Research Article

Journal of MASS SPECTROMETRY

Received: 3 September 2009 Accepted: 30 October 2009 Published online in Wiley Interscience: 24 November 2009

(www.interscience.com) DOI 10.1002/jms.1699

# High-throughput method for on-target performic acid oxidation of MALDI-deposited samples

**Brad J. Williams, William K. Russell and David H. Russell***

**An information-rich on-target performic acid oxidation method, which is compatible with alkylation for differentiation of free cysteine *versus* disulfide-containing peptides, is described. On-target oxidation is achieved using performic acid vapor to oxidize disulfide-containing peptides and/or small proteins on the matrix-assisted laser desorption/ionization (MALDI) sample deposits. The on-target oxidation method is preferred over solution-phase oxidation methods because (1) less sample handing is required, (2) oxidation throughput is drastically increased and (3) ion suppression effects are reduced because performic acid is not added directly to the MALDI spot. The utility of this method is demonstrated by simultaneous oxidation of multiple MALDI sample deposits containing model disulfide-linked peptides, intact bovine insulin and a bovine ribonuclease A proteolytic digest.** 

*Supporting information may be found in the online version of this article.*

**Keywords:** on-target oxidation; performic acid vapor; disulfide bonds; ribonuclease A; MALDI-MS

## Introduction

Disulfide bonds play important roles in stabilizing and maintaining native protein structure,[1,2] and mass spectrometry (MS) plays an increasingly important role in deciphering these processes,[3] especially protein folding/unfolding kinetics.[4–6] Disulfide bond locations within proteins can be determined using a variety of MS-based methods, including tandem MS,[7,8] database search algorithms,[9–12] chemical reduction,[13–15] chemical oxidation[16–18] and metal ion cleavage of disulfide bonds.[19,20] Xu and coworkers developed a tandem MS search engine to simplify database searches for disulfide-linked peptides fragment ion spectra,[12] but such spectra can be complicated by direct cleavage of the disulfide bond between interlinked disulfide peptides, which results in a limited number of sequence-informative fragment ions from each $\alpha$- and $\beta$-chain peptide forming the disulfide linkage.[7] Disulfide-linked peptides can also be identified through chemical reduction of the disulfide bond. For example, a reductive matrix-assisted laser desorption/ionization (MALDI) matrix, 1,5-diaminonapthalene (1,5-DAN),[14] was used by Quinton and coworkers to characterize disulfide-bridged peptides using liquid chromatography (LC) coupled off-line with MALDI-MS/MS.[15] This reductive MALDI matrix successfully reduces disulfide bonds during the MALDI process; however, 1,5-DAN also yields a high relative abundance of matrix clusters up to 1000 *m/z*, which complicates the analysis of low molecular weight disulfide and/or cysteine-containing peptides.

Chemical oxidation offers the following benefits over performing chemical reduction of the disulfide bond: (1) a 48-Da mass shift occurs per cysteine residue, (2) cysteic acid ($SO_3H$) enhances negative ion formation and (3) the performic acid simultaneously cleaves the disulfide bond and chemically modifies each peptide involved in the disulfide bridge. For example, performic acid oxidation has been used to study disulfide-containing proteins prior to proteolytic digestion and strong cation exchange (SCX) chromatography.[18] The oxidized cysteine-containing peptides undergo an elution shift owing to a decrease in peptide solution-phase charge state, because low charge state peptides are weakly retained on the SCX column. The resulting enriched oxidized cysteine-containing peptides are then subjected to off-line MALDI-MS/MS followed by peptide identification via database searching or *de novo* sequencing. This methodology utilizes a combination of oxidation, MS and database searching for analyzing the cleaved disulfide bonds of bovine serum albumin. Because performic acid cleaves the disulfide bonds prior to digestion, the native disulfide bond pattern is lost; therefore, it is important to maintain intact disulfide-linked peptides throughout proteolytic digestion to characterize the disulfide bond connectivity within proteins.

Although performic acid oxidation is widely used, there are several challenges associated with solution-phase oxidation methods. For example, typical solution-phase oxidation methods are performed on lyophilized peptide/protein samples using reaction times ranging from 2 to 4 h[18,21,22] and several steps are required following oxidation to remove the performic acid reagent prior to MS analysis.[18,21] Some solution-phase oxidation methods have decreased reaction times to 10–30 min for oxidation of cysteine/methionine-containing peptides; however, the additional clean-up steps following oxidation make this method less desirable.[17] On-target oxidation methods using solution-phase performic acid have also been successfully applied to MALDI spots containing peptides and/or proteolytic digests.[21]

* *Correspondence to: David H. Russell, Department of Chemistry, Texas A&M University, College Station, TX 77843, USA. E-mail: russell@mail.chem.tamu.edu*

*Department of Chemistry, Texas A&M University, College Station, TX 77843, USA*

For example, we showed that directly adding the performic acid solution to a prespotted MALDI deposit containing a bovine apotransferrin tryptic digest yields oxidized tryptic peptides; however, the signal-to-noise (S/N) ratios of the oxidized peptide negative ions decreased by fivefold because of ion suppression effects. The overall amino acid sequence coverage also decreased by half compared to the solution-phase oxidized sample. Thus, an on-target performic acid oxidation method that can mitigate these adverse effects would be highly beneficial. The ideal on-target oxidation method would offer the following characteristics over solution-phase oxidation methods: (1) simple experimental procedure with no post-oxidation sample cleanup; (2) decreased ion suppression effects following oxidation; (3) improved throughput over solution-phase methods and (4) maintain positive and negative ion mass spectral quality following on-target oxidation.

This report describes an on-target performic acid oxidation method using performic acid vapor to oxidize disulfide-containing peptides/proteins deposited for MALDI-MS analysis. Brown and Hartley have reported a similar approach using performic acid vapor to oxidize disulfide-linked peptides on electrophoresis paper.[23] The novel aspect of our method is found within the ability to successfully oxidize multiple MALDI spots simultaneously using performic acid vapor contained within a reaction chamber without sample cleanup. Reaction times for the on-target performic acid oxidation method (10–60 min) are also shorter than traditional solution-phase oxidation methods (2–4 h). We apply this on-target performic acid oxidation method to a model disulfide-linked peptide, intact bovine insulin and a proteolytic digest of bovine ribonuclease A. In addition, we discuss the advantages of this method over solution-phase oxidation methods and show the ability to elucidate disulfide-linked peptides from ribonuclease A.

## Experimental Section

### Chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise noted. Sequencing grade-modified trypsin was obtained from Promega (Madison, WI). Oxytocin (CYIQNCPLG-$NH_2$) was purchased from American Peptide Company (Sunnyvale, CA). Hydrogen peroxide (35% w/w) and formic acid (99% w/w) were obtained from Acros Organics (Morris Plains, NJ). HPLC grade acetone, acetonitrile ($CH_3CN$) and methanol ($CH_3OH$) were purchased from EMD Chemicals Inc. (Gibbstown, NJ). All experiments were performed with 18-MΩ water ($ddH_2O$), purified using a water purification unit (Barnstead International, Dubuque, IA).

### Sample preparation for MALDI-MS

A 15-μM stock solution of native oxytocin was prepared in 10 mM formic acid. Oxytocin was reduced by adding 2 μl of 50 mM tris(2-carboxyethyl)phosphine and incubating at 60 °C for 60 min. The synthetic mixture of native and reduced oxytocin (1 : 1 molar ratio of native : reduced oxytocin) was reacted with 2-iodoacetamide by increasing the solution pH from 3.0 to 7.1 with 40 μl of 25 mM ammonium bicarbonate ($NH_4HCO_3$) and incubated in the dark for 45 min at room temperature. The excess 2-iodoacetamide and $NH_4HCO_3$ was removed using a 10-μl $C_{18}$ resin pipette tip (Millipore, Billerica, MA) and eluted with a 60 : 40 $CH_3CN$ : $ddH_2O$ solution containing 0.1% formic acid. A 1-μl aliquot of the eluate was spotted as the overlayer (analyte deposited on prespotted MALDI matrix) to α-cyano-4-hydroxycinnamic acid (CHCA) matrix. CHCA was prepared at 5 mg/ml in a 2 : 3 $ddH_2O$ : $CH_3OH$ solution containing 10 mM dihydrogen ammonium phosphate ($NH_4H_2PO_4$) and 1 μl was prespotted using the underlayer method.[24]

Stock solutions of α-melanocyte stimulating hormone (Ac-SYSMEHFRWGKPV-$NH_2$) and α1-mating factor (WHWLQL) were prepared at 10 μM in 10 mM formic acid. The resulting solutions were then mixed 1 : 1 (v/v) with $CH_3OH$ and 1 μl (5 pmol) was spotted onto the MALDI target as an overlayer to CHCA matrix. These MALDI deposits were initially oxidized using the on-target oxidation methods described below (−20 °C for 60 min). Additional oxidation was performed on the same MALDI deposits using room temperature (+24 °C) oxidation for an additional 60 min.

A stock solution (1 mg/ml) of bovine insulin was diluted to 10 μM in 10 mM formic acid. The insulin solution was then mixed 1 : 1 (v/v) with $CH_3OH$ and 1 μl (5 pmol) was spotted onto the MALDI target as an overlayer to prespotted MALDI matrix. The on-target oxidation experiments using intact insulin were performed with three different MALDI matrices: CHCA, 2,4-dihydroxyacetophenone (2,4-DHAP) and 2,4-DHAP combined with CHCA as dual MALDI matrices. For the experiments with CHCA and 2,4-DHAP (10 mg/ml in 2 : 3 $ddH_2O$ : $CH_3OH$ with 10 mM $NH_4H_2PO_4$), the prespotted MALDI deposit was re-dissolved with an overlayer of insulin (1 μl of 5 μM insulin in 1 : 1 (v/v) $CH_3OH$ : 10 mM formic acid). For the dual MALDI matrices experiment, 2,4-DHAP was spotted as an underlayer followed by an overlayer of insulin, then subjected to on-target oxidation. Following oxidation, the 2,4-DHAP + insulin sample deposit was re-dissolved with an overlayer of CHCA prior to MS analysis. The intact insulin experiments with the varied MALDI matrices were oxidized using the on-target performic acid oxidation methods described below, with the exception of performing oxidation at room temperature (+23 °C) for 60 min.

### Ribonuclease A proteolytic digestion

Dual protease digestion (trypsin followed by chymotrypsin) of bovine ribonuclease A (RNase A) was performed using the methods described by Xu and coworkers with minor modifications.[12] Briefly, RNase A was dissolved at 1 mg/ml in a 25 mM ammonium acetate buffer adjusted to pH 6.0 with 10 mM formic acid. RNase A was initially digested with trypsin [1 : 50 (w/w) enzyme : protein ratio] for 4 h at 37 °C followed by chymotrypsin addition (1 : 50 (w/w) enzyme : protein ratio) and incubated for an additional 4 h. The RNase A proteolytic digest was alkylated following proteolytic digestion with 5 μl of 100 mM 2-iodoacetamide and incubated in the dark for 45 min at room temperature. Following alkylation, the sample was subjected to cleanup using a 10-μl $C_{18}$ resin pipette tip. The RNase A proteolytic digest was eluted with a 60 : 40 $CH_3CN$ : $ddH_2O$ solution containing 0.1% formic acid and 1 μl of the eluate was spotted as the overlayer to CHCA matrix.

### On-target and solution-phase performic acid oxidation

Performic acid was prepared using hydrogen peroxide (35% w/w) and formic acid (99% w/w) mixed at a 1 : 9 ratio, respectively, and allowed to age at room temperature for 2 h.[21] On-target performic acid oxidation using performic acid vapor was performed by placing 40 μl of a 1 : 1 (v/v) mixture of acetone : performic acid directly on the MALDI target in an area that does not contain sample (distance from the performic acid reagent to the MALDI sample deposit is not critical). The MALDI target was

Journal of MASS SPECTROMETRY

then covered in a plastic petri dish and placed in a −20 °C freezer for 10–60 min. Following on-target performic acid oxidation, the MALDI plate was allowed to reach ambient temperature then subjected to MALDI analysis without sample cleanup. Solution-phase performic acid oxidation was performed using a 1 : 19 ratio of hydrogen peroxide : formic acid as previously described.[25] The performic acid solution was used to resuspend vacuum-dried peptide samples or RNase A proteolytic digests then allowed to react at 0 °C for 4 h. Following oxidation, samples were diluted with 500 µl of $ddH_2O$ and vacuum dried. Samples were then resuspended in $ddH_2O$ and mixed 1 : 1 (v/v) with $CH_3OH$ prior to spotting on the MALDI target as an overlayer.

### MALDI-MS and MALDI-MS/MS

All MALDI-MS experiments were performed using a model 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Framingham, MA). The MALDI-MS data were acquired using the reflectron detector in both positive and negative ion modes using 1200 laser shots with external calibrants: bradykinin (2–9) (PPGFSPFR) and adrenocorticotropic hormone (ACTH) (18–39) (RPVKVYPNGAENESAEAFPLEF). Two different instrument parameters were used for MS experiments with peptides/proteolytic digests (500–4500 Da, 2100 Da focus mass) and intact bovine insulin (700–6000 Da, 3500 Da focus mass). The nonoxidized forms of RNase A proteolytic peptides, $^{118}$YPNCAY$^{123}$ and $^{73}$VHESLADVQAVCSQK$^{87}$ were used to internally calibrate the negative ion mass spectrum of the RNase A proteolytic digest. Collision-induced dissociation (CID) tandem MS spectra were acquired using 20% greater laser power than the MS spectra acquisition with atmosphere (medium pressure) as the collision gas with 1 kV of collision energy. The average coefficient of variation (CV) was calculated to be ±6% for triplicate analysis of an RNase A proteolytic digest subjected to on-target oxidation. Normalized ion signals were determined using a ratio of the single peptide ion count to the total ion count for five oxidized RNase A peptides. The normalized ion signals were then used to calculate the CV.

## Results and Discussion

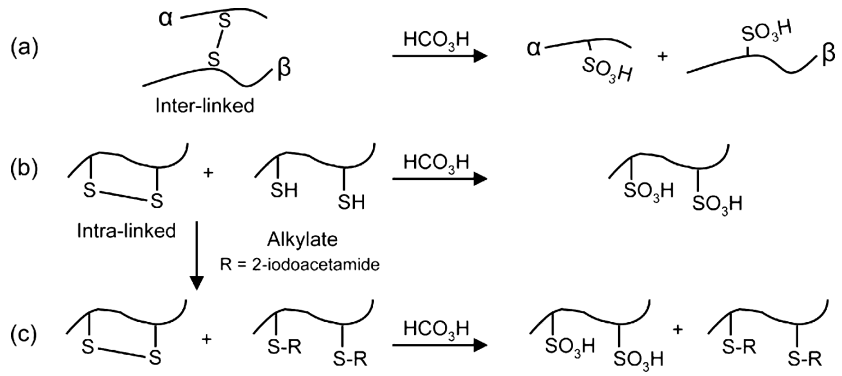
The main motivation of this work is to develop a method to simultaneously scan complex mixtures for disulfide-linked peptides and be able to distinguish between free cysteine and bridged disulfide-containing peptides. Our strategy is to use thiol alkylation chemistry followed by on-target performic acid oxidation. Scheme 1 illustrates the resulting performic acid oxidation products for both (a) interlinked and (b) intralinked disulfide-containing peptides. For example, an interlinked disulfide-bridged peptide will form oxidized products for both $\alpha$- and $\beta$-chain peptides (Scheme 1(a)). The performic acid cleaves the disulfide bond to convert each cysteine thiol to cysteic acid ($SO_3H$), whereas an intralinked disulfide-containing peptide will form a single peptide containing two cysteic acid residues (Scheme 1(b)). Note that a peptide containing two free thiol side chains will yield the same product as Scheme 1(b). Incorporating an alkylation reagent, such as 2-iodoacetamide, into the overall reaction scheme provides differentiation between peptides with two free cysteine residues or an intralinked disulfide bond. The carbamidomethyl (CAM) group protects the free cysteine residues from oxidation, while the intralinked disulfide-bridged peptide will be fully oxidized (Scheme 1(c)).

The disulfide-containing peptide oxytocin (CYIQNCPLG-$NH_2$) is used to illustrate the reaction shown in Scheme 1(c), and a synthetic mixture of native and chemically reduced oxytocin is used to show the mixture described in Scheme 1(b). The synthetic mixture of native and reduced oxytocin was reacted with 2-iodoacetamide, and Fig. 1 contains (a) positive and (b) negative ion MALDI-MS spectra for this sample taken after on-target performic acid oxidation of the native and CAM-modified oxytocin mixture. Ion signals for native oxytocin (*m/z* 1007.48 for $[M + H]^+$) and signals for alkylation products (*m/z* 1066.53 for $[M + H + CAM]^+$ and *m/z* 1123.55 for $[M + H + 2CAM]^+$) are observed in the positive ion mass spectrum after oxidation (Fig. 1(a)). These assignments were confirmed by tandem MS (Supporting Information, Fig. S1(b,c)). The intact disulfide bond of native oxytocin is cleaved and converted to cysteic acid, as evidenced by the $[M - H + O_6]^-$ ion (*m/z* 1103.37) in the negative ion mass spectrum (Fig. 1(b)). The negative ion tandem mass spectrum for fully oxidized oxytocin can be found in Supporting Information, Fig. S1(a). Following oxidation, $[M + H]^+$ ions of native oxytocin are still observed in the positive ion mass spectrum (Fig. 1(a)), which suggests an incomplete oxidation of native oxytocin. Experiments are currently underway to address the issues related to incomplete oxidation, and preliminary data for these on-target oxidation optimization studies are discussed below. Despite incomplete conversion to the fully CAM-modified oxytocin, ion signals for $[M + H + CAM + O_3]^+$ or $[M - H + CAM + O_3]^-$ are not observed in the positive or negative ion mass spectra. Therefore, the single CAM-modified oxytocin does not complicate the differentiation between a peptide that contains two free cysteine residues from one that contains an intralinked disulfide bond. Nonetheless, the 2-iodoacetamide alkylation chemistry incorporated into Scheme 1(c) allows a multiple free cysteine-containing peptide to be differentiated from an intralinked disulfide-bridged peptide such as oxytocin.

The effect of on-target oxidation on peptides that contain other oxidizable amino acids, such as histidine, methionine, tryptophan and tyrosine, was investigated using $\alpha$-melanocyte stimulating hormone (Ac-SYSMEHFRWGKPV-$NH_2$) and $\alpha$1-mating factor (WHWLQL). Figure 2(a) contains a positive ion mass spectrum for $\alpha$-melanocyte stimulating hormone taken before oxidation. Note that several oxidized peptide ion signals ($[M + H + O]^+$, $[M + H + O_2]^+$, etc.) are present in this spectrum. These oxidized ion signals are a result of oxidation owing to air exposure over time. Following on-target oxidation (1 h at −20 °C) (Fig. 2(b)), the intensities of the oxidized peptide ion signals do not increase relative to the $[M + H]^+$ ion, thus it appears that on-target oxidation does not significantly oxidize other amino acids under cold oxidation conditions. The same MALDI sample deposit was subjected to an additional hour of oxidation at +24 °C and the abundance of the oxidized ion signals do not increase relative to the $[M + H]^+$ ion signal for $\alpha$-melanocyte stimulating hormone, thus it appears that oxidation is not occurring (Fig. 2(c)). As a control experiment, native oxytocin (CYIQNCPLG-$NH_2$) was spotted on a separate sample deposit and oxidized simultaneously on the same MALDI target, and the MALDI mass spectrum from this sample contains a strong signal for the $[M - H + O_6]^-$ ion (*m/z* 1103.44) (Fig. 2(d)), indicating the oxidation reaction was successful. Similar experiments for $\alpha$1-mating factor also resulted in a minimal increase in the relative abundance of the oxidized peptide ion signals as a result of oxidation (see Supporting Information, Fig. S2), thus we conclude that the on-target oxidation






**Scheme 1.** Performic acid oxidation products of (a) interlinked and (b) intralinked disulfide-linked peptides, and (c) a mixture of an intralinked disulfide-linked peptide and the 2-iodoacetamide alkylated form.

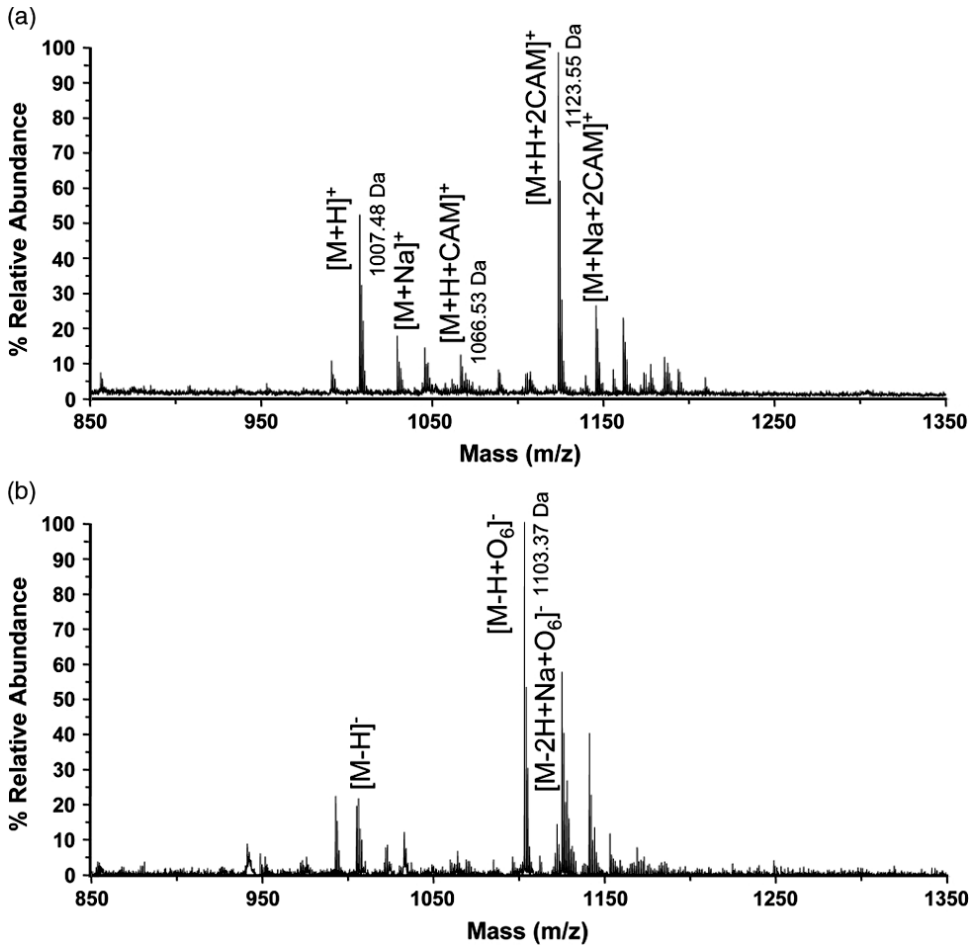



**Figure 1.** (a) Positive ion and (b) negative ion mass spectrum of native and CAM-modified oxytocin after 10 min of on-target performic oxidation performed at −20 °C.

is not complicated by reactions with H, M, W or Y amino acid containing peptides.

Bovine insulin is an excellent model system to illustrate on-target performic acid oxidation of disulfide-containing proteins, because it contains three disulfide bonds, two interchain disulfide bonds, which link the $\alpha$- and $\beta$-chains, and one intrachain disulfide bond within the $\alpha$-chain (see structure). Performic acid should cleave both the intra- and interlinked disulfide bonds to fully oxidize both $\alpha$- and $\beta$-chains.

GIVEQCCASVCSLYQLENYCN α-chain
FVNQHLCGSHLVEALYLVCGERGFFYTPKA β-chain

Figure 3 contains the negative ion mass spectra of oxidized intact insulin obtained using several different sample preparation methods. The spectrum contained in Fig. 3(a) contains a strong ion signal for the fully oxidized $\beta$-chain $[\beta-H+O_6]^-$ at $m/z$ 3492.83, but under these conditions ion signals for the fully oxidized insulin $\alpha$-chain are not observed in either positive or negative ion spectra. On

Journal of MASS SPECTROMETRY

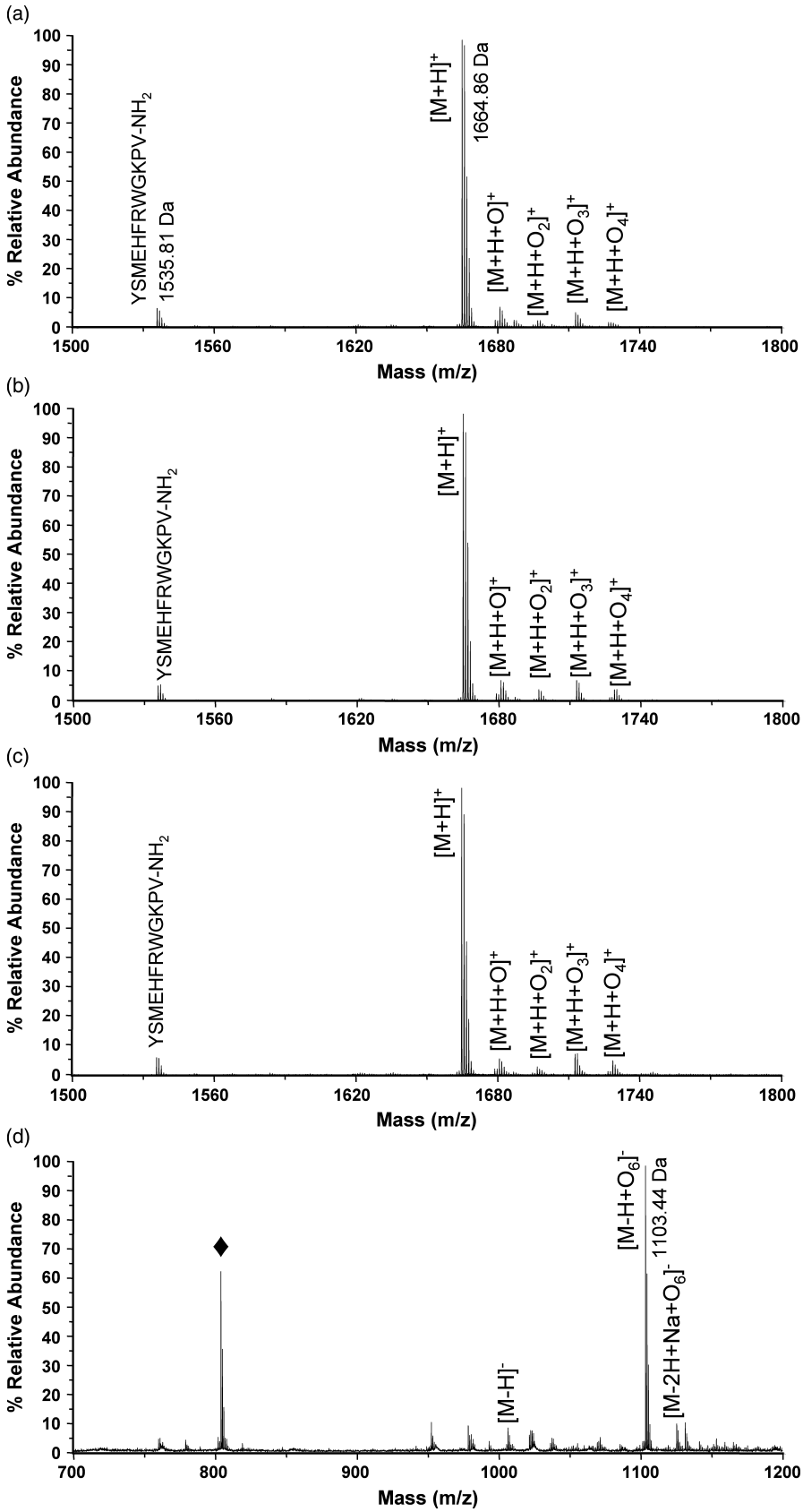



**Figure 2.** Typical positive ion mass spectra for $\alpha$-melanocyte stimulating hormone (Ac-SYSMEHFRWGKPV-$NH_2$) (a) before and (b) after an hour of on-target performic acid oxidation at $-20\,^{\circ}C$. Panel (c) represents an additional hour of on-target oxidation performed on the same sample deposit at $+24\,^{\circ}C$. Panel (d) contains a negative ion mass spectrum of a control experiment performed using native oxytocin (CYIQNCPLG-$NH_2$) oxidized in parallel with $\alpha$-melanocyte stimulating hormone on the same MALDI target. Note the change in the *m/z* scale because of the low molecular weight of oxidized oxytocin. Ion signals denoted with closed diamonds (♦) represent contaminate ions arising from the CHCA matrix.



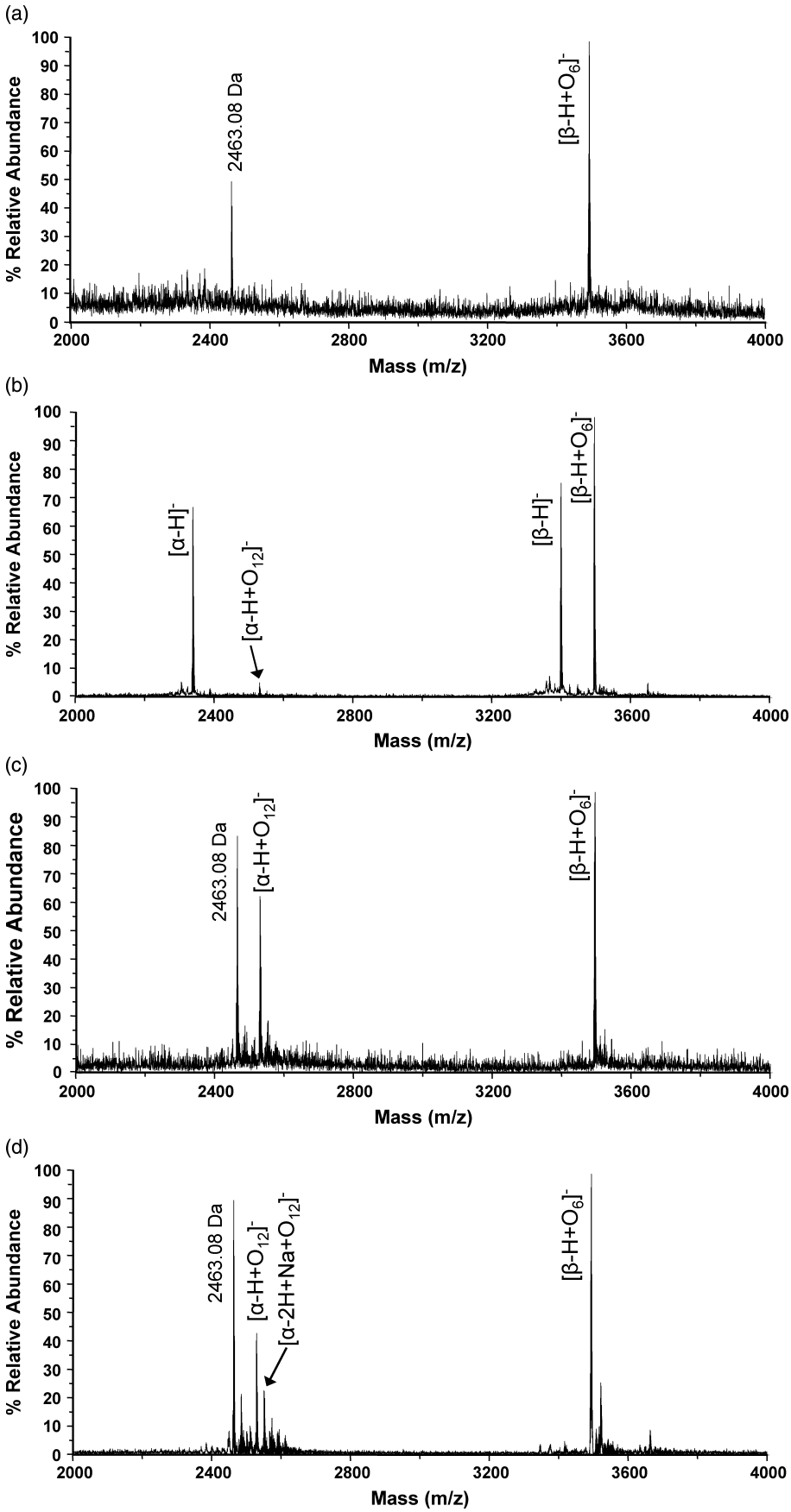



**Figure 3.** Negative ion mass spectra after on-target oxidation of (a) intact insulin and (b) chemically reduced insulin using CHCA as the MALDI matrix during oxidation. Panel (c) 2,4-DHAP was used as the MALDI matrix during on-target oxidation of intact insulin. Panel (d) 2,4-DHAP was used during oxidation followed by an overlayer addition of CHCA, which we found to improve the yield of negative ions.

Journal of MASS SPECTROMETRY

**Table 1.** Summary of negative ion MALDI-MS data resulting from on-target performic acid oxidation of RNase A tryptic/chymotryptic peptides

| Label | Peptide sequence | $[M-H]^-_{obs}$ | $[M-H]^-_{calc}$ | Mass error |
|---|---|---|---|---|
| | | | | ppm |
| A | $^{88}$NVA***C***K$^{92}$ + $O_3$ | 580.2236 | 580.2398 | −28 |
| B | $^{82}$AV***C***SQK$^{87}$ + $O_3$ | 681.2732 | 681.2875 | −21 |
| C | $^{52}$***C***NQ***MM***$^{56}$ + $O_7$ | 736.1478 | 736.1585 | −15 |
| D | $^{106}$SITD***C***R$^{111}$ + $O_3$ | 740.2736 | 740.2883 | −20 |
| E | $^{118}$YPN***C***AY$^{123}$ + $O_3$ | 776.2419 | 776.2559 | −18 |
| F | $^{93}$NGQTN***C***Y$^{99}$ + $O_3$ | 845.2562 | 845.2733 | −20 |
| G | $^{66}$***C***KPVNTF$^{72}$ + $O_3$ | 854.3588 | 854.3709 | −14 |
| H | $^{52}$***C***NQ***MM***K$^{57}$ + $O_7$ | 864.2386 | 864.2535 | −17 |
| I | $^{131}$HIIVA***C***EGNPY$^{141}$ + $O_3$ | 1261.5394 | 1261.5521 | −10 |
| J | $^{73}$VHESLADVQAV***C***SQK$^{87}$ + $O_3$ | 1659.7649 | 1659.7646 | 0 |

the other hand, ion signals at *m/z* 2463.08 are most likely partially oxidized $\alpha$-chain, $[\alpha - H + O_9 - 17]^-_{calc}$ = 2463.89 Da (−333 ppm mass error) or $[\alpha - H + O_8]^-_{calc}$ = 2463.94 Da (−353 ppm mass error), but we were unable to confirm these assignments by using tandem MS owing to the low abundance of the signals. Note that oxidation of chemically reduced insulin yields abundant ion signals for both $\alpha$- and $\beta$-chains, i.e. the fully oxidized insulin $\alpha$-chain $[\alpha - H + O_{12}]^-$ ion at *m/z* 2528.67 and $\beta$-chain $[\beta - H + O_6]^-$ ion at *m/z* 3492.34 are observed in the negative ion mass spectrum (Fig. 3(b)).

Figure 3(c) contains the negative ion mass spectra for oxidized intact insulin from 2,4-DHAP, and Fig. 3(d) contains similar spectra obtained from 2,4-DHAP prepared using an overlayer of CHCA. When on-target oxidation is performed on deposits of 2,4-DHAP, the fully oxidized insulin $\alpha$-chain $[\alpha - H + O_{12}]^-$ and $\beta$-chain $[\beta - H + O_6]^-$ ions are observed in the negative ion mass spectrum (Fig. 3(b)). Note that the negative ion yields for both $\alpha$- and $\beta$-chain oxidized forms are enhanced using 2,4-DHAP/CHCA sample preparation. The signal-to-noise (S/N) ratio of the oxidized insulin $\alpha$-chain increases 1.5-fold for the dual MALDI matrix method relative to using 2,4-DHAP as a MALDI matrix alone.

It appears that efficient oxidation of intact insulin to form oxidized $\alpha$- and $\beta$-chains is related to greater accessibility of the performic acid vapor to oxidize the insulin molecules within the 2,4-DHAP crystals. The presence of non-oxidized intact insulin ions following oxidation could indicate that insulin molecules are trapped within CHCA crystals, probably as inclusion complexes,[26] whereas with 2,4-DHAP, the insulin molecules are located on the 2,4-DHAP crystal surface and become more susceptible to oxidation (Fig. 3(c)). Further investigation of different MALDI matrices used for the on-target performic acid oxidation method may provide additional insight to explain why some cysteine and/or disulfide-containing peptides are more readily oxidized.[27]

RNase A is an excellent model protein to study disulfide bonds in proteins because it contains eight cysteine residues, each present as disulfide bonds.[12,17,25] RNase A contains four native disulfide bonds, three disulfide bonds are interlinked and the other disulfide bond is intralinked. Our experimental design involves performing proteolytic digestion under mildly acidic conditions (pH 6) to minimize disulfide scrambling[12] combined with 2-iodoacetamide alkylation chemistry to protect the free cysteine residues (i.e. disulfide-linked peptides that may become reduced during digestion) from oxidation. Following proteolytic digestion, the intact disulfide-containing peptides are then subjected to on-target performic acid oxidation, where the disulfide bond(s) are cleaved to form two peptides each containing a cysteic acid side chain (Scheme 1(a)). Each oxidized peptide chain is then subjected to tandem MS, where the amino acid sequences of the two peptide chains forming the disulfide bond can be determined.

Figure 4 contains negative ion mass spectra for the RNase A trypsin/chymotrypsin digest + 2-iodoacetamide acquired (a) before oxidation, (b) after on-target and (c) solution-phase oxidation. Fourteen new peptide ion signals resulting from performic acid oxidation are observed and those confirmed by tandem MS are labeled A–J (Fig. 4(b)), while the four additional ion signals too low in abundance to characterize by tandem MS are labeled with asterisks (*). The tandem MS confirmed amino acid sequences of the oxidized RNase A peptides are summarized in Table 1 and the corresponding negative ion tandem mass spectra are included in Supporting Information, Fig. S4. Several cysteine-containing peptides $^{52}$***C***NQ***MM***K$^{57}$, $^{93}$NGQTN***C***Y$^{99}$ and $^{88}$NVA***C***K$^{92}$ only appear after oxidation, which is most likely a result of oxidative cleavage of the intact disulfide-linked peptides. The oxidized amino acid residues are denoted in *bold italics*. Proteolytic peptides containing methionine residues (e.g. $^{52}$***C***NQ***MM***K$^{57}$ and $^{52}$***C***NQ***MM***$^{56}$) were also oxidized owing to the incorporation of two additional oxygen atoms per methionine residue. Ten total oxidized peptides accounting for all eight RNase A cysteine residues involved in disulfide bonds were detected using on-target oxidation, whereas a total of eight (six of these peptides are involved in disulfide bridging) oxidized RNase A peptides were detected using solution-phase oxidation. Comparing the mass spectral quality of the on-target (Fig. 4(b)) and solution-phase oxidation (Fig. 4(b)) method clearly illustrates an advantage of the on-target oxidation method. At first glance, the on-target oxidation method (Fig. 4(b)) appears to contain additional chemical noise above *m/z* 1000; however, the majority of these ion signals correspond to $[M - H]^-$ ions for intact disulfide-linked peptides that are present after oxidation. As for the solution-phase oxidation method (Fig. 4(c)), these ion signals from *m/z* 1000–2000 are not observed because these intact disulfide-linked peptides have undergone oxidative cleavage to produce small oxidized cysteine-containing peptides. In terms of S/N improvement, the S/N ratios for the labeled ion signals increase an average 2.5-fold for the on-target oxidation method relative to the solution-phase oxidation method. A drastic S/N enhancement occurs for peptide I ($^{131}$HIIVA***C***EGNPY$^{141}$ + $O_3$), where the ratio of the S/N values for the on-target oxidation method improves *ca.* sixfold over the solution-phase oxidation method. The decrease in relative abundance for the solution-phase oxidation method could be



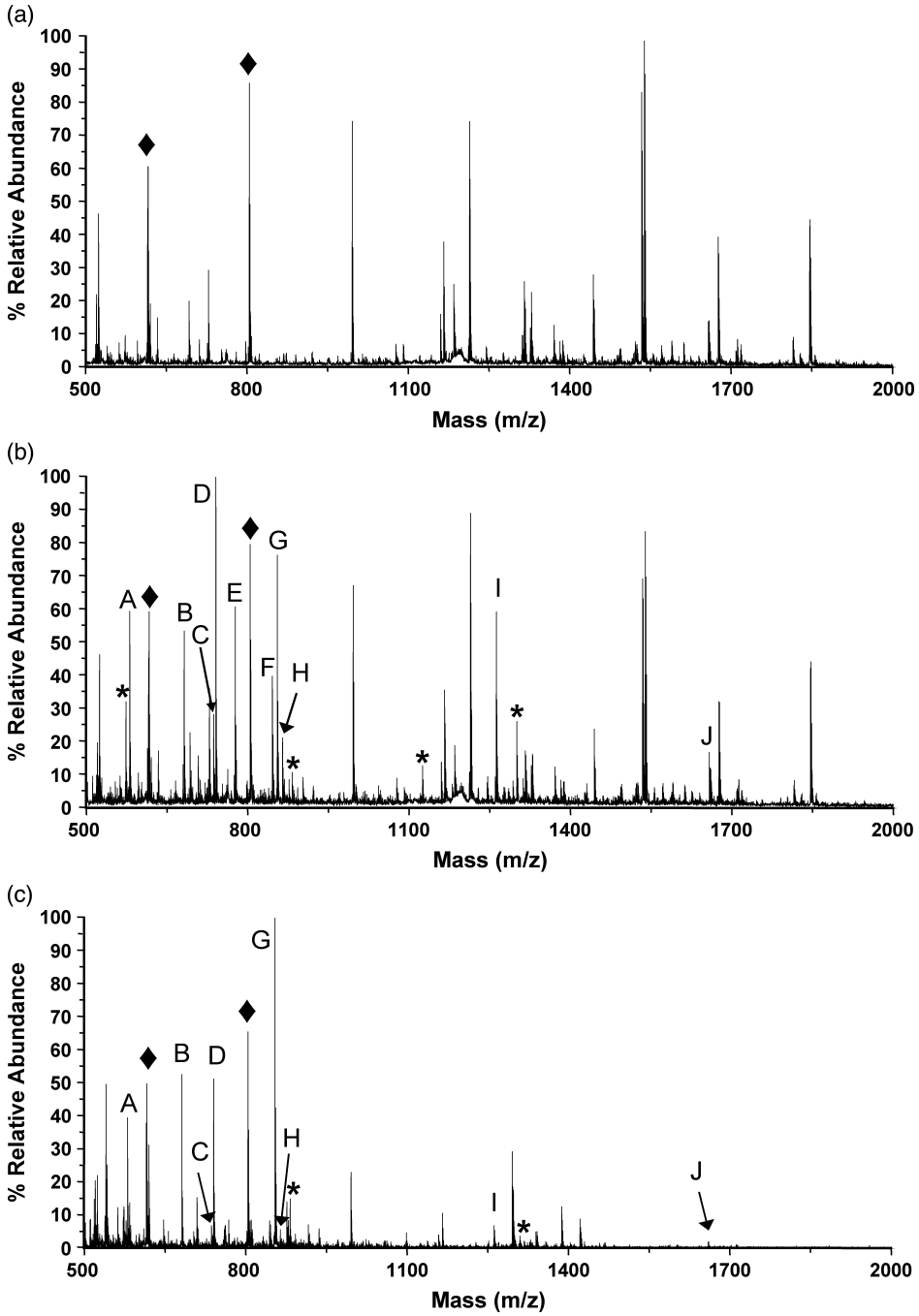


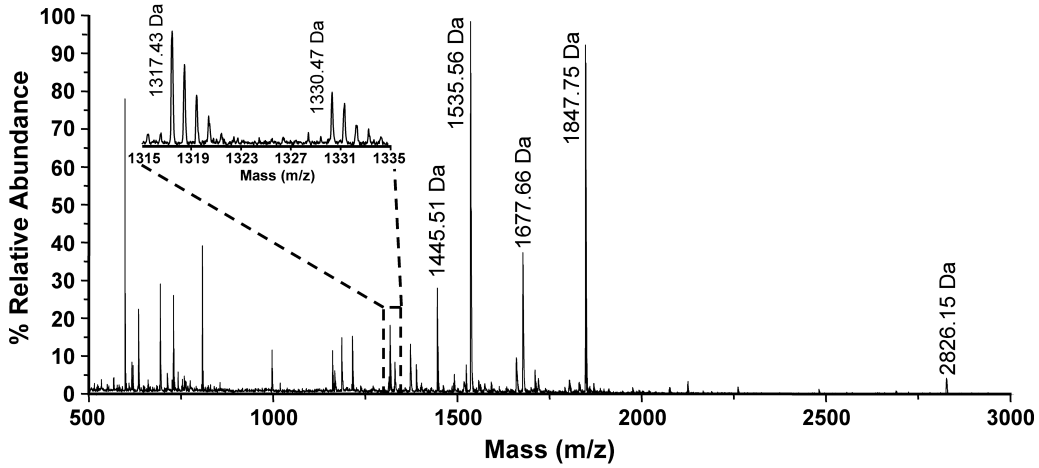

**Figure 4.** Negative ion mass spectra for an RNase A trypsin/chymotrypsin + 2-iodoacetamide digest (a) before and (b) after on-target performic acid oxidation and (c) solution-phase oxidation. For this example, the on-target oxidation was performed for 1 h at −20 °C and the solution-phase oxidation was performed for 4 h at 0 °C. The identities of the peptide ion signals labeled A–J are found in Table 1. The peptides denoted with asterisks (*) correspond to new peptide ion signals resulting from performic acid oxidation not confirmed by tandem MS. The peptide ion signals denoted with closed diamonds (♦) indicate chemical contaminates arising from the CHCA matrix.

attributed to ion suppression effects or sample loss associated with post-oxidation vacuum drying prior to MS analysis. Overall, the RNase A negative ion MS data suggest that the on-target oxidation method is suitable for proteolytic protein digests.

Several disulfide-linked peptides can be elucidated from the observed oxidized cysteine-containing peptides summarized in Table 1. The correlation of the non-oxidized peptide masses and the calculated disulfide-linked peptide masses is shown in Fig. 5(b). The peptides are arranged according to increasing mass to form an array of calculated disulfide-linked peptide masses. All the different disulfide-linked peptide bridging possibilities are calculated from the list of peptide *m/z* values accounting for the mass difference of an interlinked disulfide bond. The calculated disulfide-linked peptide masses that match an ion signal present in the positive ion mass spectrum of the oxidized RNase A trypsin/chymotrypsin digest (Fig. 5(a)) are indicated with a box around the number. For example, an ion signal at *m/z* 1535.56 is present in the positive ion mass spectrum that corresponds to the disulfide-linked peptide

Journal of MASS SPECTROMETRY

(a)




(b)

**Disulfide-bond elucidation using observed oxidized cysteine-containing peptides from RNase A**

| Cysteine Location | | | 91 | 84 | 52 | 110 | 121 | 98 | 66 | 52 | 136 | 84 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Peptide Sequence | | | | | | | | | | | |
| | | $[M+H]^+_{calc}$ | 534.25 | 635.30 | 626.20 | 694.30 | 730.28 | 799.29 | 808.39 | 754.30 | 1215.58 | 1613.78 |
| 91 | $^{88}$NVA***C***K$^{92}$ | 534.25 | | | | | | **1330.52** | | | | |
| 84 | $^{82}$AV***C***SQK$^{87}$ | 635.30 | | | | | | | | | **1847.86** | |
| 52 | $^{52}$***C***NQ***MM***$^{56}$ | 626.20 | | | | **1317.48** | | | | | | |
| 110 | $^{106}$SITD***C***R$^{111}$ | 694.30 | | | **1317.48** | | | | | **1445.58** | | |
| 121 | $^{118}$YPN***C***AY$^{123}$ | 730.28 | | | | | | | **1535.64** | | | |
| 98 | $^{93}$NGQTN***C***Y$^{99}$ | 799.29 | **1330.52** | | | | | | | | | |
| 66 | $^{66}$***C***KPVNTF$^{72}$ | 808.39 | | | | | **1535.64** | | | | | |
| 52 | $^{52}$***C***NQ***MM***K$^{57}$ | 754.30 | | | | **1445.58** | | | | | | |
| 136 | $^{131}$HIIVA***C***EGNPY$^{141}$ | 1215.58 | | **1847.86** | | | | | | | | **2826.33** |
| 84 | $^{73}$VHESLADVQAV***C***SQK$^{87}$ | 1613.78 | | | | | | | | | **2826.33** | |

Native RNase A disulfide bonds: (Cys52 - Cys110), (Cys66 - Cys121), (Cys84 - Cys136), (Cys91 - Cys98)

**Figure 5.** (a) Positive ion mass spectrum of the RNase A trypsin/chymotrypsin digest + 2-iododactamide following on-target oxidation. The peptide ion signals labeled with their corresponding *m/z* values indicate disulfide-linked peptides from RNase A elucidated from the oxidized negative ion mass spectrum. Inset figure: expanded view of mass range 1315–1335 *m/z*. (b) Table correlating the observed oxidized cysteine-containing peptides with their resulting candidate interlinked disulfide-bridged peptides. Shown are their calculated $[M + H]^+$ values for their nonoxidized counterparts. The calculated $[M + H]^+$ interlinked disulfide-bridged peptide masses highlighted with a box indicate matched *m/z* values in the above positive ion mass spectrum. The calculated disulfide-linked peptides that do not match ion signals present in the positive ion mass spectrum were removed for clarity.

($^{118}$YPN***C***AY$^{123}$/$^{66}$***C***KPVNTF$^{72}$), which is a native disulfide linkage between Cys$^{66}$ and Cys$^{121}$. This assignment was further confirmed by tandem MS by observing several b-type fragment ions and the signature fragment ions for an interlinked disulfide bond. The ion signal at *m/z* 1677.66 is most likely the disulfide-linked peptide ($^{84}$***C***SQK$^{87}$/$^{131}$HIIVA***C***EGPNY$^{141}$) with an $[M + H]^+_{calc}$ = 1677.77 Da (−67 ppm mass error) and a 170.09 Da mass difference (Ala-Val residue mass = 170.11 Da) from the *m/z* 1847.75 ion signal. All four native disulfide bonds from RNase A can be elucidated from the oxidized cysteine-containing peptides resulting from the on-target oxidation of the RNase A proteolytic digest. At this point we are not claiming a method for complete disulfide bond elucidation from a single MALDI deposit; there are several other possible RNase A disulfide-linked peptides that can be produced from different proteolytic digestion products that were not identified. Therefore, we plan to further expand on the disulfide bond elucidation capability using this on-target oxidation method in combination with LC-MALDI-MS to deconvolute the negative ion mass spectra prior to oxidation.

## Conclusions

On-target oxidation using performic acid vapor was achieved for MALDI-deposited samples containing model disulfide-linked peptides, intact insulin and a proteolytic digest of RNase A. Cysteine, cystine and methionine amino acid side chains are readily oxidized after on-target oxidation. On the other hand, other oxidizable amino acids such as histidine, tryptophan and tyrosine are not significantly oxidized as a result of on-target oxidation. The oxytocin experiments show the compatibility of the on-target oxidation method with alkylation chemistry to distinguish between a peptide containing two free cysteine residues from a peptide with a single intralinked disulfide bond. The intact insulin experiments illustrate the ability to oxidize small disulfide-containing proteins and suggest that the MALDI matrix plays a role in the oxidation process by the matrix crystals affecting the ability of the performic acid vapor to reach the analyte. All four native RNase A disulfide bonds were successfully elucidated from the negative ion MALDI-MS data from the on-target performic acid oxidation of an RNase A proteolytic digest.

The on-target oxidation method is advantageous over solution-phase oxidation methods because of the decreased sample handling and reaction time. Another major benefit of the on-target oxidation method is the ability to perform simultaneous oxidation of several MALDI sample deposits. In addition, the positive and negative ion mass spectral quality is not sacrificed following oxidation. The on-target performic acid oxidation method provides a very powerful technique to distinguish between free cysteine-containing peptides and those peptides that contain disulfide



bonds. In addition, this method will make a drastic impact for disulfide-linked peptide characterization from complex mixtures, owing to the ability to rapidly screen an entire MALDI target for sample deposits that contain enhanced negative ion signals, i.e. these are MALDI spots that may contain candidate disulfide-linked peptides. Hence, the motivation to combine the on-target oxidation method with off-line separations (e.g. capillary electrophoresis or LC) coupled with MALDI-MS[28,29] to analyze disulfide-linked peptides from complex proteomic mixtures.

### Acknowledgements

This work was supported by the Department of Energy, Division of Chemical Sciences, Offices of Basic Energy Sciences, BES DE-FG02-04ER15520 and the National Institutes of Health, RR019587.

### Supporting information

Supporting information may be found in the online version of this article.

## References

[1] C.N. Pace, G.R. Grimsley, J.A. Thomson, B.J. Barnett. Conformational Stability and Activity of Ribonuclease T1 with Zero, One, and Two Intact Disulfide Bonds, *J. Biol. Chem.* **1988**, *263*, 11820.
[2] J.M. Thornton. Disulphide Bridges in Globular Proteins, *J. Mol. Biol.* **1981**, *151*, 261.
[3] J.J. Gorman, T.P. Wallis, J.J. Pitt. Protein Disulfide Bond Determination by Mass Spectrometry, *Mass Spectrom. Rev.* **2002**, *21*, 183.
[4] X. Yu, Z. Wu, C. Fenselau. Covalent Sequestration of Melphalan by Metallothionein and Selective Alkyation of Cysteines, *Biochemistry* **1995**, *34*, 3377.
[5] J.L. Apuy, Z.-Y. Park, P.D. Swartz, L.J. Dangott, D.H. Russell, T.O. Baldwin. Pulsed-Alkylation Mass Spectrometry for the Study of Protein Folding and Dynamics: Development and Application to the Study of a Folding/Unfolding Intermediate of Bacterial Luciferase, *Biochemistry* **2001**, *40*, 15153.
[6] J.L. Apuy, X. Chen, D.H. Russell, T.O. Baldwin, D.P. Giedroc. Radiometric Pulsed Alkylation/Mass Spectrometry of the Cysteine Pairs in Individual Zinc Fingers of MRE-Binding Transcription Factor-1 (MFT-1) as a Probe of Zinc Chelate Stability, *Biochemistry* **2001**, *40*, 15164.
[7] M.F. Bean, S.A. Carr. Characterization of Disulfide Bond Position in Proteins and Sequence Analysis of Cystine-Bridged Peptides by Tandem Mass Spectrometry, *Anal. Biochem.* **1992**, *201*, 216.
[8] S.D. Patterson, V. Katta. Prompt Fragmentation of Disulfide-Linked Peptides during Matrix-Assisted Laser Desorption Ionization Mass Spectrometry, *Anal. Chem.* **1994**, *66*, 3727.
[9] R. Craig, O. Krokhin, J. Wilkins, R.C. Beavis. Implementation of an Algorithm for Modeling Disulfide Bond Patterns Using Mass Spectrometry, *J. Proteome Res.* **2003**, *2*, 657.
[10] C. Caporale, L. Bertini, P. Pucci, V. Buonocore, C. Caruso. CysMap and CysJoin: Database and tools for protein disulphides localisation, *FEBS Lett.* **2005**, *579*, 3048.
[11] S. Wefing, V. Schnaible, D. Hoffmann. SearchXLinks. A Program for the Identification of Disulfide Bonds in Proteins from Mass Spectra, *Anal. Chem.* **2006**, *78*, 1235.
[12] H. Xu, L. Zhang, M.A. Freitas. Identification and Characterization of Disulfide Bonds in Proteins and Peptides from Tandem MS Data by Use of the MassMatrix MS/MS Search Engine, *J. Proteome Res.* **2008**, *7*, 138.
[13] H.R. Morris, P. Pucci. A New Method for Rapid Assignment of S-S Bridges in Proteins, *Biochem. Biophys. Res. Commun.* **1985**, *126*, 1122.
[14] Y. Fukuyama, S. Iwamoto, K. Tanaka. Rapid seqencing and disulfide mapping of peptides containing disulfide bonds by using 1,5-diaminonaphthalene as a reductive matrix, *J. Mass Spectrom.* **2006**, *41*, 191.
[15] L. Quinton, K. Demeure, R. Dobson, N. Gilles, V. Gabelica, E.D. Pauw. New Method for Characterizing Highly Disulfide-Bridged Peptides in Complex Mixtures: Application to Toxin Identification from Crude Venoms, *J. Proteome Res.* **2007**, *6*, 3216.
[16] S.S. Thakur, P. Balaram. Fragmentation of Peptide Disulfides under Conditions of Negative Ion Mass Spectrometry: Studies of Oxidized Glutathione and Contryphan, *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 358.
[17] Y. Sun, D.L. Smith. Identification of Disulfide-Containing Peptides by Performic Acid Oxidation and Mass Spectrometry, *Anal. Biochem.* **1988**, *172*, 130.
[18] J. Dai, J. Wang, Y. Zhang, Z. Lu, B. Yang, X. Li, Y. Cai, X. Qian. Enrichment and Identification of Cysteine-Containing Peptides from Tryptic Digests of Performic Oxidized Proteins by Strong Cation Exchange LC and MALDI-TOF/TOF MS, *Anal. Chem.* **2005**, *77*, 7594.
[19] H.P. Gunawardena, R.A.J. O'Hair, S.A. McLuckey. Selective Disulfide Bond Cleavage in Gold (II) Cationized Polypeptide Ions Formed via Gas-Phase Ion/Ion Cation Switching, *J. Proteome Res.* **2006**, *5*, 2087.
[20] H. Lioe, M. Duan, R.A.J. O'Hair. Can metal ions be used as gas-phase disulfide bond cleavage reagents? A survey of coinage metal complexes of model peptides containing and intermolecular disulfide bond, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2727.
[21] J.E. Lucas. *Chemical Oxidation of Tryptic Digests to Improve Sequence Coverage in Peptide Mass Fingerprint Protein Identification*, Masters Thesis, Texas A&M University (College Station, TX), **2003**.
[22] R. Matthiesen, G. Bauw, K.G. Welinder. Use of Performic Acid Oxidation to Expand the Mass Distribution of Tryptic Peptides, *Anal. Chem.* **2004**, *76*, 6848.
[23] J.R. Brown, B.S. Hartley. Location of Disulphide Bridges by Diagonal Paper Electrophoresis, *Biochem. J.* **1966**, *101*, 214.
[24] R.D. Edmondson, D.H. Russell. Evaluation of Matrix-Assisted Laser Desorption Ionization – Time-of-Flight Mass Measurement Accuracy by Using Delayed Extraction, *J. Am. Soc. Mass Spectrom.* **1996**, *7*, 995.
[25] C.H.W. Hirs. Performic Acid Oxidation, *Methods Enzymol.* **1966**, 197.
[26] R.C. Beavis, J.N. Bridson, Epitaxial protein inclusion in sinapic acid crystals, *J. Phys. D: Appl. Phys.* **1993**, *26*, 442.
[27] B.J. Williams, W.K. Russell, D.H. Russell. Evaluation of Experimental Variables for the On-Target Oxidation of Cysteine-Containing Peptides and Proteins, *In preparation*. **2009**, (Submitted).
[28] B.J. Williams, W.K. Russell, D.H. Russell. Utility of CE-MS Data in Protein Identification, *Anal. Chem.* **2007**, *79*, 3850.
[29] G. Rosas-Acosta, W.K. Russell, A. Deyrieux, D.H. Russell, V.G. Wilson. A Universal Strategy for Proteomic Studies of SUMO and Other Ubiquitin-like Modifiers, *Mol. Cell. Proteomics* **2005**, *4*, 56.