

# Direct MALDI Glycotyping of Glycoproteins toward Practical Subtyping of Biological Samples

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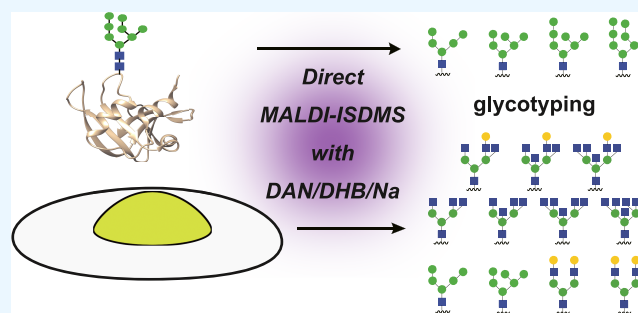
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**ABSTRACT:** The rapid analysis of glycan patterns (glycoforms) of glycoproteins can accelerate their quality control and biomarker discovery. We have focused on the direct analysis of glycoprotein glycoforms using matrix-assisted laser desorption/ionization in-source decay mass spectrometry (MALDI–ISDMS), called MALDI glycotyping. Our results show that the 1,5-diaminonaphthalene (DAN)/2,5-dihydroxybenzoic acid (DHB)/Na matrix can directly analyze the glycoforms in the femtomolar range of intact glycoproteins. The addition of DAN improved the morphology of the solid matrix due to the mixture of DAN and DHB, which significantly contribute to the high sensitivity of this direct analysis. Adding DAN significantly improved the sensitivity of the glycan precursor ions in the TOF/TOF analysis because of its enhanced fragmentation effect as an efficient UV-MALDI matrix. Further, practical glycoform analysis (glycotyping) of diluted biological samples containing glycoproteins, such as egg whites, was also successfully achieved.



## INTRODUCTION

Rapid identification of glycan patterns in glycoproteins and detection of the related changes in the proteins and biofluids can accelerate life science research and its social implementation. For example, it can aid in the development of blood transfusion medicine through the discovery of blood types and cancer medicine through the discovery of cancer antigens (or carbohydrate antigens; CAs).<sup>1,2</sup> Further, glycoforms of proteins are key factors in protein stability and pharmacokinetics; thus, their rapid identification can accelerate molecular diagnostics and quality control of biopharmaceuticals.<sup>3,4</sup> Currently, immunoassays are mainly used for diagnostics targeting glycoforms and serotypes in medical practice.<sup>5</sup> However, immunoassay methods require the prior preparation of a set of antibodies or antigens for every possible diagnostic candidate. An increase in the number of diagnostic candidates makes the process time-consuming and expensive. Therefore, matrix-assisted laser desorption/ionization (MALDI) biotyping, an innovative method used for the rapid identification of biological samples without any set of probe molecules such as antibody or oligonucleotide,<sup>6,7</sup> is ideal to rapidly identify glycoforms (glycotyping).

MALDI is a soft ionization method used for the structural analysis of macromolecules, such as glycoproteins. It is widely used in conjunction with time-of-flight mass spectrometry (TOFMS).<sup>8</sup> In particular, MALDI, which mainly produces monovalent molecular ions, is suitable for pattern analysis of glycans with structural heterogeneity owing to the ambiguity of the branching points and endpoints in the biosynthetic

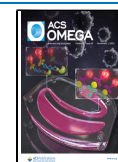
process. In MALDI–TOFMS analysis, the matrix significantly impacts the successful measurement of the analyte of interest. 2,5-Dihydroxybenzoic acid (DHB) is widely used as the first choice in MALDI–TOFMS analysis of glycans because of its high ionization efficiency.<sup>9,10</sup> However, DHB forms large needle-like crystals and localizes sites suitable for analysis, thereby limiting the versatility of MALDI–MS analysis of glycans.<sup>11</sup> Furthermore, DHB was discovered as a matrix suitable for the ionization of proteins and peptides; therefore, the ionization efficiency of the glycan moieties is relatively low when analyzing glycoproteins. In fact, complex pretreatment steps are generally required to analyze glycan patterns on glycoproteins using MALDI–TOFMS,<sup>12</sup> which increases the cost, time, and required skill.

In-source decay (ISD) is a fragmentation reaction that occurs between post-laser irradiation and ion extraction in the MALDI source. MALDI–ISD analysis of intact (glyco-)proteins is a practical protein identification technique that allows for both N- and C-terminal sequencing.<sup>13–15</sup> Matrix selection is an important factor in MALDI–ISD analysis. For example, using 1,5-diaminonaphthalene (DAN) for (glyco-

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)protein sequencing by MALDI–ISD allows for extensive sequencing by the hydrogen-donating ability to disulfide bonds and peptide bonds.<sup>16</sup>

We and Nicolardi et al. recently reported the first example of glycan-selective MALDI–ISD analysis from intact glycoprotein.<sup>17,18</sup> A sodium-doped super DHB (DHB/5-methoxy salicylic acid/Na) caused random ISD fragmentation of artificial *N*-glycans having bacterial O-antigen polysaccharide repeats to give the sequence information of the repeat units.<sup>18</sup> A solid salt matrix composed of aniline/DHB/Na<sup>19</sup> caused selective ISD at the reducing ends of *N*-glycans and glycan fragment selective ionization, making this top-down glycomic analysis possible.<sup>17</sup> However, the intensity of the glycan fragment ion signals obtained by MALDI–ISDMS analysis using this aniline/DHB/Na matrix was still insufficient to detect minor glycan components and to analyze glycoprotein containing biofluid. To solve this problem, we have focused on the structural similarities and physicochemical differences between aniline and DAN as the matrix for improving the glycan-selective MALDI–ISD analysis.

Here, we report results obtained via optimization studies targeting glycan-selective MALDI–ISD analysis for intact glycoproteins, focusing on the reducing and degradation-accelerating properties of DAN.

## EXPERIMENTAL SECTION

**Materials and Reagents.** Ribonuclease B (RNase B) from bovine was purchased from New England BioLabs, Inc. (Beverly, MA, USA). Quail egg was purchased from Muroan Uzuraen (Hokkaido, Japan), and the egg white was freeze-dried before use. Powdered chicken egg white was purchased from Kewpie Corp (Tokyo, Japan). Trypsin inhibitor sourced from chicken egg white Type III-O (ovomuroid) and DAN were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). DHB, 2,2,2-trifluoroacetic acid (TFA), sodium bicarbonate (NaHCO<sub>3</sub>), aniline, and acetonitrile (HPLC grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). TFA was purchased from Watanabe Chemical Industry Co. Ltd. (Hiroshima, Japan). NH<sub>4</sub>HCO<sub>3</sub> was purchased from Nacalai Tesque (Kyoto, Japan). Cotton wool was purchased from Suzuran Corp. (Nagoya, Japan).

**Preparation of Analyte for MALDI–ISD Analysis.** RNase B and ovomuroid were diluted to 20 μM with H<sub>2</sub>O. Powdered chicken egg white and quail egg white were diluted to 0.1, 0.5, and 1.0 g/L with H<sub>2</sub>O. These solutions were directly used for MALDI analysis.

**Matrices.** Solutions of 50 mM DAN in acetonitrile/H<sub>2</sub>O (1:1, v/v), 1000 mM aniline in acetonitrile, 500 mM DHB in acetonitrile/H<sub>2</sub>O (9:1, v/v), and 100 mM NaHCO<sub>3</sub> in H<sub>2</sub>O were prepared. The DAN/DHB/Na matrix was prepared by mixing 4 μL of 50 mM DAN, 2 μL of 500 mM DHB, and 1 μL of 100 mM NaHCO<sub>3</sub> and diluting to 100 μL with acetonitrile/H<sub>2</sub>O (1:1, v/v). The aniline/DHB/Na matrix was prepared by mixing 1.2 μL of 1000 mM aniline, 2 μL of 500 mM DHB, and 1 μL of 100 mM NaHCO<sub>3</sub> and diluting to 100 μL with acetonitrile/H<sub>2</sub>O (1:1, v/v). The DAN matrix was prepared by mixing 20 μL of 50 mM DAN and diluting to 100 μL with acetonitrile/H<sub>2</sub>O (1:1, v/v). The DHB matrix was prepared by mixing 2 μL of

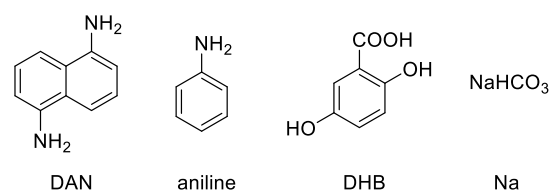
500 mM DHB and diluting to 100 μL with acetonitrile/H<sub>2</sub>O/TFA (50:50:0.1, v/v/v).

**MALDI Sample Preparation.** On an MTP AnchorChip 1536 BC target plate, 0.35 μL of the matrix solution was loaded. Then, 0.35 μL of the analyte solution was deposited on the matrix pre-spotted target plate and dried at 25 °C. On the MTP AnchorChip 400/384 TF target plate, 0.50 μL of the matrix solution was loaded, followed by depositing 0.50 μL of the sample solution and drying at 25 °C. Photographs of the dried matrix with the specimen were recorded using a monochrome industrial camera DMK 33UX264 (Imaging Source, Bremen, Germany) with a UPlanFLN 4× objective (Olympus, Tokyo, Japan) and 60 mm extension tubes.

**MALDI–TOF and TOF/TOF MS.** All spectra were acquired on an ultraflex III instrument (Bruker, Bremen, Germany) equipped with a 200 Hz Smartbeam Nd:YAG laser (355 nm). The ISD spectra from intact glycoprotein were acquired in the positive reflectron mode with 1000 laser shots and a random walk. The generated ions were accelerated to the kinetic energy of 25.0 kV. The low-mass ion deflector cutoff was set to 700 Da. In the laser-desorption/ionization (LIFT) TOF/TOF mode, the ISD fragment ions were accelerated to 8 kV in the MALDI ion source and selected in a time gate. The selected ISD ion was further accelerated to 19 kV in the LIFT cell. The metastable post-source decay (PSD) ions were analyzed without any additional fragmentation process, such as collision-induced dissociation.

## RESULTS AND DISCUSSION

**Optimization of Matrix Contents for Glycan-Selective MALDI–ISD Analysis of Intact Glycoprotein.** RNase B is a 15 kDa glycoprotein with a single high-mannose-type *N*-linked glycan.<sup>20</sup> It was used as the model analyte to optimize the matrix contents (Figure 1, Table 1) to compare the molecular



**Figure 1.** Matrix components used in this study. 1,5-Diaminonaphthalene: DAN; 2,5-dihydroxybenzoic acid: DHB.

ion spectral patterns of the corresponding fragment region of *N*-glycan (1000–1700 Da) using MALDI–ISD (Figures 2, S1–S3). From the initial aniline/DHB/Na [12:10:1 (nmol/μL)] matrix (Table 1, entry 1), aniline was replaced with DAN in various ratios (Table 1, entries 2–7). As previously reported, the mixture of RNase B with the aniline/DHB/Na (12:10:1) matrix yields the <sup>2,4</sup>A and <sup>0,2</sup>A type ISD fragment pattern of *N*-glycan with five (*m/z* 1097 and 1157), six (*m/z* 1259 and 1319), and seven mannose (*m/z* 1421 and 1481) units as the sodium adduct ions (Figure S1).<sup>17</sup> With DAN/DHB/Na (12:10:1; Table 1, entry 2), RNase B preferentially yields *c*-ions corresponding to the *N*-terminal fragment pattern as protons and sodium adduct ions in the region above *m/z* 1200 with a reduced intensity of *N*-glycan-derived *A*-ions. In addition, the intensity of the *N*-glycan ISD fragments decreased (Figure 2b). As the ratio of DAN to DHB was decreased by 20%, the intensity of the peptide signal gradually decreased (Figure S1). With DAN/aniline/DHB/Na

**Table 1. Ratio of the Matrix Mixture Tested. 1,5-Diaminonaphthalene: DAN; 2,5-Dihydroxybenzoic Acid: DHB**

entry	DAN [mM] <sup>a</sup>	aniline [mM] <sup>a</sup>	DHB [mM] <sup>a</sup>	Na [mM] <sup>a,b</sup>
1		12	10	1
2	12		10	1
3	10	2	10	1
4	8	4	10	1
5	6	6	10	1
6	4	8	10	1
7	2	10	10	1
8	10			
9	10			1
10	10		10	1
11	8		10	1
12	6		10	1
13	4		10	1
14	2		10	1
15			10	1

<sup>a</sup>Concentration of each compound in matrix solution before mixing with analyte. <sup>b</sup>Added as NaHCO<sub>3</sub> solution.

(2:10:10:1, Table 1, entry 7), RNase B gives a more robust ISD fragment pattern of *N*-glycan than with aniline/DHB/Na (12:10:1) and provides a signal pair corresponding to eight mannose residues (*m/z* 1582 and 1643) (Figure 2c,d, Tables S1,S2). These data indicate that DAN can be an excellent additive for glycan-selective ionization in the aniline/DHB/Na type matrix. However, DAN as a matrix alone enhances the formation and ionization of peptide fragments and reduces the ionization efficiency of glycan fragments.<sup>21</sup>

Interestingly, all of the major product ions corresponding to peptide fragments were assigned as proton adduct ions  $[M + H]^+$  with minor sodium adduct ions  $[M + Na]^+$  even in the presence of the sodium salt additive. Further, the product ions corresponding to glycan fragments were assigned only as sodium adduct ions  $[M + Na]^+$ . This led us to evaluate DAN (Table 1, entry 8) and DAN/Na (10:1, Table 1, entry 9) for MALDI–ISD analysis of RNase B. When DAN was used as the matrix, only RNase B-derived peptide fragments containing reduced cysteine residue were identified as proton adduct ions, and no signals indicating the formation of glycan fragments were observed.<sup>16,21</sup> The addition of sodium ions to the DAN matrix did not affect the pattern and intensity of the proton adduct ions derived from the peptide fragments, except with weak sodium adduct ions (Figure S2). However, very weak *N*-glycan-derived sodium adduct ions were also observed. These results further confirmed that DAN is a matrix that strongly induces peptide fragmentation and its proton adduct ion formation but does not contribute to the generation of glycan fragments or sodium adduct ions. Next, the effect of the DAN addition to DHB was explored. As the ratio of DAN to DHB was decreased, the peptide signal gradually disappeared, while the glycan signal was enhanced as sodium adduct ions (Figure S3, Table 1, entries 10–15). The addition of 20% DAN to DHB dramatically improved the sensitivity of glycan detection on RNase B, and the *N*-glycan fragments with eight mannose in low abundance were detected without any peptide-derived ion signals (Figure 2d, Table 1, entry 14, Table S3). Since limiting the amount of DAN to DHB improves the sensitivity of direct glycan analysis, properties of DAN other than reducibility contribute to this improvement. Without DAN,

that is, DHB/Na (10:1), the signal strength derived from the glycan fragments was markedly reduced (Figure S3, Table 1, entry 15). Furthermore, signals corresponding to the proton adduct and sodium adduct (*m/z* 1546 and 1568) derived from the *N*-terminal 13 amino acid fragments of RNase B disappeared, and only the signal from the high-mannose-type glycan fragment was observed as the sodium adduct ions.

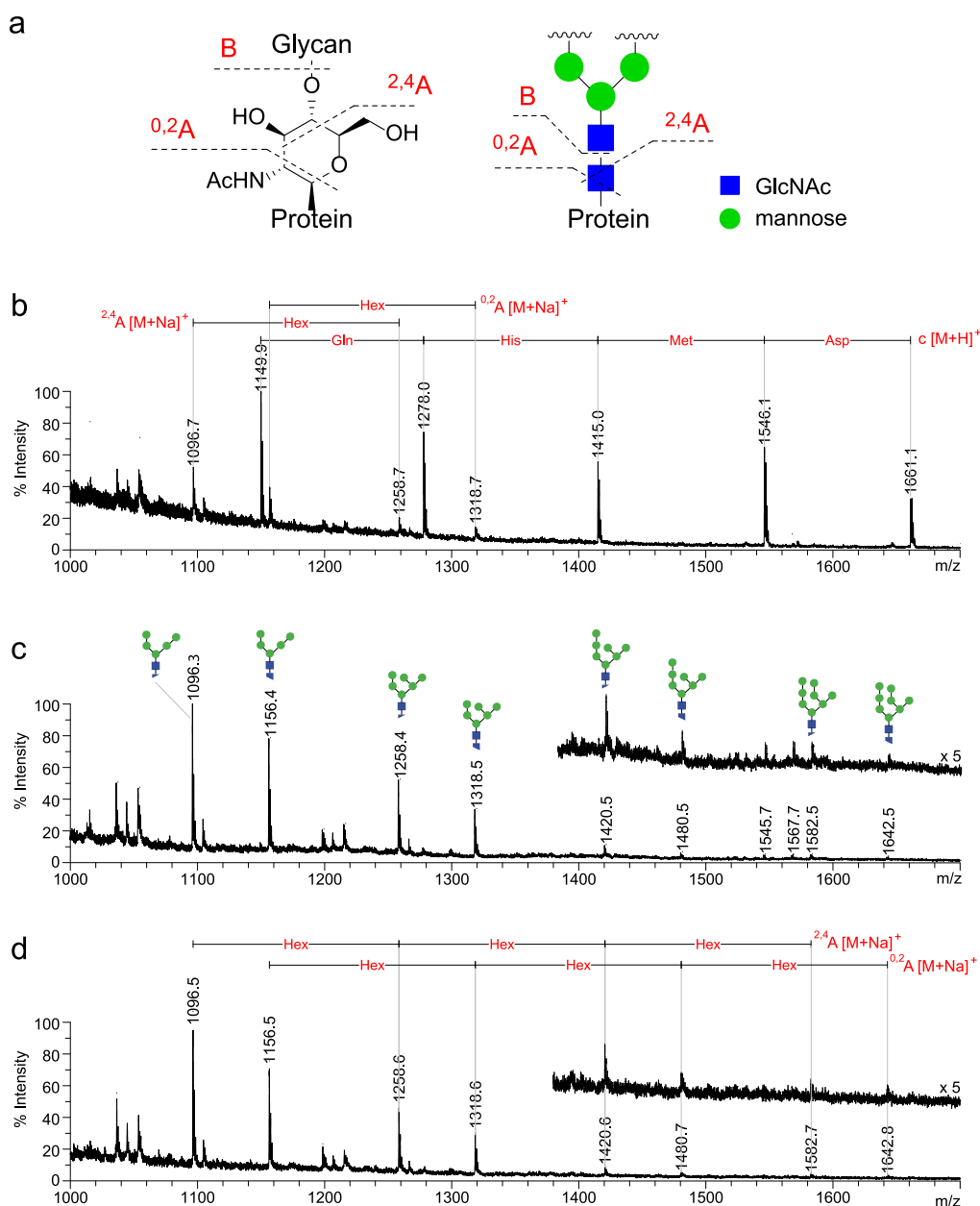
**Investigating the Effects and Mechanisms of DAN in the Direct Analysis of Glycoproteins.** To evaluate the detailed effect of DAN addition, we compared the direct detection limits of glycan on RNase B with the aniline/DHB/Na (12:10:1), DAN/aniline/DHB/Na (2:10:10:1), and DAN/DHB/Na (2:10:1) matrices. The glycan pattern detection limit of the aniline/DHB/Na matrix for intact RNase B was 1.75 pmol/spot (Figure S4). As expected, the addition of DAN markedly improved the detection limit. Thus, the glycan pattern spectra with the DAN/aniline/DHB/Na (2:10:10:1) and DAN/DHB/Na (2:10:1) matrices were directly produced from 175 fmole/spot glycoproteins (Figures S5,S6).

Next, the contribution of PSD to the ion patterns derived from glycan fragments generated by the aniline/DHB/Na (12:10:1), DAN/aniline/DHB/Na (2:10:10:1), and DAN/DHB/Na (2:10:1) matrices was investigated. Because the reflectron mode reflects molecular ions during the process of ion separation by TOF, its spectrum is affected by PSD. Therefore, the influence of PSD can be investigated by comparing the results of the linear and reflectron mode measurements. The analysis of the *N*-glycoform of RNase B by these matrices is realized because of the regioselective intracyclic cleavage reaction at the reducing terminal GlcNAc residue. The results showed that the RNase B-derived glycan fragmentation patterns between the linear and reflectron modes agreed well for all three matrices (Figure S7). In contrast to the MALDI–ISD glycan spectrum, the parent ions of RNase B glycoprotein showed matrix dependency between the linear and reflectron modes. With aniline/DHB/Na (12:10:1), the parent ions of RNase B were observed under both the linear and reflectron modes, as well as DHB of DHB/Na (10:1) as reported.<sup>17</sup> With DAN/DHB/Na (2:10:1), the parent ions of RNase B were observed under the linear mode but disappeared under the reflectron mode (Figure S8). It is speculated that the reducing ability and peptide fragmentation efficiency of DAN may contribute to the PSD-promoting ability of the glycoprotein parent ion.

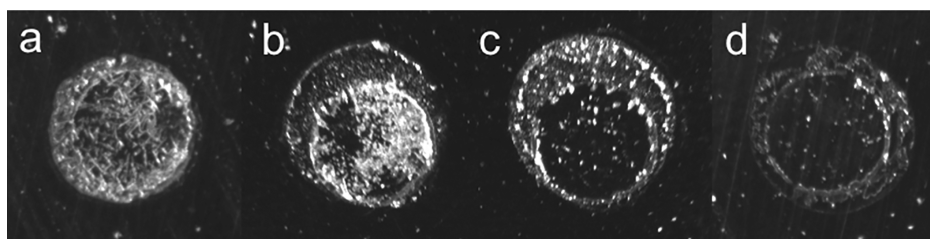
We then compared the PSD patterns in the MALDI–TOF/TOFMS measurements of mixtures of each matrix and RNase B using the sodium adduct ion derived from the most potent ISD peak at *m/z* 1096.0 as the precursor ion. Compared to aniline/DHB/Na (12:10:1), the precursor ion fragmentation was enhanced by DAN/DHB/Na (2:10:1), and a detailed analysis of the internal structure of glycans was possible (Figure S9). In all cases, a fragmentation pattern corresponding to the loss of the reducing terminal fragment site of the precursor A ion or that of hexose was observed. This indicates that the X or B ions were selectively produced by PSD. This chemoselectivity of the fragmentation positions in PSD and ISD helps to infer the glycan structure.

The morphological changes of each matrix up to its solidification on the target plate were also compared (Figures 3, S1 and S3). DHB/Na (10:1) tends to form needle-like crystals.<sup>6</sup> Aniline salts of DHB resulted in crystal refinement via an ionic-liquid-like intermediate.<sup>13</sup> The matrices with 20% DAN added to DHB, particularly sensitive to glycan detection,





**Figure 2.** (a) <sup>0,2</sup>A, <sup>2,4</sup>A, and B type ISD fragment position of *N*-glycans. MALDI–ISD spectrum of RNase B (20 pmol  $\mu\text{L}^{-1}$ ) with (b) 1,5-diaminonaphthalene (DAN)/DHB/Na (12:10:1), (c) DAN/aniline/DHB/Na (2:10:10:1), and (d) DAN/DHB/Na (2:10:1).

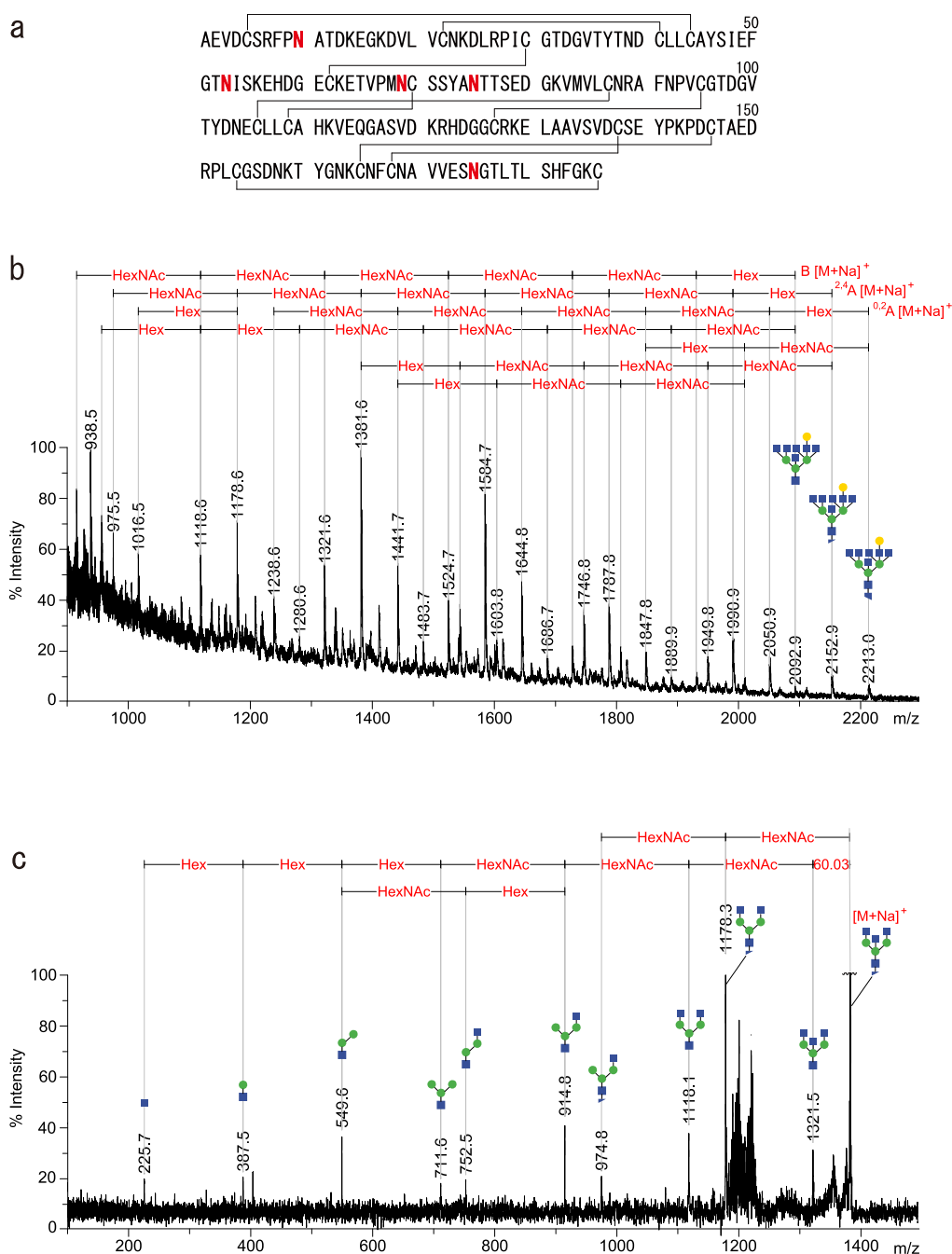


**Figure 3.** Morphological comparison of each matrix. (a) 2,5-Dihydroxybenzoic acid (DHB)/Na (10:1), (b) aniline/DHB/Na (12:10:1), (c) 1,5-diaminonaphthalene (DAN)/aniline/DHB/Na (2:10:10:1), and (d) DAN/DHB/Na (2:10:1).

formed a transparent solid layer on hydrophilic spots prepared on a water-repellent surface such as AnchorChip. In particular, aniline-free DAN/DHB/Na (2:10:1) required a shorter drying time (Figure S10).

These results indicate that DAN/DHB/Na (2:10:1) is the most practical matrix for the direct analysis of glycan patterns on glycoproteins.

**MALDI–ISD Analysis of Ovomuroid.** Ovomuroid (Figure 4a), a 23 kDa glycoprotein with five *N*-glycans derived



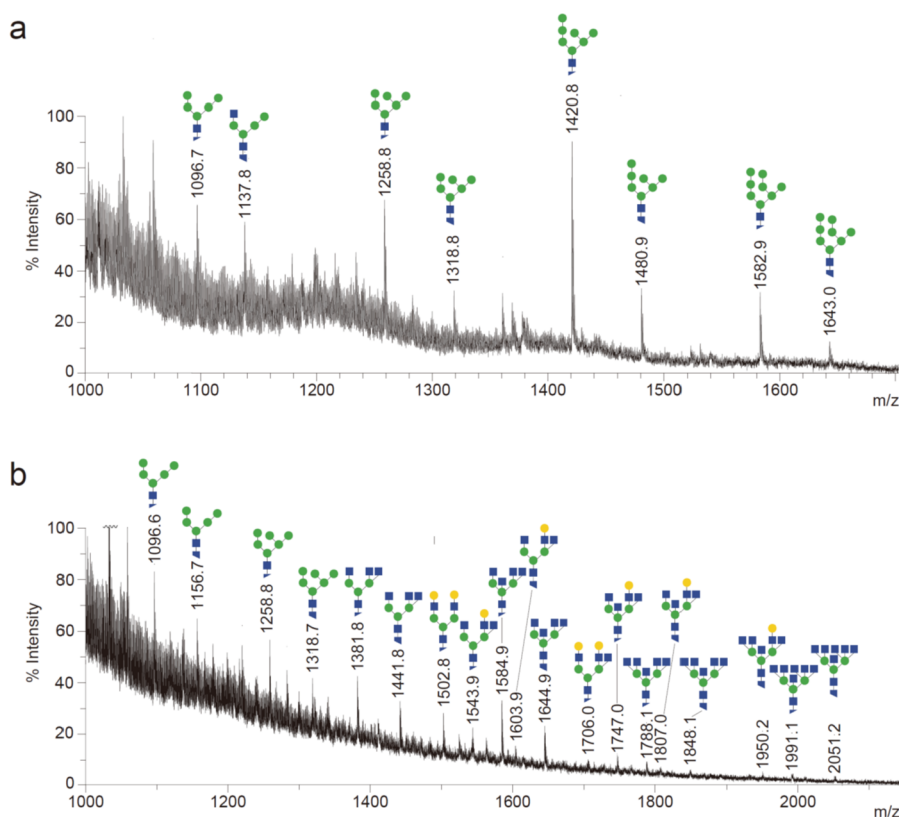
**Figure 4.** (a) Structure of ovomuroid and glycosylation site (bold red). (b) MALDI–ISD mass spectrum of ovomuroid with 1,5-diaminonaphthalene (DAN)/2,5-dihydroxybenzoic acid (DHB)/Na (2:10:1). (c) MALDI–TOF/TOF mass spectrum of precursor  $^{0.2}A$  ion ( $m/z$  1382) determined using MALDI–ISD analysis of ovomuroid with DAN/DHB/Na (2:10:1).

from chicken eggs, was also used as an analyte. Unlike RNase B, ovo-muroid has a large diversity of *N*-glycan structures, including high mannose and complex and highly branched hybrid-type glycans.<sup>22</sup> A mixture of 7 pmol of ovomuroid and DAN/DHB/Na (2:10:1) was measured to show 2–8 residues of HexNAc and 3 and 4 residues of A and B-ion patterns, respectively, corresponding to *N*-glycan fragments combined with hexoses as sodium adduct ions (Figure 4b, Table S4). This indicates that the ovomuroid has a variety of *N*-glycans ranging from hexasaccharides to five-branched complex-type glycans, which is consistent with the structures reported in conventional glycomics studies.<sup>23</sup> Ovomuroid was then mixed with DAN or DAN/Na (10:1) and measured, and a weak

glycan fragment A ion pattern was observed as the sodium adduct along with a characteristic peak at  $m/z$  1023. This peak corresponds to the proton adduct ion of the nonapeptide (AEVDCSRFP) fragment containing a reduced cysteine residue from the N-terminus of the ovomuroid (Figure S11).

Furthermore, the MALDI–TOF/TOFMS analysis of the strongest signal ( $m/z$  1381.5) selected as the precursor ion indicated that this ion was derived from a complex-type glycan chain in which both the reducing and non-reducing ends are formed exclusively of GlcNAc (Figures 4c, S12, and Table S5).

**MALDI–ISD Glycomics of Egg Whites.** Next, we performed direct MALDI–ISD analysis on 1000–10,000 times diluted chicken and quail egg whites as model samples



**Figure 5.** MALDI–ISDMS of egg whites. (a) Quail egg white with 1,5-diaminonaphthalene (DAN)/2,5-dihydroxybenzoic acid (DHB)/Na (2:10:1). (b) Chicken egg white with DAN/DHB/Na (2:10:1). All molecular ion signals were detected as sodium adduct ions.

of biofluid (Figures S13–S16). The *N*-glycans in quail egg white are mainly composed of high-mannose-type glycans and hybrid-type glycans.<sup>24</sup> When diluted quail egg white was mixed with DAN/DHB/Na (2:10:1) and measured, high mannose-type glycans with 5–8 mannose and GlcNAc residues hybrid-type glycans with one additional group were observed as sodium adduct ions (Figures 5a, S14 and Table S6). However, when a diluted chicken egg white solution<sup>23</sup> was mixed with DAN/DHB/Na (2:10:1), complex-type glycan patterns with GlcNAc counts of 5–8 were observed as the sodium adduct ions (Figures 5b, S16 and Table S7). The *N*-glycan patterns from these two egg whites were in good agreement with those observed after conventional enzymatic digestion, separation, and chemical modification to increase ionization efficiency.<sup>23,24</sup> Meanwhile, the glycan pattern peaks were attenuated when the same samples were measured in aniline/DHB/Na (12:10:1, Figures S13,S15).

## CONCLUSIONS

This study aims at improving the direct analysis of *N*-glycans on glycoproteins by the MALDI–ISD method toward practical MALDI glycotyping. As a result, the addition of DAN to a conventional matrix, DHB mixtures, improved the detection limit of the analyte to the femtomolar scale without any pretreatment. In particular, DAN/DHB/Na (2:10:1) improved the sensitivity and accumulation characteristics on water-repellent surfaces and the solidification time of the mixture with the specimen. Meanwhile, MALDI–TOF/TOF analysis, in which PSD degradation occurs at high laser irradiation energies, showed an apparent enhancement of the glycoside bond degradation to form B-ions in the DAN-doped system. This result indicates that the addition of DAN enhanced the

transfer of laser irradiation energy to the glycan fragments. Furthermore, DAN/DHB/Na (2:10:1) showed high sensitivity and reproducibility in the direct analysis of biological samples such as egg white. Therefore, DAN/DHB/Na (2:10:1) is an effective matrix with high glycan selectivity and detection sensitivity for the glycan analysis of glycoprotein and glycoprotein-containing biosamples by MALDI–ISDMS. Since this matrix is suitable for direct glycan analysis of specimens, it is ideal for pattern analysis of biological samples using glycoform as an indicator. Our results indicate that MALDI glycotyping is a practical technology that can be added to MALDI biotyping technology, currently targeting protein and peptide fragment patterns. Toward practical glycotyping, this study pursued speed and simplicity, sacrificing the comprehensiveness emphasized in conventional glycomics studies. In fact, this study was limited to the positive ion mode analysis of neutral *N*-glycans. Further innovations will be necessary for typing negatively charged glycans with sialic acid or uronic acid. The glycan sequence information by the pseudo-MS<sup>3</sup>-PSD method reported in this study would extend the current limitations of MALDI glycotyping and provide additional subtyping information of the samples. We are also investigating the direct analysis of glycoprotein *O*-glycans and glycolipid glycans hidden by matrix-derived noise regions (<1 kDa) and their application to preprocessing-free MALDI imaging.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c05429>.

MALDI-<sup>2</sup>ISD-TOFMS, MALDI-TOFMS, and MALDI-TOF/TOFMS spectrum, glycan composition assignment data of the spectrum in the manuscript, structure of RNase B, snapshots of the matrices on the target plate (PDF)

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### Author Contributions

S.U. performed experiments, H.H. designed research, and wrote the paper. All authors have given approval to the final version of the article.

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### Notes

The authors declare no competing financial interest.

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