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RESEARCH ARTICLE

(E)-Propyl α-Cyano-4-Hydroxyl Cinnamylate: A High Sensitive and Salt Tolerant Matrix for Intact Protein Profiling by MALDI Mass Spectrometry

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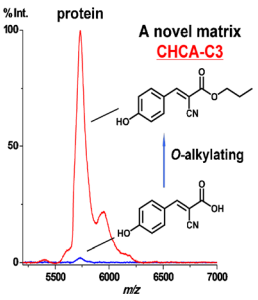

Abstract. Low-abundance samples and salt interference are always of great challenges for the practical protein profiling by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Herein, a series of carboxyl-esterified derivatives of α-cyano-4-hydroxycinnamic acid (CHCA) were synthesized and evaluated as matrices for MALDI-MS analysis of protein. Among them, (E)-propyl α-cyano-4-hydroxyl cinnamylate (CHCA-C3) was found to exhibit excellent assay performance for intact proteins by improving the detection sensitivity 10 folds compared with the traditional matrices [i.e., super2,5-dihydroxybenzoic acid (superDHB), sinapic acid (SA), and CHCA]. In addition, CHCA-C3 was shown to have high tolerance to salts, the ion signal of myoglobin was readily detected even in the presence of urea (8 M), NH_4HCO_3 (2 M), and KH_2PO_4 (500 mM), meanwhile sample washability was robust. These achievements were mainly attributed to improved ablation ability and increased hydrophobicity or affinity of CHCA-C3 to proteins in comparison with hydrophilic matrixes, leading to more efficient ionization of analyte. Furthermore, direct analysis of proteins from crude egg white demonstrated that CHCA-C3 was a highly efficient matrix for the analysis of low-abundance proteins in complex biological samples. These outstanding performances indicate the tremendous potential use of CHCA-C3 in protein profiling by MALDI-MS.

Keywords: MALDI-TOF MS, Esterified α-cyano-4-hydroxycinnamic acid, Proteins, Sensitivity, Salt-tolerance

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Introduction

Matrix assisted-laser desorption ionization (MALDI) and electrospray ionization (ESI) are two main ionization techniques of mass spectrometry (MS) widely used in proteomic analysis [1–4]. Comparison with ESI-MS MALDI-MS has shown advantages for direct analysis of proteins in complex biological samples because of its capacity of higher salt-tolerance and high throughput, as well as production of singly or lower charged ions. Tremendous interests and efforts have been stimulated to develop various strategies by using the MALDI-MS to find biomarkers in blood serum and plasma [5], urine [6], cerebrospinal fluid [7], extracts from tissues and cells [8], etc., and to investigate protein distributions in microorganisms [9, 10], foods [11], and tissue [12]. Particularly, MALDI-MS based imaging technique has been developed for directly localizing proteins on a tissue and providing post-translational modification and spatial distribution information in a single experiment [13, 14]. This exclusive technique has shown great potentials for direct investigation of biological function-related macromolecules, such as cytokines, enzymes, neuropeptide precursors, and receptor in pathological conditions.

In MALDI-MS analysis, the most common sample preparation procedure is to simply mix samples with a matrix, which generates matrix-sample co-crystals on the surface of MALDI plate after ambient drying. Upon laser irradiation, analyte ions are generated and subjected for detection. However, low-

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abundance samples and signal suppression by salt contaminants often associate with the process and influence successful MALDI-MS analysis. Earlier works have developed numbers of techniques in sample preparation procedures prior to analysis, which included using on-plate enrichment methods [15], liquid matrixes [16], additives to matrix [17, 18], spotting sample techniques [19, 20] such as vacuum sublimed method and nanoliter spots depositing technique to enhance signal intensities and detection limits, using C18 Ziptip [21], surface-functionalized nanoparticles [22] to prefractionate complex biological samples, using hydrophobic polymers such as Teflon [23] and parafilm wax [24] as sample support for on-plate peptides/proteins enrichment or desalting. Recently, we used patterned MALDI supports to obtain simultaneous enrichment, desalting, and selective peptides/proteins analysis [25, 26]. Nevertheless, these techniques require additional labor, time, and handling procedures in the fabrication or preparation process, which inevitably bring certain limits to applications for large-scale protein profiling (e.g., cancer biomarker screening and identification of microorganisms). Moreover, many previous methods are not applicable to imaging mass spectrometry because of the requirement of direct deposit of matrix onto surface of the tissue. Therefore, the development of a simple, rapid, high throughput, low cost, high sensitive, and salt-tolerant technique is still critically required in protein identification by MALDI-MS.

A fundamental field of research and improvements associated with MALDI and its application in high efficient MS analysis is of matrix design. Matrix serves as the requisite and basis for MALDI analysis. It has been generally accepted to possess some common characters, including absorbing laser energy, preventing sample aggregation, good ability for co-desorption of the analyte, and transferring charge (proton, electron or cation) to analyte, etc.[27, 28]. These characters of the selected matrix have a direct impact on the assay performance. Two conventional matrixes are α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB). Based on them, several derivatives have been formulated to improve matrix performance. Previously, 4-chloro-α-cyanocinnamic acid (Cl-CHCA), a CHCA derivative, was reported to provide lower proton affinity and obtain outstanding detection sensitivity of acidic peptides and higher sequence coverage for the identification of protein digests [29]. Recently, O-alkylated dihydroxybenzoic acid (ADHB), an esterified derivative of DHB, has been developed to improve its affinities for hydrophobic peptides [30]. ADHB as additive matrix was mixed with the traditional matrix CHCA and improved the sensitivity of hydrophobic peptides 10- to 100-fold compared with CHCA.

Herein, a series of new matrices were synthesized by modifying CHCA with hydrophobic alkyl on the carboxyl group and evaluated by analysis of various proteins. After screening the MALDI assay performance, (E)-propylα-cyano-4-hydroxyl cinnamylate (CHCA-C3) was found to be the most promising candidate for intact protein analysis. The improved detection sensitivities by one order of magnitude for protein profiling were obtained by using CHCA-C3 as the matrix, compared with super2,5-dihydroxybenzoic acid (superDHB), sinapinic acid (SA), and CHCA. Furthermore, CHCA-C3 was assessed to be able to generate legible spectra of proteins of interest even in the presence of high concentrations of contaminants. Owing to the high detection sensitivity and enhanced salt-tolerance, the practical use of CHCA-C3 matrix for protein profiling was also investigated by direct analysis of crude egg white.

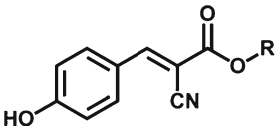
Experimental

Chemicals and Materials

α-Cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), 2,5-dihydroxybenzoic acid (DHB), 5-methoxysalicylic acid, bovine insulin, cytochrome *c*, and ubiquitin were purchased from Bruker Daltonics (Bremen, Germany). Porcine insulin, lysozyme, myoglobin, growth hormone, trypsin TPCK treated from bovine pancreas, pepsin, bovine serum albumin (BSA), transferrin from human, IgG from rabbit, acetonitrile (ACN), trifluoroacetic acid (TFA), and alkyl alcohols were purchased from Sigma-Aldrich (Beijing, China). Thermophilic histone and alkaline phosphatase were obtained as a gift from Dr. Quanshun Li (Jilin University). The chemicals were of analytical grade. All aqueous solutions were prepared using Milli-Q water by Milli-Q System (Millipore, Billerica, MA).

Synthesis of Substituted CHCA

Typically, 0.57 g of α-cyano-4-hydroxylcinnamic acid (3.0 mmol) was first suspended in 20 mL of propyl alcohol, to which 20 μL of concentrated sulfuric acid was added. The mixture was heated to reflux at 80 °C for 5 h. After cooling to room temperature, the solution was concentrated by rotary evaporation. The residue was then dissolved in ethyl acetate, washed three times with ammonium bicarbonate solution, and dried over $MgSO_4$. (E)-propyl α-cyano-4-hydroxyl cinnamylate (CHCA-C3) was obtained as yellow crystal after purification by column chromatography (0.40 g, 1.7 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19 (s, 1H), 7.98 (d, J = 8.2 Hz, 2H), 6.97 (d, J = 8.2 Hz, 2H), 5.83 (s, 1H), 4.29 (t, J = 6.5 Hz, 2H), 1.79 (dt, J = 14.1, 7.1 Hz, 2H), 1.04 (t, J = 7.3 Hz, 3H). The same method was used to acquire the substituted CHCA with linear alkyl, *i.e.*, R = C1, C2, C4, C5, C6, C8, C10, C12 (Figure 1). The substituted CHCA with R = isobutyl, isopropyl, and tert-butyl were synthesized with Knoevenagel reaction [31].



R = C1, C2, C3, C4, C5, C6, C8, C10, C12, isoButyl, isoPropyl, tert-butyl

Figure 1. Substituted CHCAs with hydrophobic substituent groups. Cn (*n* = 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, n denotes the carbon number in linear alkyl alcohols)

Sample Preparation

Proteins were dissolved in 0.1% aqueous TFA (v/v) at appropriate concentrations. Two sample deposition methods were applied in this study: the dried-droplet (DD) and two-layer (TL) method. For the DD method, matrix solutions of CHCA and SA were saturated in 50% ACN/0.1% aqueous TFA (v/v), and superDHB (DHB + 10% 5-methoxysalicylic acid) was dissolved in distilled water-ethanol (9:1, v/v) at 20 mg mL^{-1}. CHCA-C3 solution was prepared in 50% ACN/0.1% aqueous TFA (v/v) at 1 mg mL^{-1}. All matrix solutions were fleshly made each time. Equal volumes of analyte solution and matrix solution (each at 10 μL) were thoroughly mixed by repeatedly aspirating with pipet for six times, then 1 μL of the mixed solution was deposited on the steel target plate (MTP 384 target plate ground steel, Bruker) and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). For the TL preparation method, 1 μL of CHCA or SA solution (12 mg mL^{-1} in 80% acetone/ethanol) was firstly deposited on the target spot to form a first seed layer, then 1 μL of the analyte-matrix mixed solution was deposited on the top of the first layer and left to dry. The TL method was not applicable to superDHB because of the limit of solubility of DHB in organic solvent such as acetone. There were no obvious advantages in the terms of sensitivity improvement by using TL method for CHCA-C3; hence, CHCA-C3 was performed only with the DD method.

Instrumentation

MALDI-TOF MS experiments were performed on an Autoflex speed TOF/TOF mass spectrometer (Bruker Daltonics, Germany) with a pulsed Nd:YAG laser at a wavelength of 355 nm. Spectra were obtained by 500 laser shots and a delayed extraction time of 150 ns in linear and positive ion mode. An acceleration voltage of 19.38 kV (IS1) was applied for a final acceleration of 16.88 (IS2). MS/MS experiments were run in the MS/MS lift mode using collision induced dissociation gas (air). In general, laser energy was kept about 10% above threshold to obtain the best signal quality with low noise. For the evaluation of the limits of detection (LODs), laser energy was adjusted from the threshold energy of corresponding matrix ions to 100% by variable laser attenuator. The LODs for proteins was defined as the lowest quantity of analyte detected as $[M + H]^+$ with S/N ≥ 3 when each series of analyte solution at different concentration made by 10-fold serial dilutions was evaluated. After smoothing and baseline subtraction twice, the S/N ratio of analyte ion in the spectra were automatically marked by analysis software. Fresh pipet tips were used for each transfer procedure of solution. Targets for the sample deposition were processed with careful cleaning (sequential cleaning with hot water, acetone, hot water, 30% TFA, acetonitrile, and distilled water) and supersonic treatment (methanol, 40 min) before being used. Flex Analysis 3.3080 Software (Bruker Daltonics) was used to work up data. Commercial peptide or protein mixture (peptide standard II, protein standard I, or protein standard II) served as external standards for calibration of the mass weight. All experiments were carried out at least three times to ensure the reproducibility of the spectral profiles.

ESI-MS experiments were carried out in ESI positive ion mode of an electrospray ionization and quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer (microTOF-Q II, Bruker Daltonics, Germany). For MS/MS experiment, argon was used as collision gas. The collision energy was optimized for the best fragmentation pattern at 7.0 eV.

Results and Discussion

Preliminary Evaluation of Substituted CHCA Matrices

A series of substituted CHCA matrices were synthesized by modifying CHCA on the carboxyl group with hydrophobic alkyl (Figure 1). The matrices were characterized by ^1H NMR and tested to be of better solubility than CHCA in common organic solvent, such as methanol, acetonitrile, etc. UV-Vis spectra revealed that all the esterified CHCAs possessed absorption peak at around 338 nm, which is comparable to that of CHCA (Supplemental Figure S1, see Supporting Information). A preliminary test of these compounds as MALDI matrices for protein analysis was performed using bovine insulin as the analyte. As shown in Supplementary Figure S2, the signal-to-noise (S/N) ratio of different alkyl chain-length of the CHCA derivative matrices and analyte ions were evaluated based on the variation of laser energy. Broadly speaking, threshold energies required for highest and stable positive-ion signals of bovine insulin were about 5% higher than those of corresponding matrix ions. The threshold energies for matrix ions using esterified CHCA with carbon numbers of the alkyl groups n = 2–5 were at energy of 56 ± 1%, which were slightly lower than those for CHCA-Cn (n = 1 or >5) and CHCA at 60 ± 2%. Simultaneously, spectra of analyte insulin using the esterified CHCA with carbon numbers of the alkyl groups n = 2–5 showed higher S/N ratio value. Among them, CHCA-C3 exhibited the highest S/N ratio at about 1000 compared with CHCA (DD method) with S/N ratio at about 200. These data suggested that ablation of the esterified CHCA with carbon numbers of the alkyl groups n = 2–5 required less energy than ablation of esterified CHCA (n = 1 or >5) and CHCA. For analyte-M^+, a similar trend could be observed. Ion yields of insulin were obviously improved using esterified CHCA with carbon numbers of the alkyl groups n = 2–5. The energy threshold and optimal S/N ratio using CHCA-C3 were at about 60% and 700, compared with CHCA (DD method) at 66% and 100. CHCA prepared with two-layer (TL) method was also investigated. The energy threshold for both matrix and analyte-M^+ declined by 4% and optimal S/N ratio improved 2- to 3-fold. Thus, CHCA-C3 was selected as the candidate matrix and used for further evaluation.

Sensitivity, Sample Preparation Morphology/Homogeneity, and Signal-to-Noise Ratio

SuperDHB, SA, and CHCA are popular matrices for intact protein analysis [10, 18, 32, 33]. To further investigate the applicability of CHCA-C3 matrix in protein analysis, MALDI MS analysis was performed by screening a series of intact proteins with molecular weights ranging from 5734.52 to 148,420 Da in comparison with these three classic matrices. As listed in Table 1, for all the tested proteins, the LODs obtained by using superDHB are lower than using CHCA and SA with the DD method. When the TL method was applied for CHCA and SA, the LOD obtained using CHCA showed nearly the same order of magnitude as that using superDHB (100 to 10 fmol), whereas SA resulted in LODs one order of magnitude higher than that with CHCA and superDHB for the detection of four small proteins (i.e, bovine insulin, porcine insulin, lysozyme, and myoglobin). In view of these sensitivity improvements, CHCA and SA would be performed with TL method for all of the following comparisons. In contrast, CHCA-C3 enables obtaining the lowest LODs at 10 to 1 fmol, which improved the detection sensitivity 10-fold compared with these three traditional matrices.

When CHCA and SA were performed with DD method, it was noticed that hunting for "hot spots" in the matrix-analyte co-crystals was required to obtain good spectra, especially at analyte concentration closed to LOD. These might be attributed to the uneven analyte-matrix co-crystals [34]. When TL method was applied for CHCA and SA, the spot to spot reproducibility was well improved [34]. Herein, matrix-analyte co-crystal with superDHB (DD method), SA (TL method), CHCA (TL method), and CHCA-C3 (DD method) were investigated. Supplementary Figure S3 shows sample spots of these four matrices collected from MALDI camera. The crystal layer of SA and CHCA were dense and homogeneous. CHCA-C3 also exhibited a uniform but slightly thin crystal. As a result, there was little difference for signal intensities achieved from different parts of crystal spots for sample preparation with SA, CHCA, and CHCA-C3. In the case of superDHB, it formed large needlelike crystals on the rim of the spot. Thus, it was difficult to obtain assignable signal information on the center of spot with the superDHB preparation, and it required search for "sweet spots" in the rim, especially for low concentration of analyte. Furthermore, the spot-to-spot reproducibility were investigated by evaluating the relative standard deviation (RSD) obtained by calculating $[tryspin]^+$ S/N ratio variation. As shown in Supplementary Figure S4, the RSD were 81.3%, 43.5%, 33.7%, and 38.4% for superDHB, SA, CHCA, and CHCA-C3, respectively. These results were consistent with the morphology of matrix-analyte co-crystal as described above. Hence, our data suggested CHCA-C3 was a good matrix candidate for highly reproducible analysis of proteins.

The S/N ratio was also compared in detail for these four matrices. Trypsin (basic protein with isoelectric point ~10.1) and pepsin (acidic protein with isoelectric point ~1.0) were typically selected as the reference proteins. As shown in Figure 2, CHCA-C3 exhibited the highest S/N ratios among these four matrices for both tested proteins at the corresponding concentrations. Moreover, for trypsin, the detection limit was 5 fmol with CHCA-C3, whereas that was 50 fmol with superDHB and CHCA, and 100 fmol with SA. As shown in Figure 2b, rather weak signal corresponding to $[pepsin]^+$ with a S/N at about 3.2 was obtained at 100 fmol of sample loading using superDHB. Although there was no need to find "sweet spots" for CHCA and SA with TL method, the detection limit of pepsin was at only 1 pmol with S/N ratios of 7.9 and 23.4 for CHCA and SA, respectively. In contrast, CHCA-C3 exhibited excellent signal for 1 pmol pepsin with a S/N ratio at 129.4 and showed the lowest detection limit at

Table 1. Obtained LODs for Proteins Using CHCA-C3, Compared with superDHB, SA, and CHCA

#	Protein	Mass (Da)	CHCA (fmol[a])		SA (fmol[a])		superDHB (fmol[a])	CHCA-C3 (fmol[a])	Sensitivity improvement rate[b] (fold)
			DD method	TL method	DD method	TL method	DD method	DD method	
1	Bovine insulin	5734.52	10	10	100	100	10	1	10
2	Porcine insulin	5788	10	10	100	100	10	1	10
3	Ubiquitin	8586.76	100	100	1000	100	100	10	10
4	Thermophilic histone	9716	100	100	1000	100	100	10	10
5	Cytochrome *c*	12361	100	100	1000	100	100	10	10
6	Lysozyme	14260	10	10	100	100	10	1	10
7	Myoglobin	16952	100	100	1000	1000	100	10	10
8	Growth hormone	22124	1000	100	1000	100	100	10	10
9	Trypsin	23982	1000	100	1000	100	100	10	10
10	Pepsin	35000	1000	1000	1000	1000	100	10	10
11	Alkaline phosphatase[c]	56000	100	100	100	100	100	10	10
12	Bovine serum albumin	66430	100	100	1000	100	100	10	10
13	Transferrin[c]	77064	100	100	1000	100	100	10	10
14	IgG[c]	148420	1000	100	1000	100	100	10	10

[a]fmol represent the amount of sample loading on the target spot.
[b]Sensitivity improvement rate was obtained by dividing the detection limit using superDHB by that using CHCA-C3.
[c]Alkaline phosphatase, transferrin, and IgG are glycoproteins.

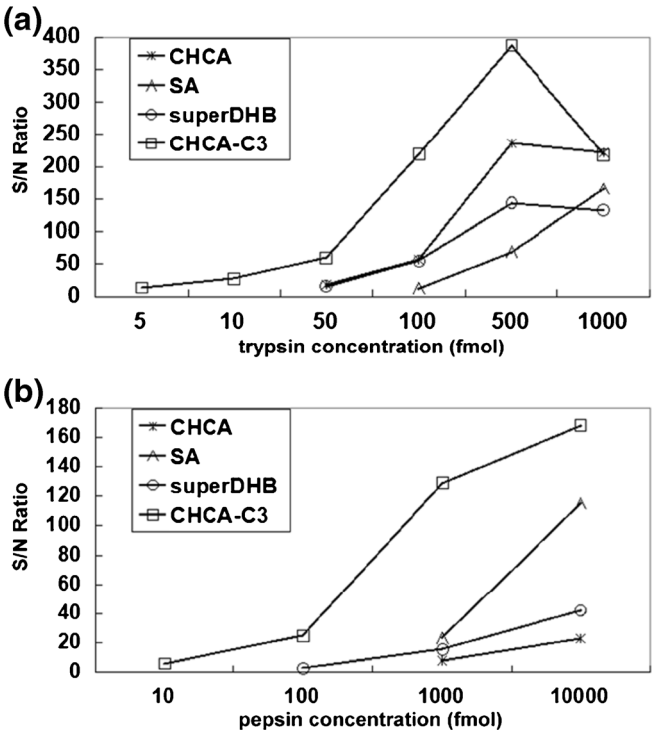


Figure 2. Average S/N ratio for molecular ion signals of **(a)** trypsin and **(b)** pepsin at different protein loading. Each S/N ratio value was an average of S/N ratio obtained from nine spectra. The matrices used for analysis are superDHB, SA, CHCA, and CHCA-C3. The CHCA and SA were performed with TL method, and the superDHB and CHCA-C3 were prepared with DD method

10 fmol with a S/N ratio at 5.9. Thus, these data further confirm that CHCA-C3 would be a promising matrix for detection of trace amount of proteins.

The sensitivity improvement efficiency was further evaluated by detecting protein mixtures of five low-concentration proteins, including bovine insulin, lysozyme, myoglobin, trypsin, and pepsin with superDHB, SA, CHCA, and CHCA-C3. The used laser energies for different matrices were kept slightly higher than the signal threshold value to achieve reliable spectra. As shown in Figure 3a–d, when the analyte loadings were 50, 50, 100, 50, and 100 fmol for insulin, lysozyme, myoglobin, trypsin, and pepsin, respectively, the signal peaks of $[insulin]^{+}$, $[lysozyme]^{2+}$, $[lysozyme]^{+}$, $[myoglobin]^{2+}$, and $[trypsin]^{+}$ were noted using superDHB and CHCA, whereas the signal peaks of $[myoglobin]^{+}$ and $[pepsin]^{+}$ were not visible. For the case of SA, only signal peaks corresponding to insulin and lysozyme were observed. In contrast, the sample preparation with CHCA-C3 revealed highly resolved spectra of signal peaks corresponding to all the five proteins. As the concentrations of five proteins further decreased to 10, 10, 50, 10, and 50 fmol (Figure 3e, f), highly resolved signal peaks for the five low-concentration proteins could still be observed with CHCA-C3. However, only weak signal peaks of $[lysozyme]^{2+}$ and $[lysozyme]^{+}$ were noted with superDHB and CHCA, and no assignable signal could be observed with SA.

Protein Charge State

It has been reported that CHCA usually has limit detection ability in analysis of high molecular weight proteins, which increases the tendency to generate multiple protonation with relatively low intensity of singly protonated ion [35]. Here, transferrin (*Mw* = 77064 Da) as analyte was investigated. Supplementary Figure S5 shows that the highest charge state observed using CHCA matrix was 8+ and the relative abundance of $[M + H]^{+}$ was far below $[M + H]^{2+}$ and $[M + H]^{3+}$ (Supplementary Figure S5a). The highest charge state with superDHB (Supplementary Figure S5c) was 4+ and $[M + H]^{2+}$ dominated the spectrum. In contrast, the highest charge state of protonated transferrin by using CHCA-C3 matrix was 3+ and the $[M + H]^{+}$ signal dominated the whole spectrum (Supplementary Figure S5d), which was similar to the spectrum using SA (Supplementary Figure S5b). The enhanced relative abundance of singly protonated protein and reduced charge number acquired by using CHCA-C3 would make it more conducive to the identification of objective peaks and prevent the interference of other multiply charged protein peaks, particularly for analysis of big and complex protein sample.

Resolution

Peak resolution was a significant parameter for accurate and reliable analysis of intact protein by mass spectrometry. SupplementaryTable S1 lists the obtained mass resolution values (full width at half maximum, FWHM) for the singly charged molecular ion of trypsin (*m/z* 23982) and transferrin (*m/z* 77064) by MALDI-MS. CHCA-C3 showed comparable resolution values with superDHB, SA, and CHCA for higher sample loading at 1 pmol. However, it should be noted that the resolution values of protein peaks obtained with superDHB, SA, and CHCA would decrease to varying degrees with the decrease of sample loading, and even become undetectable at 10 fmol of analyte loaded on the target. In contrast, there was little difference for resolution values of protein peaks by using CHCA-C3, even at the sample loading as low as 10 fmol. Hence, these data suggest CHCA-C3 should be a superior matrix for detection of low-concentration proteins.

Salt Tolerance and Sample Washability

High concentrations of contaminants (such as salts, buffers, and surfactants) are often and inevitably presented in biological sample. These existing compounds strongly suppress target ionization, which usually leads to weak ion signal available or even no detectable signal in MALDI-MS [22, 36, 37]. Salt tolerance of CHCA-C3 was evaluated by using myoglobin as the protein analyte in the presence of urea, NH_4HCO_3, and KH_2PO_4, and compared with CHCA, SA, and superDHB. As shown in Figure 4a–d, high quality spectra were obtained without any salts with all the tested matrices. In the presence of 8 M urea (Figure 4e–h), there was no objective signal observed using superDHB, SA, and CHCA. This is because

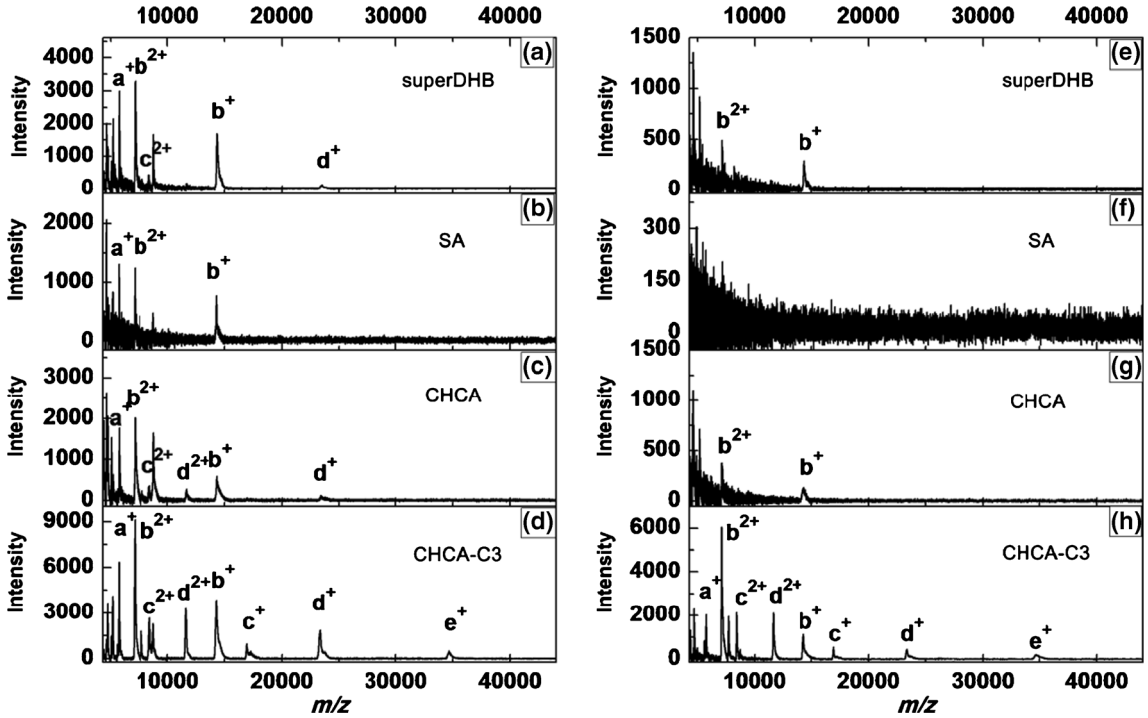


Figure 3. MALDI mass spectra of protein mixtures of five proteins: bovine insulin [**(a)**, *m/z:* 5734.5], lysozyme [**(b)**, *m/z*: 14260], myoglobin [**(c)**, *m/z*: 16952], trypsin [**(d)**, *m/z*: 23982], and pepsin [**(e)**, *m/z*: 35000]. The sample loading of the corresponding five proteins were 50, 50, 100, 50, 100 fmol for the left spectra **(a)**, **(b)**, **(c)**, **(d)**, and 10, 10, 50, 10, 50 fmol for the right spectra **(e)**, **(f)**, **(g)**, **(h)**. Different matrices were marked in the figures and different charge states were marked on corresponding peaks

urea may form a thick layer after solvent evaporation, which would severely impede UV absorption and energy transfer among molecules [38]. In contrast, a legible spectrum corresponding to the $[M + H]^+$ was clearly observed with S/N ratio at 47.8 using CHCA-C3 as the matrix (Figure 4h). Likewise, in the presence of 2 M NH_4HCO_3 (Figure 4g–i), no detectable signal could be observed using SA and superDHB, and a severely suppressed signal corresponding to $[M + H]^+$ were detected with S/N ratio at 3.8 using CHCA by searching for "hot spots," whereas a legible signal was obtained with S/N ratio at 38.5 using CHCA-C3 (Figure 4h). In addition, phosphate is one of the most commonly used buffers, yet MALDI is relatively insensitive to the phosphate [39]. Thus, KH_2PO_4 (500 mM) was selected to evaluate the tolerance for phosphate. Not surprisingly, legible signal was only obtained with CHCA-C3 matrix (Figure 4m–p). Therefore, the excellent salt tolerance makes CHCA-C3 a matrix with great potential for assay performance or direct protein analysis without tedious pretreatment.

Moreover, simple washing of the analyte/matrix on the MALDI target with deionized water is a simple and efficient procedure to improve ion signals of interest [40]. This is because the salts are mainly distributed outside the matrix crystals and readily removed by washing, whereas the biological molecules are more or less evenly incorporated in the matrix crystals (often less soluble in water). Therefore, it is necessary for the applied matrix to possess good crystal structure and properties to obtain ideal washing performance. Thus, sample washability was investigated in the presence of urea, NH_4HCO_3, and KH_2PO_4. After the analyte-matrix-contaminant was co-deposited and allowed to be completely dried on the target spot, 2.5 μL of 0.1% TFA aqueous solution was deposited on the top of the crystal for 5–10 s before being removed. As shown in Supplementary Figure S6, significant improved signal intensities were observed after one wash and limited loss of protein sample was observed even after three washes. This superior sample washability with CHCA-C3 should be ascribed to the improved hydrophobic nature of CHCA-C3 matrix. The hydrophilic salt contaminants can be easily removed by washing while the protein analytes are preserved to be embedded in the matrix crystal because of the insolubility of CHCA-C3 in washing solution.

Practical Protein Profiling in Crude Egg White

Identifications of target proteins in real organic samples are often difficult because of sample complexities. Based on the improved sensitivity and salt tolerance available with CHCA-C3 for the analysis of integrated protein, MALDI mass spectra of crude egg white was investigated to illustrate applicability. Previously, SA with TL preparation was commonly used as MALDI matrix for the detection of crude egg white [41, 42]. Thus, SA was used as the reference matrix. For direct analysis of crude egg white without pretreatment, nearly no signals could be detected with both SA and CHCA-C3, even though

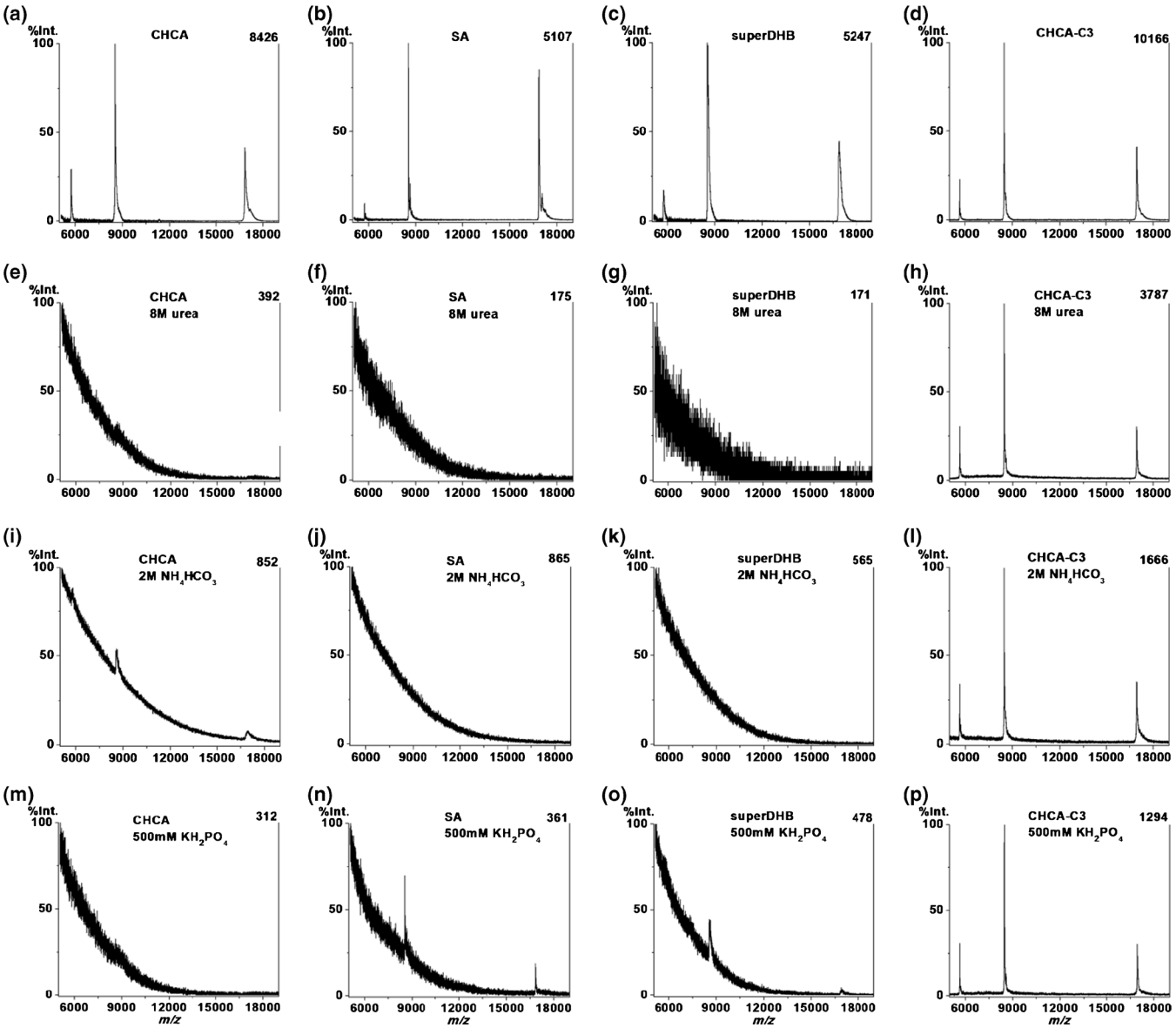


Figure 4. MALDI mass spectra for evaluating salt tolerance of matrices. Myoglobin as analyte was detected with CHCA (first row), SA (second row), superDHB (third row), and CHCA-C3 (fourth row). Sample detected in first rank without any additive salts (a, b, c, d); the second rank detected in the presence of 8 M urea (e, f, g, h); the third rank detected in 2 M NH_4HCO_3 (i, j, k, l); and the fourth rank detected in 500 mM KH_2PO_4 (m, n, o, p). The salt concentrations indicated in this work are for the protein samples prior to mixing with the MALDI matrixes. All analyte concentrations were always kept at 1 pmol. Peak intensity can be inferred from the coordinate in the upper right corner of each mass spectrum

washing steps on plate were carried out. This suggests that excess salts in crude egg white largely suppress analyte ionization. Therefore, the crude egg white samples were diluted 10 and 500 times, respectively, with 30% ACN/0.1% aqueous TFA (v/v) (TA30). The diluent solution and matrix solutions were mixed in 1:1 volume ratio and then 1 μL of mixture solution was deposited on the target. One wash with 0.1% TFA aqueous solution was made on each deposited sample to remove excess salt. Because matrix crystal might be decreased by the washing step, an additional 1 μL of pure matrix solution was used to cover the sample spot to form a uniform and dense crystal layer. In the egg white, four major proteins occupying more than 3.0% content are ovalbumin (54.0%, Mr ~45,000, pI = 4.5), ovoconalbumin (12%–13%, Mr ~77,700, pI = 6.0), ovomucoid (11.0%, Mr ~28,000, pI = 4.1), and lysozyme (3.4%–3.5%, Mr ~14,300, pI = 10.7) [43]. Figure 5a shows that three proteins were detected using SA as matrix for 10 times dilution of crude egg white. The *m/z* at 77,760, 44,542 and 14,295 are likely from ovoconalbumin, ovalbumin, and lysozyme, respectively. In contrast, apart from the comparable mass signals for the above-mentioned three proteins detected by SA matrix, an additional protein peak at *m/z* 47,989 along with a doubly charged protein ion were noted using CHCA-C3 (Figure 5c), which are likely from G2 ovoglobulin

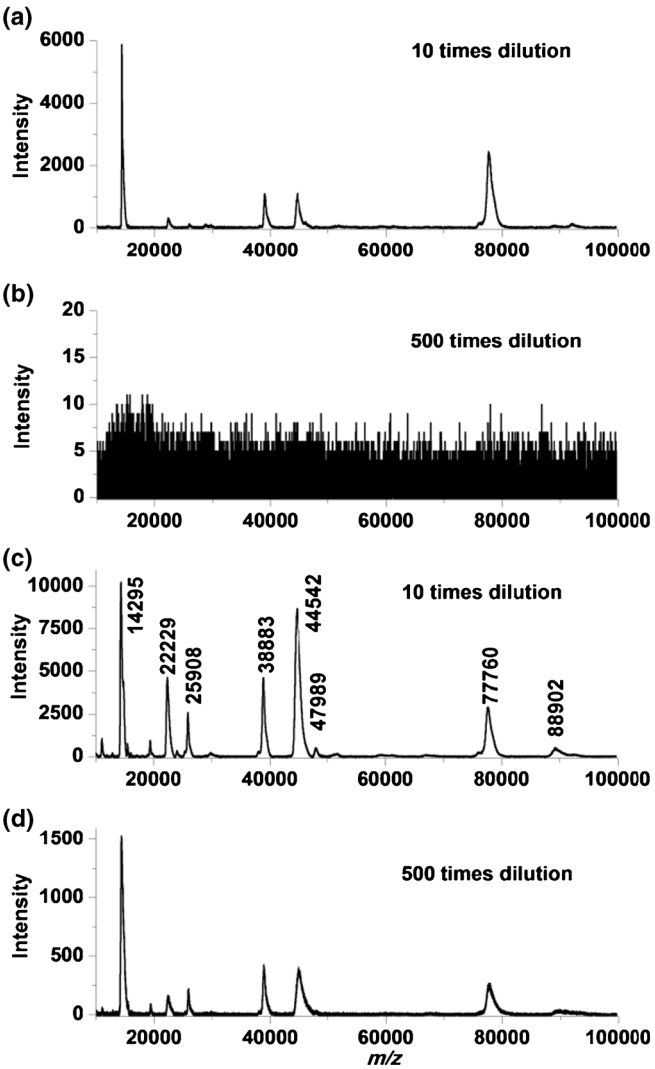


Figure 5. MALDI mass spectra of crude egg white with dilution 10 times using **(a)** SA, **(c)** CHCA-C3, and 500 times using **(b)** SA, **(d)** CHCA-C3 matrices. The *m/z* range is from 10 to 100 KDa. The *m/z* of corresponding peaks are labeled in **(c)**

(1.0%, pI = 4.9–5.3). The S/N ratio of protein signal corresponding to ovoconalbumin and lysozyme using CHCA-C3 are nearly identical to that obtained with SA. In the case of ovalbumin, the S/N ratio obtained with CHCA-C3 is 115.4 compared with 28.5 with SA. However, with 500 times dilution, no assignable signal could be detected using SA, while three proteins corresponding to ovoconalbumin, ovalbumin, and lysozyme with S/N ratio at 56.8, 22.5, and 15.4, respectively, were still clearly detected with CHCA-C3. Several repeating experiments for the egg white over the course of different hours were performed and the obtained spectra were nearly identical, indicating that the novel matrix CHCA-C3 was reproducible and sufficient to meet precise protein analysis.

Mechanism Analysis

The thermochemical properties (the proton affinity and reaction enthalpy) of esterified CHCA (from CHCA-C1 to CHCA-C6) and CHCA were investigated with B3LYP DFT molecular orbital approach in Gaussian 09 [44, 45]. (Table S2, see Supporting Information). The theoretical data demonstrated that the proton affinities of the esterified CHCAs were higher than that of CHCA, namely a lower transfer ability of proton. Furthermore, the reaction enthalpies of CHCA and esterified CHCAs had minimal difference (0.08 eV), indicating the same ability to get over the primary ionization step for all esterified CHCAs.

This inconsistency between the thermochemical properties and the practical MALDI-MS performance thus led us to obtain further insight into the mechanism of ionization processes of CHCA-C3. To this end, the MALDI mass spectrum of pure CHCA-C3 was recorded. As shown in Supplementary Figure S7a, not only the signals of protonated, sodiated, and potassiated CHCA-C3 but also protonated CHCA was observed. In order to prove whether the produced CHCA comes from esterified CHCA, protonated CHCA-C3 (*m/z*: 231.119) ion was selected to fragment in MS/MS mode with MALDI and ESI. The protonated signal peak of CHCA (*m/z* 190.057) was dominant and no protonated CHCA-C3 ion was observed in MALDI MS/MS spectrum (Supplementary Figure S7b). In the ESI MS/MS spectrum, there was only a very weak ion signal of protonated CHCA-C3 under a 7.0 eV collision energy (Figure S7c). These data demonstrated that CHCA-C3 can be easily fragmented into CHCA under considerably low energy. This transition from CHCA-C3 to CHCA can be explained to undergo a McLafferty-type rearrangement reaction in the gas phase [46] (i.e., a γ-hydrogen was transferred to the C-terminal carbonyl group to cause the elimination of an alkene). The active hydrogen on the carboxyl of CHCA produced through fragmentation has been proven to be transferred to analyte by deuterium labeling experiment [47]. Therefore, the ionization processes using CHCA-C3 as the matrix should involve both CHCA-C3 and fragmented CHCA, which endows the CHCA-C3 matrix with comparable ionization ability as CHCA in the gas phase.

It is generally accepted that the ionization in UV MALDI adopts a two-step framework (i.e., primary ionization upon laser ablation and secondary reactions in the desorbed plume that follows [48]). Although CHCA-C3 has no advantages on the secondary ionization step, the improved ablation ability along with relatively lower threshold energy for CHCA-C3 was observed compared with superDHB, SA, and CHCA, as shown in the above data and discussions. This means that the laser-induced cluster evaporation could free more matrix and analyte ions for the secondary ionization process, which may directly result in improved MALDI-MS performance toward intact protein. In MALDI-MS, the morphology of matrix-analyte co-crystal is also of great importance for obtaining a satisfied assay performance. An even-distributed and dense crystal would lead not only to good shot-to-shot reproducibility but also to the elimination of searching for a "hot-spot." Meanwhile, the data in Table 1 shows an increased sensitivity with SA and CHCA when using TL method instead of DD method. Thus, it is believed that the homogeneous CHCA-C3-

analyte co-crystal should partly contribute to the sensitivity improvement. Another benefit of using CHCA-C3 as matrix is its hydrophobic nature, which has resulted in high salt tolerance and robust sample washability in analysis of intact protein. In these cases, CHCA-C3 was proposed to have high affinity with protein, so that the protein analyte could be tightly embedded in the matrix crystal and subjected to effective ionization without the interference of salt or wash process. Additionally, it has been reported that the increased affinity between matrix molecules and analyte could dramatically improve the detection sensitivity in MALDI-MS [49–51]. Therefore, though the detailed mechanism is not yet clear, the improved MALDI-MS performance for analysis of intact protein using CHCA-C3 matrix should be generally ascribed to high laser-induced ablation ability, even-distributed matrix-analyte co-crystal, and increased affinity for protein.

Conclusions

Herein, a novel MALDI matrix CHCA-C3 has been developed to improve MALDI-MS performance for protein profiling with a broad mass range. The detection sensitivity of proteins with CHCA-C3 was improved 10 times compared with traditional matrices (i.e., superDHB, SA, and CHCA). Moreover, CHCA-C3 exhibited excellent salt tolerance and sample washability in the presence of high concentrations of salts, such as urea (8 M), NH_4HCO_3 (2 M), and KH_2PO_4 (500 mM). Based on the highly enhanced detection sensitivity and robust salt-tolerance, CHCA-C3 was successfuly used for MADLI profiling of crude egg white. A study to develop a more effective matrix based on the findings obtained is underway.

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