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Characterization of Extremely Hydrophobic Immunostimulatory Lipoidal Peptides by Matrix Assisted Laser Desorption Ionization Mass Spectrometry

Jung-Suk Jang[†], Sung-Taek Lee[‡], and Yoon-Seok Chang[*]

[*]Department of Chemistry, Hanyang University, Ansan 425-791, Korea
[†]Korea Research Institute of Chemical Technology, Taejon 305-606, Korea
[‡]Department of Biological Science, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea
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Synthetic lipoidal peptides based on viral protein sequences have been prepared. These peptides contain an N-palmitoyl group at the N-terminal residue, which is a modified cysteine, containing a S-[2,3-bis(acyloxy)-(2-R,S)-propyl] moiety. When this residue (Pam_3Cys) is at the N-terminus of a synthetic peptide, it acts as potent immunoadjuvant to enhance both IgM and IgG antibody responses to the attached peptide. Conventional analytical procedures (*e.g.*, Edman degradation and amino acid analysis) are either not applicable due to the N-terminal modification, or do not provide confirmation of the intact structure. Chromatographic analysis is also hindered by the tendency of these lipoidal Pam_3Cys peptides to form large aggregates, and in some cases to be permanently adsorbed on reversed phase columns. We have applied several mass spectrometric techniques, including fast atom bombardment (FAB), electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) to characterize the intact structures of a number of different Pam_3Cys synthetic peptides. The MALDI-MS has been found to be the most sensitive for the analysis of the structure of Pam_3Cys peptides.

Introduction

Synthetic peptides are prepared to contain an N-palmitoyl moiety at the N-terminal residue of the peptide which is a modified cysteine, containing a S-[2,3-bis(acyloxy)-(2-R,S)-propyl] moiety. When this residue is placed at the N-terminus of various synthetic peptides, it has been found to be potent immunoadjuvant which enhances both IgM and IgG antibody responses to the attached peptide.[1~6] Synthetic analogues of these compounds include those bearing palmitoyl groups (Pam_3Cys) as shown in Figure 1. These synthetic peptides have significant advantages, since the addition of other adjuvants are not required, and most importantly, the epitope can be specifically defined.

It is critical, however, that these peptides should be structurally characterized prior to their use in immunological studies.[7~8] This is most important, since the synthesis involves several steps where the peptide is exposed to conditions that can provide amino acid side chain modification and/or deacylation. And, the lipoidal nature of the peptides make them extremely difficult to be purified and analyzed. Reverse phase HPLC can lead to irreversible adsorption to the bonded phase. Although we have found that the N-methyl-2-pyrrolidone (NMP) is useful for solubilization and isocratic elution for some of these lipopeptides, it is not successful in all cases for HPLC purification.[9] The peak broadening resulting from the inherent self-aggregation of these compounds may, in even favorable cases, obscure contaminating peaks. Thus, amino acid analysis is not adequate to fully characterize these peptides prior to their use in immunological studies. Further, because the N-terminal is blocked, traditional Edman sequencing cannot be employed to determine the proper sequence of synthetic peptide.

We are currently using several mass spectral techniques to characterize the amino acid sequences of the Pam_3Cys peptides found in the envelop glycoproteins of HIV-1 and the Simian Immunodeficiency Virus (SIV).[17] Conventional FAB-MS analysis using standard matrices, such as glycerol and nitrobenzyl alcohol, is not particularly effective for these molecules, largely due to their tendency to aggregate. Here,

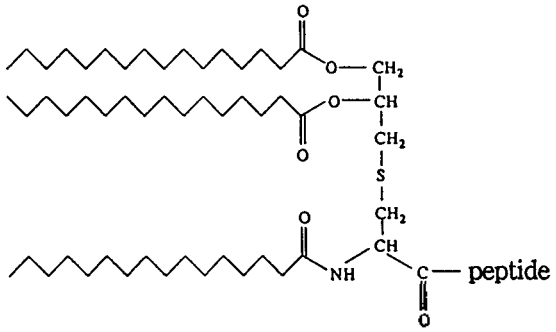

Figure 1. Structure of N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-cysteinyl (Pam_3Cys) peptide, showing the modified cysteine residue with an N-palmitoyl ester and a dipalmitoylglyceryl moiety connected via a thioether linkage. This amino acid is placed at the N-terminus of the synthetic peptide.

we report comparative studies on the the analysis of Pam3 Cys peptides by fast atom bombardment (FAB)-, electrospray ionization (ESI)- and matrix assisted laser desorption ionization (MALDI)-mass spectrometry (MS).

Experimental

A set of lipoidal Pam_3Cys peptide samples were synthesized with procedures of Metzger *et al.*[10] by Dr. Patrick Kanda at the Southwest Research Foundation for Biomedical Research, San Antonio, Texas. Following deprotection, samples were purified by HPLC with a C-18 reverse phase column before mass spectral analysis.

The MALDI mass spectra were acquired by a Bruker Reflex MALDI time-of-flight (TOF) instrument (Bruker, Instrument Inc., Billerica, USA) in positive mode, using both linear and reflector. The samples (0.5 ug) were diluted 10 : 1 in a α-cyanocinnamic acid matrix and irradiated with a nitrogen laser (337 nm) of which average power was about 10^6 W/cm^2. The generated molecular ions were accelerated in a constant electrical voltage of +35 kV. The time difference between desorption by a short laser pulse duration of 5 ns and ion detection is proportional to the square root of the mass to charge (m/z) ratio. The spectra were obtained by summing 30 laser pulse signals. An instrumental mass resolution of up to 1,000 FWHM (the ratio of the peak mass to the full width measured in mass units at half height, n/dm) can be obtained for molecular ions below 10,000 Da. The calibration was accomplished using a mixture of substance P (RPKPQQ-PPGLM-NH_2, 1347.6 Da) and insulin (5733.6 Da) as internal mass standards.

The electrospray ionization mass spectra were collected using a VG Platform (Fison Instrument, Altrincham, U.K.) benchtop single quadrupole mass spectrometer in positive mode. A Harvard model 22 syringe pump (Harvard Apparatus, South Natick, MA, USA) was used to infuse the samples into the instrument at a rate of 10 ul/min. Operating conditions were as follows; mass range, m/z 100-700; capillary, 2.24 kV; counter electrode, 0.16 kV; sampling cone, −27 V; focus lens, −34 V; source temperature, 45 ℃. Several scans were usually averaged to improve signal-to-noise ratio.

The FAB-MS was carried out in MS-1 of a JEOL HX110 A/HX110A high resolution tandem mass spectrometer (JEOL Ltd., Akishima, Japan) in positive mode. The resolution was set at either 1,500 or 3,000. 3-Nitrobenzyl alcohol (NBA) was used as the matrix, but detectable signals were obtained only when the lipoidal peptides were solubilized in N-methyl-2-pyrrolidone before MS analysis. Samples were prepared for FAB-MS analysis by mixing 1 ul of the peptide solution (1-2 nmol) with 1 ul of NBA on the probe tip. The accelerating voltage was 10 kV and the JEOL Cs^+ ion gun was operated at 25 keV. Five scans were acquired and averaged between m/z 100-3,500. The instrument was calibrated by using (CsI)$_n$$Cs^+$ cluster ions. Mass spectra were acquired with a JEOL DA 7,000 data system running on a HP 9,000 computer.

Results and Discussion

Preliminary results show that fast atom bombardment-mass spectrometry (FAB-MS) could be a useful technique for characterizing these synthetic lipoidal peptides, but conditions for successful analysis are not always predictable, since the tendency to form aggregate in matrix is somewhat dependent upon the nature of the amino acid residues present in the peptide (Figure 2 and 3). With the use of N-methyl-2-pyrrolidone (NMP) as a dopant to the matrix, we have obtained an adequate signal for some samples.[9] Most results to date have been obtained by dissolving the synthetic products in NMP and adding the solution to 3-nitrobenzyl alcohol (NBA) matrix on the probe tip (usually 1-2 nmol samples were loaded). These data have been used to confirm the purity of the synthetic lipopeptides before expensive and time-consuming immunological studies begin. The FAB-MS of lipopeptide samples have indicated in some cases that blocking groups have not been completely removed. Final structural characterization requires complete sequencing analysis by tandem mass spectrometry (MS/MS) approaches.[11] However, the weak parent MH^+ signal for these peptides requires high sensitivity instrumental conditions such as employing the array detector.[12] With sufficient sensitivity, complete sequencing analysis using the tandem instrument may be possible. For the FAB-MS analysis, it may be possible to reduce aggregate formation by running the samples in extremely diluted solutions. This method, however, has failed in many cases due to the fact that lowering the concentration approaches the detection limits.

Since the FAB-MS does not appear to be the most reliable method, matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS)[13~14] has been tried for these highly hydrophobic lipopeptides. Results show that molecular ions are well observed with much lower sample sizes than those in FAB-MS or even in ESI-MS. The actual sample size loaded on to the probe tip was 1-10 pmol. Aggregation does not seem to be a problem in this case and most common matrices work well with these samples. In most cases, we have solved the questions of sequence and purity of the samples. The protonated molecular weight of the sample Pam_3-Cys-DRPEGIEEEGGERDRSG is estimated to be at 3051.97, with the peptide portion up to the aspartic acid (D) being at 2158.97. The MALDI spectrum, however, indicates that an aspartic acid residue has been deleted from the expected synthetic sequence (Figure 4). The protonated molecular weight of another sample Pam_3-Cys-GRIQRGPGRAFVTIGK is calculated to be about 2603.99, with the peptide portion

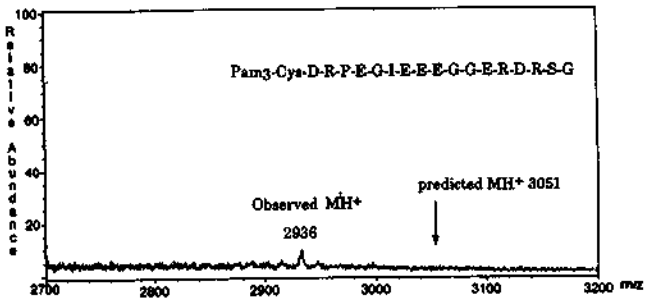

Figure 2. FAB-MS of Pam_3Cys peptide, HIV-1 gp 41 (735-752) in 3-nitrobenzyl alcohol with N-methyl-2-pyrrolidone used to solubilize the peptide. The observed M'H% peak suggests that one aspartic acid residue was deleted during synthesis. Signal intensity, however, makes detection of other components problematic.

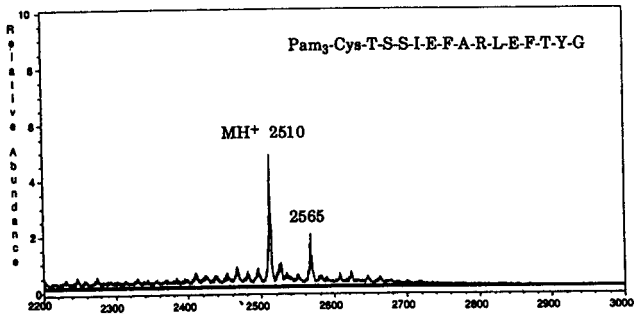

Figure 3. FAB-MS of Pam_3Cys-TSSIEFARLQFTYG synthetic peptide in NBA/NMP. The ability to obtain useful FAB spectra, even with the addition of NMP, is dependent on the structure of the peptide.

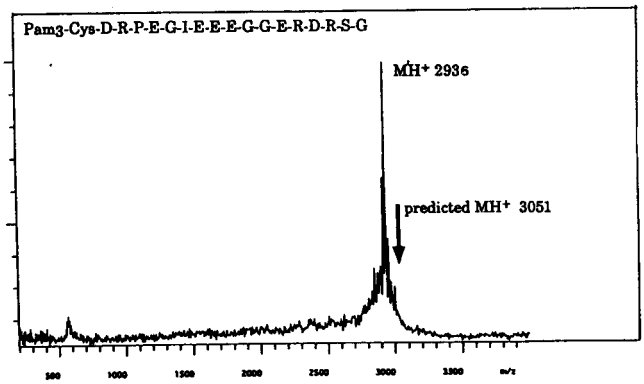

Figure 4. MALDI-MS of Pam_3Cys peptide, HIV-1 gp 41 (735-752). These data support the conclusion that the expected product was not obtained, but rather that one aspartic acid residue was not introduced in the sequence (cf. Figure 2B).

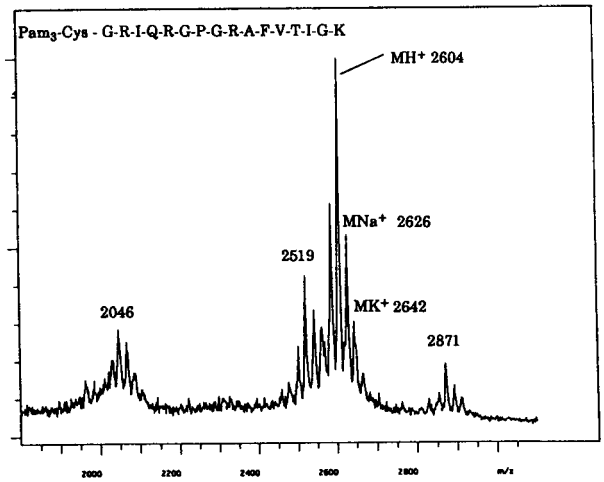

Figure 5. MALDI-MS of synthetic Pam_3Cys peptide HIV-1 gp 120 (315-329) w/G at the N-terminus. FAB-MS analysis did not show any peptide-related peaks. Here, the expected MH^+ ion was detected (m/z 2604), together with several other products and their sodium and potassium adducts.

being at 1710.99. The MALDI spectrum shows the right protonated molecular ion at 2604 (Figure 5). However, this sample includes a lot of undesired impurities that we could not identify. Another sample was claimed to be same peptide as the Pam_3-Cys-GRIQRGPGRAFVTIGK, which was obtained from a different batch. The MALDI spectrum shows two characteristic peaks at m/z 2053 and m/z 4108 (Figure 6). We could not suggest the suitable structures for these two ions, but the latter peak is expected to be dimer formation.

Finally, with some otherwise difficult Pam_3Cys lipopeptide samples we also have had some preliminary success with electrospray ionization. With a methanol/water solution (100 ng/mL) we have obtained readily interpretable spectra based on the molecular weights of corresponding peptides (Figure 7). Solubility has not yet been a problem with this technique. No peptide-related signals were detected in the FAB spectrum of this sample. The expected MH^+ peak is the largest analyte signal in the ESI spectrum, but other unidentified components also were detected. The MALDI spectrum of this sample showed significantly different responses for these and other components (Figure 6). The preliminary results with ESI-MS[15~16] suggest that this technique may be the alternative choice for analyzing this class of lipopeptides, we cannot yet exclude the possibility that differential sensitivity in ESI-MS for different components in a mixture will give misleading results for some samples. We will investigate this possibility by analyzing artificial mixtures of Pam_3Cys peptides of various sizes and degrees of acylation.

Conclusion

Chromatographic analysis is not applicable to analyze the intact Pam_3Cys lipoidal peptides because of the tendency of these hydrophobic peptides to form large aggregates, and in some cases to be permanently adsorbed on reversed phase columns. Edman sequencing method also is hindered due to N-terminal blocking by Pam_3Cys group. We have applied several mass spectrometric techniques, including fast atom bombardment, electrospray ionization and matrix assisted laser desorption ionization to characterize the intact structures of a number of different Pam_3Cys synthetic peptides. The FAB-MS cannot be used successfully with standard matrices such as glycerol, thioglycerol, 3-nitrobenzyl alcohol, but the addition of N-methyl-2-pyrrolidone (NMP) to NBA matrix does produce detectable MH^+ signals in some cases. Much better results are obtained with ESI-MS, with improved sen-

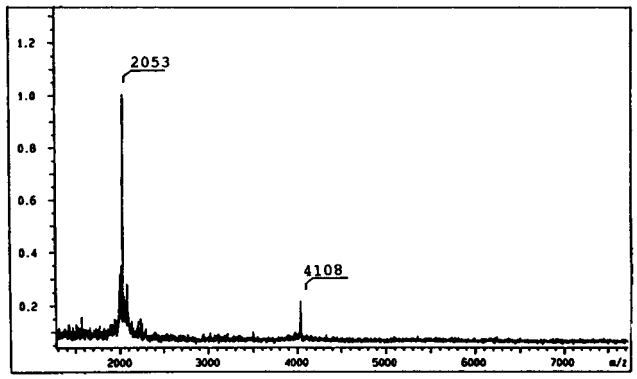

Figure 6. MALDI-MS of Pam_3Cys peptide obtained from failed synthesis of HIV-1 gp 120. The spectrum shows two characteristic peaks at m/z 2053 and m/z 4108, the latter supposedly is from dimer formation.

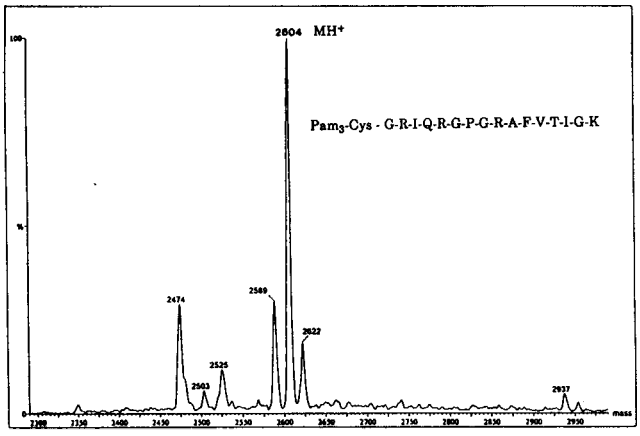

Figure 7. ESI-MS of Pam3Cys peptide, HIV-1 gp 120 (315-329) w/G at the N-terminus. No peptide-related signals were detected in the FAB spectrum of this sample. The expected MH^+ peak was the largest analyte signal in the ESI spectrum, but other components were detected. The MALDI spectrum of this sample showed significantly different responses for these and other components (cf. Figure 5).

sitivity relative to FAB-MS. Some evidence for the formation of aggregates was apparent in the ESI spectra, but most of the peaks observed were related to the monomeric peptide. The MALDI-MS was found to be even more sensitive than ESI-MS for the analysis of Pam_3Cys peptides. Lipoidal peptide monomers were readily detectable in the low picomole range by observing exact molecular masses.

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