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SHORT COMMUNICATION

Effects of Matrices and Additives on Multiple Charge Formation of Proteins in MALDI–MS Analysis

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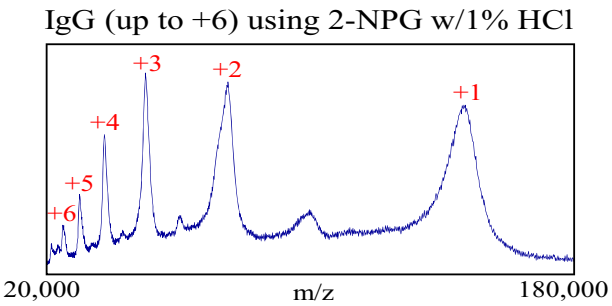

Abstract. The sinapinic acid (SA) matrix has frequently been used for protein analysis in matrix-assisted laser desorption/ionization–mass spectrometry (MALDI–MS). However, the SA matrix does not result in the formation of distinctive multiple protein charge states, whereas the 2-nitrophloroglucinol (2-NPG) matrix is capable of this. The formation of multiple charge states in the MALDI–MS analysis of proteins is advantageous in that it results in higher accuracy. In this study, the mass spectra of several common standard proteins, namely cytochrome *c*, myoglobin, bovine serum albumin (BSA), and immunoglobulin G (IgG), were compared using various matrices (2,5-dihydroxybenzoic acid, α-cyano-hydroxycinnamic acid, SA, and 2-NPG). Furthermore, the mass spectra of two large standard proteins (BSA and IgG) using various acid additives (H_3PO_4, HNO_3, H_2SO_4, HCl, and trifluoroacetic acid) with the 2-NPG matrix were also compared. Among the different matrices, 2-NPG provided the broadest range of multiple protein charge states, while, among the different additives, the 2-NPG matrix in combination with HCl generated the broadest multiple charge states as well as the most intense protein peaks.

Keywords: Matrix, Additive, MALDI–MS, Proteins, 2-Nitrophloroglucinol

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Introduction

The characteristics of matrix-assisted laser desorption/ionization–mass spectrometry (MALDI–MS) include low analyte fragmentation, the formation of single charge states, and the capability to analyze macromolecules. Therefore, MALDI–MS has frequently been used for the analysis of proteins. When a laser beam is used to irradiate a crystallized sample spot that contains an analyte-matrix mixture, the matrix, which acts as an energy mediator, helps to ionize and desorb the mixture from the MALDI plate [1]. Sinapinic acid (SA) is known to be the most suitable matrix for MALDI–MS analysis of proteins [2–4]. For example, in a comparison of the MALDI mass spectra of human milk lysozyme (molar mass = 14.693 kDa) using vanillic acid and SA matrices, significantly decreased adduct peaks relative to the molecular ion peak were observed using the SA matrix, which provided a higher quality protein mass spectrum [5]. In addition, evaluations of MALDI–MS analyses of a large number of different proteins (rabbit-muscle actin, tropomyosin, horse erythrocyte carbonic anhydrase II, and *E. coli* β-galactosidase) using a SA matrix have demonstrated that the SA matrix can be used for most types of proteins [5]. Recently, it was reported that a 2-nitrophloroglucinol (2-NPG) matrix resulted in the formation of multiple protein charge states [6], which helped to improve the accuracy of macromolecule analysis by reducing the *m/z* scan range as well as enhancing mass accuracy and sensitivity [1]. For example, even if the mass of analytes is significantly higher than the *m/z* range of the mass spectrometer, the analytes can be detectable as multiply charged ions since multiple charges decrease their *m/z* values [7]. It was reported the mass accuracy of a mass analyzer increases as the observed *m/z* region decreases [8]. In this study, we investigated the most suitable matrix and acid additive combination for the MALDI–MS analysis of proteins by comparing four different matrices (2,5-dihydroxybenzoic acid (DHB), α-cyano-hydroxycinnamic acid (CHCA), SA, and 2-NPG) and five different acid additives (H_3PO_4, HNO_3, H_2SO_4, HCl, and trifluoroacetic acid (TFA)) with a 2-NPG matrix.

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Experimental

Equine heart cytochrome *c*, equine heart myoglobin, bovine serum albumin (BSA), human serum immunoglobulin G (IgG), DHB, CHCA, SA, ammonium bicarbonate (ABC), acetonitrile (ACN), TFA (99%), nitric acid (HNO_3, 60%), sulfuric acid (H_2SO_4, 95%), and hydrochloric acid (HCl, 35–37%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2-NPG was purchased from Alfa Aesar (Ward Hill, MA, USA). Phosphoric acid (H_3PO_4, 85%) was purchased from Oriental Chemical Industries (Seoul, South Korea).

Protein stock solutions were prepared by dissolving 10 mg of each protein in 1 mL of 50 mM ABC buffer. To prepare the DHB matrix solution, 10 mg DHB was added to 1 mL of 50% ACN containing 1% phosphoric acid (PA) [9] or 1% TFA in distilled water. To prepare the CHCA matrix solution, 7.5 mg CHCA was added to 1 mL of 50% ACN and 0.1% TFA in distilled water. To prepare the SA matrix solution, 10 mg SA was added to 1 mL of 50% ACN and 0.1% TFA in distilled water. To prepare the 2-NPG matrix solution, 10 mg 2-NPG was added to 1 mL of 50% ACN and 1% TFA in distilled water. To prepare the 2-NPG matrix solutions for experiments to investigate the effects of acid additives, 10 mg 2-NPG was added to a 1 mL aqueous solution containing 50% ACN and 1% of each acid (H_3PO_4, HNO_3, H_2SO_4, HCl, or TFA), which was prepared by mixing 500 µL ACN, 10 µL acid, and 490 µL distilled water. The percentage concentration of each acid stock solution was not 100%; the actual concentrations of acids in the prepared 2-NPG matrix solutions were somewhat lower than 1%, depending on the original concentration of the acid.

Each protein stock solution (cytochrome *c*, myoglobin, BSA, or IgG) was mixed with each matrix solution (DHB, CHCA, SA, or 2-NPG) in a 1:1 (*v*/*v*) ratio. To evaluate the effects of acid additives, two of the protein solutions (BSA and IgG) were each mixed in a 1:1 (*v*/*v*) ratio with 2-NPG matrix solutions containing different acids (H_3PO_4, HNO_3, H_2SO_4, HCl, and TFA). Then, 1.5 µL of each of these mixtures was loaded onto the MALDI plate and dried at atmospheric pressure under a flow of air for 10 min. Mass spectrometric analyses of crystallized mixtures were performed using a MALDI–time-of-flight MS instrument (IDSys LT; ASTA, Korea), which was operated under the following conditions: 30 laser shots of a 349 nm Nd:YLF UV laser, 2000 ns pulse width, 100 Hz pulse repetition rate, and positive ion linear mode.

Results and Discussion

Comparison of Different Matrices for Protein Analysis

Figure 1 presents MALDI mass spectra of proteins (cytochrome *c*, myoglobin, BSA, and IgG) measured using DHB,

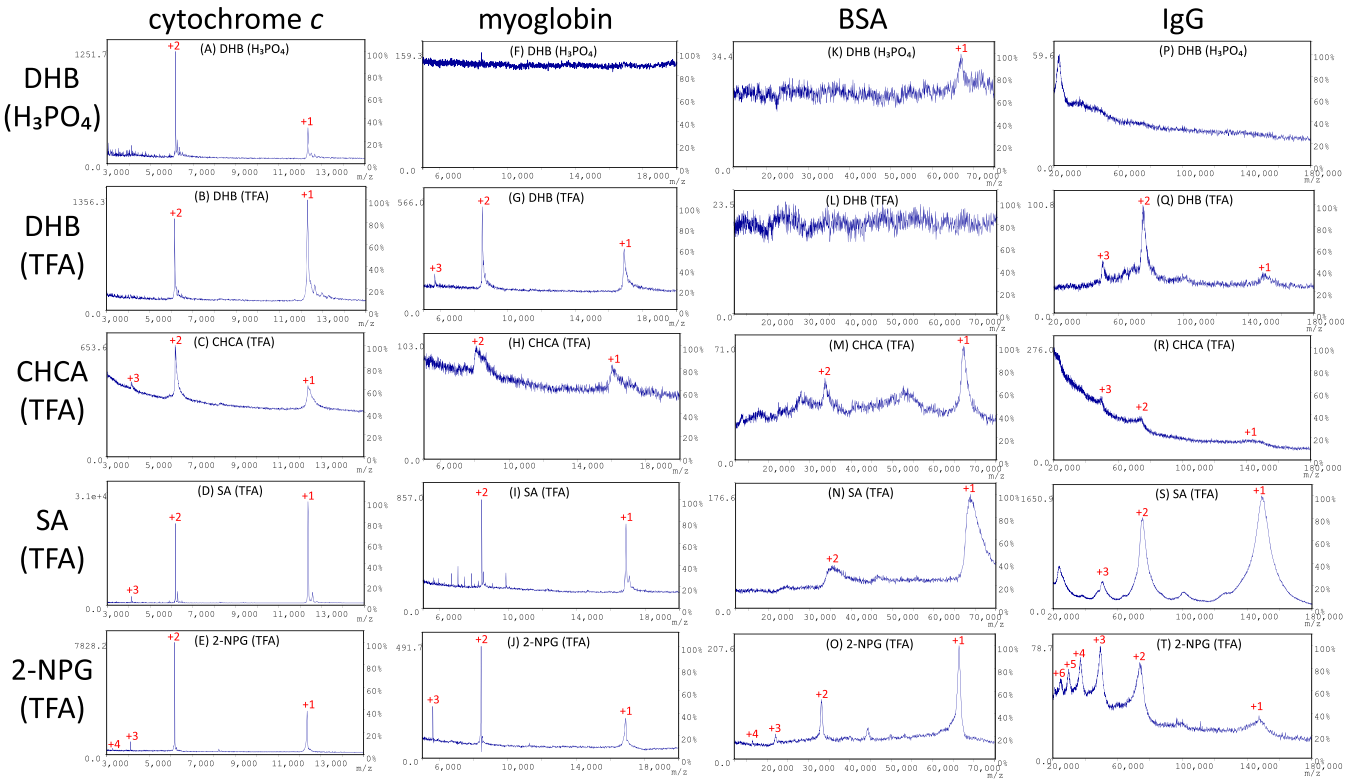

Figure 1. MALDI mass spectra of proteins (cytochrome *c*, myoglobin, BSA, and IgG) using DHB (panels A, B, F, G, L, M, Q, and R), CHCA (C, H, N, and S), SA (D, I, O, and T), and 2-NPG (E, J, P, and U) matrices. MALDI mass spectra of proteins using the DHB matrix were acquired using both H_3PO_4 (A, F, L, and Q) and TFA (B, G, M, and R) acid additives, while only TFA was used for the mass spectra of proteins using other matrices (CHCA, SA, and 2-NPG). The loadings of cytochrome *c*, myoglobin, BSA, and IgG were 61, 44, 11, and 5 pmol, respectively

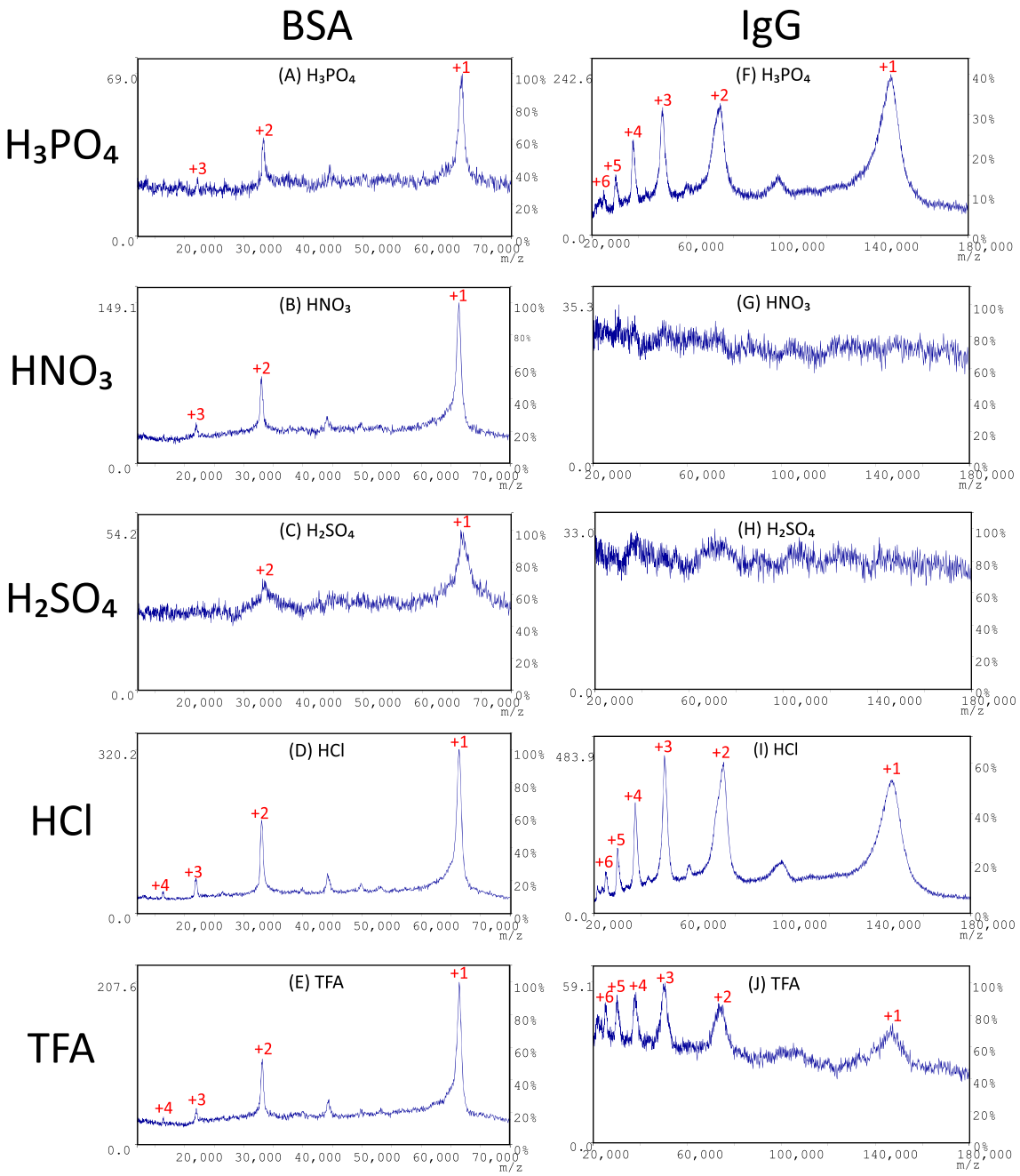

Figure 2. MALDI mass spectra of BSA (left) and IgG (right) for 2-NPG matrix solutions prepared with five different acid additives: H_3PO_4 (panels A and F), HNO_3 (B and G), H_2SO_4 (C and H), HCl (D and I), and TFA (E and J). The loadings of BSA and IgG were 11 and 5 pmol, respectively

CHCA, SA, and 2-NPG matrices. The MALDI mass spectra of proteins using the DHB matrix were acquired with one of two different acid additives, H_3PO_4 and TFA, while the mass spectra of proteins using other matrices (CHCA, SA, and 2-NPG) were acquired using only TFA as an additive. The average molar masses of cytochrome *c*, myoglobin, BSA, and IgG were 12.360, 16.951, 66, and 150 kDa, respectively. The MALDI mass spectra using the DHB matrix with addition of H_3PO_4 or TFA generated + 1 and + 2 charge states for cytochrome *c*, while CHCA and SA matrices generated + 1, + 2, and + 3 charge states. Among the matrices, the 2-NPG matrix generated the broadest range of charge states, of up to + 4 for cytochrome *c*. In the case of myoglobin, no protein peaks were observed using the DHB matrix with H_3PO_4 additive, whereas using the DHB matrix with TFA additive generated + 1, + 2, and + 3 charge states; CHCA and SA matrices generated + 1 and + 2 charge states, and the 2-NPG matrix exhibited charge states of up to + 3. In the analysis of BSA, the protein peak was observed as a + 1 charge state using the DHB matrix with H_3PO_4 additive, whereas no protein peak was observed using the DHB matrix with TFA additive; CHCA and SA matrices generated + 1 and + 2 charge states, while a broad range of

Table 1. Summary of MALDI–MS Analyses of Four Standard Proteins (Cytochrome *c*, Myoglobin, BSA, and IgG) Using Different Matrices (DHB, CHCA, SA, and 2-NPG) and Acid Additives (H_3PO_4, TFA, and HCl)

	Previous investigations				Current study		
	Matrix	Charge states	Additive	References	Matrix	Charge states	Additive
Cytochrome *c*	DHB	(+ 1)	H_3PO_4	[13]	DHB	(+ 1, + 2)	H_3PO_4
		(+ 1, + 2)	TFA	[1]		(+ 1, + 2)	TFA
	CHCA	(+ 1, + 2)	TFA	[14, 15]	CHCA	(+ 1 to + 3)	TFA
	SA	(+ 1)	TFA	[16]	SA	(+ 1 to + 3)	TFA
	2-NPG	(+ 1 to + 8)	TFA	[1]	2-NPG	(+ 1 to + 4)	TFA
Myoglobin	DHB	(+ 1)	TFA	[13]	DHB	(No peak)	H_3PO_4
						(+ 1 to + 3)	TFA
	CHCA	(+ 1, + 2)	TFA	[17]	CHCA	(+ 1, + 2)	TFA
		(+ 1 to + 3)	TFA	[18]			
	SA	(+ 1)	TFA	[19, 20]	SA	(+ 1, + 2)	TFA
		(+ 1, + 2)	TFA	[17]			
		(+ 1 to + 3)	TFA	[18]			
					2-NPG	(+ 1 to + 3)	TFA
BSA	DHB	(+ 1)	TFA	[21]	DHB	(+ 1)	H_3PO_4
						(No peak)	TFA
	CHCA	(+ 1, + 2)	TFA	[17]	CHCA	(+ 1,+ 2)	TFA
		(+ 1 to + 3)	TFA	[15]			
	SA	(+ 1)	TFA	[16]	SA	(+ 1, + 2)	TFA
		(+ 1, + 2)	TFA	[17, 20]			
					2-NPG	(+ 1 to + 4)	TFA, HCl
IgG					DHB	(No peak)	H_3PO_4
					CHCA	(+ 1 to + 3)	TFA
	SA	(+ 1, + 2)	TFA	[22, 23]	SA	(+ 1 to + 3)	TFA
		(+ 1 to + 3)	TFA	[24]			
		(+ 1 to + 4)	No additive	[25]			
					2-NPG	(+ 1 to + 6)	TFA, H_3PO_4, HCl

charge states (+ 1 to + 4) were observed using the 2-NPG matrix. In the analysis of IgG, no protein peak was observed using the DHB matrix with H_3PO_4 additive; DHB, CHCA, and SA matrices with TFA additive generated charge states of up to + 3, while among the matrices, the 2-NPG matrix provided the broadest range of charge states of up to + 6.

The most intense protein peaks were observed using the 2-NPG matrix for cytochrome *c* and BSA, and the SA matrix for myoglobin and IgG. In terms of charge states, the 2-NPG matrix generated the most multiply charged peaks: + 3 for myoglobin, + 4 for cytochrome *c* and BSA, and + 6 for IgG. Therefore, we further investigated the effects of acid additives using the 2-NPG matrix.

Influence of Different Acid Additives on Protein Analysis Using the 2-NPG Matrix

Figure 2 presents the effectiveness of different acid additives (H_3PO_4, HNO_3, H_2SO_4, HCl, and TFA) in the MALDI–MS analysis of BSA and IgG using the 2-NPG matrix. In the analysis of BSA, two additives, HCl and TFA, generated the most charge states of up to + 4, followed by H_3PO_4 and HNO_3 additives that provided charge states of up to + 3, and then H_2SO_4 that generated charge states of + 1 and + 2. In the MALDI–MS analysis of IgG, the additives H_3PO_4, HCl, and TFA provided the most charge states of up to + 6, whereas no protein peaks were observed using HNO_3 and H_2SO_4 as additives.

In terms of absolute protein peak intensities, HCl generated the most intense protein peaks among the different additives. Thus, the HCl additive provided the broadest range of charge states and the most intense protein peak among different additives in the MALDI–MS analysis of BSA and IgG. The increased protein peak intensities and charge state ranges using HCl as an additive can be explained by the high acidity of HCl compared with the other acids [10, 11]. Highly acidic matrix solutions (e.g., pH < 2.0) were reported to be advantageous in the detection of proteins using MALDI–MS with a CHCA matrix [12]. However, the inability to detect IgG using HNO_3 or H_2SO_4 as additives, but the reasonable detection of IgG using H_3PO_4 or TFA, implies that acidity is not the only factor that determines the effectiveness of MALDI–MS analyses of proteins. H_3PO_4 was reported to be advantageous in MALDI–MS analyses of peptides due to its ability to capture residual sodium ions and to passivate MALDI plates [9], which may have also contributed to the improved detection of BSA and IgG proteins using H_3PO_4 in the present work.

Comparison to Previous Studies

Table 1 compares the results of the present work and those of previous studies involving MALDI–MS analyses of standard proteins (cytochrome *c*, myoglobin, BSA, and IgG), with details of the matrices used (DHB, CHCA, SA, and 2-NPG), protein charge states, and acid additives. Previous reports were found by searching "Google" or "Web of Science" with the

keyword "MALDI" along with the individual names of the proteins. As shown in Table 1, most previous MALDI–MS investigations were performed using a SA matrix with TFA additive. Generally, the charge state distributions of the protein peaks in the present study obtained using DHB, CHCA, and SA matrices are similar to those in previous reports, while our MALDI–MS analyses of BSA and IgG using the 2-NPG matrix exhibited the broadest charge state distributions.

Conclusion

In this study, we investigated the effects of matrices and additives on MALDI–MS protein analysis. Among four different matrices (DHB, CHCA, SA, and 2-NPG), the 2-NPG matrix was found to be the most effective in generating multiple protein charge states. Among five different additives (H_3PO_4, HNO_3, H_2SO_4, HCl, and TFA), HCl was found to be the most effective in generating multiple charge states and protein peaks with increased intensities, when used in combination with the 2-NPG matrix for the analysis of BSA and IgG.

Acknowledgements

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