A New Method for Analysis of Disulfide-Containing Proteins by Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry

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A simple and high-throughput method for the identification of disulfide-containing peptides utilizing peptide-matrix adducts is described. Some commonly used matrices in MALDI mass spectrometry were found to specifically react with sulfhydryl groups within peptide, thus allowing the observation of the peptide-matrix adduct ion $[M + n + n' \text{ matrix} + H]^+$ or $[M + n + n' \text{ matrix} + Na]^+$ (n = the number of cysteine residues, $n' = 1, 2, \dots, n$) in MALDI mass spectra after chemical reduction of disulfide-linked peptides. Among several matrices tested, α -cyano-4-hydroxycinnamic acid (CHCA, molecular mass 189 Da) and α -cyano-3-hydroxycinnamic acid (3-HCCA) were found to be more effective for MALDI analysis of disulfide-containing peptides/proteins. Two reduced cysteines involved in a disulfide bridge resulted in a mass shift of 189 Da per cysteine, so the number of disulfide bonds could then be determined, while for the other matrices (sinapinic acid, ferulic acid, and caffeic acid), a similar addition reaction could not occur unless the reaction was carried out under alkaline conditions. The underlying mechanism of the reaction of the matrix addition at sulfhydryl groups is proposed, and several factors that might affect the formation of the peptide-matrix adducts were investigated. In general, this method is fast, effective, and robust to identify disulfide bonds in proteins/peptides. (J Am Soc Mass Spectrom 2009, 20, 2284–2293) © 2009 American Society for Mass Spectrometry

Post-translational modifications of protein are crucial to maintain its structure and fulfill the biological functions. Disulfide bond formation, a major determinant of maintaining the 3D structure of proteins, is one of the most notable post-translational modifications. The disulfide bridges in a protein cannot be predicted from its amino acid sequence; therefore, the determination of disulfide bonds in a protein will provide useful information on its 3D structure and stability, and contribute to the understanding of its structural-functional relationship [1–3]. There are a number of existing methods for the determination of disulfide bonds within proteins as described in the literatures. Among them, the NMR-based approach is efficient for analyzing disulfide-rich peptides [4–6]. However, it has the limitation of needing considerable amounts of the analytes. With the development of mass spectrometry, great improvements in speed and sensitivity for disulfide bond analysis have been made. The first report of insulin reduction on a mass spectrometry

probe appeared in 1986 [7]. The technique based on reduction during a mass spectrometry experiment was also employed by other groups [8, 9]. A widely accepted approach based on partial reduction and alkylation was reported [10–13]. The approach is laborious because most steps need optimizations.

Interestingly, it was found that 1,5-diaminonaphthalene (1,5-DAN) can reduce disulfide bonds in peptides in the laser plume [14, 15]. Using this feature of 1,5-DAN, disulfide linkages in the proteins were characterized by the comparison of mass differences between the reduced, partially reduced, and nonreduced molecular ions. However, in the interpretation of the obtained data, one must exercise extreme caution because 1,5-DAN can dramatically enhance in-source decay (ISD) fragmentation [16]. Recently, a screening method that uses ISD of disulfide bonds for identification of disulfide-linked peptides in mass spectra has been developed [17, 18]. Zhang and Kaltashov reported a method to detect disulfide-linked proteins using the broadband collision-activated dissociation (CAD) in the negative ion mode [19]. Selective detection of products of disulfide bond dissociation in the gas phase is difficult because of the presence of abundant ions produced by backbone cleav-

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age in broadband CAD spectra. Several recent studies have reported cleavages of the S–S or S–C bond at disulfide linkages using MALDI-TOF/TOF-MS, ESI-MS/MS, or LIFT-TOF/TOF-MS [20–26], in which a strategy for the selective elimination of hydrogen disulfide (H_2S_2) is widely employed. To enhance the selective cleavages, alkali and alkaline earth metal enolate complexes were used [20–23]. A novel analytical strategy developed by Xu used a new tandem MS database search program (MassMatrix) to identify disulfide-linked peptides [27]. The algorithm based on the statistical scoring model in MassMatrix facilitates the MS^n data processing.

In the mass spectrometric analysis of disulfide-linked peptides using MALDI-TOF-MS, we observed prominent peptide-matrix adduct ions when reduction of disulfide bonds by dithiothreitol (DTT) was performed on target plate. To further investigate this matrix addition phenomenon, we tested 12 other common matrices. A variety of factors including pH, the molar ratio of matrix to disulfide-linked peptide, and reducing reagents were also used. A mechanism of the observed adduction between sulfhydryls and α -, β -unsaturated matrices is proposed. To the best of our knowledge, this is the first detailed study on the matrix addition reaction as well as its application to the analysis of disulfide-containing proteins.

Experimental

Materials and Reagents

CHCA, 3-HCCA, sinapinic acid (SA), ferulic acid (FA), caffeic acid (CA), trans-3-indoleacrylic acid (IAA), dithranol (DT), 2-(4-hydroxyphenylazo)benzoic acid (HABA), 2,5-dihydroxybenzoic acid (DHB), 3-hydroxypicolinic acid (3-HPA), 2,4,6-trihydroxyacetophenone (THAP), 2,6-dihydroxyacetophenone (DHAP), 3-aminoquinolin, human insulin, oxytocin, and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma (St. Louis, MO, USA). Scheme 1 shows structures of all the tested matrices. Octreotide was supplied by GL Biochem Ltd. (Shanghai, China). Lysozyme was purchased from Sino-American Biotechnology Company. β -Mercaptoethanol (ME) was acquired from Dingguo Bio-Technology Co., Ltd (Beijing,

China). Trypsin (sequencing grade) and DTT were purchased from Promega (Madison, WI, USA). Acetonitrile (ACN, HPLC grade) was obtained from Fisher Chemical Company (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA, HPLC grade) and formic acid (HPLC grade) were bought from TEDIA Company (Fairfield, OH, USA). All other chemicals used in this study were analytical grade and were used without further purification. Ultrapure water (specific conductivity, $18.2 \text{ M}\Omega/\text{cm}$) was produced by a MilliQ device (Millipore, Milford, MA, USA).

Proteolytic Digestion

Human insulin and lysozyme were digested separately at 37°C by trypsin in $50 \text{ mM NH}_4\text{HCO}_3$ at pH 7.8. Enzyme/substrate ratio was adjusted to 1:50, and digestion was performed for 6 h. The digestion was terminated by freezing at -20°C without being adjusted to acidity.

Sample Preparation

Sample preparation for MALDI-MS. Sample concentration was adjusted to 1 mg/mL before analysis. The following compounds in $50 \text{ mM NH}_4\text{HCO}_3/\text{NH}_4\text{OH}$ (pH 9.0) were used as reductants: (1) DTT (40 mM), (2) TCEP (40 mM), (3) ME ($100\text{--}1000 \text{ mM}$). The following compounds were used as MALDI matrices: (1) CHCA, (2) 3-HCCA, (3) SA, (4) FA, (5) CA, (6) IAA, (7) DHB, (8) HABA, (9) THAP, (10) DHAP, (11) HPA, (12) DT, and (13) 3-aminoquinolin. All the matrices were used at a concentration of 45 mM in 50% water/acetonitrile except HABA (in ethanol) and DT (in tetrahydrofuran). On-target reduction was performed under following conditions: A $0.5 \mu\text{L}$ each of the tryptic digests of the proteins and the peptide solutions was mixed on the stainless steel target with a $0.5 \mu\text{L}$ aliquot of each of freshly prepared reductant solutions. After 20 min at room temperature, $0.5 \mu\text{L}$ matrix samples were spotted on the MALDI plate; 5% TFA water might be used to adjust the pH of analyte/reductant/matrix complex when needed. The mixture was dried at ambient temperature before MALDI analysis. For complete reduction of the disulfide bond-containing peptides in solu-

Scheme 1. Structures, abbreviations, and molecular weights of matrices used in this study.

tion, 50 μ L each of octreotide and oxytocin solutions were first mixed with the same volume of each reductant solution for 1 h at 37 °C and then incubated for different times (0.5–4 h) at 37 °C with 50 μ L or 100 μ L each of matrix solutions described above at final pH 2–8.5. Aliquots (1.5 μ L) were used for MALDI-MS analysis without further purification.

Sample preparation for HPLC-ESI-MS. An aqueous solution of octreotide (50 μ L; 1 mg/mL) was mixed with a solution (50 μ L) of DTT in 50 mM $\text{NH}_4\text{HCO}_3/\text{NH}_4\text{OH}$ buffer (pH 9.0). The final concentration of DTT was 20 mM, resulting in a molar ratio of DTT to octreotide of 41:1. After the mixed solution was incubated at 37 °C for 1 h, a solution of 45 mM CHCA in 50% water/acetonitrile (50 μ L) was added. The mixture was incubated at 37 °C for another 1 h. The molar ratio of CHCA to octreotide was 46:1. The peptide mixture was lyophilized and redissolved in 100 μ L of HPLC mobile phase for subsequent HPLC separation.

MALDI-MS

MALDI mass spectra were acquired on a MALDI-TOF mass spectrometer (Voyager DE-STR; Applied Biosystems, Foster City, CA, USA) equipped with a 337 nm nitrogen laser. The instrument was operated in positive ion reflectron mode. The accelerating voltage was 20 kV, while the grid voltage and delay time were set at 65% and 100 ns, respectively. The laser intensity, optimized for each sample, was attenuated to just above the threshold of ionization for each sample. The laser was fired at a frequency of 3 Hz, and spectra were recorded from the sum of 50 laser shots. Each preparation technique evaluated was prepared and examined in triplicate, with the best spectrum in terms of relative intensity of analyte used for comparison with other techniques. Spectra were externally calibrated using singly charged monoisotopic peaks of angiotensin I (1296.6853 Da), ACTH (clip 1–17) (2093.0867 Da), and ACTH (clip 18–39) (2465.1989 Da). Postacquisition processing of data was performed utilizing the Data Explorer software (Applied Biosystems).

HPLC-ESI-MS

High-performance liquid chromatography was performed on a Waters 2695 system (Milford, MA, USA) consisting of an automatic sampler, a column heater, and a quaternary high-pressure pump. Separations of analytes were carried out using a Diamonsil C_{18} column (250 mm $\times$ 4.6 mm i.d., 5 μ m; Dikma, Beijing, China). The chromatography was performed at 25 °C. The mobile phase consisted of acetonitrile/0.1% formic acid in water (20:80, vol/vol) at a flow rate of 0.5 mL/min. Mass spectrometric detection was carried out on a Finnigan LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with an ESI source. The spray voltage was set at 4.5 kV in the positive ion mode. The

capillary voltage was fixed at 30 V, and its temperature was maintained at 250 °C. Nitrogen was used as both the sheath gas, at a flow rate of 60 arb, and the auxiliary gas at a flow rate of 30 arb. Automated acquisition of mass spectra was executed by data-dependent scanning using Xcalibur software (Thermo Finnigan).

Results and Discussion

Observation of Adducts of Matrix and Sulfhydryl-Containing Peptide

In this study, we selected oxytocin (simplified as M_1) and octreotide (M_2) as examples, and the structures are listed in Scheme 2. Figure 1a and b present MALDI mass spectra of oxytocin with CHCA matrix before and after reduction by DTT, and Figure 1c and d correspond to those of octreotide, respectively. In Figure 1a, a base peak corresponds to $[\text{M}_1 + \text{Na}]^+$ at m/z 1029.47, and a weak one corresponds to $[\text{M}_1 + \text{K}]^+$ at m/z 1045.37; in Figure 1c, a base peak $[\text{M}_2 + \text{H}]^+$ at m/z 1019.42 and a minor peak $[\text{M}_2 + \text{Na}]^+$ at m/z 1041.37 are observed. A different situation occurred in Figure 1a and c. In the oxytocin case, the base peak is $[\text{M}_1 + \text{Na}]^+$, but the base peak is $[\text{M}_2 + \text{H}]^+$ for octreotide in Figure 1c. Comparing Figure 1a with 1b as well as Figure 1c with d, the 2-Da mass shift indicates the reduction of the disulfide bond.

Additionally, the matrix-peptide adducts are also observed as shown in Figure 1b and d. Comparison of the spectrum in Figure 1b with that in Figure 1a obtained in the absence of DTT reveals that the mass spectrum in Figure 1b contains three dominant new signals at m/z 1220.50, 1409.53, and 1387.54, which are attributed to $[\text{M}_1 + 2 + \text{CHCA} + \text{Na}]^+$, $[\text{M}_1 + 2 + 2 \text{CHCA} + \text{Na}]^+$, and $[\text{M}_1 + 2 + 2 \text{CHCA} + \text{H}]^+$, respectively. In Figure 1d, adduct ions $[\text{M}_2 + 2 + \text{CHCA} + \text{H}]^+$, $[\text{M}_2 + 2 + \text{CHCA} + \text{Na}]^+$, and $[\text{M}_2 + 2 + \text{CHCA} + \text{K}]^+$ at m/z 1210.47, 1232.44, and 1248.42, as well as $[\text{M}_2 + 2 + 2 \text{CHCA} + \text{H}]^+$ at m/z 1399.50 are also observed. The observation of $[\text{M}_{1(2)} + 2 + \text{CHCA} + \text{H}]^+$ is consistent with the result reported by Glocker's group [28, 29]. On-target reduction was performed with TCEP in the presence of the CHCA matrix in their reports. However, they only observed the adduct ion $[\text{peptide} + 2 + \text{CHCA} + \text{H}]^+$ and did not report the result with other matrices and the interpretation of the observed phenomenon. In an attempt to gain a better insight into this phenomenon, several other commonly used matrices were also tried. Among the matrices (see Scheme 1), we found that 3-HCCA, SA, FA, and CA were also able to form matrix-adducts of reduced forms of disulfide-containing peptides under pH 7.5 (Supplemental Information, Figure S-1, which can be found in the electronic version of this article). The data for the use of 3-HCCA as matrix are very similar to those for CHCA and not provided in this report.

Scheme 2. Structures and molecular weights of oxytocin, octreotide, insulin, and lysozyme examined in this study.

Generation of Peptide-Matrix Adducts in Solution or in Gas Phase

To clarify this question, we took octreotide as an example. Octreotide solution was treated with DTT and then incubated with CHCA, followed by LC/ESI-MS analysis. The LC/ESI-MS chromatogram of the incubation mixture is shown in Figure 2a. Two peaks, I and II, are obtained in Figure 2a. Figure 2b and c correspond to the mass spectra of peak I and II, respectively. As shown in Figure 2b and c, peak II corresponds to a pure compound, while peak I is a poorly separated one. The ESI mass spectrum of the fraction eluted at peak II (Figure 2c) shows a strong signal at m/z 1021.4 and a small signal at m/z 510.4, correspond-

ing to the singly charged ion and the doubly charged ion of the reduced octreotide, respectively. Peak I contains three components as indicated in Figure 2b. The most abundant ion in the full-scan mass spectrum of peak I (Figure 2b) is a singly charged adduct of reduced octreotide and one CHCA at m/z 1210.3. The two minor ions at m/z 1021.3 and 1399.3 correspond to the reduced octreotide, the adduct of reduced octreotide, and two CHCA. The evidence for the generation of peptide-matrix adducts in the solution phase during peptide reduction with DTT and incubation with CHCA was provided by LC/ESI-MS analysis.

It is commonly known among mass spectrometrists that matrix association is observed to some extent in the

Figure 1. MALDI mass spectra in positive ion mode of (a) oxytocin, (b) oxytocin after on-target reduction by DTT, (c) octreotide, and (d) octreotide after on-target reduction by DTT using CHCA as the matrix under pH 7.5. Signals labeled with an asterisk are assigned to peptide-matrix adducts. Signals corresponding to peptide-matrix 1:2 adducts are labeled with an open triangle.

Figure 2. (a) Total ion chromatogram of a solution containing 0.5 mg/mL of reduced octreotide incubated with 45 mM CHCA. (b) ESI mass spectrum associated with the TIC peak I. (c) ESI mass spectrum associated with the TIC peak II.

gas phase. The matrix adduct signals reported in the literature [30–32], most of which are formed as noncovalent species in the gas phase, are different from those observed in our report. The peptide-matrix adducts observed in our study are based on the interaction between the –SH group of Cys and an α , β -unsaturated matrix in solution.

The pH Dependence and Mechanism of the Generation of Peptide-Matrix Adducts

A solution of 0.5 μ L octreotide (1 mg/mL) after on-target reduction by DTT using the same volume of CHCA, SA, and CA as matrices under alkaline condition (pH 8.5) were recorded the MALDI mass spectra shown in Figure 3a–c, respectively. Moreover, the similar result was obtained for FA as matrix at the same pH (Supplemental Information, Figure S-2a). Curiously, in the case of IAA, although the peptide-matrix adduct

peak was not large relative to the base peak, it was present (Supplemental Information, Figure S-2b). Here, it should be noticed that no peptide-matrix adducts were observed for other matrices, even under strong alkaline conditions. Through comparison of mass spectrum in Figure 3a with that in Figure 1d, it can be readily found that the higher the pH, the higher the intensity of peptide-CHCA adducts. For the other matrices case, such as SA, CA, and FA, the matrix addition reaction seemed to be more sensitive to pH than the case of CHCA. Take cases of SA and CA; a dramatic increase of the relative abundance of not only $[M_2 + 2 + \text{matrix} + H]^+$ labeled with * (an asterisk), but also $[M_2 + 2 + 2 \text{ matrix} + H]^+$ labeled with Δ (an open triangle) is observed in Figure 3b and c.

The peptide-matrix adducts disappeared at pH < 2.5 for CHCA and pH < 6.5 for SA, FA, and CA. As for IAA, only a little adduct was observed even in extremely alkaline conditions owing to the structural difference. So alkaline conditions were favorable to the formation of peptide-matrix adducts.

Considering structures of the matrices and MS data, the following deduction can be made; this adduct formation is related to the carbon-carbon double bond adjacent to the carboxyl group. The present data appear to be consistent with current literature, which suggests alkylation of cysteine in proteins via reaction with α -, β -unsaturated compounds (notably acrylonitrile, acrylamide, and methyl acrylate) [33–37]. Based on our experimental data, we proposed a reaction mechanism of a free –SH group in proteins and an α -, β -unsaturated matrix as shown in Scheme 3. Because the proton affinity of cysteine is 216.1 kcal $\cdot$ mol $^{-1}$, which is rather basic [38], so a free –SH group of proteins releases a

Figure 3. MALDI mass spectra of octreotide obtained after on-target reduction by DTT using (a) CHCA, (b) SA, and (c) CA as the matrices at final pH 8.5. Signals labeled with an asterisk are assigned to peptide-matrix 1:1 adducts. Signals corresponding to peptide-matrix 1:2 adducts are labeled with an open triangle.

Scheme 3. A proposed reaction mechanism of a free $-SH$ group of proteins and an α -, β -unsaturated matrix.

proton only under alkaline condition. Then RS^- attacks at the double-bond of an α -, β -unsaturated matrix. Theoretically, it is difficult to have a nucleophilic addition reaction for an α -, β -unsaturated carboxylic acid. However, the nucleophilic addition reaction can occur if $-R''$ is an electrophilic group, like cyano radical $-CN$. CHCA and 3-HCCA are regarded as derivatives of acrylonitrile, which are favorable to the Michael addition reaction; thus distinct peptide-matrix adducts were observed using CHCA or 3-HCCA as a matrix. According to the reaction mechanism, the results of pH effect might be interpreted as follows: The nucleophilic addition reaction can occur easily at sulfhydryl group of cysteine under high pH due to easy release of a proton. Our experimental results show that the ability for modification of cysteine by the matrices tried is as follows: CHCA $\approx$ 3-HCCA $\gg$ SA $\approx$ FA $\approx$ CA.

Effect of Molar Ratios, Incubation Time, and Reductants

Solutions (pH 7.5) containing fixed concentration (1 mg/mL) of reduced oxytocin and two volumes (the same and 2-fold volumes of oxytocin solution) of each matrix were analyzed by MALDI-MS, i.e., the molar ratios of matrix/oxytocin of 46:1 and 92:1 were employed, and the representative spectra are shown in Figure 1b (46:1), Figure S-1a–c (46:1), Figure S-3 (92:1), and Figure 4 (92:1). From the above figures, an increase of the molar ratio of matrix to oxytocin significantly enhanced the intensities of adducts. As to IAA, under high pH and high molar ratios of matrix to peptide, it was hard to find the adduct signals (see Figure S-2b and Figure S-3c). We have conducted the experiment with different incubation times, which did not significantly affect the addition reaction.

Three common reductants for disulfide-containing peptides were evaluated in this work. When ME was used as reductant on target or in solution, peptide-matrix adducts were observed at the molar ratios of ME/oxytocin < 100 :1 (data not shown). Formation of mixed disulfides between the cysteinyl thiol-containing peptide and ME was dominant, and no matrix adducts were observed when higher molar ratios of ME to

disulfide-linked peptide (>150 :1) were used for reduction of disulfide bonds. This indicates that ME competes with an α -, β -unsaturated matrix for binding to the cysteinyl thiol in liquid phase. Our data support the report of Spiess et al., in which peptide-CHCA adduct ions were not observed at the molar ratio of ME/peptide > 150 :1 in solution [29]. Peptide-matrix adducts were observed after complete reduction of the disulfide-linked peptides in solution using TCEP or DTT, but ME in our study, because the reaction of cysteinyl thiol-containing peptide and ME, competed with the generation of peptide-matrix adduct. The former reaction may be faster than the later one, so the adducts were not observed when ME was used as a reductant. Therefore, the reductants, TCEP and DTT, were better than ME without interfering analysis of disulfide containing peptides. As a result, both TCEP and DTT are recommended to use for disulfide analysis.

Application for the Analysis of Disulfide-Rich Proteins

To prove its feasibility for identifying peptides with a disulfide linkage in complex mixtures, we examined the tryptic digests of insulin and lysozyme. Insulin is a small model protein comprised of two peptide chains with two inter-disulfide bond bridges in addition to an intra-disulfide bond on the A-chain (Scheme 2). The amino acid sequence of insulin contains two potential cleavage sites for trypsin, which is indicated by dashed lines in Scheme 2. The mass spectrum of the tryptic digests of insulin using CHCA as matrix is presented in Figure 5a, which shows two major monoisotopic peaks at m/z 859.47 and 4863.23. On-target reduction of human insulin with DTT followed by spotting 45 mM DHB solution yielded the MALDI-TOF spectrum in Figure 5b. In this case, the ion signal at m/z 4863.23 disappears, and two new ion signals for the peptide of the A-chain (A1–A21) containing 4 cysteines (m/z 2383.00) and the

Figure 4. MALDI mass spectra of oxytocin obtained after on-target reduction by DTT using (a) CHCA and (b) CA as the matrices at the molar ratio of matrix/oxytocin of 92:1 adopted final pH 7.5. Signals labeled with the asterisk are assigned to peptide-matrix 1:1 adducts. Signals corresponding to peptide-matrix 1:2 adducts are labeled with an open triangle.

Figure 5. MALDI mass spectra of insulin (a)–(c) and lysozyme (d) and (e) tryptic digests using (a) and (d) CHCA as matrix, (b) and (e) obtained after on-target reduction by DTT using DHB as matrix, and (c) and (f) obtained after on-target reduction by DTT using CHCA as matrix in alkaline conditions. Signals marked with an asterisk are assigned to peptide-CHCA 1:1 adducts. Signals corresponding to peptide-CHCA 1:2 adducts are labeled with an open triangle. The filled circle indicates the ion peaks of peptide-CHCA 1:3 adducts. The ion peak of peptide-CHCA 1:4 adduct is marked with an open square. Signals in the mass range between 2250 and 3250 are enhanced by three times to make a clear annotation. Signals in the mass range between 2220 and 3280 are enhanced by six times to make a clear annotation.

peptide of the B-chain (B1–B22) containing 2 cysteines (m/z 2487.23) are observed. Thus, all three disulfide bonds of insulin were reduced. Nevertheless, by using CHCA as a matrix in Figure 5c, the measured monoisotopic mass peak of m/z 2383.01 migrated to 3139.23 for $[M + H]^+$; and the resulting mass shift of 756 u indicates four cysteine residues. A further monoisotopic mass peak of m/z 2487.23 for $[M + H]^+$ is shifted by 378 Da, corresponding to two cysteine residues.

The mass spectra in Figure 5d–f clearly show the application of the method for mass spectrometric analysis of lysozyme. It has 129 amino acid residues containing eight cysteine residues, 17 potential cleavage sites for trypsin (Scheme 2). The observed and calculated masses of tryptic peptides related to disulfide bonds are listed in Table 1. As shown in Figure 5d–f, signals such as at m/z 289.2, 517.3, 874.4, 1045.6, 1428.7, 1676.8, and 1753.8 correspond to peptides that do not contain any cysteines. Comparing Figure 5d with Figure 5e, peaks at m/z 1168.52, 1515.72, and 3268.60 disappear, corresponding to disulfide-containing peptide fragments, and new ions at m/z 936.39 and 2337.16 are assigned to cysteine-containing peptide fragments. Comparing Figure 5f with 5e, we specifically screened for ion signals showing molecular mass shifts in increments of 189 u, corresponding to a CHCA addition to cysteine site. The fractions shown in Figure 5e and f both include a peak with a molecular mass of 2337 for $[M + H]^+$, which is shifted to m/z 2904.28 in Figure 5f, indicating three cysteine residues in the fragment. The peptide-matrix 1:1 adducts (labeled with an asterisk in the figure) are observed at m/z 439.17, 524.20, 1025.47,

1125.43, and 1457.70, which indicate that there is only one cysteine residue in each fragment, and the observed values are quite close to those shown in Table 1.

Peptide-matrix adducts were also observed for both insulin and lysozyme using SA, FA, or CA as a matrix, even though the intensities of the signals were lower than those of CHCA adducts. According to our data, CHCA is the best matrix for the identification of disulfide bonds in proteins by MALDI-MS analysis.

Conclusions

Identifying the presence of a disulfide linkage in peptides is an essential part of protein analysis. A convenient method is developed to identify the disulfide-containing protein/peptides by using a specific modification of protein sulfhydryl groups with α -, β -unsaturated matrices in on-target MALDI-MS analysis. The data presented here clearly demonstrate that CHCA and other commonly used matrices with α -, β -unsaturated carboxyl group can be used to form peptide-matrix adducts with disulfide-containing proteins/peptides after chemical reduction under alkaline conditions in the solution phase.

The on-target reduction method has the potential for simple and rapid identification of disulfide in peptides without resorting to the alkylation of disulfide bonds and HPLC separation. In the present study, we not only observed the formation of peptide-matrix adducts after chemical reduction in the MALDI mass spectra, but also discussed the mechanism of the generation of the adducts as well as the conditions to gain a better understanding and to broaden its applications. The evidence for the

Table 1. Disulfide-linked tryptic peptides generated from lysozyme before and after chemical reduction and cysteine residues with CHCA adducts

Peptide position	Peptide sequence	Measured mass (MH) ^a	Calculated mass (MH) ^a
cys 6-cys 127	GCR CELAAMK ^b	1168.52	1168.53
cys 30-cys 115	CK GYSLGNWVCAAK ^b	1515.72	1515.71
cys 64-cys 80	WWCNDGR NLCNIPCSALLSSDITASVNC ^b	3268.60	3268.56
115-116	CK ^c	250.15	250.12
126-128	GCR ^c	335.15	335.15
6-13	CELAAMK ^c	836.42	836.40
62-68	WWCNDGR ^c	936.39	936.38
22-33	GYSLGNWVCAAK ^c	1268.60	1268.61
74-96	NLCNIPCSALLSSDITASVNC ^c	2337.16	2337.12
115-116	CK+1 CHCA ^d	439.17	439.16
126-128	GCR+1 CHCA ^d	524.20	524.19
6-13	CELAAMK+1 CHCA ^d	1025.47	1025.44
62-68	WWCNDGR+1 CHCA ^d	1125.43	1125.42
22-33	GYSLGNWVCAAK+1 CHCA ^d	1457.70	1457.65
74-96	NLCNIPCSALLSSDITASVNC+1 CHCA ^d	2526.19	2526.16
74-96	NLCNIPCSALLSSDITASVNC+2 CHCA ^d	2715.24	2715.20
74-96	NLCNIPCSALLSSDITASVNC+3 CHCA ^d	2904.28	2904.24

^a Monoisotopic mass.

^b Disulfide-linked tryptic peptides from lysozyme.

^c The peptides containing cysteine residues derived from reducing disulfide-linked tryptic peptides.

^d Peptide-matrix adducts.

generation of peptide-matrix adducts occurring in solution phase instead of gas phase was provided. The mechanism of the nucleophilic addition reaction and the conditions to affect the formation of matrix adducts were proposed and studied. The pH of the solution is the most important factor to control the formation of the adducts.

All disulfide bonds in human insulin and lysozyme have been successfully examined using this new method. The number of disulfide bonds in peptides can be clearly deduced by the mass shift between the masses by using DHB matrix (or other matrices that cannot interact with protein sulfhydryl groups) and those by using CHCA matrix after reduction. The simplicity of the method makes it an attractive option for unequivocal identification of disulfide bonds in peptides/proteins.

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Appendix A Supplementary Material

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