FAB-MS/MS Spectrometry in Determining the Primary Structure of γ -Glutamyl-Containing Peptides

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Positive fast atom bombardment tandem mass spectrometry (FAB-MS/MS) was applied for peptide sequencing, particularly for determining the gamma glutamyl linkage involved in metal-binding peptides such as Cadystin (γ EC)₃G = Cadystin A and Cadystin (γ EC)₂G = Cadystin B (MW 771 and 539, respectively). The fragmentation patterns between the natural gamma glutamyl peptide and the synthetic alpha glutamyl one were clearly distinguishable. FAB-MS/MS was proved to be a good method for determining these peptides, since it needed no chemical degradation and only a small amount of the peptide was needed for determination.

Cadmium-binding peptides were found as metallothionein-like peptides in the fission yeast, *Schizosaccharomyces pombe*, by Hayashi and his colleagues in 1981.¹⁾ These peptides, Cd-BP1 (molecular weight 4000) and Cd-BP2 (MW 1800), were induced in the logarithmical growth of the yeast upon exposure to cadmium. They contained multiples of unit peptides, which were composed from multiples of cysteine and glutamic acid ending with glycine. They had high ability to bind cadmium (II) as subunit structures. The unit peptide was named "Cadystin." The sequences of Cadystin (γ EC)₃G = Cadystin A and Cadystin (γ EC)₂G = Cadystin B were determined through chemical analyses by Kondo *et al.* in 1984²⁾ to be

Cadystin (γ EC)₃G = Cadystin A

H- γ -L-Glu-L-Cys- γ -L-Glu-L-Cys- γ -L-
Glu-L-Cys-Gly-OH

Cadystin (γ EC)₂G = Cadystin B

H- γ -L-Glu-L-Cys- γ -L-Glu-L-Cys-Gly-OH

These amino acid sequences in Cadystin (γ EC)₃G = Cadystin A and Cadystin (γ EC)₂G

= Cadystin B have the common repeating unit of γ -L-Glu-L-Cys ending up with Gly at the C-terminal. This sequence is partly the same as the tripeptide, glutathione.

Glutathione H- γ -L-Glu-L-Cys-Gly-OH

The cadmium-binding peptides have attracted the attention of biochemists and were also found in plants by Grill *et al.* in 1985.³⁾ Although they have been assigned the same structures and had the same biological functions as cadystin, they named the peptides as phytochelatin. The nomenclature of the same compound should be carefully given in order to avoid unnecessary confusion, although it was found from different sources. We propose a more definitive name of "Cadystins (γ EC)_{*n*}G" for those peptides having *n* repeating dipeptidyl groups in H-(γ -L-Glu-L-Cys)_{*n*}-Gly-OH; for example "Cadystin (γ EC)₃G = cadystin A. Recently, we have found higher homologs of cadystins having *n* = 4, 5, $\dots$ etc.

Cadystin did not undergo the usual Edman degradation. It was difficult for the structural elucidation that the carboxyl group next to the gamma position of glutamic acid was linked

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to cysteine. Cadystin analogs have been synthesized in order to prove the stereostructure, and chemical degradation was the only method that was used to determine the gamma glutamyl linkage of glutamic acid.⁴⁾ We demonstrate here a more useful FAB-MS/MS method to distinguish between the alpha and gamma glutamyl linkage of cadystins.

Materials and Methods

Chemicals. Gamma Cadystin (γEC)₃G [=Cadystin A], Cadystin (γEC)₂G [=Cadystin B] and their alpha analogs were obtained by syntheses. Gamma and alpha tripeptides (Glu-Ala-Gly) were presented by Dr. Kazuharu Ienaga of Nippon Zoki Pharmaceutical Co., Ltd., Kinashi Yashirocho, Kato-gun, Hyogo 673-14, Japan.

Mass spectrometry. A JMS-DX 705L instrument (JEOL) was used for FAB-MS/MS analyses. Two double-focus mass spectrometric machines were connected as EB-EB and aligned through an interface, after which the ion was collided with helium. A mixture of 3-nitrobenzylalcohol and hexamethylphosphoramide (HMPA) was employed as the matrix for FAB measurements. A peptide (less than

Structure of Cadystins and Analogs

Cadystin (γEC)₃G (old name = cadystin A)

Cadystin (γEC)₂G (old name = cadystin B)

(EC)₃G = α analogue of Cadystin (γEC)₃G

(EC)₂G = α analogue of Cadystin (γEC)₂G

γEAG = deS-glutathione = norophthalamic acid

EAG = α -deS-glutathione

Assignment of the Fragment Pattern

1 μ g) was dissolved in 10 μ l of distilled water–MeOH or dimethyl sulfoxide. A 2 μ l of the peptide solution was mixed with the matrix (*ca.* 10 μ l). The mixture was introduced into the first mass spectrometer, which positively ionized the sample with FAB. The sample was irradiated with a xenon gun at 10^{-1} Torr for ionization in an electric field with 3 kV acceleration. The protonated molecule (MH^+) of each peptide was selected from the first mass system, and was simultaneously collided with He gas to produce daughter ions, which were directly introduced into the second mass system. The data from each measurement were averaged after accumulating about 10 times.

Results and Discussion

Positive fast atom bombardment tandem mass spectrometry (FAB-MS/MS) conveys

strong fingerprint characteristics from the daughter ions, so that it can be applied to identify quite similar compounds. The precursor ions were selected and separated from the noise with the first mass system. Each ion was collided with He gas to produce daughter ions, which were then analyzed by the second mass system. The fragmentation spectrum could be used to identify the peptide which was thermolabile within the level of a microgram.

The FAB mass spectrum of Cadystin provided little information about the fragment ion series to distinguish between the natural gamma glutamyl linkage peptide and the syn-

Table I. DETERMINATION OF THE α - AND γ -GLUTAMYL LINKAGE THROUGH FRAGMENT IONS IN THE FAB-MS/MS SPECTRA OF CADYSTIN A AND B ANALOGS

Cleavage pattern		Cadystin A		Cleavage pattern		Cadystin B	
Daughter ion	<i>m/z</i>	γ	α	Daughter ion	<i>m/z</i>	γ	α
Z6	59			Z4	59		
Y6+2	76			Y4+2	76		
X6/A1	102		*	X4/A1	102	*	**
B1	130			B1	130		
C1	145			C1	145		
Z5	162			Z3	162		
Y5+2	179	**		Y3+2	179	*	
X5/A2	205			X3/A2	205		
B2	233		*****	B2	233	***	***
C2	248			C2	248		
Z4	291	*****		Z2	291		
Y4+2	308			Y2+2	308	*	**
X4/A3	334			X2/A3	334		
B3	362			B3	362		*****
C3	377			C3	377		
Z3	394			Z1	394		
Y3+2	411	**		Y1+2	411	*****	
X3/A4	437			X1/A4	437		
B4	465	***	**	B4	465	*****	*****
C4	480			C4	480		
Z2	523		*	MH ⁺	540	540	540
Y2+2	540	***					
X2/A5	566						
B5	594		*****				
C5	609						
Z1	626						
Y1+2	643	*****					
X1/A6	669						
B6	697		*****				
C6	712						
MH ⁺	772	772	772				

* Represents the relative intensity of fragment ions.

thetic alpha one. Discrimination was achieved with the FAB-MS/MS spectrum, it providing sequence-specific fragment ions which were required to determine the structure. There were three points of peptide cleavage called A, B and C when the charge was retained at the N-terminal, and conversely X, Y and Z when the charge was retained by the C-terminal fragment of the peptide.⁵⁾

The fragment ions of "Y+2" as a result of

double protonation were observed instead of "Y", since the amino group ($\text{NH}+1$) was protonated to be an ammonium group (NH_3+1). All possible sequence ions of the Cadystin cleavage are tabulated in Table I. The protonated molecule, m/z 772 (MH^+) of Cadystin (γEC)₃G = Cadystin A [$(\gamma\text{-Glu-Cys})_3\text{-Gly}$], was selected as the precursor ion, and it was collided with helium gas to produce the daughter ions, m/z 643 (Y_1+2), 540 (Y_2+2),

FAB-MS SPECTRUM OF CADYSTIN A

FAB-MS/MS SPECTRUM OF CADYSTIN A

MW 771

Fig. 1. FAB-MS and FAB-MS/MS Spectra of Cadystin A.

FAB-MS SPECTRUM OF α -CADYSTIN A

FAB-MS/MS SPECTRUM OF α -CADYSTIN A MW 771

Fig. 2. FAB-MS and FAB-MS/MS Spectra of the Alpha Cadystin A Analog.

465 (B_4), 411 (Y_3+2) and 291 (Z_4) (Fig. 1). The fragmentation pattern of $(\gamma\text{-Glu-Cys})_3\text{-Gly}$ was clearly distinguishable from that of $(\alpha\text{-Glu-Cys})_3\text{-Gly}$ (Fig. 2). The daughter ions of the alpha glutamyl linkage were m/z 594 (B_5), 523 (Z_2), 465 (B_4), 233 (B_2) and 102 (X_6/A_1). Similarly, Cadystin $(\gamma\text{EC})_2\text{G} = \text{Cadystin B}$ [$(\gamma\text{-Glu-Cys})_2\text{-Gly}$] showed daughter ions 465 (B_4), 411 (Y_1+2), 362 (B_3), 308 (Y_2+2), 233 (B_2), 179 (Y_3+2) and 102 (X_4/A_1), while $(\alpha\text{-$

$\text{Glu-Cys})_2\text{-Gly}$ showed daughter ions 465, 362, 308, 233 and 102 (Figs. 3 and 4). The gamma linkage was easily cleaved at the N-terminal peptide bond, high intensity being observed at m/z 643 and m/z 411 for Cadystin $(\gamma\text{EC})_3\text{G} = \text{Cadystin A}$ and Cadystin $(\gamma\text{EC})_2\text{G} = \text{Cadystin B}$, respectively. On the other hand, the alpha linkage was easily cleaved at the internal peptide bond. High intensity being observed at m/z 594 and m/z 362 for Cadystin $(\gamma\text{EC})_3\text{G} =$

FAB-MS SPECTRUM OF α -CADYSTIN B

FAB-MS/MS SPECTRUM OF α -CADYSTIN B MW 539

Fig. 4. FAB-MS and FAB-MS/MS Spectra of the Alpha Cadystin B Analog.

We found great advantages in FAB-MS/MS spectrometry for a convenient identification of the glutamyl peptide between the gamma linkage, and the alpha linkages even in the tripeptide, H-Glu-Ala-Gly-OH (MW 275). The protonated molecule of the gamma linkage peptide, m/z 276, gave high intensity fragment ion at m/z 147 (Y+2) and 201 (B), while the alpha linkage peptide gave high

intensity peaks at m/z 258 ($MH^+ - 18$) and 201 (B) as shown in Figs. 5 and 6, respectively. Since the tripeptide molecule was not large, the cleavage pattern easily occurred and all possible points of cleavage were found in both of them. However, the distinction between the gamma and alpha linkage was clearly observed. This data is consistent to those of tripeptides which have been measured with

FAB-MS SPECTRUM OF NOROPHTHALMIC ACID

FAB-MS/MS SPECTRUM OF NOROPHTHALMIC ACID MW 275

Fig. 5. FAB-MS and FAB-MS/MS Spectra of the Tripeptide (Glu-Ala-Gly, Norophthalmic Acid).

B/E linked scan sputtered ion mass spectrometry (SIMS) by Nakamura *et al.*⁶⁾

According to the sequence-specific fragment ions resulting from cleavage of the peptide, the FAB-MS/MS method proved considerably useful for determining the amino acid sequencing in peptides, including the gamma and/or alpha mode in the glutamyl linkages. We have already demonstrated this technique for the

identification of pentose in a mushroom light emitter,⁷⁾ as well as in mycotoxin analysis.⁸⁾

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FAB-MS SPECTRUM OF α -DES-GLUTATHIONE

FAB-MS/MS SPECTRUM OF α -DES-GLUTATHIONE

MW 275

Fig. 6. FAB-MS and FAB-MS/MS Spectra of the Alpha Tripeptide (Glu-Ala-Gly, deS-Glutathione).

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