International Journal of Mass Spectrometry 281 (2009) 82–88

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

The combination of simple MALDI matrices for the improvement of intact glycoproteins and glycans analysis

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ARTICLE INFO

Article history:
Received 11 November 2008
Received in revised form 20 December 2008
Accepted 22 December 2008
Available online 31 December 2008

Keywords:
MALDI-TOF mass spectrometry
Binary matrices
Glycoprotein
Ribonuclease B
Free glycan

ABSTRACT

The choice of matrix has fundamental importance in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of glycoproteins. Therefore, a set of selected matrices has been tested. The attention was focused on new binary matrices, that were a combination of matrices commonly used for carbohydrates or proteins analysis such as 2,5-dihydroxybenzoic acid (2,5-DHB), 2,5-dihydroxyacetophenone (DHAP), 2,4,6-trihydroxyacetophenone (THAP), α-cyano-4-hydroxycinnamic acid (CHCA), and sinapinic acid (SA).

The binary matrices 2,5-DHB/CHCA and 2,5-DHB/SA have been proved the mass spectra with the best quality and showed an universal applicability. Application of these matrices enabled to quickly screen the microheterogeneity of glycan moieties for either intact glycoproteins, or a mixture of deglycosylated glycoproteins together with free, underivatized glycans. Moreover, these binary matrices were more tolerant to the presence of salts in studied samples. Our results suggest that a combination of two matrix compounds could be useful for an improved determination of the molecular mass values of analytes coming from complex biological samples, especially for the structural characterization of glycoproteins of pharmaceutical interest.

1. Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is an analytical technique widely used for different proteomic studies (plant proteomics [e.g., [1–5]], cancer proteomics [e.g., [6–8]], drug proteomics [e.g., [9]], *etc.*) and for the characterization of post-translational modifications of proteins. Glycosylation and glycation are very common forms of post-translational modifications that have been detected in both biological and synthetic samples. Special attention has been focused on the analysis of glycoproteins and their glycans, because they play many important roles in living organisms (intracellular transport, biological recognition, formation of organs, fertilization, infections, tumour formation, *etc.*) [10].

Despite of increasing knowledge about MALDI-TOF-MS and application of this technique for the analysis of proteins and glycoproteins, an optimization of the experimental conditions is still required [11]. Especially the search of new matrix compounds is an active area in MALDI-MS. Generally, MALDI matrices must fulfil several requirements: (a) be able to embed and isolate analytes (e.g., by co-crystallization), (b) be soluble in solvents compatible with analyte, (c) be vacuum stable, (d) absorb the laser wavelength, (e) cause co-desorption of analyte upon laser irradiation, (f) promote analyte ionization [12]. Ionization mechanism depends on the physico-chemical properties of both matrix and sample and on sample-matrix crystals [11,13].

The matrices used for glycoproteins, forming the bridge between carbohydrates and proteins, have to be chosen carefully according to the molecular mass of the investigated glycoprotein. In fact, due to resolution of adducts, fragment ions, salts, and other components such as glycoforms, the problems with the determination of accurate molecular masses arise for larger glycoproteins. The correct choice of the matrix may reduce the production of these unresolved components. Generally, 2,5-dihydroxybenzoic acid (2,5-DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) ionize glycopeptides with masses below 5000 Da. For intact glycoproteins (above limit about of MW > 5000 Da), it is more appropriate to use sinapinic acid (SA), 2-(4-hydroxyphenylazo)benzoic acid (HABA), 2,5-dihydroxyacetophenone (DHAP), or 2,4,6-trihydroxyacetophenone (THAP) [14–17]. Nevertheless, Keller and Liang used CHCA as matrix for low nanomolar concentration of proteins and observed better results than the ones obtained using 2,5-DHB, HABA, or SA [18].

An application of binary matrices or different co-matrices is an alternative way to improve the matrix abilities and the quality of mass spectra. The co-matrices usually containing 2,5-DHB, are com-

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doi:10.1016/j.ijms.2008.12.017

Table 1
Preparation of matrices.

Matrix	Abbreviation	Concentration [mg/mL]	Solvent
2,5-Dihydroxybenzoic acid	2,5-DHB	15	Deionized water
2,4,6-Trihydroxyacetophenone	THAP	100	Acetone
α-Cyano-4-hydroxycinnamic acid	CHCA	Saturated	Acetone
2,4-Dihydroxyacetophenone	DHAP	50	Acetonitrile: 0.1% trifluoroacetic acid (1:1, v/v)
Sinapinic acid	SA	20	Acetonitrile: 0.1% trifluoroacetic acid (7:3, v/v)
2,5-DHB/THAP	Mixture 1:1 (v/v)		
2,5-DHB/CHCA			
2,5-DHB/DHAP			
2,5-DHB/SA			
THAP/CHCA			
THAP/SA			

monly applied to analyse free carbohydrates [e.g., [19–27]]. Karas et al. improved the resolution of mass spectra of carbohydrates and glycoproteins using a mixture of 2,5-DHB and 2-hydroxy-5-methoxybenzoic acid [22]. The addition of 1-hydroxyisoquinolin to 2,5-DHB improved the toleration of mass spectrometer to presence of buffers and other contaminants [19]. 2,5-DHB with glycerol produced a homogenous MALDI sample especially for the analysis of compounds that need to be stabilized in glycerol [25]. A mixture of THAP and diammonium citrate was found to overcome the suppression of phosphorylated peptides by nonphosphorylated ones [28]. Spermine or α-L(−)-fucose combined with 2,5-DHB created other co-matrices [29,30]. The binary matrix CHCA/2,5-DHB was successfully used to improve of the MALDI-TOF-MS results of both peptide mass mapping and intact glycoproteins [23]. Zhong and Lin found that mixture of two conventional MALDI matrices (CHCA and 9-aminoacridine) provided less background in detection of small molecules [31].

We report here the results of an application of several binary MALDI matrices for the characterization of intact glycoproteins as well as mixture of proteins and glycans obtained after deglycosylation. The mixtures of matrices have been tested using ribonuclease B (RNase B) that is a glycoprotein commonly used as a standard.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased in Sigma (St. Louis, MO, USA) or Fluka (Buchs, Switzerland) Corporations. Solvents were bought from Riedel de Haehn (Seelze, Germany) or Fluka.

2.2. Standard glycoprotein

A water solution of bovine pancreatic RNase B (Sigma; 0.5–5.0 mg/mL) was used to optimize MALDI-TOF-MS experimental conditions during intact glycoprotein analysis.

2.3. In-solution deglycosylation of RNase B

500 μL of reduced RNase B (1 mg/mL) was prepared by adding 50 μL reduction solution (50 mmol/L dithiothreitol) to 500 μL of glycoprotein solution [5×10^{-4} g of RNase B in 450 μL NH_4HCO_3 (20 mmol/L; pH 8)] followed by heating to 56 °C for 45 min. After cooling, reduced RNase B was deglycosylated with Peptide-*N*-glycosidase *F* (PNGase *F*; *Flavobacterium meningosepticum*, Sigma; 2 U) at 37 °C, overnight. Control samples were prepared in the absence of PNGase *F*. The mixture obtained after overnight deglycosylation was analyzed by MS without any further treatment.

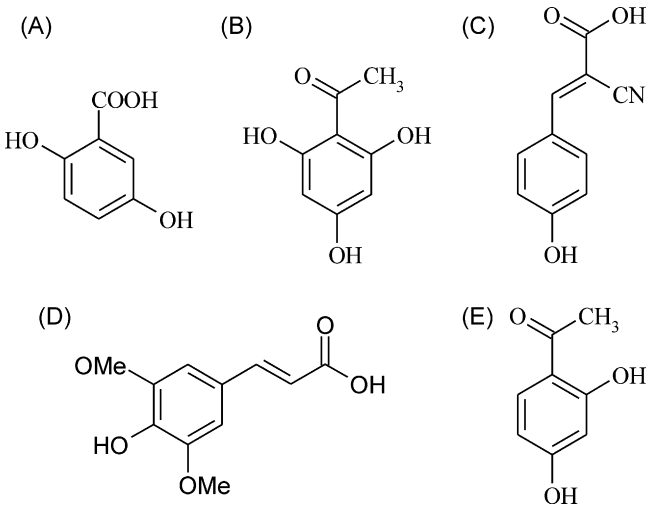

Fig. 1. Structural formulas of used MALDI matrices: (A) 2,5-dihydroxybenzoic acid (2,5-DHB), (B) 2,4,6-trihydroxyacetophenone (THAP), (C) α-cyano-4-hydroxycinnamic acid (CHCA), (D) sinapinic acid (SA) and (E) 2,4-dihydroxyacetophenone (DHAP).

2.4. MS analysis

All mass spectra were recorded using MALDI-TOF/TOF 4700 Proteomics Analyzer (Applied Biosystems, Framingham, USA)

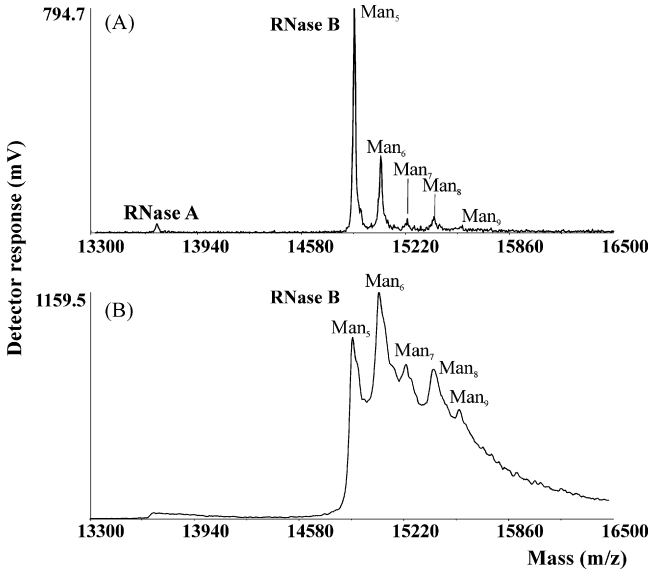

Fig. 2. +LIN MALDI-TOF mass spectra of intact RNase B. The experimental conditions: 0.5 mg/mL of RNase B, matrix THAP with laser power (A) 6000, (B) 7000 relative units were used.

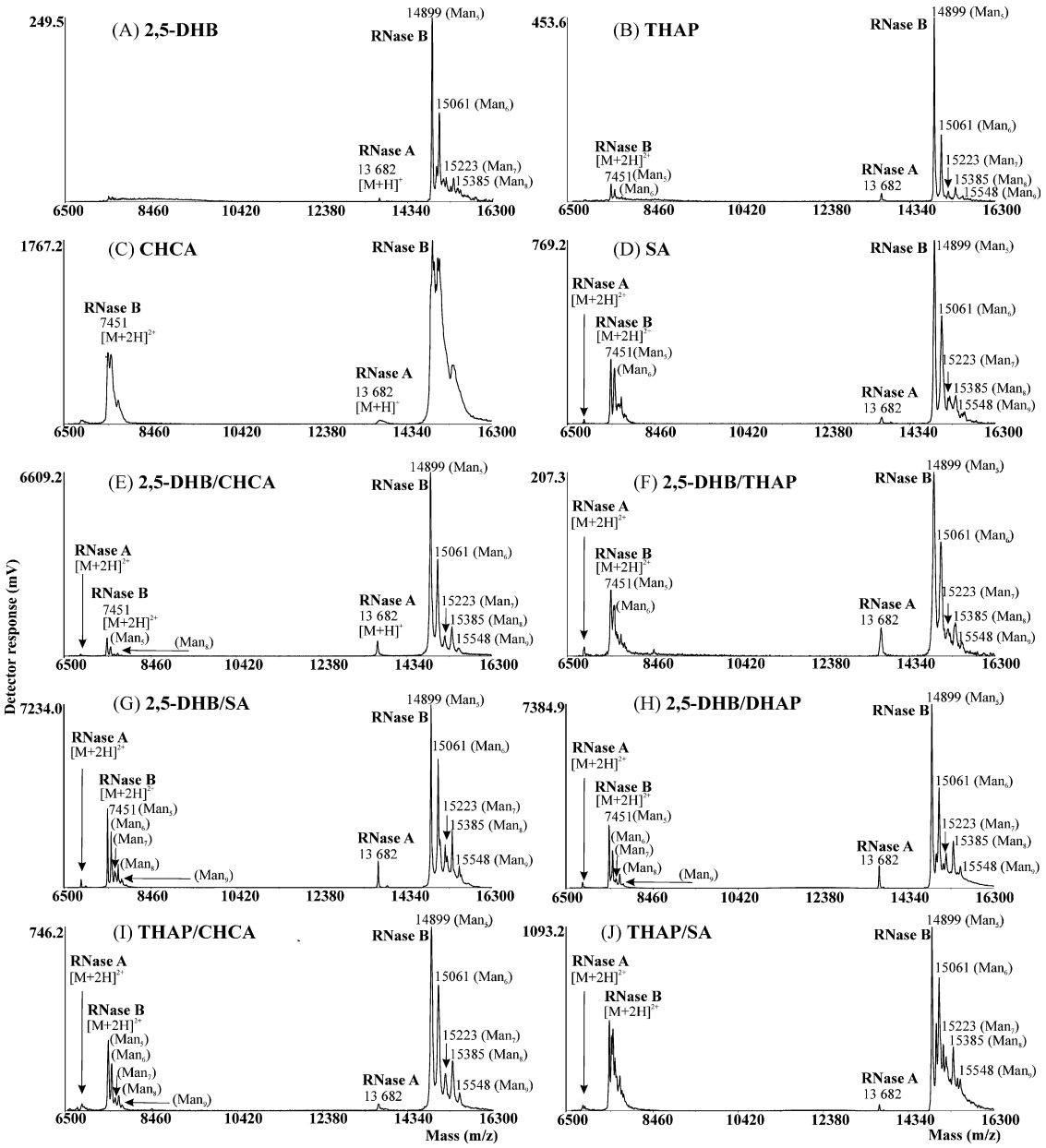

Fig. 3. +LIN MALDI-TOF mass spectra of intact RNase B. The experimental conditions: 0.5 mg/mL of RNase B, matrix: (A) 2,5-DHB, (B) THAP, (C) CHCA, (D) SA, (E) 2,5-DHB/CHCA, (F) 2,5-DHB/THAP, (G) 2,5-DHB/SA, (H) 2,5-DHB/DHAP, (I) THAP/CHCA, (J) THAP/SA, and DD slide preparation technique were used.

equipped with Nd:YAG laser (355 nm). The optimal laser power was selected from the relative scale 0–8800. The ion acceleration voltage was set at 20 kV. The experiments with intact proteins were performed in positive linear (+LIN) mode. The external calibration was carried out using protein mixture (Applied Biosystems) containing horse heart cytochrome C, myoglobin and bovine trypsinogen. The sample solutions (0.4 μL) were deposited on a MALDI plate by dry-droplet method. The preparation of MALDI matrices and their structural formulas are summarized in the Table 1 and Fig. 1.

3. Results and discussion

As was indicated before, the selection of an adequate matrix is fundamental for sample desorption during MALDI-TOF-MS analysis of all kind of analytes. Since glycoproteins consist of peptide and glycan parts, and glycans can be composed by both neutral as well as charged oligosaccharides, the choice of convenient matrix and their complete MS analysis is more complicated. Therefore, we looked for a universal matrix suitable for the analysis of both intact glycoproteins, and mixture of deglycosylated proteins and their released, underivatized glycans. RNase B was selected as a model protein for the demonstration of matrix ability and for the optimization of experimental conditions and instrumental parameters. It is a simple glycoprotein containing single glycosylation site at Asn_{34} binding the high-mannose *N*-glycans. The glycans varies from five to nine mannoses (Man) residues attached to the chitobiose core (=two β-D-*N*-acetylglucosamines–$GlcNAc_2$), so the molecular mass of intact RNase B range is from 14.9 to 15.5 kDa [32–34].

3.1. Application to intact glycoprotein analysis

The compounds 2,5-DHB, CHCA, THAP, DHAP, and SA are "one-component" matrices commonly used for the MS analyses of carbohydrates, proteins, and peptides. Therefore they were selected as criteria for the comparison of abilities of our new binary matrices.

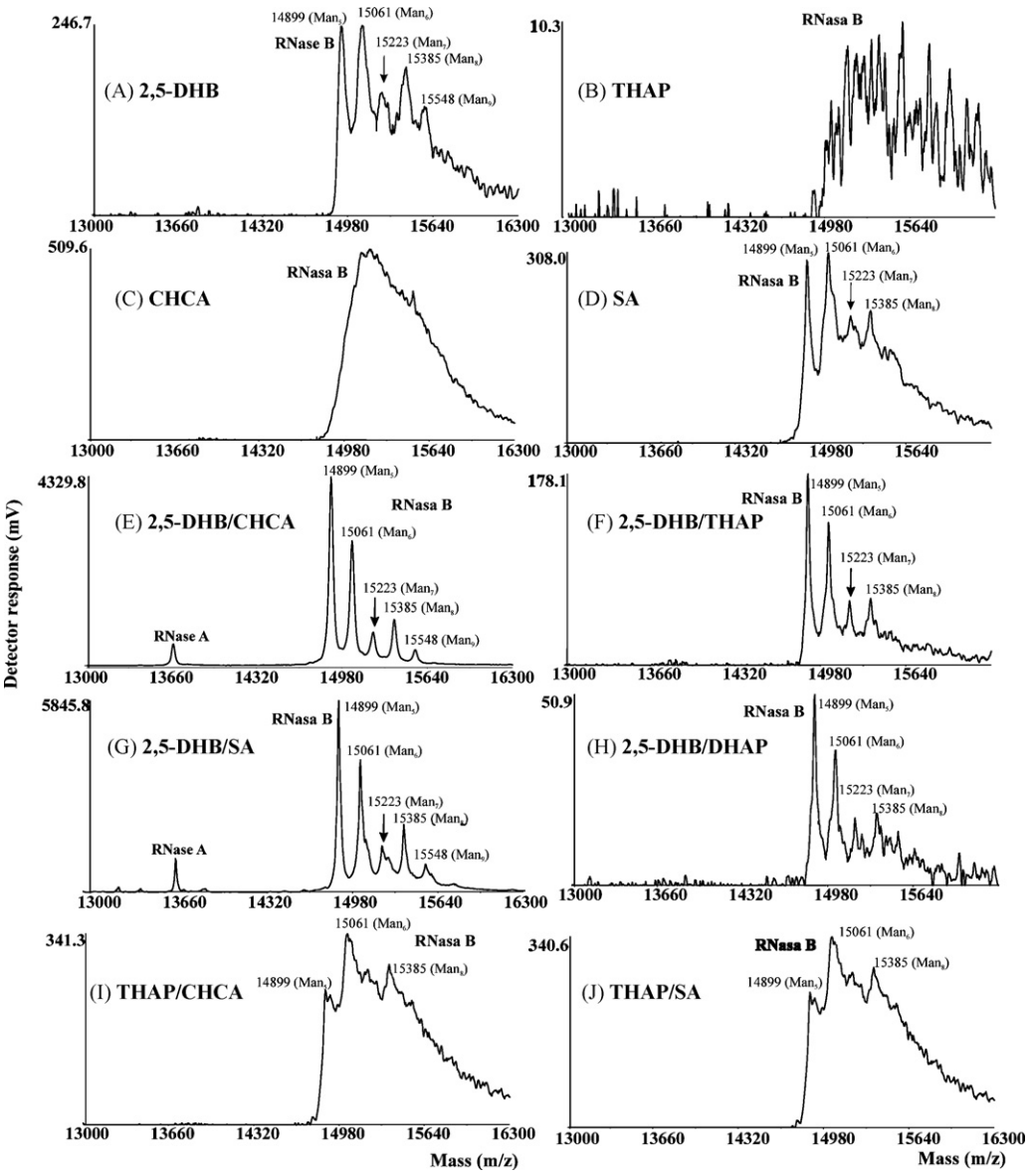

Fig. 4. +LIN MALDI-TOF mass spectra of intact RNase B dissolved in 20 mmol/L NH_4HCO_3. The experimental conditions: 1 mg/mL of RNase B, matrix: (A) 2,5-DHB, (B) THAP, (C) CHCA, (D) SA, (E) 2,5-DHB/CHCA, (F) 2,5-DHB/THAP, (G) 2,5-DHB/SA, (H) 2,5-DHB/DHAP, (I) THAP/CHCA, (J) THAP/SA, and DD slide preparation technique were used.

The mass spectra were acquired with matrices prepared according to the procedure published previously [23,35] and summarized in the Table 1. An optimization of sample concentration was accomplished to obtain the best mass spectra. An adequate concentration of RNase B for intact protein analysis was determined at 0.5 mg/mL. Except DHAP, all matrices resulted in satisfactory protein signals for this concentration of RNase B. DHAP did not provide any mass spectra, until the protein concentration was 3 mg/mL or more, therefore this matrix was not used for this study.

It is well known that mass spectra quality also depends on the laser power [35]. An example of biased laser power is shown in Fig. 2, where THAP was used as a matrix. The broadening of peaks was caused presumably by non-differentiated adducts (Fig. 2B) and this impeded the determination of accurate molecular masses of RNase B glycoforms. The dependence of mass spectra quality on the increasing laser power had the same tendency for all matrices, but the optimal laser intensity had to be carefully chosen especially for binary ones.

The quality of mass spectra was evaluated in respect of signal to noise ratio, intensity of peaks and the resolution of individual glycoforms. A good quality mass spectra should present five sharp signals that come from five glycated forms of RNase B, separated by 162 Da representing addition of Man unit.

All matrices provided mass spectra with signals of pseudo molecular ions $[M+H]^+$ (M represents molecule of protein) at m/z about 14,900–15,548 and doubly charged ions $[M+2H]^{2+}$ at m/z about 7450–7856 corresponding to intact RNase B glycoforms with good signal:noise ratio. In addition, low abundance signal of ion $[M+H]^+$ at m/z 13,682, corresponding to RNase A (non-glycosylated form of RNase with an identical amino acid sequence) was also observed in all mass spectra. RNase A is present as a contaminant, in variable amounts, in commercial samples of RNase B. The mass difference between RNase A and RNase B is about 1217 Da, which corresponds to $GlcNAc_2Man_5$ glycan.

We observed that one-component matrices differed in their ability to produce signals of the individual glycoforms (Fig. 3A–D). Although it was published [15,17] that RNase B could be resolved with THAP or CHCA, but only difficultly with SA, we gained acceptable results using THAP and SA (Fig. 3B and D). The matrices 2,5-DHB (Fig. 3A) and CHCA (Fig. 3C) resulted in mass spectra with

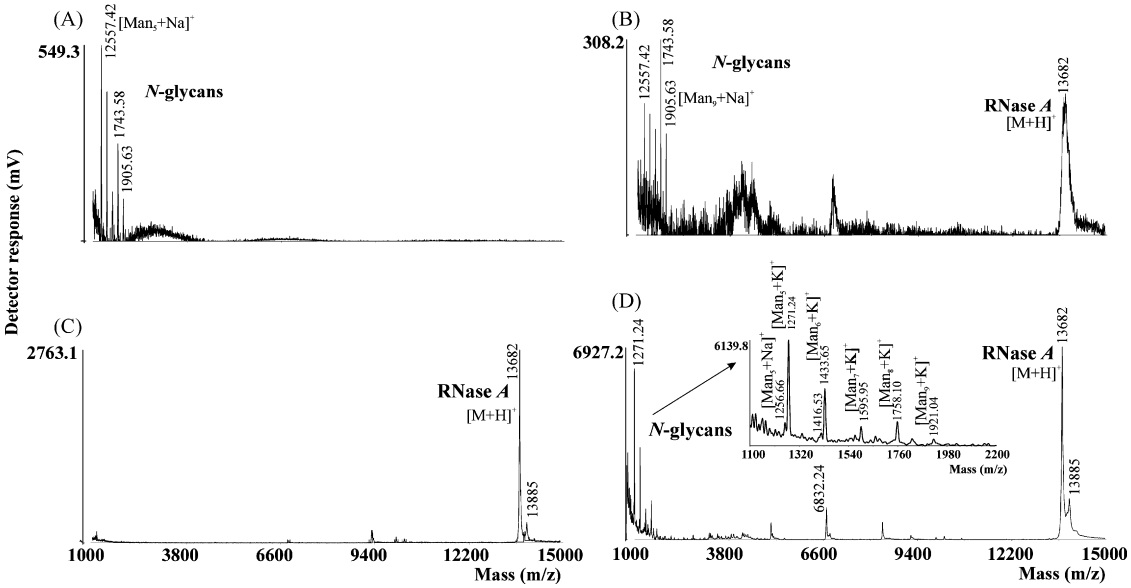

Fig. 5. +LIN MALDI-TOF mass spectra of the reaction mixture of deglycosylated RNase B and its released *N*-glycans obtained after overnight deglycosylation with matrices (A) 2,5-DHB; (B) 2,5-DHB/CHCA; (C) SA; (D) 2,5-DHB/SA. Man_{5-9} represents molecules of glycans containing from five to nine Man residues attached to the chitobiose core.

unsatisfactory resolution or non-resolved signals of adduct ions and glycoforms, respectively. All five glycoforms, separated by 162 Da (Man), were recognized only in THAP and SA mass spectra. Nevertheless, the intensity of the peaks did not exceed 800 mV, which was not satisfactory.

In order to enhance the quality of the mass spectra of glycoproteins, selected binary matrices (2,5-DBH/CHCA; 2,5-DBH/THAP; 2,5-DBH/DHAP; 2,5-DBH/SA; THAP/CHCA; THAP/SA) created by mixing of common ones, have been tested. Mass spectra obtained using optimal settings (Fig. 3E–J) showed a characteristic set of peaks corresponding to five RNase B glycoforms (Man_5 to Man_9). In all cases, except 2,5-DHB/THAP (Fig. 3F), the intensities of the proteins signals were noticeably enhanced. We also observed improved resolution of glycan microheterogeneity, comparing to the ones obtained with one-component matrices. An application of THAP/CHCA matrix (Fig. 3I) resulted in clear mass spectra with well defined all five glycoforms of RNase B, but the disadvantage was low repeatability of measurements. The addition of 2,5-DHB decreased the detection limit of DHAP more than six times thus allowed to detect the signal of RNase B of concentration 0.5 mg/mL (Fig. 3H), which was not achieved with simple DHAP. The matrices 2,5-DHB/DHAP and THAP/SA (Fig. 3H, J) enabled to observe not only microheterogeneity of glycosylation, but also peaks corresponding to adduction of 98 Da. This mass difference is generally ascribed to the modification with phosphate [36,37]. This makes mass spectra more complex, but on the other hand, these matrices could probably find an application in phosphoproteins or phosphopeptides MALDI-TOF-MS analysis.

The best results were obtained using binary matrices 2,5-DHB/CHCA and 2,5-DHB/SA (Fig. 3E and G). Their mass spectra were clear, peaks had very good resolution and excellent intensity (about 26 times–2,5-DHB/CHCA, and 29 times–2,5-DHB/SA more than common 2,5-DHB). Application of those binary matrices provided good both spot-to-spot and sample-to-sample reproducibility. An additional advantage of 2,5-DHB/CHCA matrix was satisfying results obtained by using low laser power (about 3000 units, in comparison with e.g., 2,5-DHB about 5500 units, 2,5-DHB/THAP about 6000 units), which was economical for the instrument and decreased ion source contamination.

Laugesen and Roepstorff [23] supported the hypothesis that one of the reasons of signal enhancement was better biphasic crystallization resulted in a homogenous crystallization of binary matrices on whole sample spot [23]. Therefore the differences in crystallization behaviour were monitored and this presumption was visually confirmed (data not shown). 2,5-DHB formed long crystal needles at the rim, while the mixture of two matrices caused an improvement of surface crystallization, which increased the signal intensity and spot to spot reproducibility.

3.2. Matrices tolerance towards salt and buffers

Higher concentration of salts and buffers causes problems during MALDI-MS analysis (e.g., inhibition of MALDI matrix crystallization, production of adducts with analyte molecules during desorption/ionization process) [38]. Therefore the next step was the analysis and the comparison of salt tolerance of matrices. RNase B was dissolved in NH_4HCO_3 solution (20 mmol/L) and analyzed by MALDI-TOF-MS without any desalting step. Ammonium bicarbonate solution was selected because it is commonly used during deglycosylation process. Generally, the presence of salts in the sample caused marked decrease of the intensity and the resolution of protein peaks (Fig. 4). Particularly an application of CHCA and THAP separately and in the binary matrices (THAP/CHCA and THAP/SA) resulted in broad undifferentiated protein peaks (Fig. 4B, C, I, J). The mass spectrum of the sample mixed with 2,5-DHB kept the signals intensity (but only about 250 mV), nevertheless the resolution of glycoforms was deteriorated (Fig. 4A). SA, 2,5-DHB/THAP, and 2,5-DHB/DHAP displayed signals only four glycoforms Man_{5-8} (Fig. 4D, F, H). Only the mixtures 2,5-DHB/CHCA (Fig. 4E) and 2,5-DHB/SA (Fig. 4G) passed this test, showing the peaks of all five RNase B glycoforms. Their resulted mass spectra were of similar quality as the ones obtained without presence of buffer in the protein sample, keeping both the resolution and the signal intensity.

3.3. Analysis of deglycosylated mixture

Generally, further elucidation of glycan structures involves usually their enzymatic release from the native glycoprotein, subsequent separation or isolation (e.g., by lectin affinity chro-

matography, graphite carbon HPLC [39,40]) and the detection by MS. Therefore MALDI-TOF-MS analysis of reaction mixture obtained after the in-solution deglycosylation was also optimized and the impact of a matrix choice on the quality of mass spectra was studied again. PNGase *F* was selected for the deglycosylation, because it is the most universal deglycosylating enzyme. It specifically cleaves *N*-glycans between chitobiose core and asparagines residue in the polypeptide backbone [41,42].

The reaction mixture obtained after overnight deglycosylation was investigated by MALDI-TOF-MS without any further purification (Fig. 5). The complete deglycosylation was indicated by disappearance of the peak at *m*/*z* 14,899 belonging to native RNase B, respectively its shift to lower mass value giving a MS signal of RNase A (the aglyco from of RNase). The deglycosylated protein was represented by $[M+H]^+$ ion at *m*/*z* 13,682. A minor peak at *m*/*z* 13,885 corresponded to the protein-GlcNAc molecule was also present in the mass spectrum. Liu et al. [43] has observed the same phenomenon. A possible explanation is that endo-β-*N*-acetylglucosaminidase *F* (Endo *F*) often co-exists with PNGase *F*. Endo *F* cleaves *N*-linked glycans between the GlcNAc–GlcNAc bonds in chitobiose core, thus it causes appearance of the $[M+GlcNAc+H]^+$ ion in the mass spectra. Released *N*-glycans formed $[Man_{5-9}+Na]^+$ and $[Man_{5-9}+K]^+$ ions, where Man_{5-9} represents molecules of high mannose glycans containing from five to nine Man residues attached to the chitobiose core.

Although it is recommended to separate deglycosylated proteins to obtain high-quality mass spectra of released glycans, our experiments confirmed that it was not necessary. As we showed, some matrices can supplement the other ones in regard of obtaining complete information about investigated sample. The signals of protein were suppressed and glycans were favoured when 2,5-DHB (Fig. 5A), THAP and THAP/SA (data not shown) were used. On the other hand, the application of SA provided better results for the proteins (Fig. 5C). The binary matrices enabled to detect both deglycosylated proteins and released glycans in the same MALDI spot (as the example Fig. 5B and D). The best results were gained with 2,5-DHB/SA (Fig. 5D), which as the only one resulted in mass spectra with excellent signal intensity (more than 6900 mV) and resolution of both protein and free glycans.

4. Conclusion

A set of MS data of intact glycoproteins, together with MS analysis of glycopeptides and glycans, generated by enzymatic digestion, provide complete information about glycoprotein structure. This work reports MALDI-TOF-MS procedure with application of binary matrices that can facilitate this demanding task. As the first step, we aimed our effort at the development of salt tolerant matrices for intact glycoproteins analysis. The binary matrices 2,5-DHB/CHCA and 2,5-DHB/SA has been proved to be the most providential in this case. We have also introduced 2,5-DHB/SA as an universal MALDI matrix, suitable for the analysis of free glycans as well as intact proteins obtained after deglycosylation. The binary matrices are promising mainly because it allows to skip the purification steps (desalting steps prior to analysis of intact proteins from real samples and the separation of deglycosylated protein prior to glycan analysis), which can cause the loss of analyte. Thus the application of binary matrix is not time and sample consuming and it is very useful approach especially for the rapid profiling of glycan mixtures released from unknown glycoproteins.

We believe that the application of binary matrices is a promising way for the MALDI-MS analysis of glycoproteins, and also for the determination other post-translationally modified proteins (e.g., glycated proteins, phosphoproteins).

Acknowledgements

Corresponding author (M.L.) want to dedicate this article to Dr. Josef Chmelík (in memoriam † 16.7.2007) as an expression of gratitude for sharing his knowledge during her PhD studies. Authors would also like to thank Dr. Richard Cmelik and Dr. Inga Petry-Podgorska for their constructive critics and reviewing of this publication.

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (Research Centre for the Study of Extract Compounds of Barley and Hop No. 1M0570), Grant Agency AS CR (IAA600040701), and Research Plan of Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic (AV0Z40310501).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2008.12.017.

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