In-Source Decay Characteristics of Peptides in Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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In-source decay (ISD) of peptides, coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, has been examined to determine the influence of the matrix, the susceptibility of amino-acid residues to ISD, and the effect of extraction delay times. Out of nine di- and tri-hydroxybenzoic acids and three cinnamic derivatives tested, the most suitable matrix for ISD was 2,5-dihydroxybenzoic acid. The amine bond at Xxx–Gly and Xxx–Val residues was less susceptible than other amino-acid residues to ISD; however, the more sensitive residue(s) were not as clear. Using a peptide that gave the y_n- and (z_n + 2)-series product ions, it was confirmed that amide-bond cleavage (formation of the y_n-series ions) accompanied metastable peaks, whereas metastable peaks were never observed with amine-bond cleavage [formation of the (z_n + 2)-series ions]. Furthermore, abundant c_n-series ions, which originate from amine-bond cleavage on the peptide backbone, were observed whenever a minimum delay time of 38 ns or continuous extraction was used to obtain spectra. These data indicate that amine-bond cleavage in ISD takes place on the ionization time scale before the energy randomization is completed. (J Am Soc Mass Spectrom 2001, 12, 420–427) © 2001 American Society for Mass Spectrometry

In-source decay (ISD) coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) presents us with a fascinating method for sequencing peptides and proteins of low picomole levels. Brown and co-workers first reported the MALDI-TOF-ISD method [1], and more recently presented it as a strategy for proteome research [2]. One of the advantages of ISD, compared with conventional mass spectrometric degradation methods such as collision-induced dissociation (CID) [3] and post-source decay (PSD) [4], is that the amino-acid sequence of proteins can be determined without predigestion [5]. Another advantage is that the product ions observed are nearly always c_n-series ions (using Biemann's nomenclature [6]), which are useful for the carboxyl (C)-terminal sequencing of peptides. The observation of c_n-series ions of peptides is limited to mass spectra obtained by energy-sudden desorption (ESD) methods such as fast-atom bombardment (FAB) [6, 7] and plasma desorption (PD) [8]; c_n-series ions are rarely observed in either CID or PSD. The c_n-series ions are formed through specific cleavage of the amine bond NH–C^{α}. However, the product ions that form strongly depend on the basic and acidic nature of the constituent amino acids [9, 10], as has been well documented from CID and/or PSD experiments [3, 11].

ISD is undoubtedly the most convenient method for the assignment of product ions from proteins and peptides because the ISD products are limited to c_n-, b_n-, a_n-, y_n-, and/or (z_n + 2)-series ions [1, 9]. In addition, the b_n-, a_n-, and (z_n + 2)-series ions tend to disappear from the spectra of large molecular mass peptides [10]. Over-degraded products such as d_n-, v_n-, and w_n-series ions are not often observed, nor are the product ions produced by the loss of ammonia NH_3 or water H_2O molecules from y_n- and/or b_n-series ions, as is seen with PSD [4, 12]. It would be of interest for peptide and protein research to determine which amino-acid residues are more or less susceptible to ISD. Katta et al. [13] found that c_n-series ions originating from amine-bond cleavage between Xxx–Gly residues were relatively weak in abundance by ISD. Furthermore, they pointed out the lack of c_n-series ions originating from Xxx–Pro bonds in the work of Brown et al. [14]. Here I have further investigated the susceptibility of different amino-acid residues to ISD for the identification of protein and peptide sequences.

The matrix has an important role on the ISD processes. According to Brown et al. [14, 15], excess energy from the matrix is deposited in the analyte by an exothermic proton transfer via hydrogen bonds between basic site(s) of the analyte and the matrix hydroxyl group(s). Brown et al. [14] compared ISD spectra

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Table 1. ISD product ions observed for angiotensin I (Mr 1296.5) in three matrices, by using the linear TOF mode

	2,4-DHB			2,5-DHB			2,4,6-THB		
Residue	a_n	b_n	c_n	a_n	b_n	c_n	a_n	b_n	c_n
Asp1									
Arg2									
Val3									
Tyr4	a4		c4	a4		c4	a4		c4
Ile5	a5		c5	a5		c5	a5		c5
His6	a6	b6		a6	b6		a6	b6	
Pro7	a7		c7	a7		c7	a7	b7	c7
Phe8	a8		c8	a8		c8	a8	b8	c8
His9	a9	b9	c9	a9	b9	c9	a9	b9	c9
Leu10									

of the oxidized B chain of bovine insulin that were obtained using six different organic matrices. They found that the most suitable matrix for the formation of ISD products was 2,5-dihydroxybenzoic acid (2,5-DHB) [14, 15]. We previously compared the ISD spectra of three different peptides obtained using 2,5-DHB and α-cyano-4-hydroxycinnamic acid (CHCA) and also found that 2,5-DHB was the most suitable matrix [9]. Here I have further investigated the effect of matrix on ISD.

In this study, I have compared ISD spectra obtained in nine isomers of di- and tri-hydroxbenzoic acid and three cinnamic acid derivatives. I confirm again that 2,5-DHB is the best matrix for ISD determination of peptides and protein. I also report that amine bonds at Xxx–Gly and Xxx–Val residues have a low susceptibility to ISD, and that amine-bond cleavage in ISD occurs rapidly on the ionization time scale.

Experimental

Materials

Peptides. Angiotensin I (Mr 1296.5): DRVYIHPFHL; substance P (Mr 1347.7): RPKPQQFFGLM-NH_2; bombesin (Mr 1619.9): Pyr-QRLGNQWAVGHLM-NH_2; adrenocorticotropic hormone 18-39 (ACTH18-39, Mr 2465.7): RPVKVYPNGAEDESAEAFPLEF; ACTH1-24 (Mr 2931.6): SYSMEHFRWGKPVGKKRRPVKVYP; chromogranin A (Mr 1819.0): EEEEEMAVVPQGLFRG-NH_2 were purchased from the Peptide Institute (Minoh, Osaka, Japan). Trifluoroacetic acid (TFA) and acetonitrile were purchased from Wako Pure Chemical Industry (Osaka, Japan). The matrix materials α-cyano-4-hydroxycinnamic acid (CHCA), 2,3-dihydroxybenzoic acid (2,3-DHB), 2,4-DHB, 2,5-DHB, 2,6-DHB, 3,4-DHB, 3,5-DHB, 2-hydroxy-5-methoxybenzoic acid (HMBA), 2,3,4-trihydroxybenzoic acid (2,3,4-THB), 2,4,6-THB, 3,4,5-THB, sinapinic acid, ferulic acid, and caffeic acid were purchased from Sigma (Milwaukee, WI). All reagents were used without further purification.

MALDI-TOF Mass Spectra

MALDI-TOF mass spectra were acquired on a PerSeptive Biosystems Voyager-DE STR TOF reflectron mass spectrometer (PE Biosystems, Framingham, MA) equipped with a N_2 laser (337 nm, 3 ns pulse length; Laser Science, Franklin, MA) and a dual microchannel plate detector (Galileo, Sturbrige, MA). The spectra were acquired in positive ion mode. The instrument was used in the delayed extraction linear mode, the reflector mode, or continuous extraction. The ion accelerating voltage and the delay time for the linear TOF mode were 20 kV with the grid voltage set at 95.0% and 198 ns, respectively. The ion accelerating voltage and the delay time for the reflector TOF mode were 20 kV with the grid voltage set at 73.5% and 238 ns, respectively. The analyte peptide was dissolved in water at a concentration of 200 pmol/μL, and 1 μL of the analyte solution was mixed with a 9 μL saturated matrix of 0.1% TFA in water/acetonitrile (3:2 v/v). A 0.5 μL of the solution was deposited onto a gold-coated target plate, and the solvents were removed by drying in air at room temperature.

Results and Discussion

2,5-Dihydroxybenzoic Acid Is the Most Suitable Matrix for ISD

Initially, dihydroxy- and trihydroxy-substituted benzoic acids were used as the matrix to obtain the appropriate ISD spectra for analytical use. For the ISD of angiotensin I and substance P, examined using linear TOF mode, six dihydroxybenzoic acids were compared: 2,3-DHB, 2,4-DHB, 2,5-DHB, 2,6-DHB, 3,4-DHB, and 3,5-DHB. In addition, three trihydroxybenzoic acids 2,3,4-THB, 2,4,6-THB and 3,4,5-THB were compared for angiotensin I. Another isomer 2,4,5-THB was not commercially available. For angiotensin I, although all spectra showed an intense peak corresponding to the protonated molecule $[M + H]^+$, the useful ISD product ions were observed only when 2,4-DHB, 2,5-DHB, or 2,4,6-THB was used. The isomer 3,4,5-THB was a poor

Table 2. ISD product ions observed for substance P (Mr 1347.7) in three matrices, by using the linear TOF mode

	2,3-DHB			2,4-DHB			2,5-DHB		
Residue	a_n	b_n	c_n	a_n	b_n	c_n	a_n	b_n	c_n
Arg1									
Pro2									
Lys3									
Pro4									c4
Gln5	a5		c5	a5		c5	a5		c5
Gln6	a6		c6	a6		c6	a6		c6
Phe7	a7		c7	a7		c7	a7		c7
Phe8	a8		c8	a8		c8	a8		c8
Gly9			c9			c9			c9
Leu10	a10		c10	a10		c10	a10		c10
Met11									

matrix in both ISD and normal mass spectra. Table 1 summarizes the product ions observed using Biemann's nomenclature [6] (a_n-, b_n-, c_n-, y_n-, and z_n + 2-series ions). Because of the interference from matrix peaks, product ions lower than c3 ion have not been listed. A c6 ion was not observed because of the presence of a proline residue at position 7; instead a6 and b6 ions were observed. For substance P, the useful product ions were observed with 2,3-DHB, 2,4-DHB, or 2,5-DHB (Table 2). The c_n-series ions often were observed together with the corresponding a_n- and/or b_n-series ions in the spectra of both angiotensin I and substance P. But an a9 ion originating from the cleavage at the C^{α}(Gly9)–CONHC^{α}(Leu10) bond was not observed in the spectra of substance P with any matrix. The formation of a_n-series ions may be explained by the successive loss of the carbonyl group from b_n-series ions, and not from direct cleavage at the C^{α}–CONH bond [9].

These results indicate that the isomers with a hydroxyl group at the 2-position are effective for the formation of ISD products. Such isomers differ from 3,4-DHB and 3,5-DHB because the 2-position hydroxyl group can form a six-membered ring by hydrogen bonding with the 1-position carboxyl group. Krause and Schlunegger [16] pointed out that in normal MALDI-TOFMS experiments, ortho-hydroxybenzoic acid isomers have higher matrix activity than their corresponding meta and para isomers in the meaning of stronger $[M + H]^+$ signals. They reported the molar absorptivities (ϵ_{337}) at a wavelength of 337 nm for 26 matrix materials, which indicated that the ability of the matrix to provide prominent mass spectra does not depend necessarily on molar absorptivity. For example, 2,6-DHB (ϵ_{337} = 700) had the same ability as 2,5-DHB (ϵ_{337} = 4250); although 3,5-DHB with a low molar absorptivity (ϵ_{337} = 100) was clearly poor in ability [16]. However, I found that neither 2,6-DHB nor 3,5-DHB was effective in ISD experiments, which indicates that ISD may need more limiting conditions for the matrix material, as compared with standard MALDI-TOFMS experiments. A large molar absorptivity, such as that of 2,5-DHB, might be a requirement of ISD.

To examine the influence of the 5-hydroxy group in the matrix material, I used 2-hydroxy-5-methoxybenzoic acid (HMBA) as a matrix to obtain ISD spectra of angiotensin I and ACTH18-39. The angiotensin I spectrum showed an intense $[M + H]^+$ peak, and only a few c_n-series ion peaks (data not shown). The spectrum of ACTH18-39 showed an intense $[M + H]^+$ peak and many c_n-series ion peaks, but not more than in spectra obtained using 2,5-DHB as the matrix. The matrix HMBA has a higher molar absorptivity (ϵ_{337} = 5000) than that of 2,5-DHB [16], and it differs from 2,5-DHB only in the functional group at the 5-position. From this, I conclude that the 5-hydroxy group in 2,5-DHB may have an important structural role in the formation of abundant ISD products.

It has also been reported by some workers that cinnamic acid derivatives such as sinapinic acid [5, 13] and ferulic acid [3] are suitable for ISD experiments of proteins. The molar absorptivity of sinapinic acid is surprisingly high (ϵ_{337} = 14,600) [17]. However, I found that 3,4-dihydroxy cinnamic acid (caffeic acid) was much poorer than 2,5-DHB and HMBA as a matrix material in the ISD spectra of ACTH18-39 (Table 3), and that sinapinic acid and ferulic acid were not useful at all (data not shown).

Residues of ACTH18-39 that are Less Susceptible to ISD

Biemann [6] has reported that ACTH18-39 gives c_n- and a_n-series ions in a normal FAB mass spectrum, but that the spectrum lacks c6 and c18 ions owing to the presence of proline at residues 7 and 19. That was the first recorded observation of c_n-series ions produced under an ESD condition. Here I obtained MALDI-TOF-ISD spectra of ACTH18-39 using 2,4-DHB, 2,5-DHB, and 2,4,6-THB, which I had shown to be suitable matrices for ISD. The use of the reflector TOF mode allowed each product ion to separate into isotope peaks. All spectra showed the complete series of the c_n and/or a_n ions (n = 4–21), except for c6 and c18 ions (data not shown). All three matrices were effective in forming sequence-related products; however, it was hard to find

Table 3. ISD product ions observed for adrenocorticotropic hormone 18–39 (Mr 2465.7) in three matrices, by using the reflector TOF mode. Abundance of each ion designated in parentheses is represented by the count obtained by subtracting background levels on the vertical axis of the spectra

	2,5-DHB			HMBA			Caffeic acid		
Residue	a_n	b_n	c_n	a_n	b_n	c_n	a_n	b_n	c_n
Arg1									
Pro2									
Val3	a3 (5170)		c3 (9078)						
Lys4	a4 (2107)		c4 (2394)						
Val5	a5 (5304)	b5 (1379)	c5 (8206)						
Tyr6	a6 (6603)	b6 (2315)							
Pro7	a7 (1405)		c7 (6184)			c7 (3722)			c7 (984)
Asn8	a8 (3432)	b8 (1067)	c8 (3698)	a8 (958)		c8 (1910)			c8 (362)
Gly9			c9 (8640)			c9 (2427)			c9 (1572)
Ala10	a10 (1873)		c10 (5457)	a10 (661)		c10 (2445)	a10 (341)		c10 (778)
Glu11	a11 (2705)		c11 (7367)	a11 (1456)		c11 (3306)			c11 (1252)
Asp12	a12 (1163)	b12 (2173)	c12 (5099)			c12 (2225)			c12 (954)
Glu13	a13 (1897)		c13 (4947)	a13 (1078)		c13 (1408)			c13 (1996)
Ser14	a14 (1117)		c14 (3942)	a14 (623)		c14 (1935)		c14 (731)	
Ala15	a15 (1590)		c15 (3353)	a15 (468)		c15 (1408)			c15 (1342)
Glu16	a16 (1622)		c16 (3235)	a16 (749)		c16 (1566)			c16 (1428)
Ala17	a17 (1080)		c17 (4174)	a17 (478)		c17 (2302)			c17 (2370)
Phe18	a18 (2130)	b18 (1520)		a18 (720)	b18 (508)		a18 (667)	b18 (476)	
Pro19	a19 (710)		c19 (2632)	a19 (234)		c19 (1235)	a19 (398)		c19 (850)
Leu20	a20 (1532)		c20 (3590)	a20 (618)		c20 (970)	a20 (602)		c20 (995)
Glu21									
Phe22									
$[M + H]^+$	(67260)			(55900)			(66881)		

the so-called "sweet spot" for 2,4-DHB and 2,4,6-THB for the ISD phenomenon in the crystal area of 1 mm in diameter. The sweet spot for 2,5-DHB was easy to find. The ISD spectrum of ACTH18-39 obtained with 2,5-DHB and the reflector TOF mode is shown in Figure 1. The c8 ion at *m/z* 971 originating from amine-bond cleavage between residue 8 and Gly9 was weaker in abundance than the rest. The abundance of the c8 ion at *m/z* 1046 of substance P, which originates from cleavage at the amine bond Xxx–Gly9, was also relatively weak in the spectra obtained by using the linear TOF mode. This feature has also been observed in the negative-ion MALDI-TOF-ISD spectrum of the peptide porcine pancreastatin 33–49, including Gly9 and Gly13 residues [9].

To further examine the influence of the matrix on the susceptibility of residues to ISD, I compared the MALDI-TOF-ISD spectrum of ACTH18–39 obtained with 2,5-DHB to those obtained with caffeic acid and HMBA matrices (Table 3). The spectrum with 2,5-DHB showed abundant c_n-series ions (n = 3–20), together

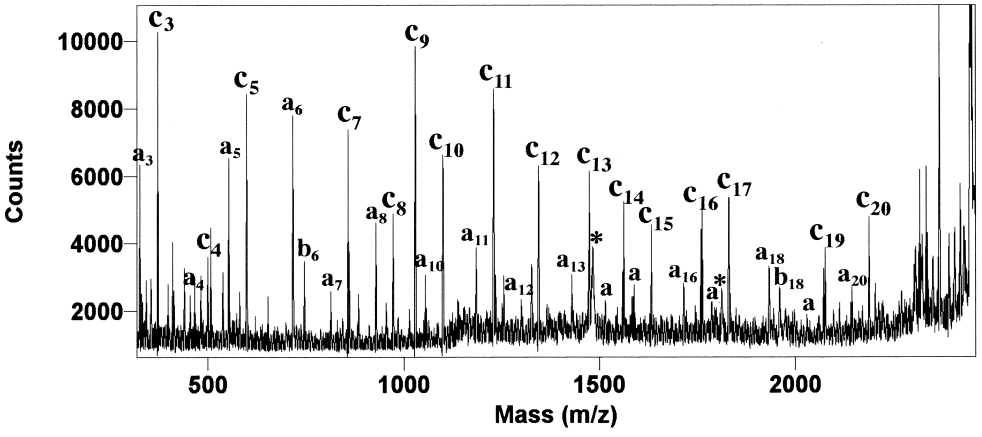

Figure 1. MALDI-TOF-ISD spectrum of adrenocorticotropic hormone 18–39 (Mr 2465.7) obtained with 2,5-dihydroxybenzoic acid, using a 238 ns extraction delay time and reflector TOF mode. Asterisk indicates metastable peaks. The c4 and c8 ions originating from amine-bond cleavage at Xxx6–Val5 and Xxx8–Gly9 residues are relatively lower than the rest in abundance. The abundance of each product ion is summarized in Table 3.

with a_n- and/or b_n-series ions; but the a9 ion originating from cleavage at the C^{α}(Gly9)–CONHC^{α}(Ala10) bond was missing (Figure 1). Furthermore, both c4 and c8 ions originating from cleavage at Xxx6–Val5 and Xxx8–Gly9 bonds, respectively, were lower in abundance than other ions. Although spectra obtained with caffeic acid and HMBA also showed useful c_n-series ions (see Table 3), these spectra lacked product ions smaller than b6. The low susceptibility of Xxx–Val and Xxx–Gly bonds to ISD is supported by the ISD spectra of intact bovine serum albumin obtained with ferulic acid by Reiber et al. [2], of bovine superoxide dismutase obtained with sinapinic acid by Lennon et al. [5], and of r-metHuLeptin obtained with sinapinic acid by Katta et al. [13], although these authors did not point this out in their respective reports. I therefore conclude that the amine bonds at Xxx–Gly and Xxx–Val residues are insensitive to ISD.

Confirmation of Residues that are Less Susceptible to ISD

To confirm which residues are less susceptible to ISD, I obtained ISD spectra of other peptides containing both valine and glycine residues, using the reflector TOF mode. The ISD spectra of bombesin and ACTH1–24 were obtained with 2,5-DHB (data not shown), and the observed product ions are listed in Table 4a and 4b. The ISD data of angiotensin I and substance P obtained using the reflector TOF mode are listed in Table 4c and 4d, respectively.

Bombesin, which contains Gly5, Val10, and Gly11, showed c_n-series ions and the corresponding a_n-series ions, except for a5 and a11. The c4, c9, and c10 ions, which originate from amine-bond cleavage at Xxx–Gly and Xxx–Val, were clearly lower than the rest in abundance (see ion count in Table 4a). Coupled with the lack of a5 and a11 ions, this indicates that the bonds on both sides of glycine residues, i.e. Xxx–Gly and Gly–xxX, have a low susceptibility to ISD. In contrast, the ISD spectrum of ACTH1–24, which contains Gly10, Val13, Gly14, Val20, and Val22, showed y_n, $(z_n + 2)$-, and abundant c_n-series ions, but the a_n-series ions were hardly observed. As expected, the c9 and c13 ions originating from amine-bond cleavage at Xxx–Gly were lower in abundance than the rest. Furthermore, the c12, c19, and c21 ions originating from cleavage at Xxx–Val were also relatively low in abundance (see ion count in Table 4b).

Nonergodic Amine-Bond Cleavage of Peptide Backbone

The amine-bond cleavage of peptide backbone, which leads to the formation of c_n- and/or $(z_n + 2)$-series ions, seems to be limited to the use of ESD methods such as FAB [6, 7], PD [8], and MALDI. McLafferty's group [18, 19] recently demonstrated that electron capture dissociation (ECD) of multiply protonated proteins produced under electrospray ionization also produces c_n- and z_n-series ions. The formation of these product ions has been rationalized by a nonergodic dissociation [20]. A characteristic common to both ESD and ECD methods may be a prompt fragmentation that occurs on the ionization time scale and before the energy randomization is completed. To understand the mechanism of ISD, which has different decay characteristics from CID and PSD, it is important to estimate the time frame in which ISD occurs after the laser irradiation. I therefore conducted experiments that varied the delay time, or that used continuous extraction without a delay time, while observing the ISD products.

The ISD spectra of chromogranin A, which has a five glutamic acid cluster at the N-terminus, was obtained with both a 39 and a 238 ns delay time, which indicated that formation of the y_n- and $(z_n + 2)$-series ions occurred within at least 39 ns (the minimum delay time in the DE system used here was 38 ns). Interestingly, the ISD spectrum of chromogranin A obtained using 2,5-DHB, a 238 ns delay time and a somewhat higher laser fluence than the threshold showed broadened metastable peaks (indicated by an asterisk in Figure 2), as well as the y_n- and $(z_n + 2)$-series ions. The metastable decay of an analyte ion, which occurs in the field-free drift region in the reflector TOF analyzer, can be detected as the product ions in the PSD spectrum [21, 22]. The PSD spectrum of protonated chromogranin A was obtained using a CHCA matrix. The CHCA matrix brings about preferential cleavage of the amide bond to form y_n-, b_n-, and/or a_n-series ions rather than the amine-bond cleavage to form c_n- and/or $(z_n + 2)$-series ions in ISD experiments [9]. The PSD spectrum of protonated chromogranin A showed y10, y11, y12, y13, y14, and y15 ions (Figure 3a). Furthermore, as expected, the ISD spectrum with CHCA showed only y_n-series ions and metastable peaks, and not $(z_n + 2)$-series ions (Figure 3b). These metastable peaks were at almost the same mass as those in the ISD spectrum with 2,5-DHB (Figure 2); therefore, the metastable peaks in Figure 2 must correspond to y_n-series ions. It is important to recognize that the amine-bond cleavage that forms $(z_n + 2)$-series ions does not essentially accompany metastable decay (on the μs time scale), whereas the amide-bond cleavage leading to the formation of y_n-series ions does accompany metastable decay. This suggests that MALDI-ISD is a nonergodic dissociation.

Furthermore, the ISD spectra of ACTH18–39 were obtained with 2,5-DHB using the minimum delay time of 38 ns (Figure 4a) and continuous extraction (Figure 4b), under the same laser fluence. Continuous extraction deteriorated the mass resolution of the product ions, but c_n-series ions were clearly observed with high abundance. Although I cannot tell from these data whether amine-bond cleavage in ISD occurs before or after the formation of protonated molecules, these data suggest that the formation of c_n-series ions occurs

Table 4. ISD product ions observed for (a) bombesin (Mr 1619.9) (b) adrenocorticotropic hormone 1–24 (Mr 2931.6), (c) angiotensin I (Mr 1296.5), and (d) substance P (Mr 1347.7) in 2,5-dihydroxybenzoic acid, using the reflector TOF mode. Abundance of each ion designated in parentheses is represented by the count obtained by subtracting background level on the vertical axis of the spectrum

(a) Bombesin

Residue	a_n	b_n	c_n
Glu1			
Gln2			
Arg3			c3 (7602)
Leu4			c4 (1403)
Gly5			c5 (5608)
Asn6	a6 (2205)		c6 (3410)
Gln7	a7 (2041)		c7 (3918)
Trp8	a8 (2205)		c8 (4134)
Ala9	a9 (1320)		c9 (2560)
Val10	a10 (1980)		c10 (2576)
Gly11			c11 (4951)
His12	a12 (1650)		c12 (6671)
Leu13	a13 (1855)		c13 (5560)
Met14			
$[M + H]^+$	(67150)		

(b) ACTH1–24

Residue	c_n	y_n	$z_n + 2$
Ser1			
Tyr2			
Ser3			
Met4			
Glu5			
His6	c6 (783)	y19 (966)	
Phe7	c7 (609)		
Arg8	c8 (1188)		
Trp9	c9 (705)		
Gly10	c10 (1762)		
Lys11			
Pro12	c12 (965)		
Val13	c13 (647)		
Gly14	c14 (1387)	y11 (721)	z11 (420)
Lys15	c15 (1277)	y10 (710)	z10 (861)
Lys16	c16 (1120)	y9 (622)	z9 (657)
Arg17	c17 (1305)	y8 (587)	z8 (572)
Arg18		y7 (583)	z7 (913)
Pro19	c19 (622)		
Val20	c20 (931)		
Lys21	c21 (685)		
Val22	c22 (854)		
Tyr23			
Pro24			
$[M + H]^+$	(63365)		

(c) Angiotensin I

Residue	a_n	b_n	c_n
Asp1			
Arg2			
Val3			
Tyr4	a4 (1942)		c4 (1297)
Ile5	a5 (2299)		c5 (2515)
His6	a6 (2412)	b6 (1848)	
Pro7	a7 (1095)		c7 (2316)
Phe8	a8 (1452)		c8 (2454)
His9	a9 (1584)		c9 (5755)
Leu10			
$[M + H]^+$	(63528)		

(continued)

Table 4. *(continued)*

(d) Substance P

Residue	a_n	b_n	c_n
Arg1			
Pro2			
Lys3			
Pro4	a4 (337)		c4 (382)
Gln5	a5 (331)		c5 (838)
Gln6	a6 (482)		c6 (1144)
Phe7	a7 (366)		c7 (1160)
Phe8	a8 (517)		c8 (830)
Gly9			c9 (1290)
Leu10	a10 (430)		c10 (1696)
Met11			
$[M + H]^+$	(66680)		

during on the ionization time scale, and is thus much faster than a metastable process.

Conclusions

A useful property of ISD, coupled with ESD methods such as FAB, PD, and MALDI, is the generation of the c_n- and $(z_n + 2)$-series ions originating from amine-bond cleavage on the peptide backbone. ISD differs from other post-source degradation methods such as CID and PSD, which primarily bring about amide-bond cleavage to form b_n- and y_n-series ions. The unusual amine-bond cleavage may be due to the fast transfer of energy to peptides from the excited matrix molecules caused by energy-sudden impinging. The mechanism of the formation of c_n- and $(z_n + 2)$-series ions in ISD is interesting because in ECD it has shown to be a nonergodic process.

The most important factor that affects the quality of ISD peptide spectra is the matrix. I found that 2,5-DHB was far superior to other dihydroxybenzoic acids, some trihydroxybenzoic acids and three cinnamic acid derivatives that I tested here. The low susceptibility of Xxx–Gly and Xxx–Val residues to ISD was independent of the matrices used. The knowledge that glycine and valine are less susceptible to ISD will be useful for the sequence analysis of both proteins and peptides.

Observation of ISD product ions does not depend on the delay time in a 39 to 238 ns time range, nor on the use of the continuous extraction without delay time; however, the optimum delay time observed for ISD products seems to be in agreement with standard MALDI-TOFMS experiments. The prompt fragmentation seen in ISD is an unusual phenomenon that occurs when energy-sudden impinging contacts condensed-phase samples, such as the liquid matrix in FAB and the solid matrix in PD and MALDI. Fast energy transfer without energy randomization may be required for amine-bond cleavage on the peptide backbone. In the ISD spectrum of chromogranin A, the amine-bond cleavage leading to formation of the c_n- and $(z_n + 2)$-series ions was not accompanied by any metastable

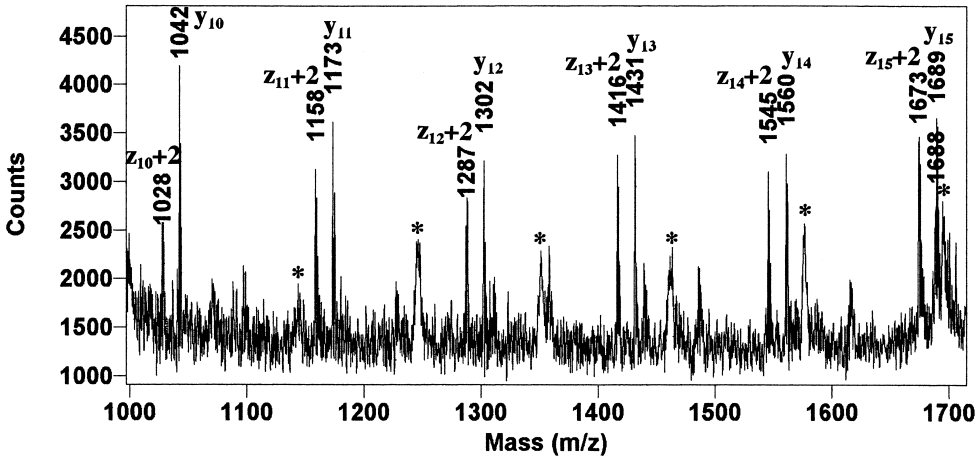

Figure 2. MALDI-TOF-ISD spectrum of chromogranin A (Mr 1819.0) obtained with 2,5-dihydroxybenzoic acid, using a 239 ns extraction delay time, reflector TOF mode, and a relatively higher laser fluence. Asterisk indicates metastable peaks.

peaks, whereas the amide-bond cleavage leading to formation of the b_n- and y_n-series ions showed broadened metastable peaks. This indicates that the amine-bond cleavage in ISD occurs rapidly and immediately after laser irradiation. Further insight into the mechanism of ISD will be gained from an increased understanding of FAB and PD processes, as well as MALDI. But it is already becoming practical to apply MALDI-

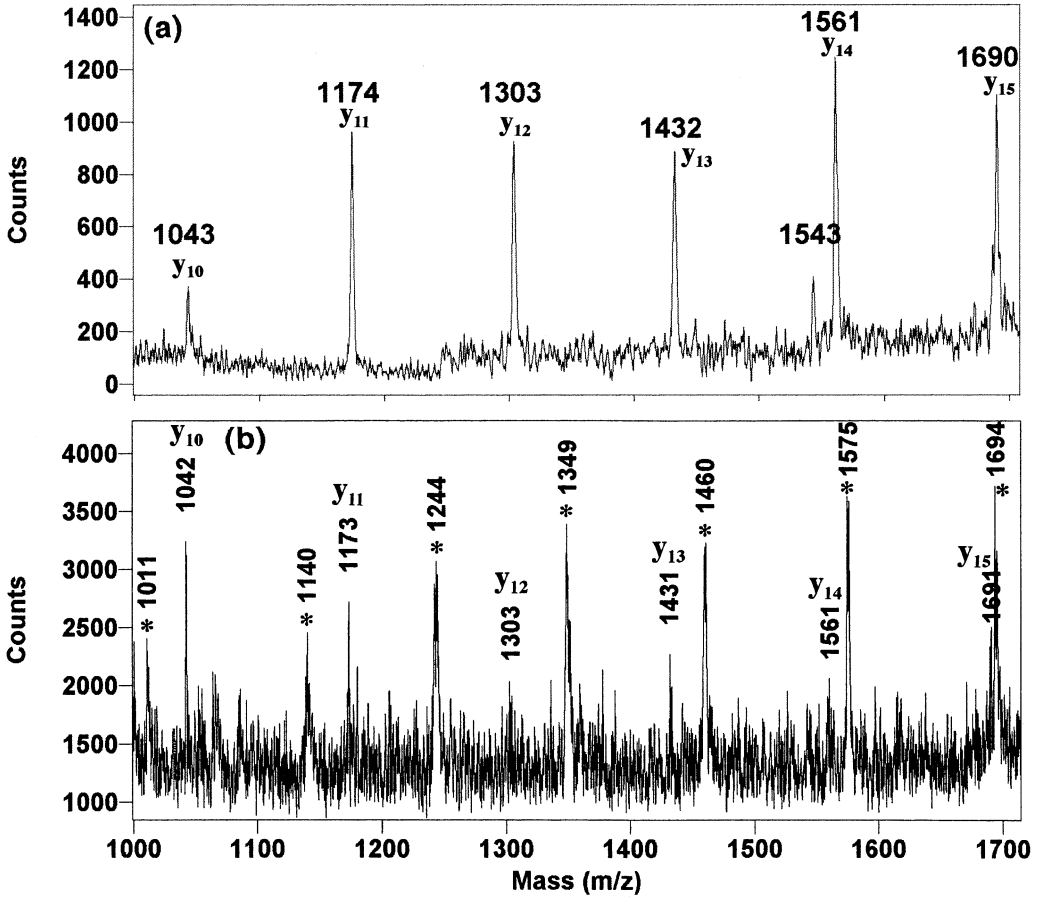

Figure 3. (**a**) MALDI-TOF-PSD spectrum of the protonated molecule $[M + H]^+$ of chromogranin A (Mr 1819.0) and (**b**) ISD spectrum of chromogranin A, obtained with α-cyano-4-hydroxycinnamic acid which gives b_n- and y_n-series rather than c_n- and $(z_n + 2)$-series ions [9]. Asterisk indicates metastable peaks corresponding to y_n-series ions.

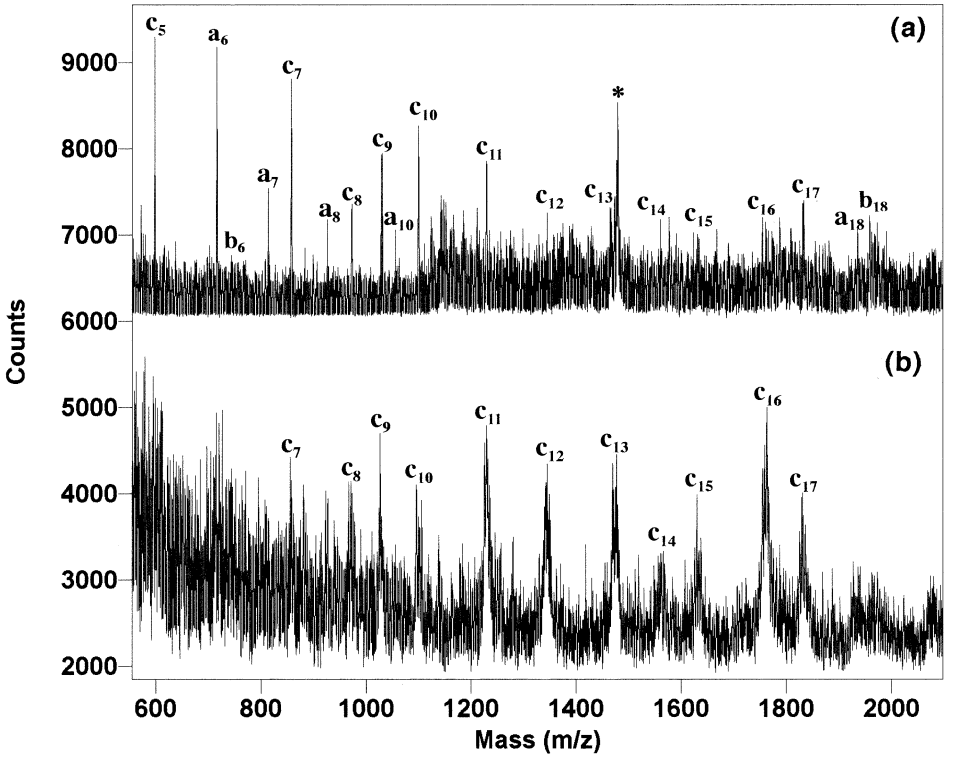

Figure 4. MALDI-TOF-ISD spectra of adrenocorticotropic hormone 18–39 (Mr 2465.7) obtained with 2,5-dihydroxybenzoic acid, with (**a**) 38 ns extraction delay time and (**b**) continuous extraction. Asterisk indicates metastable peaks.

TOF-ISD to the identification of the proteome and to the analysis of post-translational modifications, as shown by Reiber et al. [2] and Lennon et al. [23]. In addition, the rapid identification and sequence determination of proteins without predigestion using ISD is currently in progress.

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