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Coordination of Alkali Metal Ions to Glycans Dictates Fragment Yield in MALDI In-Source Decay with Hydrogen Abstraction

Daiki Asakawa,[†*] Nicolas Smargiasso and Edwin De Pauw

Dear Sir,

Matrix-assisted laser desorption/ionization (MALDI) in-source decay (ISD) is a powerful method for top-down sequencing of intact proteins.[1] MALDI-ISD is initiated by hydrogen atom transfer from matrix to analyte in a matrix crystal.[2] The resulting "hydrogen-abundant" peptide radical preferentially produces the *c'/z•* fragment pair by radical-induced cleavage.[3] Subsequently, radical *z•* fragments undergo a variety of radical reactions.[3, 4] The *a* and *y'* ions are alternatively formed by fragmentation of "hydrogen-abundant" peptide radicals during MALDI-ISD processes and *a* and *y'* ions formation cannot be explained by the N–C_α bond cleavage model alone. Although the mechanism of *a* and *y'* ions formation is not fully understood, the yield of these ions is related to the proton affinity of the matrix used.[5] Since the protonated analytes are generated by proton transfer from the protonated matrix to the analyte, the internal energy of analyte ions increases as the matrix proton affinity decreases.[5] Therefore, a matrix with a low proton affinity gives more internal energy to the peptide by proton transfer reaction and thereby favors the formation of *a* and *y'* ions.[5] This indicates the thermochemistry of peptide ionization to be important in the MALDI-ISD process.

On the other hand, MALDI mass spectrometry has also been used for the analysis of glycans.[6] 2,5-Dihydroxybenzoic acid is emerging as the good matrix for this purpose[6] and it works as a hydrogen radical donor during the MALDI-ISD process[1]. However, the formation

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of "hydrogen-abundant" glycan radicals is not efficient, because most of the glycan does not contain an unsaturated bond. Fragmentation of glycans in MALDI-ISD therefore requires much higher laser fluence than peptide fragmentation due to lack of a radical-induced fragmentation pathway. In fact, the fragment ions are observed as a weak intensity in the MALDI mass spectrum of glycan, even when the N_2 laser is used at full power.[7] The use of ultrahigh laser irradiation (50 mJ/pulse) for MALDI produced abundant glycan fragments.[8]

Recently, the use of 5-nitrosalicylic acid (5-NSA) for the MALDI-ISD of peptides and glycans was reported to promote fragmentation pathways involving "hydrogen-deficient" radical precursors.[9, 10] In the case of peptides, hydrogen abstraction resulted in the production of a nitrogen-centered "hydrogen-deficient" radical, leading to an *a*•/*x* fragment pair via radical-induced C_α–C cleavage.[11] For MALDI-ISD analysis of glycan, the hydrogen atom of the hydroxyl group in glycan is abstracted by 5-NSA, generating a "hydrogen-deficient" glycan radical.[10] Both glycosidic and cross-ring cleavages subsequently occur due to the fragmentation of "hydrogen-deficient" glycan radicals.[10] Interestingly, isobaric glycans could be distinguished by structure-specific ISD ions, and the molar ratio of glycan isomers in a mixture was estimated from the abundance ratios of their fragment ions.[10]

In contrast to fragment formation, the oxidized analytes, [M–2H] are alternatively formed by further hydrogen abstraction from "hydrogen-deficient" intermediate radicals.[9, 10] The formations of fragments and [M–2H] from the [M–H]• radical can be regarded as competing reactions:

$$[M–H]• + 5\text{-NSA} \rightarrow [M–2H] + [5\text{-NSA}+H]• \quad (1)$$

$$[M–H]• \rightarrow \text{fragments} \quad (2)$$

The hydrogen abstraction from the [M–H]• radical occurs in the MALDI plume, when numerous collisions between the radical and matrix molecules take place (reaction 1).[12] In contrast, fragment formations can be explained by radical-induced cleavage of the [M–H]• radical.[9, 10, 11] Therefore, fragments would be formed by unimolecular dissociation of [M–H]•

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radicals (reaction 2). It was previously shown that the yield of $[M–2H+H]^+$ is proportional to the initial velocity of analyte ions.[12, 13] Since the initial velocity of analytes is proportional to the collision rate,[14] the results obtained indicate that the collision rate between analyte and matrix in the MALDI plume is a predominant factor mediating reactions of the "hydrogen-deficient radical", [M–H]•. In other words, the high abundance of fragment ions with lower initial velocity can be understood as resulting from a low collision rate in the MALDI plume allowing the formation of fragments allowed by an extended lifetime of the "hydrogen-deficient radical", [M–H]•. Consequently, the yield of ISD fragments and [M–2H] would be influenced by its lifetime and dissociation rate.

In the present work, we investigated the MALDI-ISD efficiencies of glycans cationized by various alkali cations. Fragmentation efficiency was found to be enhanced by decreasing the size of the alkali metal cation. The alkali metal cation with the smallest radius, *i.e.* Li^+, facilitated cross-ring and glycosyl bond cleavages due to decreasing the activation barrier for fragmentation. By contrast, the use of K^+ produces a "hydrogen-deficient" intermediate with a prolonged lifetime, allowing further hydrogen atom abstraction to yield [M–2H] via collision with matrix molecules in the MALDI plume.

5-NSA was purchased from Kanto Kagaku (Tokyo, Japan). LNDFH-I, LNDFH-II, and alkali metal chlorides (LiCl, NaCl and KCl) were purchased from Sigma-Aldrich (St. Louise, MO, USA). All reagents were used without further purification. All of the solvents used were HPLC grade in quality except for water from the Milli-Q purification system (Millipore; Billerica, MA, USA). The 5-NSA was dissolved in water/ACN (1/1, v/v) at 10 mg/mL. The metal salts (2.5 mM for LiCl, 0.5 mM for NaCl and KCl, final concentration) were added to the 5-NSA solution. A volume of 0.5 μL of analyte glycan solution (20 μM in water) was deposited onto a MALDI target plate, and a 0.5 μL portion of the 5-NSA solution was then added. MALDI-ISD mass spectra were recorded using an UltraFlex II TOF/TOF mass spectrometer (Bruker Daltonics, Germany). The analyzer was operated in the reflectron

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mode with delayed extraction. The laser fluence was set to about 10–20% higher than the detection threshold of the intact molecular ion, and was optimized in order to obtain MALDI-ISD mass spectra that had high signal-to-noise ratios of the ISD ion peaks. The relative intensities of fragment ions of glycans were evaluated by averaging 4 sequential measurements.

Herein, we used LNDFH-I and LNDFH-II as model glycans. The fragmentation details of these glycans are described in a previous article.[10] The assignments of fragment ions observed in MALDI-ISD with 5-NSA are summarized in Scheme 1. Figures 1 and 2 show the comparison of MALDI-ISD spectra of LNDFH-I and LNDFH-II obtained using LiCl or KCl, respectively, as cationization reagents. As shown in the right inset of Figures 1 and 2, the use of KCl highly favored the formation of $[M–2H+C]^+$, where C indicates alkali metal cation. Conversely, a lower abundance of $[M–2H+C]^+$ was observed when LiCl was used. The ratio of the signal intensity of the intact cationized glycan $[M+C]^+$ to that of the oxidized one $[M–2H+C]^+$ is summarized in Table 1. The order of oxidized glycan yield was KCl > NaCl > LiCl, which corresponded to increasing cation sizes.

Next, we focused on the relationship between the fragmentation yield and the nature of alkali metal chloride used for the MALDI-ISD with 5-NSA. Observed fragment ions in Figures 1 and 2 were similar to MALDI-ISD with NaCl used as the cationization reagent.[10] Notably, the fragment ion yield in MALDI-ISD with LiCl was clearly higher than that with KCl. As described in the introduction, the “hydrogen-deficient” radical intermediate undergoes either further hydrogen abstraction (reaction 1) or fragmentation (reaction 2). To examine the fragmentation efficiency of glycan radical intermediates, the yield of each ISD fragment ion was defined by the ratio of the intensity of the oxidized glycan, $[M–2H+C]^+$, because these products were competitively formed from the glycan radical intermediate, [M–H]•. The fragment ion yields of LNDFH-I and LNDFH-II are summarized in Tables 2 and 3, respectively. The results indicate the order of the fragmentation yield to be the inverse of the

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alkali metal size order, $Li^+ > Na^+ > K^+$. This trend was generally observed for ISD fragment ions involving both glycosidic and cross-ring cleavages. It should be noted that the relationship between closed shell glycan fragmentation and metal ion size has been reported previously, indicating the complexes of glycans with a small metal cation to be more labile than those with a larger metal cation.[15, 16, 17]

The fragmentation behavior of the "hydrogen-deficient" glycan radical with a different alkali metal was studied in further detail. As described in the introduction, oxidized glycans such as $[M–2H+C]^+$, form via collision between a "hydrogen-deficient" radical intermediate and a 5-NSA molecule in the MALDI plume. According to the first author's previous reports, the oxidized peptide yield depends on the initial velocity of the analyte ion, which reflects the collision rate between analytes and matrix molecules in the MALDI plume.[12, 13] Initial velocities of analyte ions produced by MALDI are dependent on the matrix used, whereas analyte mass and ion species do not influence velocity.[18] In the present work, all MALDI-ISD experiments were performed using 5-NSA as a matrix. The only differences among the conditions were the alkali metal salts used. Therefore, the collision rate in the MALDI plume was expected to have a similar value under every condition and it would not influence on the fragmentation yield. As a consequence, differences in the fragment ion yields obtained with various alkali metal chlorides would be expected to originate from different dissociation rates of glycan radical intermediates under each of the conditions employed. In other words, Li^+ in the complex mediates the fragmentation of "hydrogen-deficient" glycan radicals by increasing their dissociation rate, indicating the thermochemistry of glycan ionization and structures of the resulting ions to be important in the MALDI-ISD process.

The metal cation is coordinated with several oxygen atoms of the glycan to make a metal-glycan complex.[17] For the fragmentation of closed shell glycans, glycosidic cleavage occurs when a cation associates with a lone electron pair of a glycosidic oxygen atom.[19] The

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individual interaction between an alkali metal cation and one oxygen atom in glycans is significantly stronger for the small alkali metal cations, which are associated with the glycosidic oxygen weakening the glycosidic bond.[17] As a result, coordination of the glycan and a small metal cation contributes to decreasing the dissociation barrier.[17] In fact, the dissociation rate of the metal-glycan complex decreases as the alkali cation size increases.[16]

As shown in Figures 1 and 2, the binding of Li^+ to glycan promoted fragmentation of the "hydrogen-deficient" glycan radical as in the case of closed shell glycans. The existence of "hydrogen-deficient" glycan radicals containing enough internal energy for fragmentation would yield a strong intensity of fragment ions. Since the Li^+-glycan radical interaction facilitated that fragmentation, it contributed to increasing the dissociation rate of the "hydrogen-deficient" radical intermediate by decreasing the required energy for fragmentation. Inversely, the yield of the oxidized glycan, $[M–2H+C]^+$, was simultaneously suppressed with a radical intermediate, [M–H]•, which has a short lifetime.

In contrast, the use of K^+ favored the formation of $[M–2H+K]^+$, and the fragmentation yield was thus lower under this condition. These observations indicate that the K^+-glycan complex produces a "hydrogen-deficient" radical intermediate with a prolonged lifetime, allowing further hydrogen atom abstraction to yield [M–2H] by collision with the matrix in the MALDI plume. The coordination of glycan to K^+ would not sufficiently contribute to decreasing the activation barrier for fragmentation. The differences among MALDI-ISD spectra with various alkali metals were reflected in the different stabilities of the "hydrogen-deficient" glycan radical. The stability order of alkali metal-glycan radical complexes is concluded to be proportional to the size of the alkali metal cation, $K^+ > Na^+ > Li^+$.

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Yours,

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Figure Caption

Figure 1. MALDI-ISD mass spectra of LNDFH-I obtained with 5-NSA matrix. (a) LiCl and (b) KCl were used as cationization reagents

Figure 2. MALDI-ISD mass spectra of LNDFH-II obtained with 5-NSA matrix. (a) LiCl and (b) KCl were used as cationization reagents

Scheme 1. Molecular structure and fragmentation schemes of (a) LNDFH-I and (b) LNDFH-II

Tables

Table 1. Ratio of the signal intensity of the oxidized ion $[M-2H+C]^+$ to that of the intact ion $[M+C]^+$ of Glycans (%).

	Li^+	Na^+	K^+
LNDFH-I	23.7 ± 8.2	28.7 ± 1.6	47.2 ± 8.9
LNDFH-II	24.6 ± 5.2	33.1 ± 3.1	45.6 ± 5.0

Table 2. Ratio of the signal intensity of fragment ions to that of the oxidized ion $[M-2H+C]^+$ of LNDFH-I (%). Mass values represent fragments without an alkali cation.

Fragment	Mass	Li^+	Na^+	K^+
$Z_{3\alpha}/C_4$	511.2	92.1 ± 10.7	20.2 ± 2.5	3.7 ± 0.5
$Z_{3\alpha}/Y_{3\beta}$	527.2	40.8 ± 4.5	9.1 ± 1.5	1.4 ± 0.6
B_3	657.2	47.1 ± 9.7	11.9 ± 2.5	3.9 ± 0.5
$Z_{3\alpha}$	673.2	52.0 ± 7.7	14.0 ± 2.6	6.5 ± 0.9
$Y_{3\alpha}$	691.2	37.8 ± 7.3	13.7 ± 1.6	3.1 ± 0.4
$^{1,3}X_{4\alpha}$	793.3	20.6 ± 4.9	7.3 ± 0.8	1.4 ± 0.3
B_4	819.3	21.8 ± 5.9	8.1 ± 0.9	3.6 ± 0.5
C_4	837.3	15.9 ± 3.6	12.9 ± 1.2	4.9 ± 0.5
$Y_{4\alpha}$ or $Y_{3\beta}$	853.3	52.1 ± 16.4	26.0 ± 2.4	9.2 ± 1.4
$^{2,4}A_5$	879.3	30.6 ± 7.9	23.9 ± 2.4	3.5 ± 0.6
$^{1,3}A_5$	939.3	72.5 ± 17.9	47.1 ± 2.7	6.9 ± 1.5

Table 3. Ratio of the signal intensities of fragment ions to that of the oxidized ion $[M-2H+C]^+$ of LNDFH-II (%). Mass values represent fragments without an alkali cation.

Fragment	Mass	Li^+	Na^+	K^+
$Y_{2\alpha}$	488.2	46.2 ± 9.6	9.5 ± 1.6	3.5 ± 0.6
$B_{2\alpha}$	511.2	115.9 ± 52.4	18.8 ± 5.1	6.5 ± 0.9
$B_{3\alpha}/Y_{3\alpha''}$	527.2	40.7 ± 16.1	12.0 ± 3.6	6.9 ± 0.3
$B_{3\alpha}$	673.2	29.5 ± 4.0	9.7 ± 2.7	5.3 ± 0.5
$C_{3\alpha}$	691.2	55.7 ± 15.2	33.0 ± 7.3	11.6 ± 1.8
$Y_{1\beta}/Y_{3\alpha''}$	707.2	11.1 ± 3.0	4.5 ± 1.3	1.2 ± 0.1
$^{1,3}A_4$	793.2	9.0 ± 2.7	4.8 ± 1.2	2.9 ± 0.6
$Y_{1\beta}$ or $Y_{3\alpha''}$	853.2	47.3 ± 7.8	30.3 ± 3.7	15.6 ± 2.8

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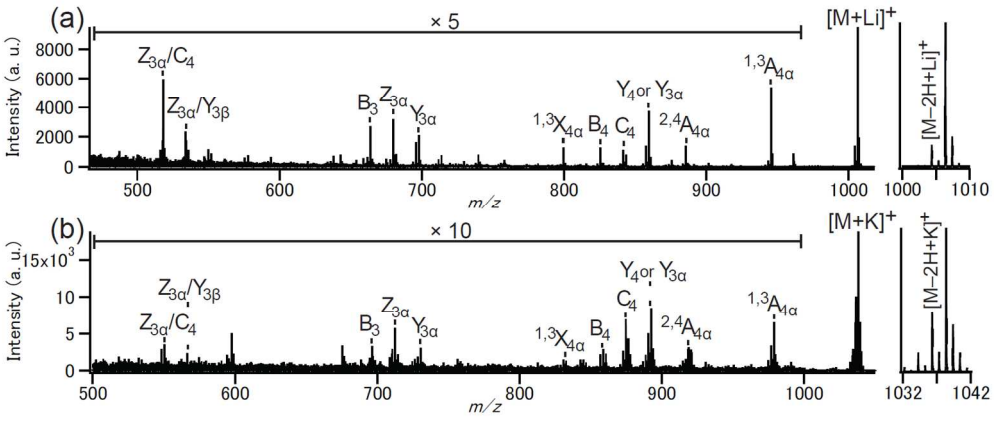

MALDI-ISD mass spectra of LNDFH-I obtained with 5-NSA matrix. (a) LiCl and (b) KCl were used as cationization reagents
452x194mm (96 x 96 DPI)

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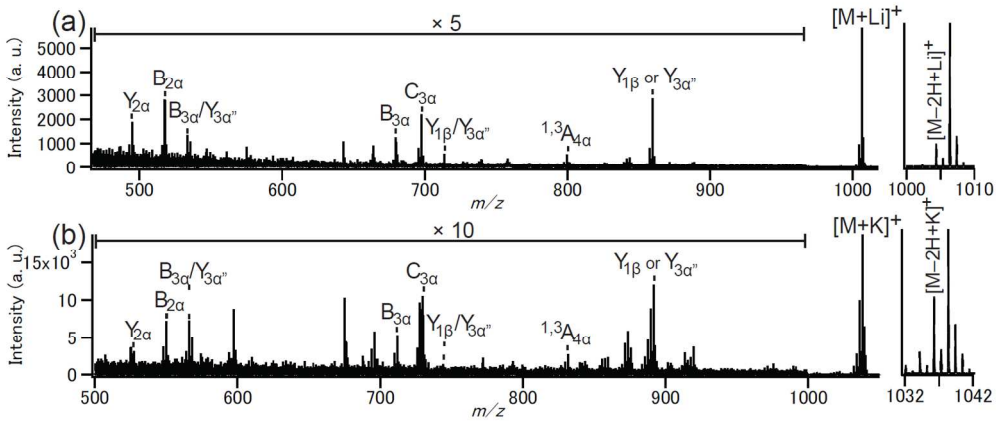

MALDI-ISD mass spectra of LNDFH-II obtained with 5-NSA matrix. (a) LiCl and (b) KCl were used as cationization reagents
450x194mm (96 x 96 DPI)

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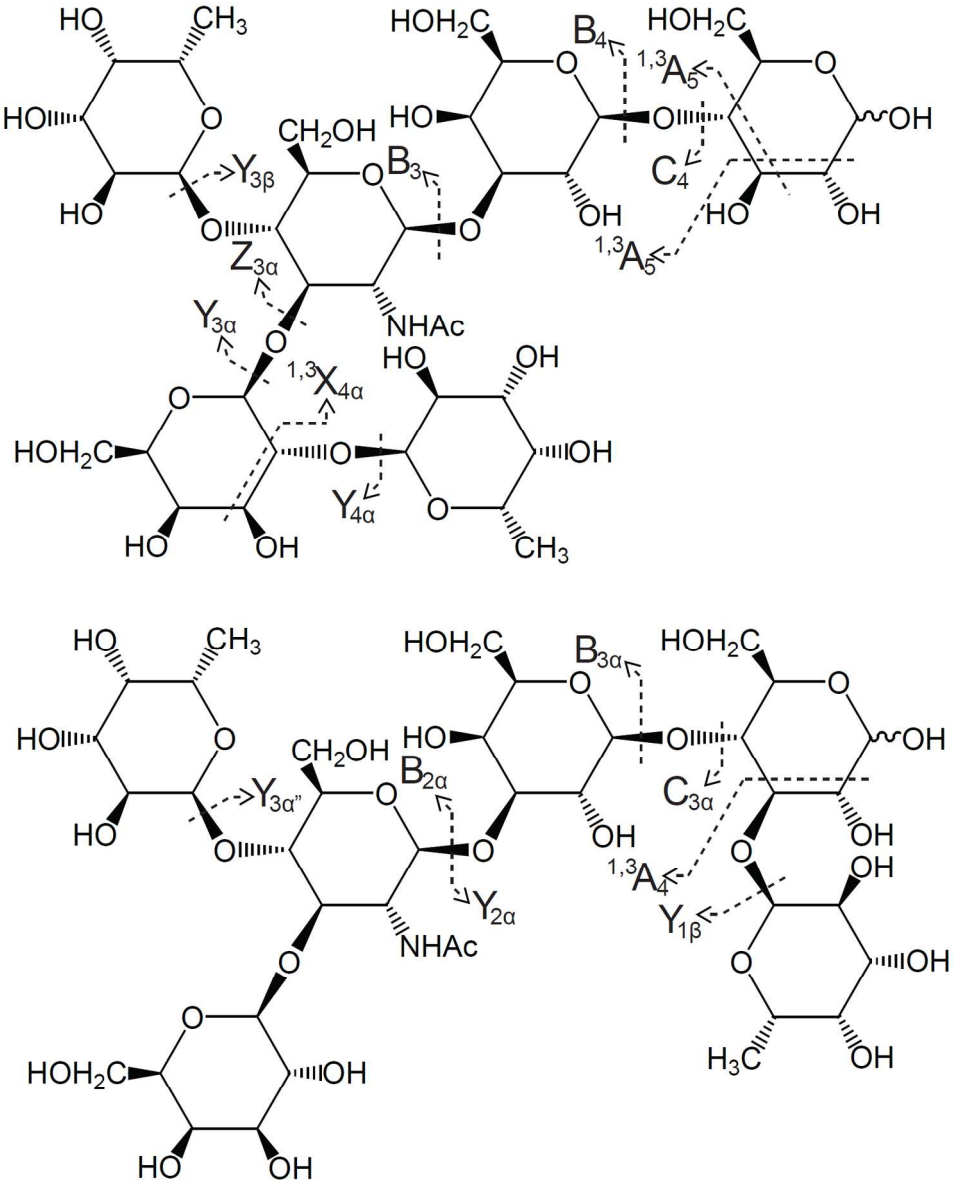

Molecular structure and fragmentation schemes of (a) LNDFH-I and (b) LNDFH-II
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