeared from the spectrum, only the charged-derivatives of these peptides appear and gave good responses in the MALDI-MS spectrum.

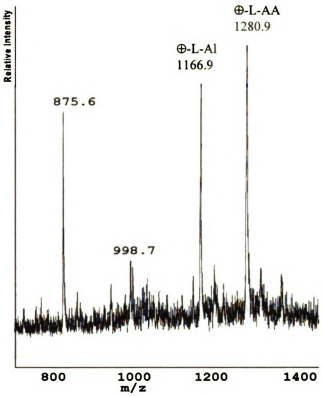


Figure. 5.6. MALDI-MS spectrum of allatostatin II (Al, FW 1067.2) and des-Asp-angiotensin (AA, FW 1181.4) derivatization products. The derivatization reaction was completed after 2 hours of reaction with chloroacetyl chloride and 2 hours with trimethylamine in the gas phase.

In fact, the reagents we used are very active. Here is a spectrum taken after 10 minutes of reaction with chloroacetyl chloride and then 10 minutes with trimethylamine in gas phase (Figure 5.7). The whole reaction consumed only 20 minutes in total. Not all peptide molecules reacted and were derivatized; we can still observe peaks of protonated peptides that remained unreacted. But we already get more than 50% derivatization yield, estimated from the relative height of derivative peaks and original peptide peaks, for all of the peptides in the mixture.

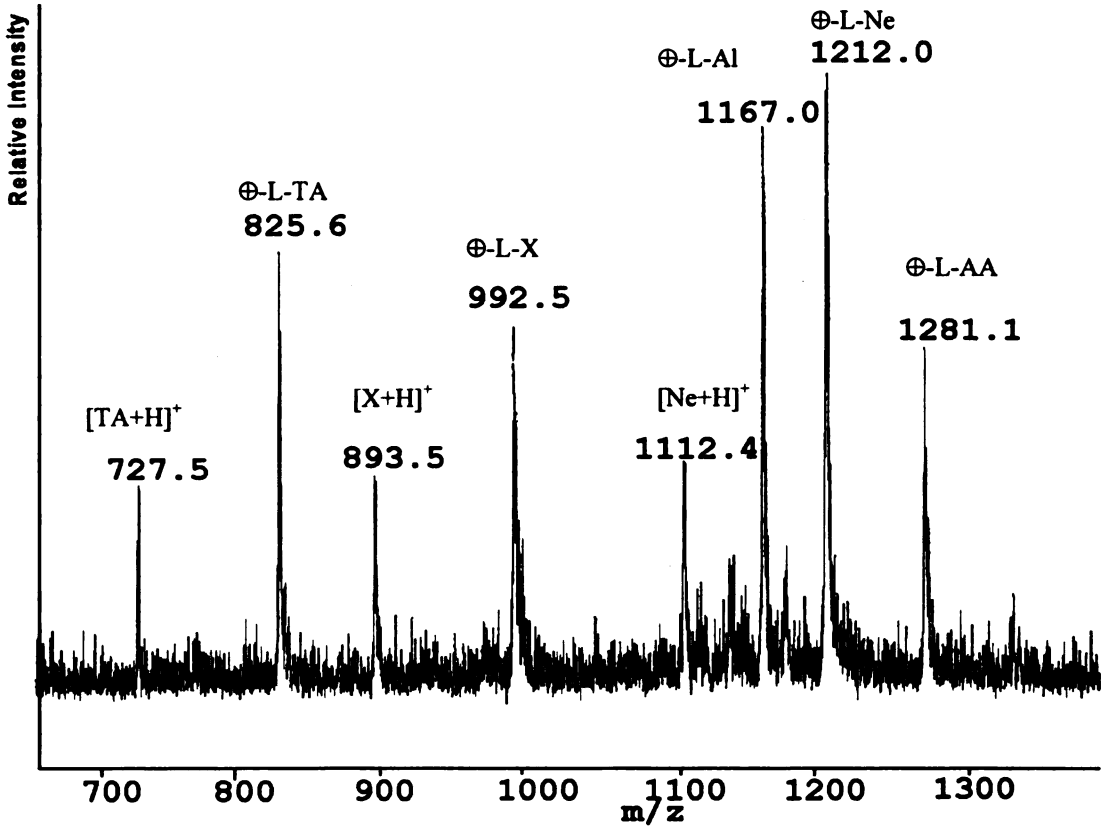


Figure. 5.7. MALDI-MS spectrum of peptide mixture derivatization products on a membrane. After a 20-minute reaction, with first chloroacetyl chloride, then trimethylamine, the 50% yield of the reaction was obtained.

With low quantities, some peptides will not give good response, occasionally no response at all in a MALDI-MS spectrum. For example, 500 fmol des-Asp-angiotensin (AA, FW 1181.4) was not detectable directly from a membrane in the spectrum as Figure 8a. This may be caused by the hydrophobic interactions between peptides and membrane surface, so not enough peptide molecules have been desorbed from the surface to be ionized and give a signal in the spectrum.

After we added onto the peptide N-terminus, a polar chloroacetyl group, the chloroacetyl

derivative of the peptide gave higher response than the original peptide (Figure 5.8.b). At the end, peptide molecules have been derivatized as the trimethylamino acetyl derivative, and we can identify it represented by a peak at 100 m/z units higher than the m/z value of the original peptide (Figure 5.8.c).

In another case, the analyte is a small peptide, VGVAPG (FW 498.9). Usually, small and hydrophobic peptides do not form as peaks at the expected m/z values in MALDI-MS spectra 5.9(a). There exist difficulties in desorption and ionization of these molecules from the membrane in the MALDI-MS technique. After the charged-derivatization process, the derivative shows good response at m/z 599.3 in Figure 5.9(b).

Other amine compounds that have been employed instead of trimethylamine in the derivatization are dimethylamine and cyclohexylamine. The resulting spectrum of derivatization of neoromedin U-8 (m/z 1111.3) with dimethylamine is shown in Figure 5.10. The dimethylamine group substituted the chloride on the other end of the chloroacetyl peptide and the total m/z value shift of the derivative from the original peptide m/z is 86.

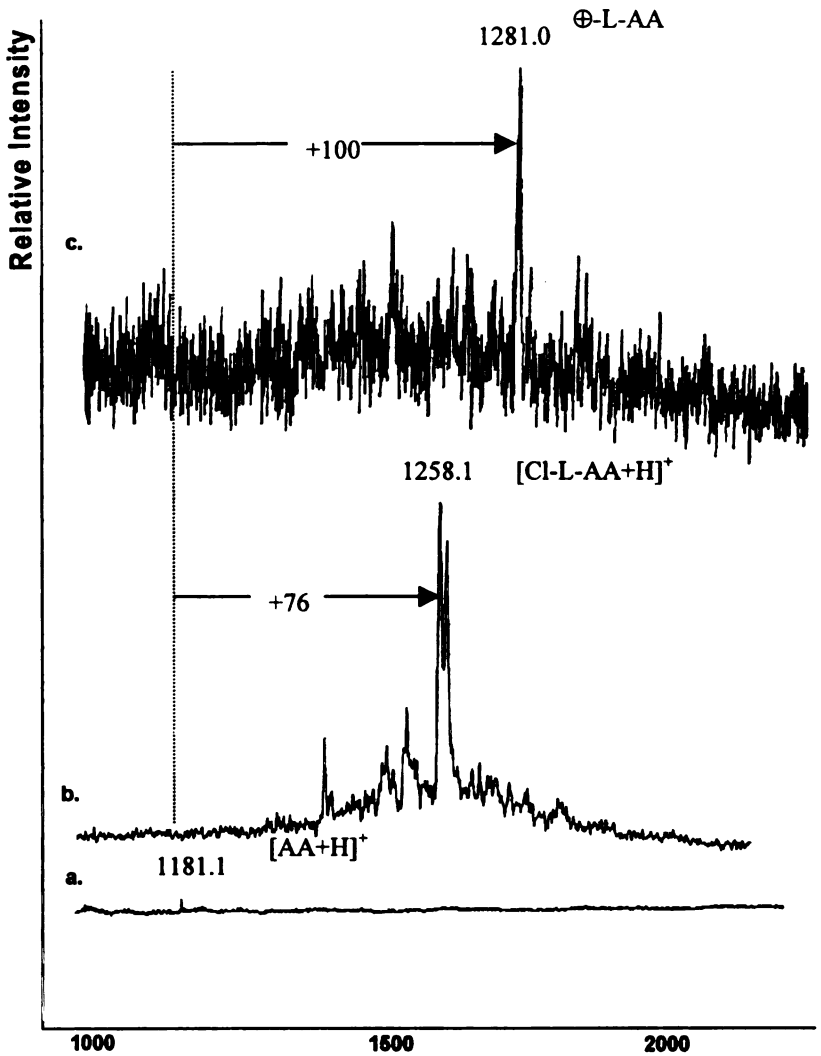


Figure. 5.8(a). In MALDI, 500 fmol of a peptide on a membrane is hardly detectable. 5.8(b). MALDI-MS spectrum of the first step derivatization product, which is 76 m/z units higher than the original peptide, indicating the presence of the low amount of peptide on the membrane. 5.8(c). MALDI-MS spectrum of the two step derivatization product, which is 100 m/z unit higher in the spectrum from the m/z value of peptide.

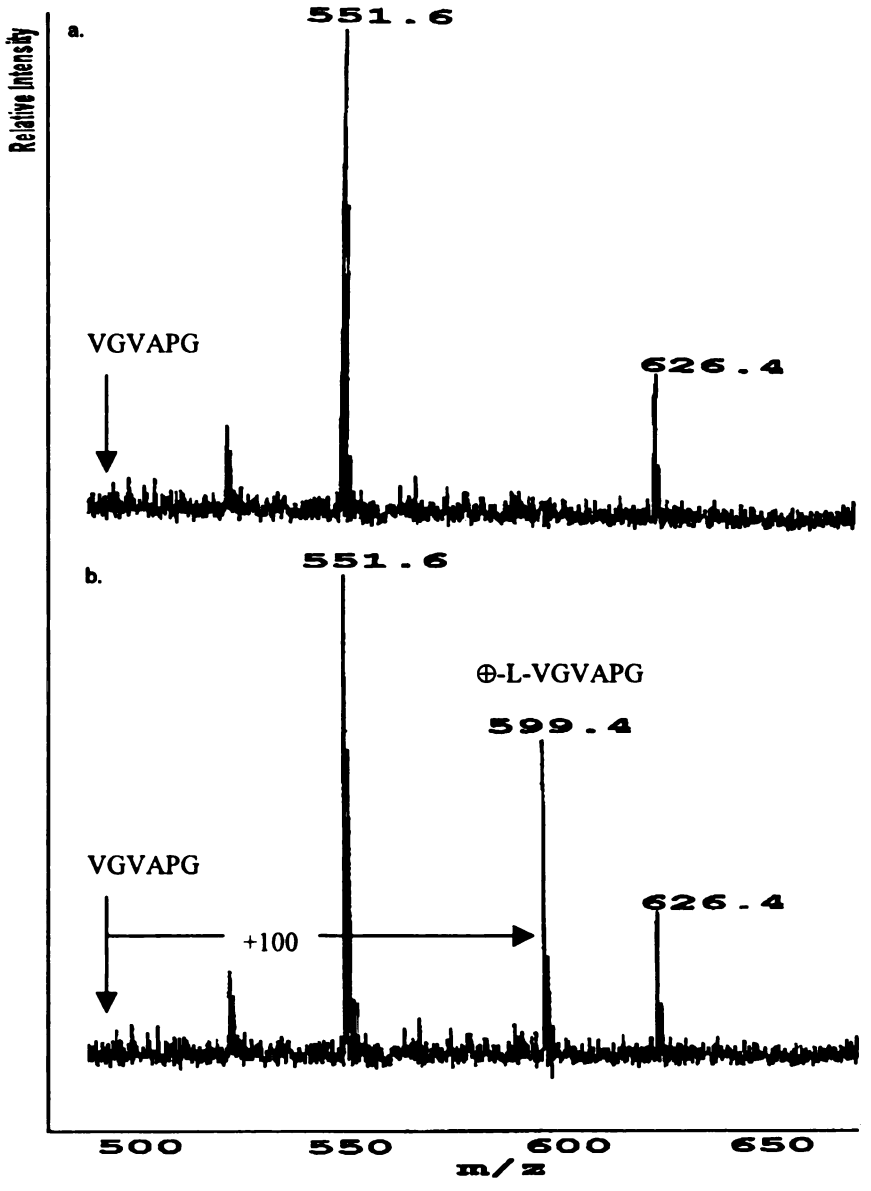


Figure. 5.9(a). 50 pmol VGVAPG (FW 498.9) on a membrane is not detectable in MALDI-MS. 5.9(b). MALDI-MS spectrum of charged-derivative of VGVAPG. Charge derivatization enhances the response of VGVAPG (FW 498.9), which has no response as an underivatized peptide in MALDI-MS.

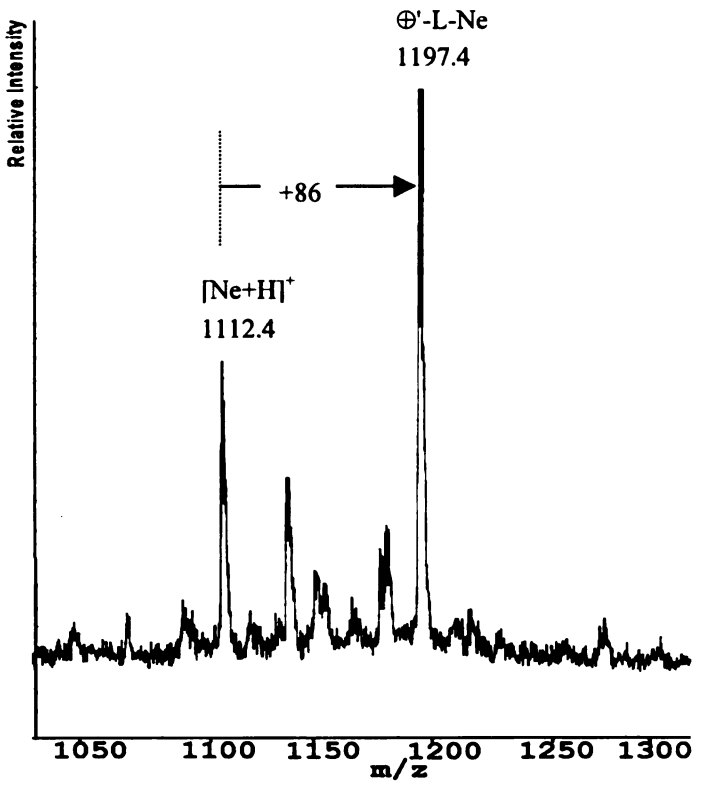


Figure. 5.10. MALDI-MS spectrum of dimethylamineacetyl derivative of neoromedin U-8. Derivatization with chloroacetyl chloride and dimethylamine reagent vapors of neoromedin U-8 (Ne, FW 1111.3) produces the charged-derivative (⊕'-L-Ne), which in the MALDI-MS is at m/z 1197.3.

However, the derivatization is not successful in the case with cyclohexylamine. In the MALDI-MS spectrum of the final mixture for the same peptide, only the chloroacetyl peptide was observed at 76 m/z units higher from the original m/z value of the peptide, as shown in Figure 5.11.

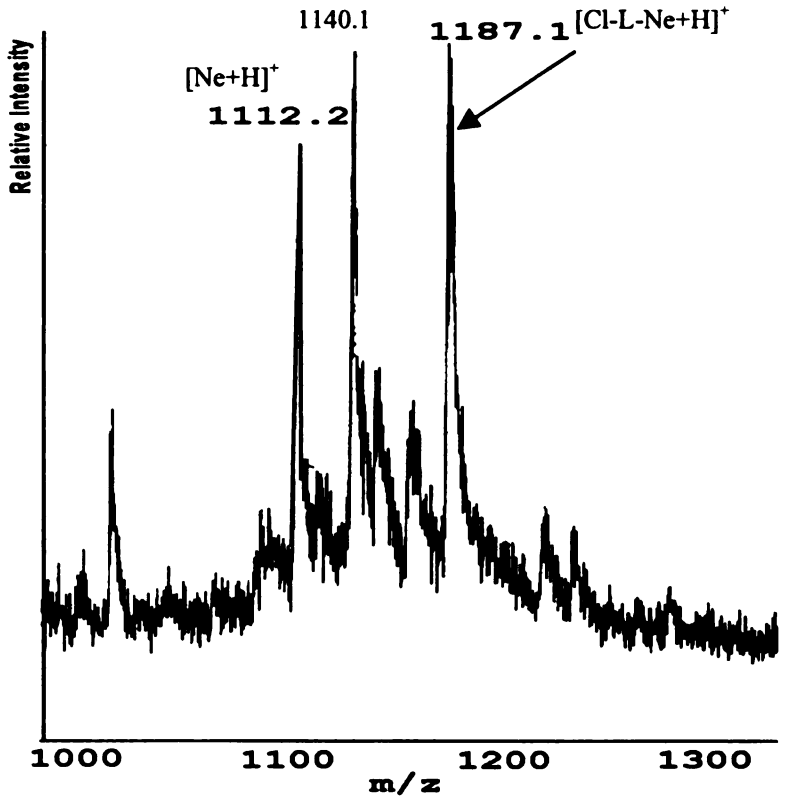


Figure. 5.11. MALDI-MS spectrum of the result of an unsuccessful neoromedin U-8 (Ne, FW 1111.3) derivatization with chloroacetyl chloride and cyclohexylamine reagent vapors, only chloroacetyl derivative $[Cl\text{-}L\text{-}Ne+H]^+$ is observed at m/z 1187.1.

A possible reason for unsuccessful derivatization with cyclohexylamine is the lower vapor pressure of the reagent [16].

Post-source decay (PSD) spectra have been taken for the trimethylamino acetyl derivative and dimethylamine acetyl derivative of neoromedin U-8. The base peaks in the spectra are the charged-derivatives in both cases. Extensive fragmentation within the charged moiety on the N-terminus of peptide was observed, as shown in Figures 5.12 and 5.13. Little structural information can be obtained from these PSD spectra.

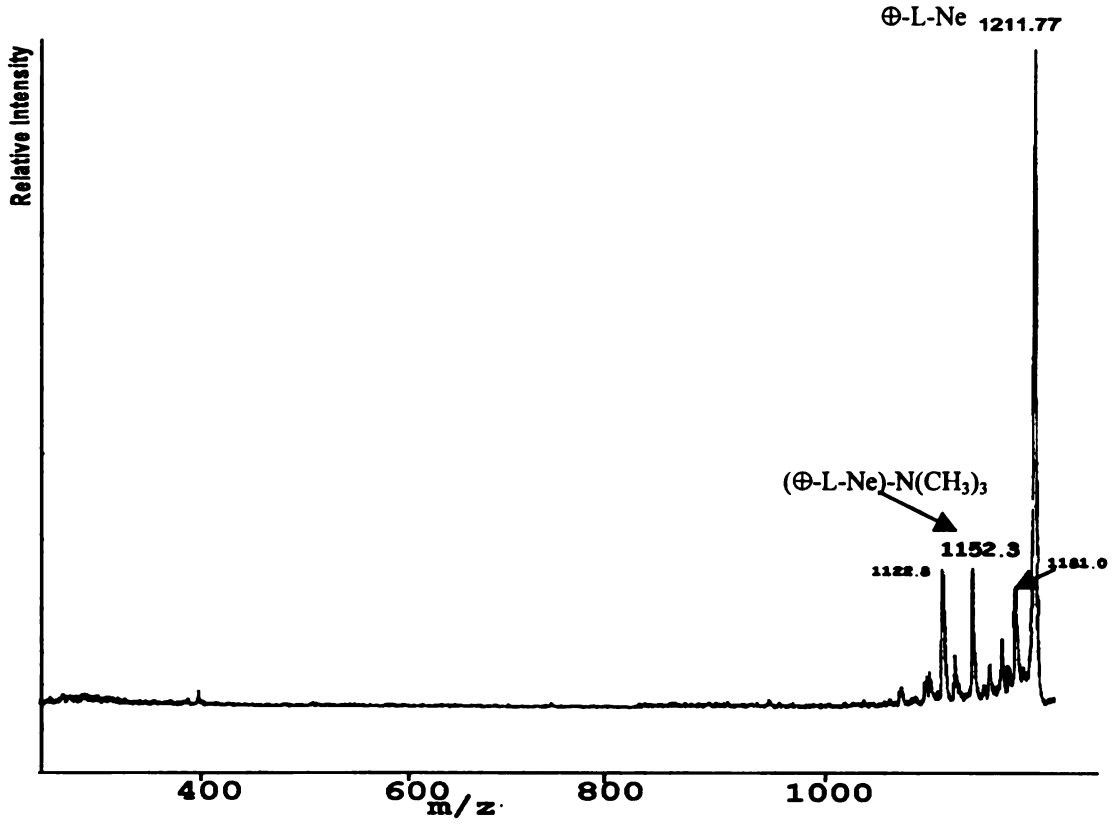


Figure. 5.12. Post-source decay spectrum of trimethylamino acetyl neoromedin U-8. The base peak is trimethylamino acetyl neoromedin U-8, at m/z 1211. The peak at m/z 1152 is from the fragmentation and loss of trimethylamine group from 1211.

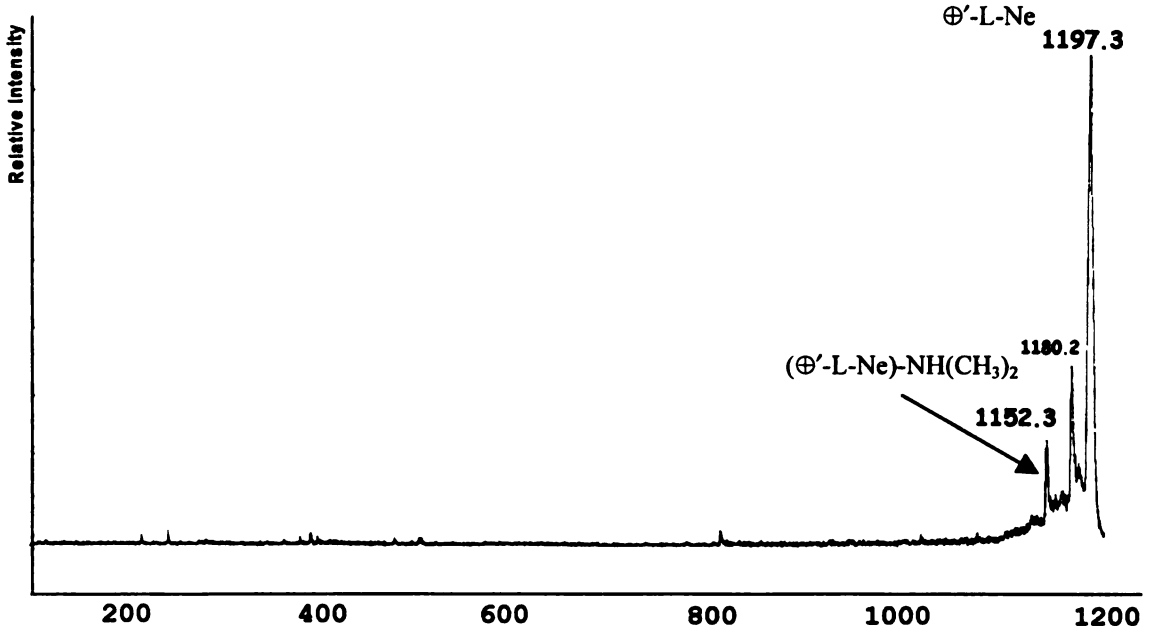


Figure. 5.13. Post-source decay spectrum of dimethylamine acetyl neoromedin U-8. The base peak is dimethylamine acetyl neoromedin U-8, at m/z 1197. The peak at m/z 1152 is from the fragmentation and loss of dimethylamine group from 1197.

Although the derivatization was successful for most of the peptides used in the experiments, there were some peptides that have no peptide signals in MALDI-MS spectra, also gave no signals after derivatization, such as Thr-Ser-Lys (m/z 334.4), Pro-Phe-Gly-Lys (m/z 507.6), and α-neurokinin (m/z 1133.3). Some times the derivatization product detected on membrane shows both the chloroacetyl derivative and the trimethylamino acetyl derivative. Long hours of trimethylamine reaction have been adopted, but the yield of the reaction still needs to be improved.

Matrix crystal formation is critical to MALDI-MS spectra. α-cyano-4-hydroxycinnamic acid matrix is usually applied by adding into the peptide (either dried or still in droplet) an equal volume of saturated matrix solution. The α-cyano-4-hydroxycinnamic acid matrix solution is acidic and has a high percentage of organic solvent (1:1 acetonitrile/water). The application of matrix solution can cause the protein to spread out on the PVDF. A spot can become a ring if a drop of matrix is placed on a protein spot and the concentration of the protein per mm^3 of membrane is reduced. Improvements in the composition and application of matrix solution to reduce protein and peptide spreading will assist in the development of the direct MALDI-TOF analysis of these biopolymers from PVDF.

Another contribution to the low resolution and low intensity of the spectra taken from the membrane is from PVDF itself. The laser intensity required to reach the ionization threshold on PVDF was higher than that required for a metal target. This was attributed to the porosity of PVDF, which permits a distribution of the analyte and matrix within the

membrane [13]. Larger variance, lower resolution in flight times was observed with PVDF due to the spatial distribution of sample within the pores. In Figure 5.14 (adopted from reference 13), the electron micrograph of PVDF membrane shows us that the matrix crystals are embedded in the pores on the membrane [13].

The main advantage of charge derivatization is that the PSD spectra of the derivatives should be much simpler and identification characterization should be done by interpreting the fragment peaks in PSD spectra [17, 18]. Unfortunately in the PSD spectra obtained for the trimethylamino acetyl derivative, extensive fragmentation was in the charged group. The charged moiety is not as stabilized on the peptide as a TMPP group in the $TMPP^{+}$-Ac derivatives [5]. Further study needs to be conducted to find a proper base or a linker (between the charged group and N-terminus of peptides, in this study, chloroacetyl chloride) of a different structure as derivatization reagents in gas phase, in order to enhance the PSD spectral quality.

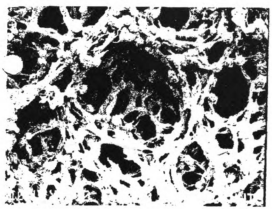


Figure. 5.14(a). Electron micrograph of PVDF membrane obtained at a ×4800 magnification. (taken from reference [13])

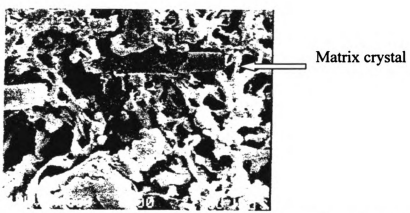


Figure. 5.14(b). Electron micrograph of PVDF after deposition of sample and matrix solution. The matrix crystals are embedded into the membrane surface. (taken from reference [13])

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