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Rational Selection of the Optimum MALDI Matrix for Top-Down Proteomics by In-Source Decay

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In-source decay (ISD) in MALDI leads to c- and z-fragment ion series enhanced by hydrogen radical donors and is a useful method for sequencing purified peptides and proteins. Until now, most efforts to improve methods using ISD concerned instrumental optimization. The most widely used ISD matrix is 2,5-dihydroxybenzoic acid (DHB). We present here a rational way to select MALDI matrixes likely to enhance ISD for top-down proteomic approaches. Starting from Takayama's model (Takayama, M. *J. Am. Soc. Mass Spectrom.* 2001, *12*, 1044−9), according to which formation of ISD fragments (c and z) would be due to a transfer of hydrogen radical from the matrix to the analyte, we evaluated the hydrogen-donating capacities of matrixes, and thus their ISD abilities, with spirooxazines (hydrogen scavengers). The determined hydrogen-donating abilities of the matrixes are ranked as follows: picolinic acid (PA) > 1,5-diaminonaphtalene (1,5-DAN) > DHB > sinapinic acid > α-cyano-4-hydroxycinnamic acid. The ISD enhancement obtained by using 1,5-DAN compared to DHB was confirmed with peptides and proteins. On that basis, a matrix-enhanced ISD approach was successfully applied to sequence peptides and proteins up to ∼8 kDa. Although PA alone is not suitable for peptide and protein ionization, ISD signals could be further enhanced when PA was used as an additive to 1,5-DAN. The optimized matrix preparation was successfully applied to identify larger proteins by large ISD tag researches in protein databases (BLASTp). Coupled with an adequate separation method, ISD is a promising tool to include in a top-down proteomic strategy.

Matrix-assisted laser desorption/ionization (MALDI)[1,2] is a widely used ionization method for the identification of peptides and proteins. In MALDI, the analyte is cocrystallized in a large excess of a host matrix. The matrix is an aromatic compound of low molecular mass that absorbs the UV laser irradiation, preventing the analyte from direct degradation. The ionization step in MALDI is not completely understood.[3] But the actual models converge all on a two-step mechanism, the primary and the secondary ionization.[4] The primary ionization concerns the formation or the separation of the ions. During the desorption/ablation in the MALDI plume, reactions of ion−molecule occur, forming the secondary ions, which will reach the detector. The secondary ionization concerns the formation of these secondary ions and becomes less controversial. In fact, it is increasingly accepted that in typical MALDI conditions the local thermal equilibrium is reached in the MALDI plume, which is generally dense or long enough. Under those conditions, conventional kinetic laws may be applied.[5−9] However, the primary ionization is still in debate and poorly understood. Two actual approaches are considered. The cluster model of Karas et al. considers that ions are largely preformed in the solid matrix.[10,11] During the desorption/ablation process, these preformed ions should be embedded in aggregates of various sizes and should undergo various charge reductions into these clusters leading to molecular ions of lower charge. The second model, the photoexcitation/pooling model, proposes that a combination of the migration of the excitation energy between two neighboring excited molecules in the matrix, followed by the concentration of this energy in pooling events permitting higher energy processes to occur.[12−14] These two models suffer limitations and are still subject to debate. Other possible contributors to the primary ionization in MALDI are also considered but with variable acceptance: e.g., the excited-state proton transfer, polar fluid model, pneumatic assistance, secondary reactions, and matrix ions.[1,15−18] Ions generated by a MALDI source are usually analyzed in a time-of-flight (TOF) analyzer.

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Intact molecular ions are the most abundant ions detected by MALDI. Nevertheless, two types of fragmentations can be distinguished, based on where they occur in the mass spectrometer. (1) In-source decay (ISD)[19,20] is a fragmentation process occurring in the source, rapidly after the laser shot. ISD fragments are therefore detected at the correct m/z in full scan MS mode (either in linear or in reflectron mode). ISD of peptides and proteins generally leads to c- and z-fragment ions, according to Biemann's nomenclature.[21,22] These fragments correspond to the cleavage of the $N-C_\alpha$ bonds on the peptide backbone. y-fragments can be observed with peptides in the MS mode if they are formed before the extraction. However, y-fragments are not necessarily formed by the same mechanism as c- and z-fragments: they can be formed by collision-induced fragmentations in the hot MALDI plume, whereas c- and z-fragments have been proposed to follow chemical activation by hydrogen radical transfer.[23] It has been proposed by Takayama that the $N-C_\alpha$ bond cleavage may occur independently of the ionization process and that whether c-ions or z-ions are formed depends on the charges of amino acid residues on the N-terminal side and C-terminal side of the cleavage, respectively.[24] (2) Postsource decay (PSD)[25] is a metastable decay activated by collisions in the MALDI plume. The fragmentation occurs during the time-of-flight of the ions, in a field-free region. The fragment ions' velocity remains the same as that of the parent ion, and their kinetic energy is therefore reduced. The metastable ions can be detected by varying the reflector potential in a reflectron TOF instrument. PSD generally leads to the formation of b- and y-fragment ions, corresponding to the cleavage of the peptidic bonds, plus neutral losses (principally H_2O and NH_3) and some a-fragment ions.

The main advantage of ISD compared to PSD is that ISD is theoretically not limited in mass and thus permits the elaboration of top-down sequencing approaches (i.e., direct sequencing of intact proteins, without prior digestion into shorter peptides) even for large proteins, as demonstrated already.[26−28] Previous efforts to improve of ISD signals were oriented toward instrument tuning and sample preparation (desalting). The most commonly used matrix for ISD is 2,5-dihydroxybenzoic acid (DHB).[29−31] The behavior of sinapinic acid (SA) is intermediate:[32] it can be used for ISD, but DHB usually gives more intense ISD fragments. The matrix α-cyano-4-hydroxycinnamic acid (CHCA), which is the best matrix for producing PSD fragments or intact molecular ions,[32] is however inefficient for ISD,[24,32] suggesting that ISD and PSD have different origins.[33]

Despite the recent development of detailed models describing MALDI desorption, ablation, and ionization processes,[5,6,8−10,12,14,34−38] the role of the matrix in analyte fragmentation is still incompletely understood.[20,24,33] There is nevertheless accumulation of evidence that ISD is due to hydrogen radical-transfer reactions in the MALDI plume. Observation of ISD fragments in positive and negative ion modes led to the elaboration of radical-based mechanisms, as in Takayama's model.[23,30] This model explains the formation of ISD fragments in the positive ion mode by a hydrogen radical-transfer mechanism, from the matrix to the carbonyl group of the analyte (peptide or protein). The transient analyte radical formed breaks rapidly to give c- and z-fragments. Kocher et al. suggested that ISD cannot be induced by a direct electron capture (as in ECD) but rather by a hydrogen radical transfer.[30] Scott et al. had indeed observed that atomic hydrogens with high velocities are present in the MALDI plume.[39] ISD can be viewed as a "chemically assisted" fragmentation method, the radical ions being more fragile as compared to closed-shell ions.

Starting from this hydrogen radical-transfer model, our strategy was to search new matrixes better suited to transfer hydrogen radicals, first onto test molecules and then on peptides and proteins. We chose spirooxazines because Calba et al. have demonstrated that spirooxazines are observed as $M^{+\bullet}$ in matrix-free laser desorption/ionization and with adducts up to $[M + 3H]^+$ in MALDI using nicotinic acid as matrix, meaning that hydrogen radicals are transferred by nicotinic acid during the ionization step.[40,41] Then, peptides were used to test the ISD ability of matrixes that gave more intense hydrogen adducts on spirooxazines. Excellent correlation between hydrogen-donating properties of the matrix and ISD-enhancing properties were found, demonstrating (1) the validity of the hydrogen radical-transfer model that was proposed for DHB[23] and (2) the validity of our rational design approach for optimizing matrixes enhancing ISD. This approach resulted in the rational selection of a matrix preparation that significantly enhances ISD of peptides and proteins.

EXPERIMENTAL SECTION

Reagents. CHCA, 2,4-dihydroxybenzoic acid (DHB), picolinic acid (PA), SA, 5-chloro-1,3-dihydro-1,3,3-trimethylspiro[2*H*-indole-2,3′-(3*H*)naphth[2,1-*b*](1,4)oxazine], 1,3-dihydro-1,3,3-trimethylspiro-[2*H*-indole-2,3′-(3*H*)naphth[2,1-*b*](1,4)oxazine], formic acid (FA), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Steinheim, Germany). 1,5-Diaminonaphthalene (1,5-DAN) was purchased from Acros. The peptides substance P (1346.7 Da), fibrinopeptide B (1569.7 Da), renin substrate (1757.9 Da), and oxidized insulin β-chain (3493.6 Da) were purchased from Sigma-

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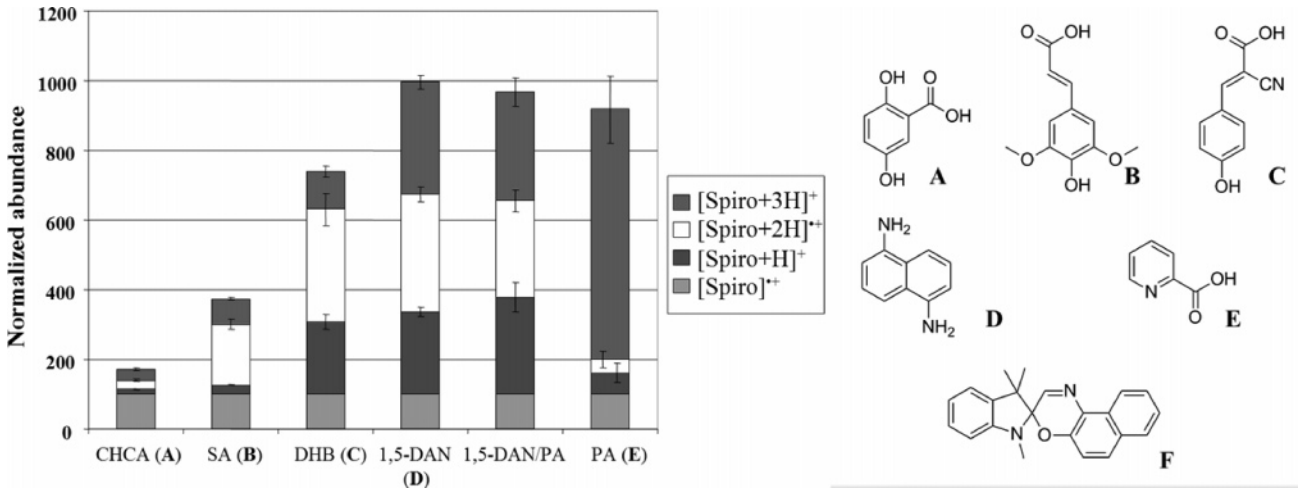

Figure 1. Diagram of hydrogen adducts onto spirooxazine (F, the analyte) for several matrixes. The amounts of hydrogenated spirooxazine species are normalized to the amount of spirooxazine radical cation (100%). (A) 2,5-dihydroxybenzoic acid (DHB); (B) sinapinic acid (SA); (C) α-cyano-4-hydroxycinnamic acid (CHCA); (D) 1,5-diaminonaphthalene (1,5-DAN); (E) picolinic acid (PA) for comparing reductive abilities of these compounds.

Aldrich. Proteins ubiquitin (8.6 kDa), myoglobin (17 kDa), and bovine serum albumin (BSA) (66 kDa) were purchased from Sigma-Aldrich. All the solvents used were HPLC grade quality (purity >99.9%).

Solutions Preparation. DHB solutions were prepared in H_2O/ACN (1/1) at 20 mg/mL. CHCA and SA solutions were prepared in H_2O/ACN (1/1) at saturation (<20 mg/mL). 1,5-DAN solutions were prepared in H_2O (0.1% TFA or 0.1% FA)/ACN (1/1) at saturation (<20 mg/mL). The 1,5-DAN solutions were always prepared shortly before MS experiments because of the instability of 1,5-DAN in acetonitrile.[42] The mixture 1,5-DAN/PA was prepared from a 1,5-DAN solution at saturation (<20 mg/mL) and a PA solution at 20 mg/mL, both in H_2O (0.1% TFA or 0.1% FA)/ACN 50/50 (v/v). The 1,5-DAN and the PA solutions were then mixed in a 75/25 ratio (v:v). Peptides and proteins were prepared in bi-distilled water at concentrations of 10^{-5} and 2×10^{-5} M, respectively. Spirooxazine solutions were prepared in THF at a concentration of 3×10^{-5} M.

Mass Spectrometry. All MALDI mass spectrometry experiments were carried out in the reflectron mode on an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Nd:YAG Smartbeam laser (MLN 202, LTB) and controlled by the Flexcontrol 2.4 software package. Each spot was prepared using the dried droplet method. One droplet of 0.75 μL of analyte solution and one droplet of 0.75 μL of matrix solution were deposited on the MALDI target and allowed to dry at ambient temperature or by heating (for DHB), leading to the crystallization of the sample. The exact same sample preparation was used for positive and negative ion mode experiments. For spirooxazines, only 0.25 μL of analyte solutions was deposited to limit the solvent effect of the spirooxazine solutions on the crystallization of the different matrixes.

Spectra are the sum of 2500 laser shots for reISD spectra and 1000 laser shots for spirooxazine spectra. For each matrix or matrix mixture used, the fluence used was 10% above threshold for the acquisition of ISD spectra. For comparison, the same laser fluence was used in the two ion modes and for spirooxazine experiments. Experiments were repeated at least 6 times on different fresh sample spots. The spectra of 2500 or 1000 laser shots are the sum of 100 shots on different places on the spot. For 1,5-DAN matrix-based preparations, only the needle-shaped crystals were used for the mass spectral acquisition.

Data Analysis Software. Biotools 3.0 (Bruker Daltonics) was used here both for fragment ion assignement (knowing the sequence) and for top-down sequencing (assuming the sequence is not known). For de novo approaches, the software assists the researcher in the following way. Interesting fragments are manually picked and the software determines, from mass differences between all selected peaks and given a tolerance threshold, several probable sequence tags of various lengths. Calculated tags have an error score that helps choosing the most relevant ones. The error score is calculated based on deviations from the theoretical mass differences (amino acid residues). Next, a sequence homology research (BLASTp) is realized with the determined tags in a protein database (Swissprot + TrEMBL) (http://www.expasy.ch/tools/blast/) by entering sequence tags determined (one tag by search line).

RESULTS AND DISCUSSION

Hydrogen Radical-Transfer Abilities. Starting from Takayama's model, potential ISD enhancing matrixes were selected by studying their abilities to form hydrogen adducts onto analytes. For this purpose, spirooxazines (F in Figure 1) were used as test molecules because they possess well-known hydrogen scavenging properties.[40,41] Recently, Tanaka's group has used a novel MALDI matrix, 1,5-DAN, for reducing intra- and intermolecular disulfide bonds of proteins.[43] The different isomers of the diaminonaphthalene are able to reduce the two intermolecular bonds of the two chains composing the bovine insulin plus the intramolecular disulfide bond of the chain α. They showed that the 1,5 isomer of diaminonaphthalene is the most reductive. They also observed that DHB is unable to realize such disulfide bond reductions in the same experimental conditions, and as a consequence, 1,5-DAN

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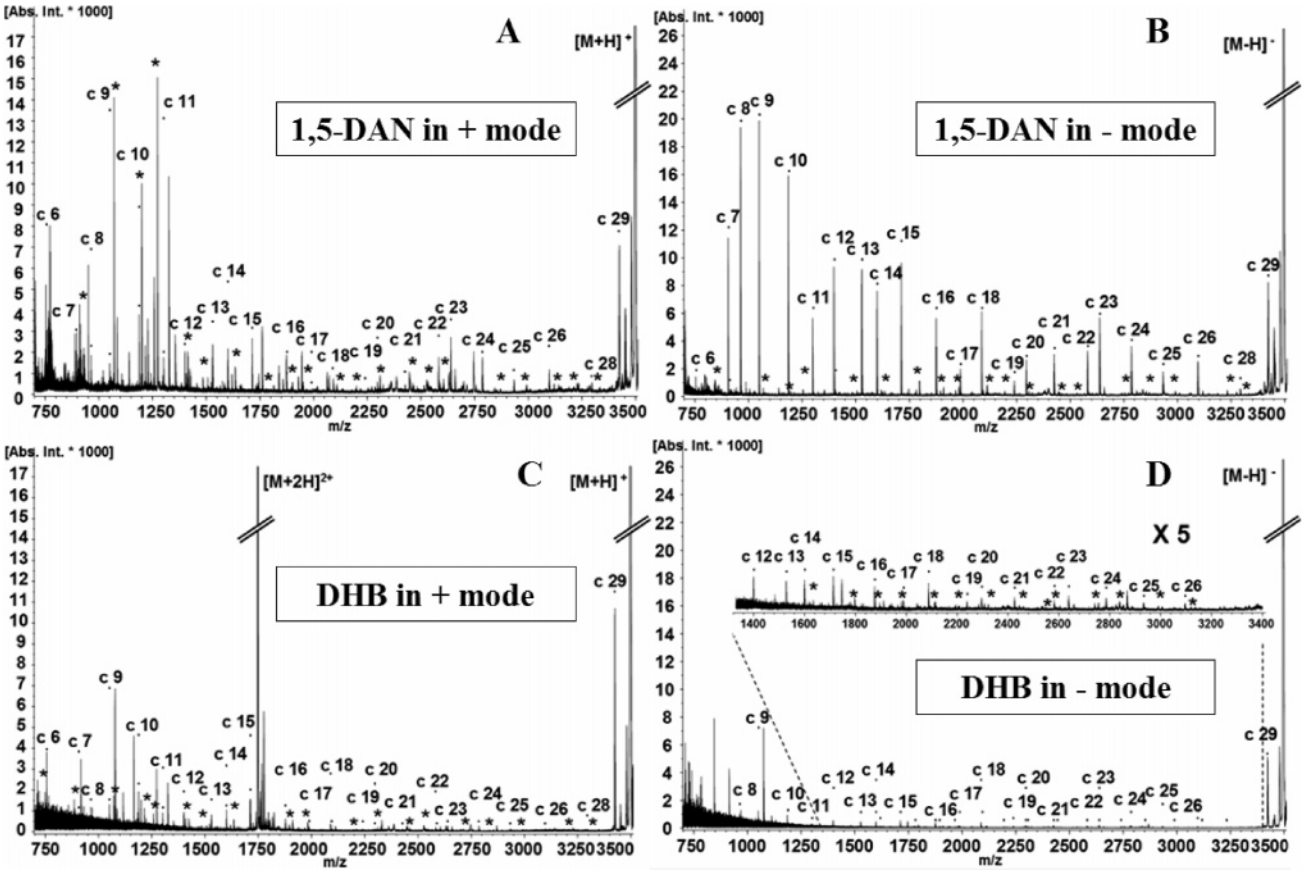

Figure 2. reISD spectra of oxidized insulin β-chain (A) in DHB and positive ion mode, (B) in DHB and negative ion mode, (C) in 1,5-DAN and positive ion mode, and (D) in 1,5-DAN and negative ion mode. 1,5-DAN matrix was prepared in H_2O (0.1% TFA)/ACN (1/1). z-Fragments are indicated by an asterisk.

might be able to induce more hydrogen transfer than DHB; it was tested with spirooxazine to confirm this hypothesis. Other matrixes have already been reported for the study of small analytes only. But even if such matrixes cannot be used alone in peptides and proteins analysis, they could still be useful as matrix additives. Hydrogen radical-transfer (hydrogen-donating) abilities of other potential ISD matrixes or additives such as PA, nicotinic acid (NA), 1,2,4-benzenetriol, or hydroquinone were therefore evaluated with the spirooxazines. Hydrogen-donating abilities of 1,5-DAN, PA, and three other standard matrixes (CHCA, DHB, SA) to the spirooxazine (F in Figure 1) have been compared. A second spirooxazine was used to confirm that the hydrogen adduct patterns depend on the matrix and not on the particular analytes (data not shown). After taking into account the isotopic distributions of the different spirooxazine species ($M^{+\bullet}$, $[M + nH]^+$, $n = 1-3$), the relative amounts of the different adduct species were calculated, and the results are shown in Figure 1. Matrix ranking, from the least to the most able to transfer hydrogen radicals to spirooxazines, is as follows: CHCA < SA < DHB < 1,5-DAN < PA. Among the three standard matrixes, DHB (C in Figure 1) gives the most hydrogen adducts onto spirooxazine, and CHCA (A in Figure 1) induces the less adduct formation. Interestingly, the hydrogen radical-transfer abilities of 1,5-DAN and of PA (respectively, D and E in Figure 1) are superior to that of DHB. The difference between DHB, 1,5-DAN, and PA is observed on the $[Spiro + 3H]^+$ species. 1,5-DAN induces more $[Spiro + 3H]^+$ than DHB, and with the sum of all hydrogenated species, 1,5-DAN is a better hydrogen-donating matrix than DHB. PA induces essentially the $[Spiro + 3H]^+$ species indicating that PA gives more easily the more hydrogenated species than the other matrixes and therefore possesses the best hydrogen-donating capacity. According to Takayama's model, the ISD yield should follow the same trend, and PA should be the best ISD matrix of them all, but PA cannot be used as a pure matrix for peptides because no ionization is observed. However, PA might still be interesting as a matrix additive to further enhance the hydrogen-donating capacity and, therefore, the ISD ability, or to help the ionization during the MALDI process of a peptide-ionizing matrix (e.g., DHB or 1,5-DAN). In conclusion, if hydrogen radical transfer is at the origin of ISD, our simple spirooxazine test allows predicting that 1,5-DAN and PA will induce more ISD fragmentation than DHB. According to Figure 1 and to the fact that PA cannot be used alone, 1,5-DAN and 1,5-DAN/PA should be the most ISD efficient matrixes used in this paper. Spectra of peptides and proteins were acquired in the different matrixes to confirm this hypothesis.

ISD of Oxidized Insulin β-Chain Using 1,5-DAN and 1,5-DAN/PA. First tests were performed on oxidized insulin β-chain (cysteine oxidized in cysteic acid). This was chosen as a model peptide because it has been often used in the past for ISD fragmentation studies using DHB.[20,44] Our new matrix preparations can therefore be easily compared with the typical ISD matrix DHB. As shown in Figure 2, 1,5-DAN produces more intense ISD

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Table 1. Ratio of ISD Fragments Total Area Relative to the Parent Ion Area, in % of Total Peak Area[a]

	ion mode	DHB	1,5-DAN	1,5-DAN/PA (75/25)
insulin β-chain, oxidized [FVNQH LC_{ox}GSH LVEAL YLVC_{ox}G ERGFF YTPKA]	positive	2% ± 0.2	12.8% ± 1.4	8.8% ± 1.8
		($n_{c,z}$ = 43)	($n_{c,z}$ = 44)	($n_{c,z}$ = 44)
	negative	1.6% ± 0.2	16.3% ± 1.5	17.5% ± 1.4
		($n_{c,z}$ =36)	($n_{c,z}$ = 44)	($n_{c,z}$ = 44)
fibrinopeptide B [EGVND NEEGF FSAR]	positive	0.7% ± 0.2	6.6% ± 0.4	5.6% ± 0.3
		($n_{c,z}$ = 14)	($n_{c,z}$ = 11)	($n_{c,z}$ = 11)
	negative	($n_{c,z}$ = 0)	9.5 ± 0.5	11.8% ± 0.8
			($n_{c,z}$ = 14)	($n_{c,z}$ = 14)
substance P [RPKPQ QFFGL M]	positive	0.7% ± 0.1	1.4% ± 0.1	2.7% ± 0.6
		($n_{c,z}$ = 4)	($n_{c,z}$ = 4)	($n_{c,z}$ = 4)
	negative	($n_{c,z}$ = 0)	4.4% ± 0.1	6.3% ± 0.4
			($n_{c,z}$ = 4)	($n_{c,z}$ = 4)
renin substrate [DRVYI HPFHL LVYS]	positive	1.2% ± 0.2	4.9% ± 0.2	5.6% ± 1.1
		($n_{c,z}$ = 12)	($n_{c,z}n_{c,z}$ = 12)	($n_{c,z}$ = 12)
	negative	0.21% ± 0.02	1.1% ± 0.2	5.5% ± 0.9
		($n_{c,z}$ = 6)	($n_{c,z}$ = 4)	($n_{c,z}$ = 14)

[a] Number of c- and z-fragment ion peaks having a signal-to-noise ratio of >3 that were taken into account for the total area calculations are given in parentheses. C_{ox}, oxidized cysteine (cysteic acid). 1,5-DAN and 1,5-DAN/PA matrixes were prepared in H_2O (0.1% FA)/ACN (1/1).

fragments (c- and z-fragments) of oxidized insulin β-chain than DHB in both ion modes, facilitating the assignment and sequencing. The absolute intensity scale used for Figure 2 are the same in positive mode (for DHB and 1,5-DAN) and negative mode, allowing comparison of the absolute intensities observed in the different matrixes. Relative ISD efficiencies (percent of ISD fragments peaks relative to the parent ion peak) of the different matrixes are summarized in Table 1. Analyzing each fragment separately, we noticed that the area of a few particular fragments remains unchanged (e.g., c_{29}), whereas the area of the most ISD fragments is increased in both ion modes when using 1,5-DAN compared to DHB. For some fragments, absolute peak area is increased by a factor of up to 10 in positive ion mode (Figure 2A and C) and by a factor of up to 20 in negative ion mode (Figure 2B and D). In the two matrixes, c- and z-fragments are observed in both ion modes but with different c/z ratios. Takayama's model can explain the ISD fragmentation in both ion modes because ionization and fragmentation are considered independent.[20] But explaining the large increase of ISD fragmentation in 1,5-DAN compared to DHB in the negative ion mode needs supplementary experiments with well-chosen synthetic peptides bearing localized charges. The presence of acidic or basic side chains, the physicochemical behavior of the 1,5-DAN matrix, and the role of additives (acids, bases, including their counterions) are factors to be investigated because each of them could influence the outcome of ISD fragmentation. Integrating fragmentation and ionization in a single model is clearly a challenge for a more thorough understanding of MALDI. Future work will be devoted to better understanding the ISD negative ion formation mechanism(s) using model peptides.

As we saw with spirooxazines, the hydrogen-donating character of PA is even higher than that of 1,5-DAN, and PA or NA (an isomer of PA) alone does not ionize large peptides efficiently. Different mixtures of PA and NA with 1,5-DAN were used to test whether even more effective ISD fragmentation than in pure 1,5-DAN could be obtained. The total area of c- and z-fragments relative to the parent ion is reported in Table 1 for the different matrix preparations in both ion modes. The results show that the efficiency of ISD fragmentation of oxidized insulin β-chain is increased in 1,5-DAN and 1,5-DAN/PA (75/25 in v/v) compared to DHB. This is in line with the increased hydrogen-donating abilities that were determined with spirooxazines (Figure 1). 1,5-DAN and 1,5-DAN/PA possess quite similar hydrogen-donating capacities. 1,5-DAN matrix-based preparations possess lower fluence thresholds than DHB, proving that the laser fluence is not at the origin of the increase of ISD efficiencies in 1,5-DAN matrix-based preparations. By using 1,5-DAN with PA, higher ISD fragments are observed in the negative ion mode compared to pure 1,5-DAN but not in the positive ion mode as shown in Table 1. As expected, the use of NA as additive to 1,5-DAN shows results similar to 1,5-DAN/PA (data not shown). Comparisons were made between the reflector mode and the linear mode. The same result was obtained: 1,5-DAN and 1,5-DAN/PA are more ISD efficient than DHB. In the reflector mode, the parent ions are the molecular ions that survived at least until they reach the reflector. The fragments are well resolved if they are formed before the ion extraction and survive at least until they reach the reflector. Interestingly, in linear mode, the proportions of ISD fragments were found superior to those of the reflector mode (data not shown) suggesting that a part of the ISD fragments are metastable and dissociate in the flight tube.

1,5-DAN/PA matrix preparation (Figure 3B) produces larger and tougher crystals than 1,5-DAN. The thin needle-shaped 1,5-DAN crystals (Figure 3A) are easily moved by the laser shots, and this leads to the loss of signal after a few successive shots at one location. 1,5-DAN/PA matrix preparation therefore greatly facilitates mass spectral acquisition, making it easy to find a needle-shaped crystal producing intense parent and fragment ion peaks. DHB crystals are shown in Figure 3C for comparison.

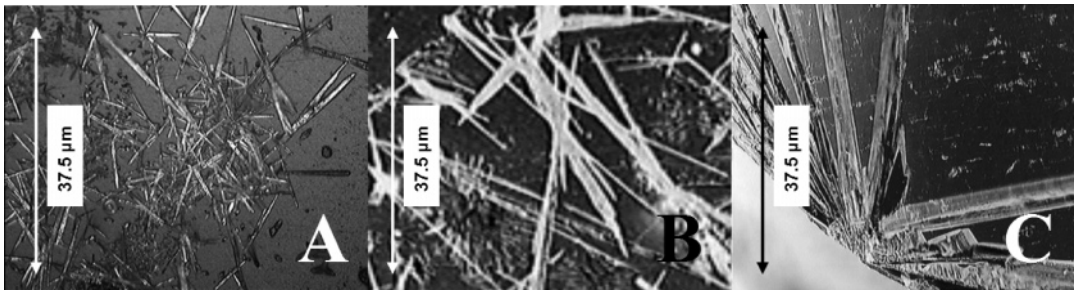

Figure 3. (A) 1,5-DAN crystals (200×), (B) 1,5-DAN/PA crystals (200×), and (C) DHB crystals (200×).

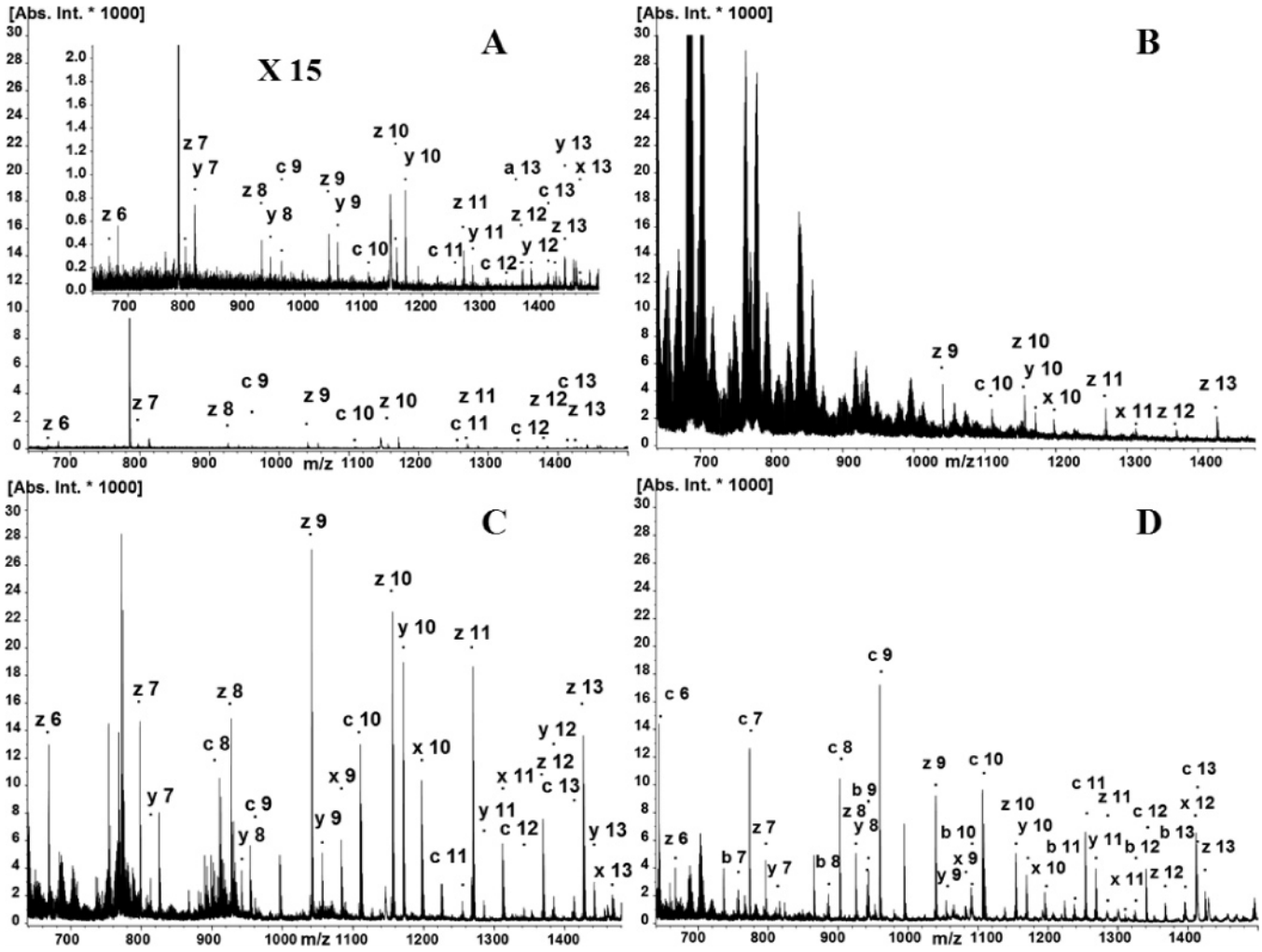

Figure 4. reISD spectra of fibrinopeptide B in positive ion mode in DHB (A), in 1,5-DAN (B), in 1,5-DAN/PA 75/25 (C), and in negative ion mode in 1,5-DAN/PA (D).

ISD of Other Short Peptides Using 1,5-DAN and 1,5-DAN/PA. Next, several other peptides were used to test the ISD ability and applicability of 1,5-DAN and 1,5-DAN/PA matrix preparations.

For small peptides, a large influence of the acid (FA or TFA) used in the matrix solutions preparations was observed. When TFA was used, ISD spectra of peptides could be obtained only in the positive ion mode for 1,5-DAN, but in both ion modes for 1,5-DAN/PA, as can be seen in the Figure 4. The absolute intensity scale used for this figure is the same for the four spectra, permitting comparison of the absolute intensities observed in the different matrixes. In this figure are represented the typical spectra obtained with fibrinopeptide B in the positive ion mode in DHB (Figure 4A), in 1,5-DAN (Figure 4B), and in 1,5-DAN/PA (Figure 4C) prepared in H_2O (0.1% TFA)/ACN (1/1), c- and z-fragment ion intensities increase in the latter preparation. Intense ISD fragments are also observed in the negative ion mode in 1,5-DAN/PA (Figure 4D).

When FA was used for the matrix solution preparations, more reproducible results in 1,5-DAN and 1,5-DAN/PA were obtained in both ion modes. ISD spectra obtained with H_2O (0.1% FA)/ACN (1/1) matrix solution preparations are highly similar (spectra not shown) to those obtained with 1,5-DAN/PA prepared in H_2O (0.1% TFA)/ACN (1/1) in Figure 4C and D. This result supports the suggested hypothesis that the acids or additives (as well as their anions and counterions) used for MALDI matrix preparations

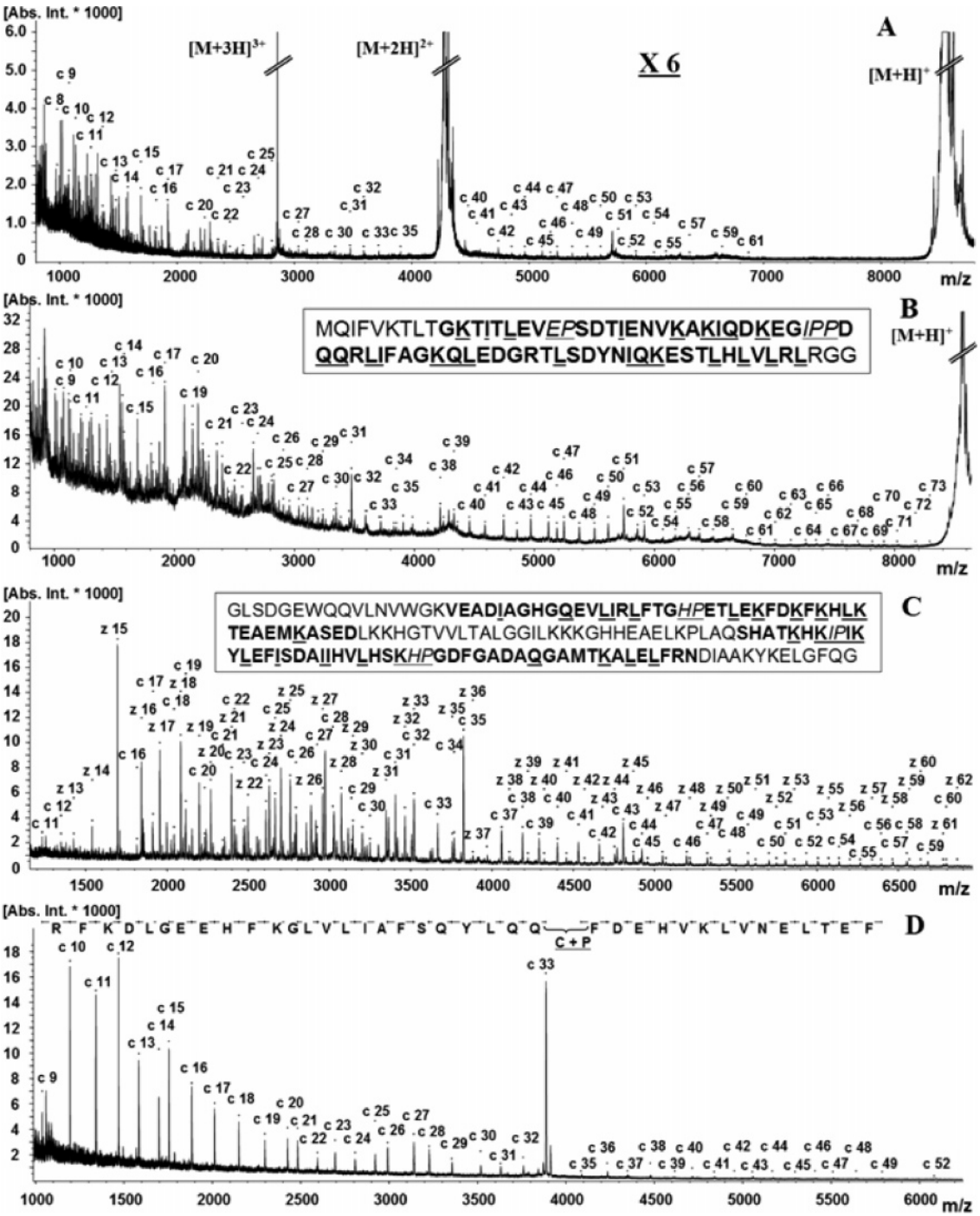

Figure 5. reISD spectra in the positive ion mode of ubiquitin in DHB (A) and in 1,5-DAN/PA (B), and of myoglobin (C) and BSA in 1,5-DAN/PA (D). In the boxes (B, C), complete sequences of ubiquitin and myoglobin are indicated, boldface amino acids are determined except isobaric amino acids (boldface underlined) and italic underlined amino acids are determined by mass difference calculations (proline residue).

can be influent on MALDI results. To compare the ISD efficiencies (percent of ISD fragments peaks relative to the parent ion peak) of the different matrix preparations in both ion modes, FA was therefore used for all matrixes and their ISD efficiencies are indicated in Table 1.

When the 1,5-DAN matrix-based preparations were prepared in H_2O (0.1% TFA)/ACN (1/1), large differences were observed in the presence or absence of PA. However, PA could have a role different from enhancing the hydrogen-donating capacity of the matrix mixture as suggested above and by Figure 1. PA keeps the hydrogen-donating capacity of the mixture similar to pure 1,5-DAN, but PA could influence the ionization process as well as TFA and FA do. These observations suggest that the acids and the additives used could have an impact, by their acidity, the proton affinity of their anions or counterions, onto the ionization or the fragmentation process, by reactions with the matrix allowing reactions in the gas phase, or by denaturing of the analyte, leading to different MALDI results.

In DHB, ISD fragments are present but with very low relative intensities, whereas enhanced intensities are observed in 1,5-DAN and in 1,5-DAN/PA prepared with FA for all peptides used as shown in Table 1.

In 1,5-DAN matrix-based preparations (FA), the sequence coverage is almost complete for all peptides with the use of c- and z-fragments, except for substance P (55%), which is the smallest peptide analyzed (1346.7 Da or 11 amino acids). In fact, detecting fragments under 1000 Da is difficult because of the presence of an intense background at low m/z due to matrix cluster ion peaks (see Figure 4C and D) that sets the limit below which ISD sequencing becomes impractical. Nevertheless, other types of fragments are often observed (y, a, x, b, and internal fragments) with short peptides. For example, intense x-fragments are observed for fibrinopeptide B as shown in Figure 4C and D. The multiple extra fragments are helpful to confirm or complete a sequence obtained with the c- and z-fragments. By using y-fragments in addition, complete sequence coverage is obtained for all studied peptides except for substance P (82%). Moreover, for short peptides, PSD fragmentation remains a useful MALDI application that can complete the mass range for peptides sequencing.

The relative ISD fragmentation efficiencies are summarized in Table 1. The ratios of total ISD fragments area relative to the molecular ion area are indicated in percent with mean errors. In parentheses are indicated the number of c- and z-fragments observed.

Application of the New Matrix to Top-Down Protein Analysis by ISD. Three proteins (ubiquitin, 8.6 kDa; myoglobin, 17 kDa; BSA, 66 kDa) were used to test the applicability of 1,5-DAN matrix-based preparations in a top-down approach and compare the results to those obtained in the classical ISD matrix DHB. For proteins, only positive ISD spectra can be obtained in DHB as well as in 1,5-DAN matrix-based preparations. We have chosen to use the 1,5-DAN/PA mixture as matrix because of its larger and tougher needle-shaped crystals facilitating mass spectral acquisition. As shown in Figure 5B, ISD fragment ion intensity for ubiquitin (8.6 kDa) is highly superior in 1,5-DAN/PA compared to DHB (Figure 5A). The long sequence tag of 65 consecutive c-ions obtained in 1,5-DAN/PA covers 84% of the ubiquitin sequence (Figure 5B). For myoglobin (Figure 5C), both c- and z-ion fragments are observed until ~8 kDa. c-ions become more intense than z-ions at m/z >3500 Da. In the case of BSA (Figure 5D), only c-ions are observed, giving a sequence tag of 40 consecutive amino acids (c_9-c_{49}). Thus, fragment ion assignment and sequence determination become simpler as the mass of the intact protein increases.

It is important to note that isobaric amino acids cannot be distinguished in MALDI-TOF−MS. The resolution is not sufficient to differentiate lysine (K) and glutamine (Q) residues with masses, respectively, of 128.095 and 128.059 Da as well as leucine (L) and isoleucine (I) residues (113.084 Da). Also, as in ECD and ETD, the presence of proline induces gaps in c- and z-fragment ion series because of its cyclic structure.[45,46] The $N-C_\alpha$ bond of proline is cleaved but the $-(CH_2)_3-$ link keeps the proline residue and the next amino acid on the N-side bonded. Because of the high sequence coverage offered by ISD fragment ion series in 1,5-DAN/PA, a gap in the sequence can be confidently interpreted as indicative of a proline residue. The missing amino acid can therefore be determined by measuring the mass difference between the c_{j-1} and the c_{j+1} ions, subtracting the mass contribution of the proline. In Figure 5D, the ISD spectrum of BSA in 1,5-DAN/PA clearly shows a gap in the c-type ions series (lack of c_{34}). By measuring mass difference between c_{33} and c_{35}, the $C_{33}-P_{34}$ tag is easily determined. There are other examples with ubiquitin and myoglobin that are represented in the sequence boxes, in Figure 5B and C, respectively, by italic underlined amino acid residues. This illustrates the easiness of de novo sequencing by ISD. We recently illustrated the use of 1,5-DAN in a LC-MALDI approach for the determination of toxins from crude venom and in the ISD top-down sequencing of snake cardiotoxins (>6500 Da).[47]

In cases where only protein identification is requested, identification by large ISD tags obtained from the intact proteins is easy and rapid (Figure 4C and D). For the three proteins studied here, c-tags, z-tags, or both obtained possessed sufficient unambiguous information (nonisobaric amino acids) to retrieve the protein identity by a BLASTp in SwissProt + TrEMBL protein database with E values as low as 5×10^{-23} or 5×10^{-41}. The E value corresponds to the theoretical probability that an alignment with a higher score of similarity occurs randomly (the lower is better). The score is a function of the length of the tag (number of amino acids) and of the similarities between consecutive amino acids of the tag with those of proteins from databases (the higher is better). With the scoring scheme used here, fewer ambiguous amino acids (including isobaric ones) result in a higher score for a given sequence tag length. Our new matrix preparation therefore increases capacities of top-down protein identification by in-source decay approaches.

CONCLUSIONS

The correlation of hydrogen-donating abilities of the matrix preparations measured on spirooxazines with their ISD efficiency further supports that hydrogen radical transfer from the matrix to the analyte is at the origin of the increase of the ISD fragmentation process. This correlation helps rationalizing the choice of a matrix for ISD. The optimal ISD matrix preparations found during this work are the 1,5-DAN and its mixture with PA. These matrix preparations, by their high hydrogen-donating capacities, favor the "chemically" assisted ISD leading to an improvement of c- and z-fragments that greatly facilitates the use of ISD in top-down approaches, as demonstrated for the sequencing of short peptides (1 kDa) to large proteins (66 kDa).

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