MALDI TOF/TOF Tandem Mass Spectrometry as a New Tool for Amino Acid Analysis

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This is the first report of an application of collisionally induced fragmentation of amino acids (AA) and their derivatives by MALDI TOF/TOF tandem mass spectrometry (MS). In this work, we collected the data on high-energy fragmentation reactions of a large group of protonated amino acids and their derivatives with the goal of determining which product ions are analyte specific and if yields of these fragment could be used for quantitative analysis. From 34 different amino acids (20 α-amino acids, β-amino acids, homocysteine, GABA, and modified AA Met sulfone and sulfoxide, hydroxyproline, etc.) we observed that high yields of the target specific immonium ions and fragmentation patterns are most similar to EI or FAB CID on sector instruments. The major exceptions were two highly basic amino acids, Arg and Orn. It is noted that neither β-, γ-, nor δ-amino acids produce immonium ions. As might be predicted from high-energy CID work on peptides from the sectors and TOF/TOF, the presence of specific indicator ions in MALDI tandem MS allows distinguishing isomeric and isobaric amino acids. These indicator ions, in combination with careful control of data acquisition, ensure quantitative analysis of amino acids. We believe our data provide strong basis for the application of MALDI TOF/TOF MS/MS in qualitative and quantitative analysis of amino and organic acids, including application in clinical medicine. (J Am Soc Mass Spectrom 2007, 18, 279–284) © 2007 American Society for Mass Spectrometry

Since their introduction in the late 1980s, the soft-ionization techniques, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), have became the major ionization methods for the analysis of biological molecules. While analysis of large biopolymers is successfully performed by both techniques, analysis of low molecular weight (LMW) molecules such as amino acids, pharmaceuticals, hormones, etc. is predominantly performed by ESI mass spectrometry with quadrupole mass analyzers. The major reasons not to use MALDI have been the abundance of matrix ions in the low mass region (100 to 600 u) and the lack of reasonable precursor selection in a MALDI tandem instrument. Also, until recently, MALDI was not considered to be a technique capable of providing robust quantitative data. Current developments in MALDI hardware [1, 2] and increased attention paid to sample preparation have changed the situation. A number of recent publications describe application of MALDI TOF for quantitative studies [3–8], including favorable comparisons of MALDI TOF with the more traditional ESI LC/MS quantitation schemes [9–11]. When installed on QqQ instruments, a MALDI source demonstrated good comparability with an ESI source on the same instrument in quantitative analysis of LMW compounds [12].

Amino acid analysis (AAA) is one of the areas where ESI LC/MS/MS was successfully applied. ESI LC/MS/MS has become a standard analytical tool in the analysis of low molecular weight physiological or therapeutic molecules of interest [13–16].

We decided to examine if the combination of high-energy collision and prompt ion detection characteristic for MALDI TOF MS/MS could be applied for AAA. The proposed application of MALDI TOF for AAA contains some advantages over ESI LC/MS/MS or GC/MS: an absence of a need for AA modification; a simplified separation/presentation of sample (e.g., minimal desalting and no LC) with demonstrated quantitative ionization [4], and rapid acquisition of data from 90 plus samples in one "session" for high throughput analysis [10].

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In this study, we have established patterns of high-energy fragmentation of amino acids and their derivatives by MALDI TOF/TOF tandem MS and demonstrated quantitative character of that fragmentation. We have discovered that fragmentation of all α-amino acids and their derivatives (except arginine) consistently produces amino acid specific immonium ions as the major fragments. We have also demonstrated that specific indicator fragmentation ions of isobaric and isomeric compounds can be used for relative quantitation of their ratios in an amino acid mixture. These results provide strong evidence for the application of MALDI TOF/TOF MS/MS in qualitative and quantitative analysis of amino and organic acids.

Materials and Methods

Chemicals

All amino acids were purchased from Sigma (St. Louise, MO). α-Cyano-4-hydroxycinnamic acid (CHCA) was obtained from Aldrich (Milwaukee, WI). HPLC grade acetonitrile and trifluoroacetic acid were purchased from Fisher Scientific.

Preparation of Amino Acid Standards

All 36 amino acid solutions were prepared at concentration of 200 μM from 10 mM stock solutions in deionized water (Tyrosine stock solution was 3 mM). Amino acid mixtures were prepared as follows. Gln/Lys solutions were prepared in molar ratios 10:1, 5:1, 1:1, 1:5, and 1:10 from 200 μM stock solutions, and Orn/Asn/$(Gly)_2$ solutions were prepared in ratios 1:1:1, 1:3:1, 1:1:3, 3:1:1, 1:4:1, 1:1:4, and 4:1:1 from 200 μM stock solution; 1 μL of the obtained solutions in each experiment was loaded on the target plate.

Mass Spectrometry

Mass spectra were acquired on Applied Biosystems (Foster City, CA) 4700 Proteomics Analyzer positive MS-MS mode at 2KV. Following instrument parameters were used: collision gas, air; metastable suppressor, on (with optimized precursor function); 4500 total shots (60 shots per subspectra and 75 subspectra); laser intensity for MS and MS-MS data collection, 4500; precursor mass range, 76 to 205.

The peak intensity values were determined using Data Explorer version 4.5 (Applied Biosystems); only fragment ions with S/N Ratio higher then 10 were included in the table.

CHCA was prepared as saturated solution in 85% acetonitrile (ACN)/0.03% trifluoroacetic acid (TFA); 1 μL of this solution was pipetted on 192-well plate (Applied Biosystems) and air-dried; after that 1 μL of amino acid solution was spotted on the target.

Results and Discussion

Qualitative Aspects of Amino Acids Fragmentation Analysis

Tandem mass spectrometry of amino acids received much attention and has been studied extensively over the years [13–26]. The overall fragmentation patterns for the majority of amino acids were established by Biemann and Gapp in the early 1960s with examples of ethyl esters of amino acids by electron impact (EI) mass spectrometry [17]. Later, fundamentally the same fragmentation patterns were confirmed to be formed by other types of ionization such as fast-atom bombardment (FAB) [18] and ESI [13, 14, 19]. Fragmentation results obtained in our study by MALDI TOF/TOF tandem MS are presented in Table 1 and Supplemental Figure S1 (which can be found in the electronic version of this article) . We have analyzed 34 different amino acids in this study and they are presented in Table 1 classified as follows: the twenty amino acids commonly occurring as protein building blocks, nine frequently encountered modified amino acids, and five amino acids not occurring in proteins, but found in free form and, finally, carnitine, a product of amino acid metabolism.

We observed that the immonium ion ($R - C = NH_2^+$) was a dominant peak formed during fragmentation of protonated aliphatic and aromatic amino acids (Table 1 and Supplemental Figure S1). There were essentially no other ions formed. In the case of isomeric Leu and Ile, characteristic fragmentation patterns included an additional apparent elimination of NH_3 from Ile immonium ion and the butyl radical (*m/z* 57) allowing clear distinction between these amino acids (Table 2). This is consistent with earlier observations made using EI MS [17], high energy CID [20], and FAB [18]. Interestingly, fragmentation patterns obtained at low energy using ESI MS/MS were highly dependent on collision energy with a wide distribution in the occurrence of abundant immonium ions, e.g., abundant from α- and δ-amino acids while not observed in β- and γ-amino acids [14]. In contrast, the high-energy CID spectra from the MALDI MS/MS of the α-amino acids formed immonium ions while β-, γ-, and δ-amino acids did not. Additional side-chain indicative fragments observed here are not listed in the low collision energy spectra.

Among AA analyzed in this study were four hydroxyl-containing amino acids (Ser, Thr, Hse, and Hyp). In the case of FAB on a sector and ESI on a triple quadrupole, decomposition of these amino acids is dominated by ions reflecting elimination of H_2O and CO_2H_2 [14, 18, 21, 22]. We observed immonium ions as the most abundant peak with a modest H_2O loss from the immonium ion and no ion corresponding to loss of H_2O from the precursor (Table 1 and Supplemental Figure S1).

The most distinct differences in fragmentation patterns between high-energy MALDI MS/MS and low-

Table 1. Results of protonated amino acids fragmentation by MALDI TOF/TOF tandem mass spectrometry

AA	MH^+	$MH^+ - CO_2H_2$	$MH^+ - H_2O$	$MH^+ - NH_3$	$MH^+ - CO_2H_2 - NH_3$	$MH^+ - CO_2H_2 - H_2O$	Other fragments
Gly	76	**30**					
Ala	90	**44**					
Ser	106	**60**			43	42	
Pro	116	**70**					43
Val	118	**72**					
Thr	120	**74**				56	45
Cys	122	**76**		105	59		112
Leu	132	**86**					44
Ile	132	**86**			69		41, 57
Asn	133	**87**					
Asp	134	**88**					74
Gln	147	**101**		130	**84**		44, 56, 75
Lys	147	**101**		130	**84**		56, 74
Glu	148	**102**				**84**	56, 75
Met	150	**104**					61
His	156	**110**					82
Phe	166	**120**					
Arg	175						30, 43, **60**, 70, **116**, 130
Tyr	182	**136**					
Trp	205	**159**		**188**			**130**
$(CH_3)_2$Gly	104	**58**					42, 44
Hse	120	**74**				56	31, 44
Hyp	132	**86**				68	
Hcy	136	**90**	**118**				
Cit	176	**130**		**159**	113		30, **70**, 116
S-(carboxymethyl) Cys	180	**134**		**163**			
Met sulfone	182	**136**					56
Met sulfoxide	166	**120**		149		102	75, 74
Gly-Gly	133	30					**76 (y-type ion)**
Orn	133		115	116			30, 43, **70**
Ala	90		72				**30**, 45
Abu	104	**58**					
AIB	104		86		41		**30**, 45
GABA	104		**86**	**87**	41		**30**, 45, 69
δ-Aminolevulinic acid	132		**114**				
Carnitine	162						**58**, **60**, **103**

The most abundant ions are marked by **bold** font and are greater than 75% of the base peak. All other ions included in the table are greater than 10% of the base peak.

Table 2. List of isomeric and isobaric groups of molecules analyzed in this study

Molecules	Specific ions, *m/z*	Comments
Isomeric Compounds		
Ala vs. β-Ala	44 (Ala); 30 (β-Ala)	Both ions are the most abundant ions
Leu vs. Ile	44 (Leu); 41, 57, 69 (Ile)	Ion *m/z* 69 is most reliable and located in the region with no extraneous peaks
Abu vs. AIB vs. GABA	58 (Abu); 87, 69 (GABA)	Ions with *m/z* 58 and 87 are the dominant fragmentation peaks
Isobaric Compounds		
Gln vs. Lys	75 (Gln); 74 (Lys)	Formation of these ions observed only in MALDI MS/MS
Tyr vs. Met sulfone	56 (Met sulfone)	
Phe vs. MetO	74, 75 (MetO)	Formation of ion with *m/z* 75 observed only in MALDI MS/MS
Thr vs. Hse	45 (Thr); 31, 44 (Hse)	Formation of ion with *m/z* 45 observed only in MALDI MS/MS
Leu/Ile. vs. Hyp vs. δ-aminolevulinic acid	69 (Ile); 68 (Hyp); 114 (δ-aminolevulinic acid)	
Asn vs. Orn vs. $(Gly)_2$	87 (Asn); 70, 115, 116 (Orn); 76 [$(Gly)_2$]	
Abu/AIB/GABA vs. $(CH_3)_2Gly$	58 (Abu); 87, 69 (GABA); 42, 44 [$(CH_3)_2Gly$]	

energy types of MS/MS were observed with sulfur-containing amino acids. All of the six analyzed S-containing AA (Cys, Hcy, S-(carboxymethyl)-Cys, Met, Met sulfoxide, and Met sulfone) uniformly produced the immonium ion as the major fragmentation ion (Table 1 and Supplemental Figure S1). That was not the case for FAB and ESI MS/MS, where in some cases the immonium ion was not observed as in the case of fragmentation of Cys under metastable ion conditions in FAB [18], or fragmentation of Met sulfoxide by ESI MS/MS where no immonium ion was formed and Met sulfone's immonium ion intensity was half of that of *m/z* 56 ion intensity [14].

The fragmentation patterns of the acidic amino acids exhibit a dominant loss of H_2O + CO and the formation of an immonium ion (Table 1 and Supplemental Figure S1). As for corresponding amides, asparagine shows a behavior similar to its acid counterpart, while glutamine produces rather a complex profile of fragments (Table 1). One of these fragment ions with an *m/z* 75 was not observed in fragmentation spectra produced by other types of ionization and activation [13, 14, 17, 18, 21]. Our data suggest that the presence of this ion allows distinguishing between two isobaric amino acids, Gln and Lys (Table 1 and Table 2). The main fragmentation reactions of protonated Lys, according to our observation, are the loss of NH_3 from immonium ion leading to formation of an ion at *m/z* 84, and the loss of NH_3 from the parent ion leading to an ion with *m/z* 130 (Table 1). Low-energy ESI CID and FAB MS/MS of protonated Lys do not lead to the formation of an immonium ion, while EI and MALDI MS/MS fragmentation patterns show its presence [14, 17, 18, 21].

Protonated arginine exhibits the most complex fragmentation pattern that differs significantly from fragmentation patterns exhibited by other amino acids. First is an absence of any ions characteristic for fragmentation of other amino acid and second, an absence of a dominant fragmentation reaction (Table 1). To some degree, such patterns were not surprising since Dookeran et al. [18] have shown that in FAB MS/MS fragmentation, the loss of H_2O and NH_3 decreases rapidly with the increase in the collision energy. On the other hand the *m/z* 70 ion is the dominant fragment ion in low- and high-energy CID FAB as well as in ESI MS/MS [14, 18, 22] but not in MALDI CID MS/MS (Table 1). Dookeran et al. [18] rationalized the formation of this ion by assuming that, after the neutral guanidine separates from protonated arginine, the remaining residue cyclizes to protonated proline (*m/z* 116) that further decomposes to a corresponding immonium ion at *m/z* 70 [18]. The indirect indications that this assumption is correct and can be extrapolated to MALDI MS/MS of arginine are the data on tandem MS of citrulline and ornithine, which share the 2-aminopentanoic moiety with arginine and only differ in the end group (Arg side-chain ends with guanidino group, citrulline with ureido group, and ornithine with amino group). The MS/MS spectra of all three amino acids exhibit formation of the same fragmentation ions at *m/z* 70 and 116 (Table 1).

MS/MS Analysis of Isobaric and Isomeric Amino Acids

The existence of isobaric and isomeric amino acids and their derivatives has always been a problem in the application of mass spectrometry to amino acid analy-

sis. A common solution in case of ESI MS/MS is a preliminary separation of AA [13, 14, 23]. In this respect, it would be interesting to see if MALDI TOF/TOF MS/MS provides the means to qualitatively distinguish isobaric compounds.

Three groups of isomeric molecules and seven groups of isobaric compounds were selected among 36 molecules analyzed in our study (Table 2). The data in Table 2 and Supplement Figure S2 show that components of all of those groups of compounds could be distinctly identified by the availability of unique fragment ion in their MS/MS spectra.

Quantitative Aspects of MALDI TOF/TOF Tandem MS

To determine the feasibility of amino acids quantitation by MALDI TOF/TOF tandem MS, we performed a relative quantitation of isobaric amino acids in their mixtures using indicator fragment ions. Two mixtures of isobaric compounds were used for proof-of-concept experiments. The first mixture consisted of Gln and Lys mixed in ratios ranging from 1:10 to 10:1, and the second mixture contained three components, two amino acids (Asn and Orn) and one dipeptide (Gly-Gly) mixed in ratios ranging from 1:5 to 5:1. In case of Gln/Lys, their molar ratio was determined by measuring minor fragmentation indicator ions while for tricomponent mixture, major fragmentation peaks were used. Nevertheless, in both cases the data obtained demonstrate good correlation between the mixture component molar ratios and indicator ions intensity ratios (Supplemental Figure S3).

Additional Applications of MALDI TOF/TOF Tandem MS

Ordinarily, amino acids and other organic acids in blood and urine are measured after deproteinization of plasma or urine samples by precipitation with sulfosalicylic acid or acetonitrile, or after methanol extraction of the analytes from dried blood spots. We have explored the use of MALDI TOF/TOF in performing MS/MS analysis directly from biological samples. To this end, the sample of human serum was diluted four times and 1 μL of this solution was loaded on a MALDI target plate and a ion with a mass corresponding to Arg was subjected to MS/MS. The data obtained clearly identify this ion as Arg (presence of characteristic signals at *m/z* 60, 116, and 130, Supplemental Figure S4A).

We have also explored the applicability of MALDI TOF/TOF in the MS/MS mode to other organic acids of physiological value. The organic acid chosen for this purpose was carnitine. Carnitine is essential for β-oxidation of long-chain fatty acids in mitochondria and is indicative of inherited metabolic disorders of fatty and organic acid metabolism [23]. The method of choice for this analysis is ESI LC/MS/MS [16, 23]. Our data show that MALDI TOF tandem MS can be successfully used for this type of analysis, too. MALDI MS/MS spectrum of 200 fmol of carnitine exhibits strong signals of both expected structural fragments at *m/z* 60 and 103 formed after breaking of N—C bond in carnitine (Supplement Figure S4B).

Conclusions

We believe this to be the first detailed study of the collisionally induced fragmentation reactions of amino and organic acids by MALDI TOF/TOF tandem MS. In this work, we have observed patterns of high-energy fragmentation reactions after MALDI ionization of a large group of protonated amino acids and their derivatives. We have found that fragmentation after MALDI ionization is most similar to EI spectra or FAB CID on sector instruments. The characteristic feature of MALDI MS/MS is the formation of immonium ion as the dominant fragmentation reaction for all α-amino acids. This includes substituted and modified protonated α-amino acids (with the exception of two basic amino acids, Arg and Orn). We assume this occurs as a result of prompt ionization and activation with high energy with the short observation time frame afforded by TOF detection. We think our data provide a strong basis for the application of MALDI TOF/TOF MS/MS in qualitative and quantitative analyses of amino and organic acids, including application in clinical medicine.

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