Anal. Chem. **2002,** *74,* 4980−4988

Screening for Disulfide Bonds in Proteins by MALDI In-Source Decay and LIFT-TOF/TOF-MS

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An automated screening method is presented that uses MALDI in-source decay (MALDI-ISD) of disulfide bonds for identification of disulfide-linked peptides in MALDI mass spectra. Peptides released by ISD of a disulfide bond can be detected at an *m*/*z* ratio that corresponds to the singly protonated peptide with a reduced cysteine residue. Therefore, screening of peak lists for signal patterns that fulfill the equation, *m*/*z* (peak A) + *m*/*z* (peak B) − *m*/*z* (H_2 + H^+) = *m*/*z* (peak C), facilitated identification of putative ISD fragments of disulfide-linked peptides (peaks A and B) and their precursors (peak C). Signals (peak C) from putatively disulfide-linked peptides were subjected to LIFT-TOF/TOF-MS to confirm the existence of a disulfide bond. Using this method, we identified all 4 disulfide bonds in RNAseA and 8 two-disulfide clusters comprising 16 out of the 17 disulfide bonds in BSA. The presented screening method accelerated the identification of disulfide bonds in RNAseA and BSA, because the number of MS/MS spectra to be acquired was reduced by 1 order of magnitude. Less than 5% of the signals selected for LIFT-TOF/TOF-MS did not correspond to disulfide-linked peptides. Furthermore, the number of possible assignments for disulfide-linked peptides was reduced by 2−3 orders of magnitude, because knowledge of the mechanism of disulfide bond fragmentation by ISD permitted use of stricter rules for the interpretation of mass spectra. Therefore, interpretation of MS/MS spectra of disulfide-linked peptides was considerably simplified in comparison to conventional approaches.

Disulfide bond formation is a posttranslational modification that strongly stabilizes the 3D structure of proteins. Therefore, identification of disulfide bonds or free cysteine residues can give valuable information on the structure and function of a protein.[1] Modern mass spectrometric methods such as MALDI-MS[2] and ESI-MS[3] are well suited to identify proteolytic fragments and to assign disulfide-linked peptides, because these techniques offer fast analysis and high sensitivity. However, to apply these methods, the protein of interest must be accessible to proteolysis with specific proteases to release linear and disulfide-linked peptides. If disulfide-linked and protease-resistant proteins are reduced with TCEP and alkylated under acidic conditions in the presence of denaturing agents, disulfide bonds are partially reduced.[4] The addition of denaturing agent ensures that the reduction of disulfide bonds occurs independently of their accessibility in the native protein.[4] The alkylating agent CDAP efficiently quenches TCEP, which is useful to stop the reduction in a controlled manner.[5] NEM can be added as a second alkylating agent to block reduced cysteine residues that were partially modified by CDAP.[5] The protein derivatives generated by partial reduction and alkylation are amenable to proteolysis.[6−8] If proteolysis is performed below pH 7, the exchange of disulfide bonds during proteolysis is inhibited, which is essential to correctly elucidate the disulfide structure of the investigated protein.[5,9,10] The proteolytic mixtures are fractionated by HPLC and analyzed by mass spectrometry. The existence of disulfide bonds is usually confirmed by fragmentation of putatively disulfide-linked peptides by CID or PSD.[11,12] If a larger protein is to be analyzed, it is often impossible to decide from *m*/*z* alone which ions correspond to disulfide-linked peptides and which do not. One possibility to identify signals of cross-linked peptides is to partially incorporate ^{18}O during proteolysis in 50% ^{18}O water. As disulfide-linked peptides usually contain more than one cleavage site, they can be distinguished from single-chain peptides by their different isotope pattern.[13] Another method is to combine data of automated LC−MS and LC−MS/MS runs[14−16] of protein digest mixtures

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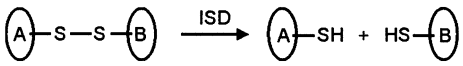
10.1021/ac025807j CCC: $22.00
Published on Web 08/23/2002

before and after reduction and alkylation to identify disulfide-linked peptides.[17] However, automatic interpretation of MS/MS data of cross-linked peptides including disulfide-linked peptides remains a challenging task.[17,18]

It is possible to avoid the reduction and alkylation step of the digested native protein and to facilitate data interpretation by using information provided by MALDI-ISD of disulfide bonds between peptide chains. MALDI-ISD of intact proteins produces c- and z-type fragment ions by breakage of the N−C_α bond. As this fragmentation occurs virtually independently of the neighboring amino acid residues, MALDI-ISD has been successfully applied to characterize N-terminal sequences of proteins, thereby identifying these proteins.[19−22] Usually, a higher laser power than that necessary to generate signals of the intact protein is applied to generate a substantial amount of c- and z-type fragment ions of the protein. According to the "lucky survivor model",[23] multiply protonated protein ions are partially neutralized by low-energy electrons, which accounts for the observation of predominantly singly charged ions in MALDI mass spectra.[23,24] The electrons could be released by charge separation during the desorption process[23] or by ejection of photoelectrons from the target surface upon laser irradiation.[25] Consequently, MALDI-ISD can be induced by those electrons that hit multiply protonated analyte molecules and should, therefore, be closely related to ECD that is used for top-down characterization of proteins in FTICR instruments.[26−28] Disulfide bonds are preferentially cleaved by ECD.[29,30] Consistent with this observation, fragmentation of disulfide bonds in the MALDI ion source is often observed, too, if disulfide-linked peptides are analyzed with standard laser intensities.[12,31−33] However, it is still being debated whether bond cleavage by ECD occurs via a hydrogen radical formed by neutralization of a proton that was attached to the protein[29] or by direct dissociative electron attachment.[34−36] In contrast to ECD, where the species R−SH and R′−S· are detected after electron irradiation of R−S−S−R′,[29] no signals of the odd electron species R′−S· are observed after cleavage of a disulfide bond by MALDI-ISD. Probably R′−S· is able to pick up a proton due to the higher density of molecules in the matrix/sample plume than in a FTICR cell. MALDI-ISD of peptides that contain one disulfide bond is illustrated in Scheme 1.

Scheme 1. ISD Fragmentation Pathway for Peptides Containing One Disulfide Bond



Peptides are usually detected as singly charged molecules in MALDI mass spectra. Therefore, the disulfide-linked precursor, A−S−S−B (Scheme 1), is detected at an m/z ratio that equals the sum of the m/z ratios of both ISD fragments minus the mass of two hydrogen atoms and one proton. The subtraction of the mass of two hydrogen atoms accounts for reduction of the disulfide bond during ISD to release A−SH and B−SH (Scheme 1). Thus, screening of MALDI mass spectra for disulfide-linked peptides and their ISD fragments should be possible, if signals for these three species are observed.

To evaluate the reliability of this proposed screening method, we used RNAseA and BSA as model systems containing 8 and 35 cysteine residues, respectively. RNAseA and BSA were partially reduced and modified, to achieve a high yield of proteolytic peptides. Proteolysis was carried out at pH 6 to avoid disulfide scrambling. Peak lists of MALDI mass spectra of HPLC-separated digestion mixtures of BSA were automatically screened using an EXCEL macro for signal triplets that fulfilled the following equation:

$$m/z\ (\text{peak A}) + m/z\ (\text{peak B}) - m/z\ (H_2 + H^+) = m/z\ (\text{peak C}) \quad (1)$$

The putatively disulfide-linked peptides were then subjected to MALDI-PSD or LIFT-TOF/TOF-MS to confirm the presence of a disulfide bond.

EXPERIMENTAL SECTION

Materials. α-Cyano-4-hydroxycinnamic acid, bombesin, bovine ribonuclease A, bovine serum albumin, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate, chymotrypsin (TLCK-treated), human adrenocorticotropic hormone fragment (18−39), human angiotensin II, *N*-ethylmaleimide, tris(2-carboxyethyl)phosphine, and trypsin (TPCK-treated) were purchased from Sigma-Aldrich (Deissenhofen, Germany). Ultrapure water (specific conductivity, 18.2 MΩ/cm) was produced by a MilliQ device (Millipore,

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Eschborn, Germany). Endoproteinase GluC (sequencing grade) was purchased from Roche Diagnostics (Mannheim, Germany). Acetonitrile (gradient grade), ammonium bicarbonate, (ethylenedinitrilo)tetraacetic acid dipotassium salt, guanidinium chloride for biochemistry, Lichrosorb C_8, 10-μm particle size, methanol (HPLC-grade), disodium hydrogen phosphate, sodium acetate, and trifluoroacetic acid (Uvasol) were bought from Merck (Darmstadt, Germany). Grom-Sil 300 Butyl C_4, 5-μm particle size, was purchased from GROM (Herrenberg, Germany). Somatostatin-28 was from Bachem (Basel, Switzerland).

Chemical Modifications. Partial reduction of BSA (180 μg) was performed with a final protein concentration of 4 μg/μL in 50 mM phosphate buffer, pH 4, containing 6 M GuaCl and 5 mM EDTA. A 4.5-μL aliquot of a freshly prepared TCEP stock solution (8.3 μg/μL, 29 mM) in 300 mM phosphate buffer, adjusted to pH 4, was added (molar ratio of TCEP/BSA = 48). After 10 min at 25 °C, 5 μL of a CDAP stock solution (24.4 μg/μL, 112 mM) in ACN (molar ratio CDAP/BSA = 192) was added. Cyanylation was carried out for 10 min at 25 °C in a thermomixer. A 5.7-μL aliquot of an NEM stock solution (1 M freshly prepared in ACN) was added to a final NEM concentration of 0.1 M. pH was raised to 5 by addition of 1.3 μL of 300 mM phosphate buffer, pH 6.3. NEM modification was carried out for 20 min at 25 °C in a thermomixer. RNAseA was modified under identical conditions except that the molar ratios of TCEP/RNAseA and CDAP/RNAseA were 24 and 96, respectively.

Removal of Reagents. Reagents were removed from BSA derivatives using C_4 microcolumns. C_4 microcolumns were prepared in 10-μL Eppendorf filter tips by applying 30 μL of a GROM-SIL 300 Butyl slurry (75 mg/mL in methanol). After sedimentation of RP material, excess methanol was removed. The column bed was washed with two 20-μL aliquots of 0.1% TFA in water. Aliquots of the reaction mixture containing 60 μg of BSA were applied to one microcolumn. Reagents were removed by washing with five 30-μL aliquots of 0.1% TFA in water. Protein derivatives were eluted in two steps using 7.5-μL aliquots of 80% ACN/0.1% TFA. All liquid handling on the column was performed by air pressure produced with a 2-mL disposable plastic syringe. In all steps except the last elution step, care was taken to prevent dry running of the microcolumns. The eluate was diluted with 0.1% TFA to reduce acetonitrile content to less than 10%, and an aliquot was analyzed by RP-HPLC to determine protein concentration. The main part of the eluate was stored at −20 °C until proteolysis. Reagents were removed from RNAseA in the same way except that Lichrosorb C_8 was used as sorbent. *NEM is highly toxic; acetonitrile and methanol are toxic and volatile. All these substances must be handled in a hood and disposed of properly. Suitable protective clothing and gloves should be worn at all times.*

Proteolytic Digest. BSA microcolumn eluates were diluted to a volume of 40 μL with 50 mM phosphate buffer, pH 6, for proteolysis. Aliquots of 15−20 μg of modified BSA were used. Trypsin/chymotrypsin and trypsin/GluC digests were performed overnight at 37 °C. Proteases were added simultaneously from stock solutions (trypsin and chymotrypsin 0.1 μg/μL in 1 mM HCl, GluC 0.1 μg/μL in MilliQ water). The enzyme/substrate ratio was 1/50 for each protease. Aliquots of 10 μg of modified RNAseA were digested under identical conditions with trypsin/GluC.

HPLC Separation. Protein and peptide separation was carried out on an HP 1100 chromatographic system (Agilent Technologies, Waldbronn, Germany) using a Zorbax RP18 (5 μm, 300 Å, 2.1 × 150 mm) column. A binary gradient with 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) was applied at a flow rate of 0.3 mL/min. BSA derivatives and RNAseA digest mixtures were analyzed using a linear gradient from 5 to 45% solvent B in 25 min after 5 min of equilibration at 5% B. Total gradient time was 45 min. A total of 24 fractions (0.7 min each) of the RNAseA digest mixture were collected starting 10 min after injection. Fractions were collected in 96-well microtiter plates using a MAPII robot (Bruker Daltonik, Bremen, Germany) under control of the MAP-Control 3.0.4 software package. Proteolytic digest mixtures of BSA were separated using a linear gradient from 5 to 32% or 37% solvent B in 100 min after 5 min of equilibration at 5% B. Total gradient time was 120 min. A total of 96 fractions (1 min each) were collected starting 6 min after injection. Fractions were dried in a vacuum concentrator and redissolved in 10 μL of 33% ACN/0.1% TFA.

MALDI-MS. Dried droplet sample preparation of HPLC fractions for MALDI-MS with HCCA as matrix (saturated solution in 33% ACN/0.1% TFA) was performed by the MAPII robot. MALDI and MALDI-PSD spectra were automatically recorded in positive mode on a Bruker Reflex III Scout 384 instrument under control of TOF-Software release 5.0 (XACQ 4.0.4). LIFT-TOF/TOF spectra were recorded on a Bruker Ultraflex TOF/TOF mass spectrometer controlled by the Flexcontrol 1.1 software package. Metastable ions were analyzed that were generated by laser-induced decomposition of the selected precursor ions. No additional collision gas was applied. Precursor ions were accelerated to 8 kV and selected in a timed ion gate. The fragments were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after the ion reflector passage. Masses were automatically annotated using XMASS 5.1.2 NT.

External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a mixture of human angiotensin II (m/z 1046.542), bombesin (m/z 1619.823), ACTH (18−39) (m/z 2465.199), and somatostatin-28 (m/z 3147.472). Parallel to the measurements, calibration was validated on three different 3 × 3 spot squares. The mixture of calibration peptides was measured using external calibration, which was carried out on the central spot of a 3 × 3 square. For the Reflex III, we observed a maximum mass error of 123 ppm for externally calibrated spectra. To achieve mass accuracy better than 60 ppm, internal calibration was carried out by doping the matrix solution with a mixture of the above-mentioned calibration peptides. Calibration of these mass spectra was carried out automatically by employing a customized macro of the XMASS 5.1.2 NT software package. This macro used the monoisotopic singly charged peaks of the above-mentioned peptides for calibration. MALDI-PSD and LIFT-TOF/TOF spectra were annotated with the BioTools 2.1 software package.

Screening of Peak Lists for ISD of Disulfide Bonds. Each peak list was screened for signal patterns fulfilling eq 1 by an EXCEL 2000 macro written in Visual Basic for Applications. All peaks, A and B, that fulfilled the equation within a mass tolerance of 100 ppm were considered putative ISD fragments of a disulfide-

Table 1. Summary of Results Obtained for All Peak Lists of HPLC Fractions of Tryptic/Chymotryptic and Tryptic/GluC Digest Mixtures by Different Ways of Data Acquisition/Interpretation

		conventional data interpretation[a]		screening method[b]	
proteases used	no. of nonredundant peaks	no. of peaks corresponding to putatively disulfide-linked peptides	no. of possible assignments	no. of peaks selected for LIFT	no. of possible assignments[a,b]
trypsin/chymotrypsin	264	169	81 634	30	17137
trypsin/GluC	277	209	245 826	25	90322

[a] Peak lists were interpreted by SearchXLinks using the conventional parameter set (see Experimental Section). [b] Only those signals that fulfilled eq 1 as peak C (= putatively disulfide-linked peptide) were interpreted.

linked peptide and were collected in a subpeak list. Identification of putative ISD fragments of disulfide-linked peptides and their precursors in peak lists of 50 HPLC fractions was performed within a few seconds on a standard PC (PIII 933 MHz). Subpeak lists were then interpreted by the program SearchXLinks[37] with a strict parameter set as described in the next paragraph. All peaks C that represented putatively disulfide-linked peptides were stored in a separate EXCEL sheet. These peaks were selected as precursor ions for MALDI-PSD or LIFT-TOF/TOF fragmentation in order to confirm the presence of disulfide bonds.

Semiautomatic Interpretation of Mass Spectra by SearchXLinks. MS peak lists were interpreted semiautomatically using the SearchXLinks program (a detailed discussion of SearchXLinks and the manual can be accessed via the SearchXLinks website at http://www.caesar.de/searchxlinks). Cleavage, modification, and link rules were specified for data interpretation. All peptide combinations matching m/z values within a mass error of 150 ppm for externally calibrated spectra and 60 ppm for internally calibrated spectra were generated, taking a maximum number of two disulfide bonds into account. Cyanylation of cysteine residues and formation of pyroglutamate during proteolysis were chosen as modification rules. If peak lists were interpreted in the conventional way to identify putatively disulfide-linked peptides, all possible assignments that contained at least one disulfide bond and at most two disulfide bonds were generated. This parameter set will be referred to as the conventional parameter set. If peak lists of putative ISD fragments of disulfide-linked peptides (peaks A or B from eq 1) were interpreted, the presence of at least one free thiol group was enforced by a special modification rule. Additionally, the maximum number of disulfide bonds per assignment was restricted to one. In the following, this parameter set will be referred to as ISD parameter set. Both parameter sets for SearchXLinks are available as Supporting Information. The set of possible assignments for a fully disulfide-linked peptide (ISD precursor, peak C) was generated by combining each element of the set of assignments that was generated for peak A (eq 1) with each element of the set of assignments that was generated for peak B (eq 1). If a single peak C (eq 1) happened to fulfill eq 1 for two different fragment pairs, (peak A, peak B) and (peak A′, peak B′), simultaneously, a corresponding set of assignments for the common precursor ion (peak C) was set up manually by intersecting the two original sets of assignments.

RESULTS AND DISCUSSION

Screening for Putatively Disulfide-Linked Peptides. RNAseA was partially reduced, cyanylated, and NEM modified to facilitate proteolytic digestion.[5] To check whether ISD was useful to predict which signals in a MALDI mass spectrum corresponded to disulfide-linked peptides, we screened peak lists of MALDI mass spectra of HPLC-separated digest mixtures of RNAseA for peaks fulfilling eq 1. Additionally, a nonredundant peak list for all MALDI mass spectra was generated by counting individual signals only once that repeatedly appeared in consecutive HPLC fractions. Out of 167 nonredundant peaks of the tryptic/GluC digestion mixture of RNAseA, 16 signals were identified as putatively disulfide-linked peptides (peaks C). Indeed, all selected signals corresponded to disulfide-linked peptides and all four disulfide bonds of RNAseA were identified. As the presented screening method relies only on the fulfillment of eq 1, disulfide-linked peptides generated by unspecific cleavage were also identified (data not shown). Furthermore, no false positive hits were generated.

The presented method was then used to screen for disulfide bonds in BSA. The amino acid sequence of BSA[38] contains 35 cysteine residues, 77 potential cleavage sites for trypsin, 108 potential cleavage sites for chymotrypsin, and 84 potential cleavage sites for GluC, which makes the identification of disulfides in BSA with conventional methods a much more challenging task. Furthermore, 16 out of the 35 cysteine residues form eight pairs of adjacent cysteine residues.[38] As a disulfide bond between adjacent cysteine residues is unlikely to occur, the presence of two disulfide bonds in one peptide has to be considered for data interpretation. To generate BSA derivatives that were readily digested in buffer solutions at pH 6, BSA was partially reduced, cyanylated, and modified with NEM. Proteolysis was performed overnight with mixtures of trypsin and chymotrypsin or trypsin and GluC, respectively. The digestion mixtures were separated by C_{18}-RP HPLC.

Efficient Acquisition of MS/MS Data by ISD Screening. To identify signals of putatively disulfide-linked peptides, peak lists of MALDI spectra of the collected HPLC fractions were first interpreted in the conventional way by using SearchXLinks. All possible assignments that contained one or two disulfide bonds were generated. As summarized in Table 1, ~70% of the ions

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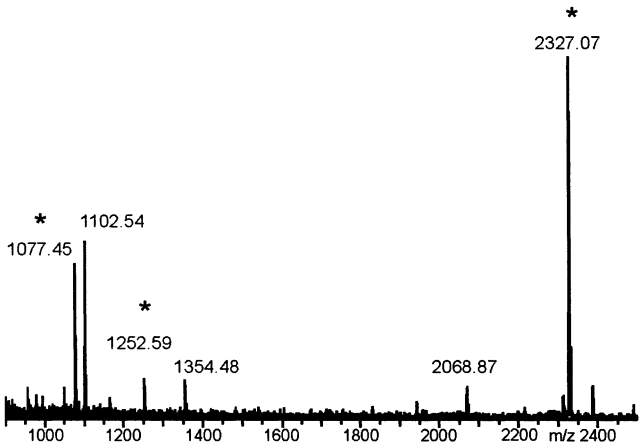

Figure 1. MALDI mass spectrum of an HPLC fraction of the tryptic/chymotryptic digest mixture of BSA eluting at 12% solvent B. Signals fulfilling eq 1 are indicated with asterisks.

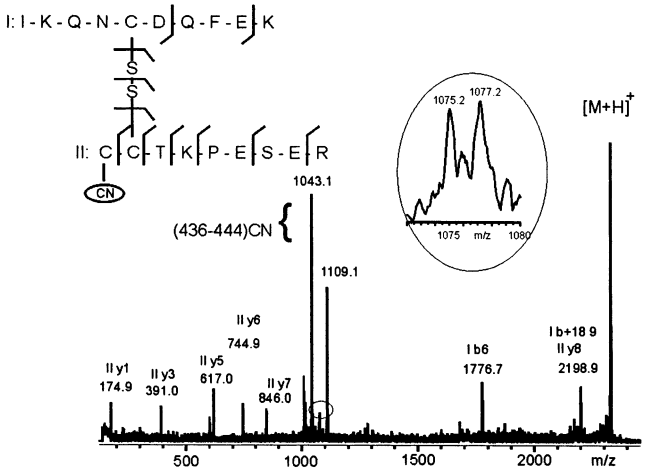

Figure 2. LIFT-TOF/TOF spectrum of the precursor ion detected at *m*/*z* 2327.07. Fragment ions that are formed by fragmentation of bonds within chain I (387−396) are labeled "I" preceding the observed ion type. Likewise, fragment ions of chain II (436−444) are labeled "II". Inset: zoom between *m*/*z* 1072 and 1080.

detected in the mass spectra were assigned to putatively disulfide-linked peptides and, therefore, were candidates for MS/MS experiments using the conventional approach. To reduce the number of MS/MS spectra to be recorded, we screened MALDI-MS peak lists for signal patterns that fulfilled eq 1. As a result, the number of signals that represented putatively disulfide-linked peptides was reduced by nearly 1 order of magnitude without using any information about the sequence of BSA and the protease specificity. However, as is obvious from Table 1, the number of possible assignments generated by SearchXLinks for these signals was still too large for manual interpretation of MS/MS data to identify the correct assignment. Therefore, additional information had to be provided to facilitate data interpretation.

Assignment of Disulfide Bonds (Singly Connected Peptides, One Solution of Eq 1). The knowledge about the mechanism of ISD of disulfide bonds was used to reduce the number of possible assignments for putatively disulfide-linked peptides. A MALDI mass spectrum of an HPLC fraction of the tryptic/chymotryptic digest mixture of BSA is presented in Figure 1. The spectrum contains three signals at *m*/*z* 1077.45, 1252.59, and 2327.07, that fulfill eq 1.

Interpretation of *m*/*z* 2327.07 by SearchXLinks using the conventional parameter set generated 67 possible assignments within a mass error of 60 ppm. If the signal detected at *m*/*z* 2327.07 corresponded to a disulfide-linked peptide, each of the fragments that were detected at *m*/*z* 1077.45 and 1252.59 would have to contain at least one free cysteine residue that was generated during ISD of the disulfide bond. Therefore, the ISD parameter set was used for interpretation of *m*/*z* 1077.45 and 1252.59 by SearchXLinks. As a result, cyanylated (436−444)−S−H and (387−396)−S−H were the only possible explanations for *m*/*z* 1077.45 and 1252.59.

The precursor ion detected at *m*/*z* 2327.07 was subjected to LIFT on the ultraflex TOF/TOF instrument. Interpretation of the LIFT-TOF/TOF spectrum was straightforward as only one assignment had to be verified. The LIFT-TOF/TOF spectrum of *m*/*z* 2327.07 shown in Figure 2 contained a signal triplet at *m*/*z* 1043.1, 1077.2, and 1109.1 that is characteristic for the asymmetric and symmetric fragmentation of a disulfide bond.[12,39] The triplet was assigned to cyanylated (436−444) containing a dehydroalanine residue, a reduced cysteine residue, and a reduced dithiocysteine residue. The signal detected at *m*/*z* 1075.2 (Figure 2, inset) was assigned to cyanylated (436−444) with the thiol group of the second cysteine residue transformed to a thioaldehyde moiety. Additionally, several y-ions of cyanylated (436−444) were observed. Fragmentation after Asp-392 accounted for the intense signal at *m*/*z* 1776.7. Thus, the suggested assignment of *m*/*z* 2327.07 to (387−396) linked to cyanylated (436−444) was confirmed.

Interpretation of the rather complex peak list of the MALDI mass spectrum shown in Figure 3 by the described screening method identified eight signals as putatively disulfide-linked peptides (peaks C in eq 1) at *m*/*z* 1877.76, 1907.72, 2043.82, 2302.14, 2869.08, 2899.09, 3157.42, and 3533.61. All of these signals could be assigned to disulfide-linked peptides (see Supporting Information), which demonstrates that our screening method can be applied to identify disulfide-linked peptides in peak lists of fairly complex spectra. This is helpful if discrete fractions are collected for MALDI-MS, which makes complete separation of all components in the mixture difficult to accomplish.

Application of the ISD parameter set to putative ISD fragments of disulfide-linked peptides proved very effective in reducing the number of possible assignments for a putatively disulfide-linked peptide. As summarized in Table 2, the number of possible assignments was reduced by a factor of ∼50 if data interpretation by SearchXLinks was carried out with the ISD parameter set that accounted for the mechanism of ISD of disulfide bonds.

Assignment of Disulfide Bonds (Doubly Connected Peptides, Two Solutions of Eq 1). As mentioned before, BSA contains two adjacent cysteine residues at eight different positions in the amino acid sequence. Hence, peptides containing two disulfide bonds should be present in the proteolytic mixtures. ISD of such peptides can occur at both disulfide bonds and can,

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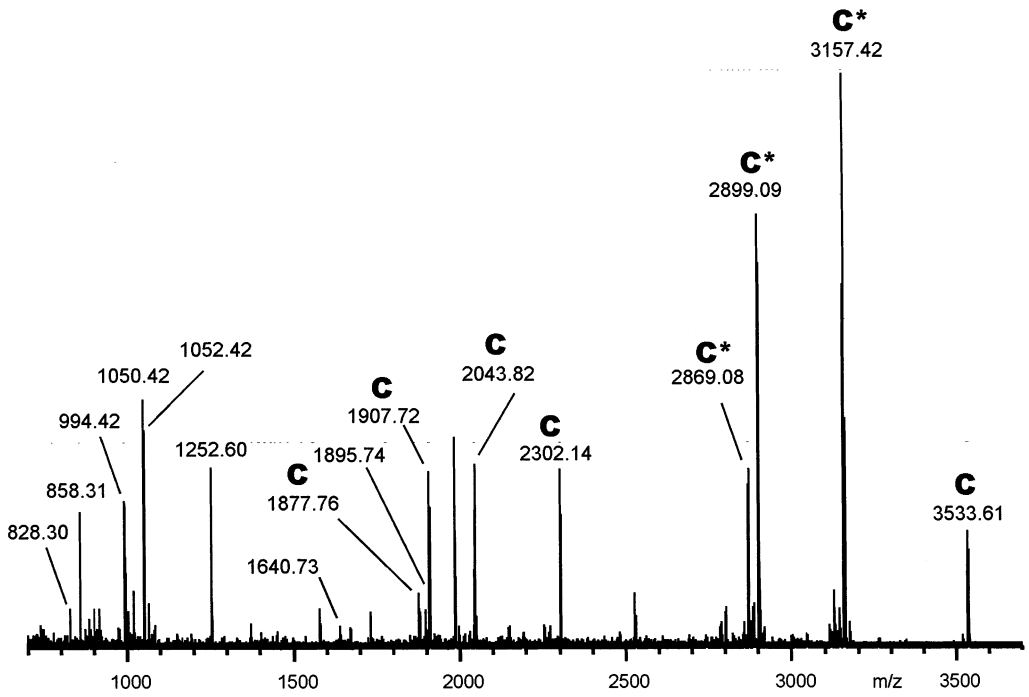


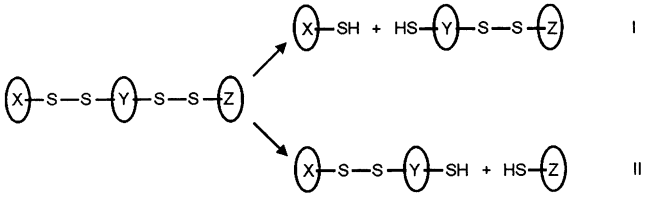
Figure 3. MALDI mass spectrum of an HPLC fraction of the tryptic/chymotryptic digest mixture of BSA eluting at 15% solvent B. Signals corresponding to disulfide-linked peptides or their ISD fragments were annotated. Signals that fulfilled eq 1 as peak C are indicated by a boldface C above the *m*/*z* ratio. An asterisk indicates that two different signal patterns were found that fulfilled eq 1.

Table 2. Number of Possible Assignments for Putatively Disulfide-Linked Peptides as a Function of the Parameter Set Used for Interpretation

proteolysis condition	no. of ions selected for LIFT[a]	no. of possible assignments: conventional parameter set	no. of possible assignments: ISD parameter set[b]
trypsin/chymotrypsin	30	17137	288
trypsin/GluC	25	90322	1641

[a] See Table 1. [b] Number of possible assignments for each peak C (see eq 1) was calculated by multiplying the numbers of possible assignments that were found for peaks A and B (see eq 1).

Scheme 2. ISD Fragmentation Pathways for Peptides Consisting of Three Peptide Chains Connected by Two Disulfide Bonds



therefore, generate two pairs of ISD fragments out of one precursor molecule, as illustrated in Scheme 2.

The mass spectrum shown in Figure 3 provides three examples of peaks C, detected at *m*/*z* 2869.08, 2899.09, and 3157.42, for each of which two different solutions of eq 1 were found. In these cases, the number of possible assignments for peak C (see eq 1) can be further reduced by assuming that peak C represents only one species. As indicated in Scheme 2, the fully disulfide-linked peptide consists of three peptides X, Y, and Z. These peptides are either directly detected as ISD fragments in the mass spectrum like peptide X (Scheme 2, pathway I) and peptide Z (Scheme 2, pathway II). Alternatively, they are part of a fragment consisting of two peptides linked via a disulfide bond and are, therefore, not directly detected, cf. peptides Y and Z following pathway I and peptides X and Y following pathway II of Scheme 2. If peak C represented only one species, the correct assignments for these disulfide-linked fragments would have to contain the common peptide Y (Scheme 2) connected by a disulfide bond to another peptide.

As mentioned in the Experimental Section and in Table 2, the set of possible assignments for peak C (eq 1) was generated by combining each possible assignment for peak A (eq 1) with each possible assignment for peak B (eq 1) if the ISD parameter set was used for data interpretation by SearchXLinks. Thus, two different sets of possible assignments were generated for the two different signal patterns that fulfilled eq 1 for an identical peak C. If peak C represented a single species, possible assignments for peak C would have to consist of those peptides that were present in the intersection of the two sets of possible assignments for the respective signal patterns.

Interpretation of the signal detected at *m*/*z* 3157.42 in the MALDI mass spectrum shown in Figure 3 illustrates the effect of this filter. Conventional interpretation of *m*/*z* 3157.42 yielded 732 possible disulfide-linked assignments. ISD screening revealed that either *m*/*z* 858.31 and 2302.14 or *m*/*z* 1252.60 and 1907.72 could be connected to form *m*/*z* 3157.42. The signals at *m*/*z* 858.31 and 1252.60 were unequivocally assigned by SearchXLinks to (445−451)−S−H and (387−396)−S−H, respectively. For the signals detected at *m*/*z* 1907.72 and 2302.14, SearchXLinks generated 6 and 11 possible assignments, respectively. Thus, the two different sets of assignments contained 6 (ISD fragments detected at *m*/*z*

Table 3. Number of Possible Assignments for Disulfide-Linked Peptides Depending on the Mode of Data Interpretation

proteolysis condition	no. of ions with two solutions of eq 1	no. of possible assignments: conventional parameter set	no. of possible assignments: ISD parameter set[a]	no. of possible assignments: ISD parameter set and intersection check[b]
trypsin/chymotrypsin	10	7007	178	9[c]
trypsin/GluC	7	28099	943	14

[a] All elements of both assignments sets for each disulfide-linked tripeptide were added. [b] Only those assignments that were present in the intersection of both sets of assignments. [c] For one signal (*m*/*z* 2869.08) no assignment was found with the parameter set used for data interpretation by SearchXLinks (see Supporting Information).

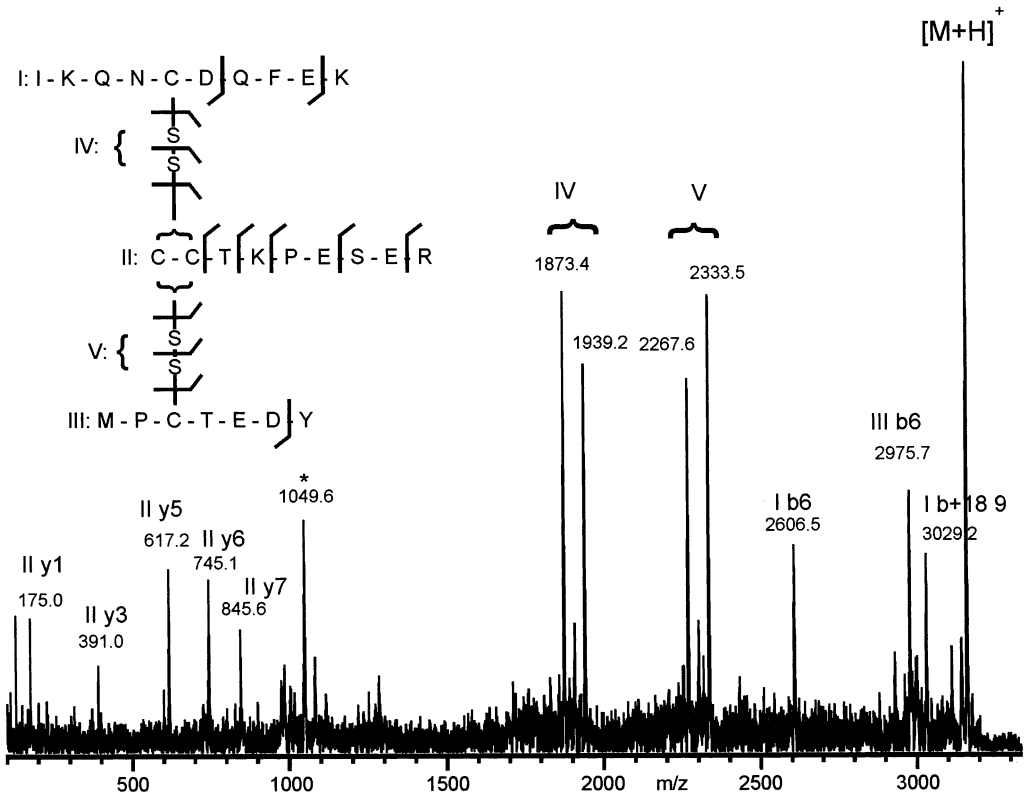


Figure 4. LIFT-TOF/TOF spectrum of the precursor ion detected *m*/*z* 3157.42. Fragment ions that are formed by breakage of (387−396), (436−444), and (445−451) are indicated by I, II, and III, respectively. Fragment ions indicated by IV and V correspond to symmetric and asymmetric fragmentation of the disulfide bonds between (387−396) and (436−444) and between (436−444) and (445−451). The signal below the asterisk was assigned to (436−444) that was released by fragmentation of two disulfide bonds.

1252.60 and 1907.72) and 11 (ISD fragments detected at *m*/*z* 858.31 and 2302.14) elements if the ISD parameter set was applied. However, only one assignment was present in the intersection of both sets of assignments. Thus, *m*/*z* 3157.42 was assigned to (387−396)−S−S−(436−444)−S−S−(445−451).

The numbers of possible assignments that were generated for all peaks C, for which two different solutions of eq 1 were found depending on the strategy used for data interpretation are listed in Table 3. These data demonstrate that the number of possible assignments was reduced by 3 orders of magnitude if the intersection of the two corresponding sets of assignments was determined compared to data interpretation by SearchXLinks using the conventional parameter set.

The assignment for *m*/*z* 3157.42 that was present in the intersection of the two different sets of assignments was verified by LIFT-TOF/TOF-MS, as illustrated in Figure 4. The presence of disulfide bonds in *m*/*z* 3157.42 is indicated by two intense signal triplets centered at *m*/*z* 1907.4 (IV, Figure 4) and 2301.9 (V, Figure 4). These fragment ions are released by symmetric and asymmetric fragmentation of disulfide bonds. For example, the peaks at *m*/*z* 1873.4, 1907.4, and 1939.2 originate from heterolytic elimination of R−S−S−H leaving dehydroalanine, homolytic cleavage leaving R−SH, and heterolytic cleavage leaving R−S−SH, respectively. In the lower mass region of the spectrum, several y-ions for peptide (436−444) were observed with high intensity. Additionally, two intense b-ions resulting from fragmentation of the disulfide-linked peptide after Asp-392 (*m*/*z* 2606.5) and Asp-450 were detected at *m*/*z* 2606.5 and 2975.7, respectively. Thus, the proposed assignment for *m*/*z* 3157.42 was verified. However, no fragment ions were identified that allowed us to decide whether Cys-391 is connected to Cys-436 or to Cys-437. To resolve this uncertainty, further experiments such as MS^n or

Table 4. Nonredundant List of Disulfide-Linked Peptides and ISD Fragments That Were Identified by the Presented Screening Method in HPLC Fractions of Tryptic/Chymotryptic Digest Mixtures

m/*z* (prec)	*m*/*z* (frag 1)	assigned to	*m*/*z* (frag 2)	assigned to	cysteine residues[a]
3557.67	701.38	(174−180)−S−H	2859.19	(115−126)−S−S−(161−173)−S−H	123, (167, 168), 176
	1391.59	(115−126)−S−H	2168.97	(174−180)−S−S−(161−173)−S−H	123, (167, 168), 176
2587.13	649.35	(199−204)−S−H	1940.85	(240−256)−S−H	199, (244, 245, 252)
3814.73	1015.50	(286−294)−S−H	2802.31	(262−273)−S−S−(274−285)−S−H	264, (277, 278), 288
	1386.58	(262−273)−S−H	2431.19	(286−294)−S−S−(274−285)−S−H	264, (277, 278), 288
3369.34	1184.50	(309−318)−S−H	2187.85	(351−369)−S−H	315, (359, 360, 368)
2899.09	994.42	(pE389−396)−S−H	1907.72	(445−451)−S−S−(436−444)−S−H	391, (436, 437), 447
	858.31	(445−451)−S−H	2043.82	(pE389−396)−S−S−(436−444)−S−H	391, (436, 437), 447
2012.91	841.50	(459−465)−S−H	1174.49	(475−480)−S−S−(484−487)−S−H	460, (475, 476), 486
	522.28	(484−487)−S−H	1493.61	(459−465)−S−S−(475−480)−S−H	460, (475, 476), 486
2873.20	1342.59	(509−520)−S−H	1533.56	(554−563)−S−S−(564−567)−S−H	513, (557, 558), 566

[a] Parentheses symbolize that cysteine residues are located in one peptide

Table 5. Nonredundant List of Disulfide-Linked Peptides That Were Identified by the Presented Screening Method in HPLC Fractions of Tryptic/Glu-C Digest Mixtures

m/*z* (prec)	*m*/*z* (frag 1)	assigned to	*m*/*z* (frag 2)	assigned to	cysteine residues[a]
3323.40	1601.71	(101−114)−S−H	1724.64	(74−76)−S−S−(82−93)−S−H	75, (90, 91), 101
3555.63	1244.54	(115−125)−S−H	2314.01	(160−180)−S−H	123, (167, 168), 176
2587.13	649.33	(199−204)−S−H	1940.82	(240−256)−S−H	199, (244, 245, 252)
3088.39	659.33	(286−291)−S−H	2432.096	(262−273)−S−S−(276−284)−S−H	264, (277, 278), 288
	1386.58	(262−273)−S−H	1704.74	(286−291)−S−S−(262−273)−S−H	264, (277, 278), 288
2605.06	707.33	(311−316)−S−H	1900.75	(359−375)−S−H	315, (359, 360, 368)
2621.00	994.38	(pE389−396)−S−H	1629.61	(436−444)−S−S−(445−449)−S−H	391, (436, 437), 447
	580.23	(445−449)−S−H	1077.45	(pE389−396)−S−S−(436−444)−S−H	391, (436, 437), 447
3314.53	713.31	(459−464)−S−H	2604.25	(484−499)−S−S−(472−478)−S−H	460, (475, 476), 486
	1823.91	(484−499)−S−H	1493.63	(459−464)−S−S−(472−478)−S−H	460, (475, 476), 486
3709.71	921.46	(565−573)−S−H	2791.24	(512−520)−S−S−(549−564)−S−H	513, (557, 558), 566
	1019.50	(512−520)−S−H	2693.18	(565−573)−S−S−(549−564)−S−H	513, (557, 558), 566

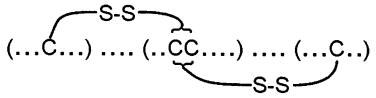
[a] Parentheses symbolize that cysteine residues are located in one peptide

additional chemical processing[4] of the collected HPLC fractions would have been necessary.

Assignment of Disulfide Bonds in BSA by Tryptic/Chymotryptic Digest. Automated screening of all peak lists of MALDI mass spectra of the HPLC separated tryptic/chymotryptic digest and subsequent acquisition of LIFT-TOF/TOF or PSD spectra of the putatively disulfide-linked peptides enabled the identification of disulfide-linked peptides that contained 28 of the 35 cysteine residues of BSA. These disulfide bonds were located in groups of four cysteine residues that form seven consecutive two-disulfide clusters in the amino acid sequence. Due to missed cleavage sites, more than one disulfide-linked peptide was identified for each two-disulfide cluster, which further confirmed the assignments. A maximum of redundancy was found for the two-disulfide cluster containing cysteine residues 391, 436, 437, and 447. Six different peptides were assigned to this two-disulfide cluster. To avoid redundant information, only one disulfide-linked peptide was listed in Table 4 for each disulfide cluster. Out of 30 signals that were selected for LID, only one was not assigned to a disulfide-linked peptide and, thus, represented a false positive hit.

Assignment of Disulfide Bonds in BSA by Tryptic/GluC Digest. Peak lists of MALDI mass spectra of HPLC fractions of the trypsin/GluC digest mixture of partially reduced, cyanylated, and NEM-modified BSA were screened for the ISD pattern of disulfide bonds in the same way as mentioned above. Table 5 contains a list of peptide ions that were assigned to disulfide-linked peptides and their ISD fragments. The data of the tryptic/GluC digest confirmed the assignments based on the tryptic/chymotryptic digest data. In addition, one further two-disulfide cluster was identified that contained cysteine residues 75, 90, 91, and 101. Out of 25 signals of the tryptic/GluC digestion selected for LID, only one was not assigned to a disulfide-linked peptide.

Scheme 3. Disulfide Bond Pattern Observed in Two-Disulfide Clusters of BSA



In summary, 32 of 35 cysteine residues were identified in eight two-disulfide clusters. Six of the identified two-disulfide clusters consisted of three peptides that were connected via two disulfide bonds. With regard to the amino acid sequence of BSA,[38] the peptide with two adjacent cysteine residues is located between two peptides with one cysteine residue each. The disulfide bond pattern of the remaining two two-disulfide clusters was characterized by partial reduction and alkylation of corresponding HPLC fractions. After reduction and alkylation of the intrachain disulfide bond, MALDI-PSD spectra of these peptide derivatives indicated that the disulfide bond pattern is identical to the two-disulfide clusters mentioned before (data not shown). Thus, the connectivity of all disulfide clusters can be described as illustrated in Scheme 3.

Cysteine residues 34, 53, and 62 were not present in peptides that were identified by the screening method described above.

Therefore, all peaks that were still unassigned were interpreted with SearchXLinks using a different parameter set. The parameter set allowed for formation of disulfide bonds only between cysteine residues 34, 53, and 62, respectively. A disulfide bond between Cys-53 and Cys-62 in the peptide (52−64) was assigned by SearchXLinks for an ion detected at m/z 1347.53. This assignment was verified by reduction and alkylation of the corresponding HPLC fraction (see Supporting Information). The remaining cysteine residue 34 was identified in its cyanylated form (data not shown) but not in any of the assigned disulfide bonds, which indicates that Cys-34 is present as a free cysteine residue in native BSA.

Thus, the state of all cysteine residues of BSA was characterized with a small number of MS/MS spectra acquired. Furthermore, knowledge about the mechanism of ISD of disulfide bonds facilitated interpretation of LID spectra. The application of the ISD parameter set for interpretation by SearchXLinks reduced the number of possible assignments by a factor of 50. If four ISD fragment ions of the two-disulfide cluster were observed and the sets of assignments were checked for consistency, the number of assignments could be reduced by 3 orders of magnitude, which enabled straightforward verification of disulfide bonds by LIFT-TOF/TOF-MS.

CONCLUSIONS AND PERSPECTIVES

As the initial chemical step of partial reduction and alkylation had to be carried out at high protein concentrations, minimizing sample volumes is essential if biologically relevant proteins shall be analyzed that are only available in small amounts and protease resistant in their native form. Therefore, the partial reduction and modification steps could be performed in a microfluidic device[40] ideally followed by proteolysis in a microreactor-on-a-chip.[41,42] As the offline interfacing of nano-LC or capillary electrophoresis with MALDI-MS has been accomplished,[43] MALDI-ISD screening for disulfide bonds seems to be feasible also for samples that are only available in small amounts.

Due to the low percentage of false positive hits, the presented screening method can be used to efficiently acquire MS/MS data of those signals that contain information about the disulfide structure of a protein. If performed on a MALDI TOF/TOF mass spectrometer, high-throughput identification of disulfide bonds in proteins is feasible. As the first step of the presented screening method does not depend on any information about the protease specificity or the sequence of the investigated protein, it is possible to identify putatively disulfide-linked peptides in proteins that possess yet unknown modifications or to allow for unspecific cleavage of the protease used. Provided that suitable de novo sequencing capabilities are available, assignment of disulfide bonds seems to be achievable in these cases also. Furthermore, the presented method can be used in protein−protein interaction studies to efficiently identify peptides that were cross-linked by agents containing a disulfide bond.

GLOSSARY

ACN	acetonitrile
ACTH (18−39)	adrenocorticotropic hormone human fragment 18−39 (CLIP)
BSA	bovine serum albumin
CID	collisionally induced dissociation
CDAP	1-cyano-4-dimethylaminopyridinium tetrafluoroborate
ECD	electron capture dissociation
EDTA	(ethylenedinitrilo)tetraacetic acid
ESI-MS	electrospray ionization mass spectrometry
e/s	enzyme/substrate ratio
TFA	trifluororacetic acid
GuaCl	guanidinium chloride
HCCA	α-cyano-4-hydroxycinnamic acid
ISD	in-source decay
LID	laser-induced decomposition
LIFT-TOF/TOF-MS	MALDI-TOF-MS/MS employing a potential lift to accelerate fragment ions
NEM	*N*-ethylmaleimide
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
pE	pyroglutamate
PSD	postsource decay
RNAseA	bovine ribonuclease A
TCEP	tris(2-carboxyethyl)phosphine
TLCK	*N*α-tosyl-L-lysine chloromethyl ketone
TPCK	tosyl-l-phenylalanine chloromethyl ketone

ACKNOWLEDGMENT

The authors thank Ute Linder for fruitful discussions and Gerhard Treitz and Martin Zabe for critically reading the manuscript.

SUPPORTING INFORMATION AVAILABLE

MALDI mass spectra of an HPLC fraction containing peptide (52−64) before and after reduction and alkylation: m_z_1347_red_alk.tif. Parameter file for conventional data interpretation with SearchXLinks: SXL_convent_par.txt. parameter file for data interpretation with SearchXLinks considering ISD mechanism: SXL_ISD_par.txt. Subroutine used for identification of putatively disulfide-linked peptides in peak lists of MALDI mass spectra: Search_disulfides_subroutine.pdf. Detailed description for all assignments of disulfide-linked peptides present in Figure 3: Fig_3_assign_details.pdf. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review May 28, 2002. Revised manuscript received July 15, 2002. Accepted July 31, 2002.

AC025807J

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