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RESEARCH ARTICLE

Unique Fragmentation of Singly Charged DEST Cross-Linked Peptides

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Abstract

It has previously been shown that when cross-linking reagent diethyl suberthioimidate (DEST) reacts with primary amines of proteins to yield amidinated residues, the primary amines retain their high basicity, and cross-linked species can be enriched by strong cation exchange. It is now demonstrated that collisional activation of singly-charged DEST cross-linked peptide ions leads to preferential cleavage at the cross-linked sites. The resulting product ions facilitate the detection and identification of cross-linked peptides.

Key words: Cross-linking, Fragmentation pattern, Collisional induced dissociation, MALDI ion trap

Introduction

Chemical cross-linking combined with mass spectrometry is becoming a popular approach for studying the tertiary and quaternary structures of proteins and protein complexes [1–5]. Nevertheless, unambiguous, reliable, and fast identification of cross-links poses a significant challenge. Normally, mass spectrometric identification of the cross-linked peptides is performed after proteolytic digestion of a reaction mixture that contains cross-linked proteins. Identification of the cross-linked peptides in a proteolytic digest of derivatized sample is impeded by the complexity of the created peptide mixture. Cross-linked species are often present in low abundances, and are outnumbered by unmodified peptides and those modified with partially hydrolyzed reagent (so-called "dead-ends"). To address this issue, several strategies have been investigated, including isotope-coding [6–9], enrichment of reagent-modified peptides by affinity purification [10–12], and the use of cross-linking reagents that produce marker ions during low-energy CID [11, 13]. These approaches facilitate the detection of cross-linked peptides over unmodified peptides. However, they encounter problems with dead-ends, since these species are also reagent modified.

Identification of cross-linked peptide ions from their CID tandem MS or MS^n spectra is another challenge. These spectra are often very complex as they contain a number of b- and y-type product ions, originating from both peptides involved in the cross-linking product. Several search algorithms have been developed to interpret these spectra [14, 15], but search times are usually long and manual validation of results is often required. One method to solve this problem is to design a cross-linking reagent that incorporates a labile covalent bond within the linker region, that is selectively and preferentially cleaved by collisional activation in the gas phase [16–22]. Dissociation of the linker leads to the formation of indicative mass-shifted product ions and neutral losses arising from cross-linked species and dead-ends. Based on the observed mass shifts, different types of cross-linking products can be easily recognized.

Previously, we synthesized a novel homobifunctional thioimidate cross-linking reagent, diethylsuberthioimidate (DEST), which effectively modifies proteins via amidination under physiological conditions [23]. One advantage of this reagent is that the product amidinated residues have pKa values about two units higher than the already basic primary amines. Therefore, the modification preserves the positive charge that amines exhibit at physiological pH. Protein tertiary and quaternary structure are thus unlikely to be disturbed by the modification. Further, if trypsin is used in the digestion of cross-linked protein, the resulting cross-linked peptides typically contain six basic sites (two N-termini, two C-terminal basic residues (lysine or arginine), and two amidinated residues from the cross-linking reaction). In an

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acidic solution, these cross-linked peptides tend to bear six positive charges, while unmodified and dead-end modified peptides typically have only two or three positive charges. We have previously demonstrated that DEST cross-linked peptides can be easily separated from other components of tryptic digests using strong cation exchange (SCX) chromatography [23, 24].

An interesting hypothesis that arose during previous work with DEST is that amidine bonds of a linkage are labile in the gas phase when deprotonated. We observed that when DEST cross-linked peptides had been ionized by ESI and dissociated via CID, low abundance ions corresponding to cleavage of the amidine bonds were sometimes observed, most often in MS/MS spectra of lower charge state precursors [23]. Given that amidine bonds are known to be unstable when deprotonated by extremely basic solutions (i.e., pH>12) [25], we believed that fragmentation of the amidine bonds of a cross-link would become more significant in MALDI-MS/MS spectra, since MALDI tends to produce singly-protonated peptide ions. These are expected to be destabilized and thus preferentially dissociate upon CID. In this work, we examine this hypothesis using a tryptic digest of DEST cross-linked cytochrome *c*. Fragmentation patterns of MS-MS spectra of cross-linked peptides were analyzed and evaluated and conclusions about fragmentation properties are reported.

Experimental

Materials

Acetonitrile (ACN), hydrochloric acid, sodium hydroxide, and trifluoro acetic acid (TFA) were purchased from EMD Chemicals (Gibbstown, NJ, USA). Horse heart cytochrome *c* (C-7752) and proteomics grade trypsin (T-6567) were purchased from Sigma (St. Louis, MO, USA). Formic acid (FA) and O-methylisoureahemisulfate were obtained from Acros Organics (Pittsburg, PA, USA). Calcium chloride dihydrate, dichloromethane, sodium chloride, and sodium phosphate monobasic were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). RapiGest SF Surfactant was purchased from Waters (Milford, MA, USA). Cross-linking reagent diethylsuberthioimidate (DEST) was synthesized as previously described [23].

Protein Cross-Linking

Horse heart cytochrome *c* was reacted with DEST. Reactions were carried out in 20 mM sodium phosphate/150 mM sodium chloride (pH 7) with the protein at a final concentration of 5 μM and reagent to protein molar ratios of 100:1. After proceeding at room temperature for 12 h, reactions were quenched by adding 0.5 M Tris buffer to a final concentration of 50 mM. After another 12 h, the resulting reaction mixtures were desalted and cleared of hydrolyzed reagent using Microcon YM-10 centrifugal filter devices with a mass cut-off of 10 kDa (Millipore, Eschborn, Germany). The concentrate of cross-linked cytochrome *c* (25 μg) was digested with proteomics grade trypsin (1.25 μg) in 100 mM Tris and 10 mM calcium chloride (pH8). Since structures of cross-linked proteins are more rigid and resistant to digestion, 100 ug RapiGest Surfactant was added to a final concentration of 0.1 % (wt/vol). Each digest reaction was allowed to proceed at 37 °C for 24 h and was subsequently quenched by adding TFA to a final concentration of 0.5 %. Reaction mixtures were incubated at 37 °C for 40 min to precipitate the RapiGest Surfactant. Samples were centrifuged at 13,000*g* for 10 min, and the resultant supernatants were transferred to new microcentrifuge tubes.

SCX Enrichment of DEST Interpeptide Cross-Links

SCX enrichment of cross-linked peptides was performed using the method reported in previous work [23] with modifications as noted below. Tryptic digest of cross-linked cytochrome *c* (25 μg) was loaded onto an SCX column (TSKgel SPNPR,4.6×35 mm; Tosoh Bioscience, Montgomeryville, PA, USA) using 0.1 % TFA in water as mobile phase and a flow rate of 0.3 mL/min. Non-cross-linked species were removed in 10 column volumes of mobile phase containing 300 mM NaCl. Interpeptide cross-links were then eluted from the SCX column onto a C18 trapping column (Thermo Hypersil-Keystone Javelin, 1.0×20 mm; Bellefonte, PA, USA) in 10 column volumes of mobile phase containing 1000 mM NaCl. After desalting with mobile phase A at 0.3 mL/min for 10 min, the contents of the C18 trapping column were eluted in a 10 min isocratic hold at 95 % 0.1 % TFA in ACN (flow rate 50 μL/min) with an injection of 16 μL 50:50 IPA and ACN. This eluate fraction enriched for interpeptide DEST cross-links was dried under vacuum and resuspended in 25 μL of 0.1 %TFA in H_2O for subsequent analysis.

Peptide Guanidination

To improve MALDI ionization intensities, tryptic peptides were guanidinated using O-methylisourea in certain experiments where noted [26, 27]. A solution of guanidination reagent was made by dissolving 0.05 g of O-methylisourea in 51 μL of water. For each derivatization, cross-linked peptides enriched from the digest of 150 μg cross-linked cytochrome *c* were dissolved in 5 μL of water. This peptide solution was mixed with 5.5 μL of ammonium hydroxide (7 N) and 1.5 μL of the guanidination reagent. The pH of the reaction solution was approximately 10.5. The reaction was incubated at 65 °C for 5–10 min before being terminated by adding 15 μL of 10 % TFA (vol/vol). Reaction mixtures were dried using a speedvac and resuspended in 25 μL of 0.1 % TFA.

Reversed-Phase Liquid Chromatography (RPLC) Separation and MALDI Spotting

Enriched cross-linked peptides were separated by nanoscale reversed-phase liquid chromatography and then directly spotted onto a MALDI plate using a robot (Eksigent, Dublin, CA, USA). Two μL of the peptide mixture (5 μg peptides) was injected onto a homemade C18 column (150 mm×75 μm, 5 μm particle size, 300 Å pore size). Purified water and HPLC grade ACN (each containing 0.1 % TFA) were used as mobile phases A and B, respectively. A linear gradient from 3 % to 45 % of B over 75 min at a flow rate of 300 nL/min was used for peptide elution. Effluent was directly mixed with 5 g/L CHCA matrix solution (75 % ACN, 25 % H_2O and 0.1 % TFA) at a flow rate of 600 nL/min. A spot was deposited every 60 s during the elution gradient, creating 120 spots in total.

MALDI-LTQ Mass Spectrometry

Peptide ions were created, isolated, and collisionally fragmented (35 % activation energy) on a MALDI-LTQ XL mass spectrometer (Thermo Scientific, FL, USA). All cross-linked peptide mixtures from model proteins were first analyzed without fragmentation to obtain a list of precursor ion masses. The 20 most intense peaks in each mass spectrum were isolated for MS-MS experiments. Fragment ions of interest were further isolated in the trap for MS^3 experiments.

Capillary LC-ESI-MS/MS of Cross-Linked Peptides

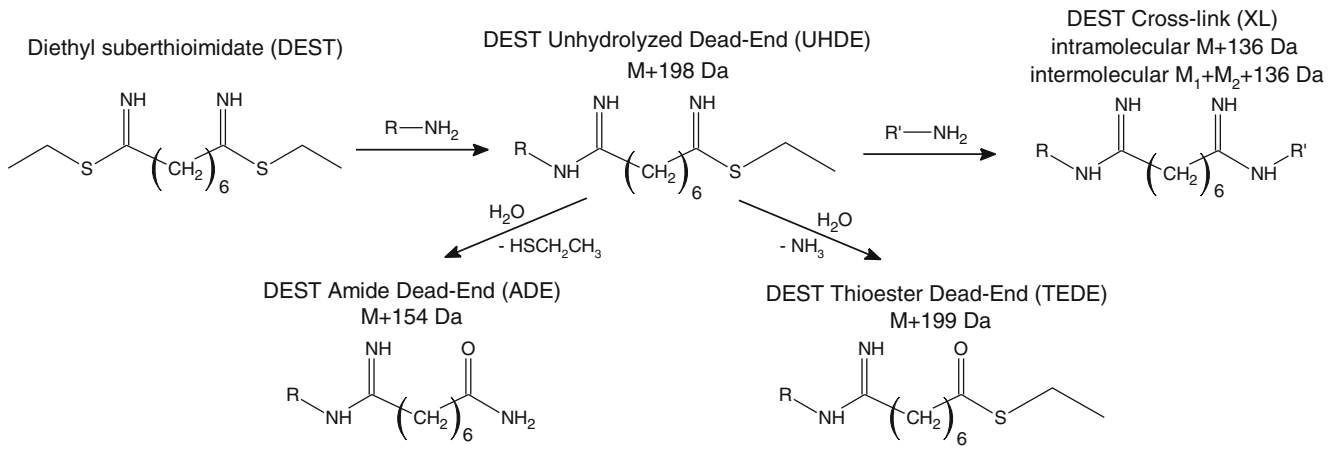
Capillary LC of peptides was performed using an Integra Frit capillary trapping column packed with 1.5 cm of C18 (150 μm×11 cm; New Objective, Woburn, MA, USA; Magic C18, 5 μm, 200 Å; MichromBio Resources, Auburn, CA, USA), a capillary analytical column packed with 15 cm of C18 (75 μm×15 cm; Magic C18, 5 μm; 100 Å, MichromBio Resources, Auburn, CA, USA), and a Dionex chromatography system (Ultimate3000; Dionex, Sunnyvale, CA, USA). In each experiment, approximately 1 μg of protein digest was injected onto a trapping column to remove salts and contaminants by flushing for 10 min with mobile phase A (0.1 % FA in 97:3 water/ACN) at a flow rate of 10 μL/min. The flow rate was then reduced to 0.3 μL/min, effluent from the trapping column was directed to the capillary LC column, and a 75-min gradient between 0 and 40 % mobile phase B (0.1 % FA in ACN) was implemented. Eluting peptides were electrosprayed into a Thermo LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) operating in data-dependent mode to acquire a full MS scan (300–2000 *m/z*) and subsequent CID MS/MS scans of the five most intense precursor ions in the LTQ at 35 % normalized collision energy. Charge state rejection was enabled for 1+ charge states. MS and MS/MS spectra were subjected to data reduction using Mascot Distiller.

Results and Discussion

Unique Fragmentation: Mechanism, MS2 and MS3

DEST modifies the primary amines of proteins, thereby introducing a set of amidine linked reaction products, including cross-links and so-called "dead-ends." As noted in Scheme 1, cross-links between two amines add 136 Da to the sum of the masses of the linked peptides. Dead-ends, which form when one end of the reagent reacts with an amine while the other end is hydrolyzed, shift a peptide's mass by 154 or 199 Da, depending on whether hydrolysis eliminates the thiol to form an amide (ADE) or eliminates ammonia to form a thioester (TEDE).

The structure of a cross-linked peptide that might be formed by proteolysis of a DEST cross-linked protein is displayed in Figure 1. In previous work [23] with DEST, it was observed that the amidine bonds at the linkage of such cross-linked peptides are prone to dissociation for lower charge states (≤3+). Therefore, we hypothesized that the amidine bonds would be preferentially cleaved when cross-



Scheme **1**. Major reaction products of DEST cross-linking

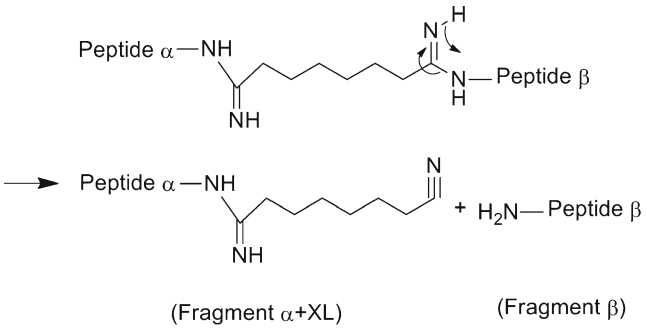


Figure 1. Mechanism for the preferential cleavage at the amidine cross-link bond

linked peptides are deprotonated. The proposed mechanism for this fragmentation process is presented in Figure 1. The hydrogen atom on the imine nitrogen moves to the nearby amine nitrogen. As a result, a cyano group is formed at the end of the linker and an intact peptide is cleaved off. The charge, which is not specifically indicated in this figure, could be on either fragment and determines which is observed. In theory, both of the amidine bonds of the linkage can be cleaved and four different fragments can be formed. These are labeled as α peptide, α+XL, β peptide, and β+XL. The mass of α+XL is that of the α peptide plus 136 Da.

To investigate our hypothesis, the tryptic digest of DEST-modified cytochrome *c* was analyzed on a MALDI-LTQ. Cross-linked peptides were spotted onto a MALDI plate after nanoscale reversed-phase liquid chromatography. MS-MS and MS^3 spectra were generated with the MALDI-LTQ mass spectrometer. Cross-linked species were identified from these spectra by manual interpretation and reference to previous work [23].

To study the effect of charge state on the fragmentation of cross-linked peptides, [73]**K**YIPGTK[79] cross-linked to [54]N**K**GITWK [60] was analyzed by both ESI and MALDI. The letters in bold here indicate the cross-linking sites. Figure 2a is the ESI MS-MS spectrum of the +3 charged molecule. The spectrum is dominated by the presence of b- and y-type sequence ions indicating the backbone fragmentation of each peptide chain. There is no obvious peak corresponding to cleavage at the amidine bonds formed by the cross-linking reaction. Figure 2b is the MALDI MS-MS spectrum of the same molecule in the +1 charge state. With only one proton on the cross-linked peptide, there is a dramatic change in the fragmentation pattern. Instead of many sequence ions as observed in the ESI MS-MS spectrum, there are only a few major fragment peaks of significant intensity. The most intense peak is associated with the loss of ammonia from the precursor. Another peak corresponds to b_n+H_2O ions that are known to form through a charge-remote mechanism when peptide ions with C-terminal basic residues are proton-deficient [23, 28]. This particular cross-link also yielded a $b_{2\beta}$ ion. It is formed by the cleavage of the peptide backbone next to the modified

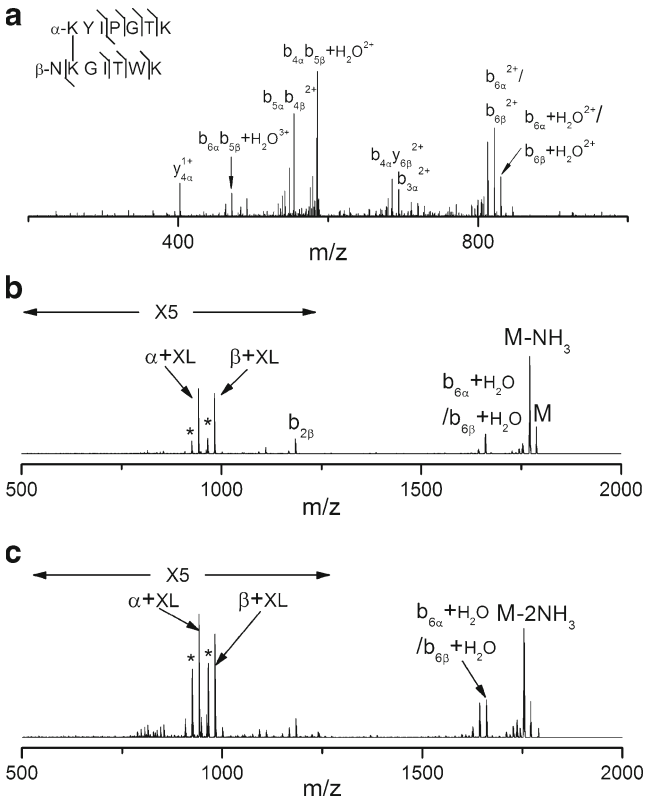


Figure 2. MS/MS spectrum of [73]**K**YIPGTK [79] cross-linked to [54]N**K**GITWK[60] **(a)** in ESI-Orbitrap (3+ precursor ion); **(b)** in MALDI-LTQ (1+ precursor) without WideBand activation, and **(c)** with WideBand activation (peaks labeled by * correspond to the loss of ammonia from α+XL or β+XL)

lysine, which is commonly observed for cross-linked peptides [5, 29]. The mechanism of this phenomenon is not yet clear. The other two intense peaks correspond to α+XL and β+XL, resulting from cleavages at the linkage points. The two small peaks labeled with asterisks are associated with the loss of ammonia from α+XL or β+XL. Protonated peptide α and β ions are not observed. Apparently the unamidinated peptides are less basic than the α+XL and β+XL fragments.

The present work involved only singly-charged MALDI-generated cross-linked peptide ions. The observed preferential cleavage at DEST cross-linked sites differs somewhat from the behavior of other CID cleavable cross-linking reagents, for example, sulfoxide (DSSO) [19], urea [22], and cyclic amine [16] containing cross-linkers. In these cases, cross-linked peptides can easily dissociate in different charge states. This indicates that the amidine bonds formed at DEST cross-linked sites are not especially labile.

WideBand activation can be used to further dissociate the $M - NH_3$ and improve the signal for the α+XL and β+XL fragment ions. WideBand activation involves the application of resonance excitation over a mass range that extends 20 Da below the selected ion [30]. This ensures that both the parent ion and any subsequent water or ammonia loss ion will be activated. The MS-MS spectrum for the same cross-linked

species, now obtained using WideBand activation, is shown in Figure 2c. Another ammonia loss is induced by the WideBand activation from the M – NH_3 fragment ion. The absolute intensity of both α+XL and β+XL ions improve. The noise level in the spectrum increases as fragment ions with ammonia or water loss appear or become more intense.

α+XL and β+XL fragment ions can be isolated in the trap and collisionally activated. Results are shown in Figure 3a and b. In both spectra, assignments to fragments containing the linker are labeled with a cross. We observe intense peaks associated with neutral loss of the linker as well as peaks corresponding to b_6+H_2O ions formed through a charge-remote mechanism [23, 28]. Besides these two fragment ions, b and y sequence ions are also observed. These can be used to identify the sites of cross-linking. Since trypsin cannot cleave at a DEST cross-linked lysine residue, the cross-linked site is usually quite obvious. In these two cases, the majority of b-type ions contain the linker, indicating that the cross-linking site is the lysine residue at or next to each peptide N-terminus.

Fragment Ion Intensities and Guanidination

Fragmentation patterns of other DEST cross-linked peptides from cytochrome *c* and the peak intensities of the fragments in their MS-MS spectra were analyzed and evaluated. For each cross-linked pair, four kinds of fragments can be formed by cleavage at a linkage point: α peptide, α+XL, β peptide, and β +XL. From our experimental results, it is believed that the appearance of the fragment ions and their intensities in MS-MS are mainly determined by the basicities of the fragments. For example, a peptide with the linker attached is usually more basic compared with the peptide itself, since it always has a basic amidinated site. Therefore, α+XL and β+XL are always observed in MS-MS spectra, whereas peptides α and β are only

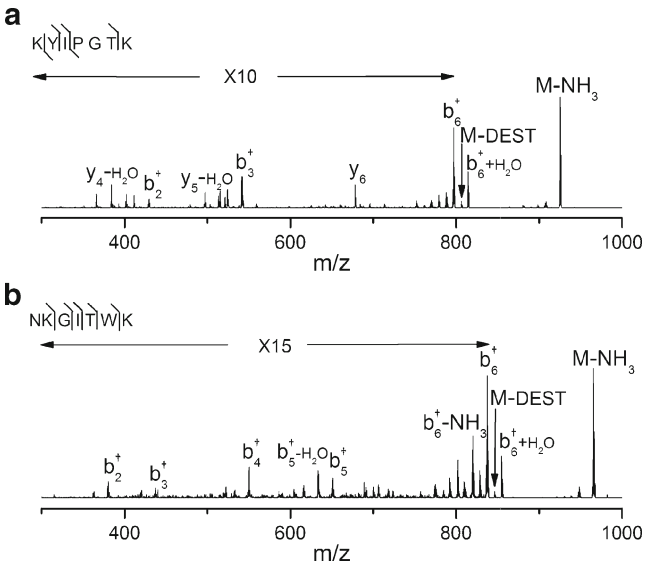


Figure 3. MALDI-LTQ MS/MS/MS spectrum of **(a)** α+XL ([73]**K**YIPGTK [79]) and **(b)** β+XL([54]N**K**GITWK[60])

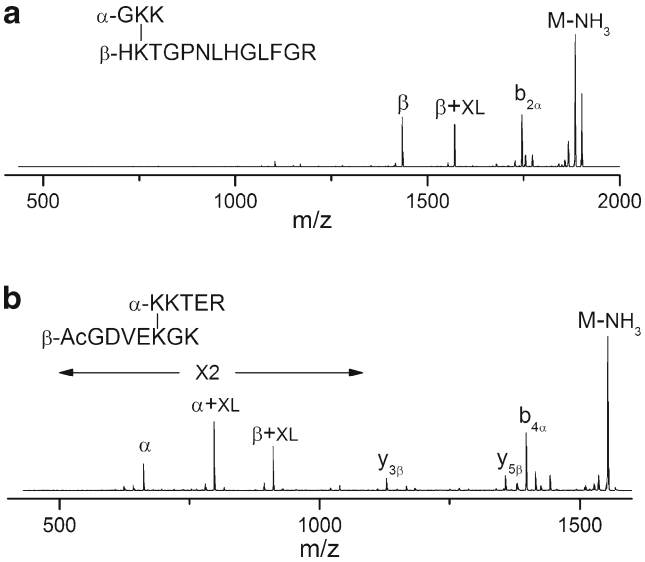


Figure 4. MALDI-LTQ MS/MS spectrum of **(a)** [6]G**K**K[8] cross-linked to [26]H**K**TGPNLHGLFGR[38], and **(b)** [87] **K**KTER [91] cross-linked to [1]AcGDVE**K**GK[7]

seen occasionally. Whether peptide α or peptide β is detectable depends on their relative basicities.

Most MALDI MS-MS spectra of DEST cross-linked peptides contain both (α+XL) and (β+XL) as in the example of Figure 2b. This is usually the case when two peptide chains have similar basicity. However, occasionally, one peptide is much more basic than the other. In this case, only this chain is observed in the MS-MS spectrum. Figure 4a shows the MS-MS spectrum of [6]G**K**K[8] cross-linked to [26]H**K**TGPNLHGLFGR[38]. The β peptide (HKTGPNLHGLFGR) contains far more basic residues (five basic sites: one arginine, one lysine, one N-terminus, and two histidines). In this case, the spectrum was dominated by β fragment ions: β and β+XL. This particular cross-link also

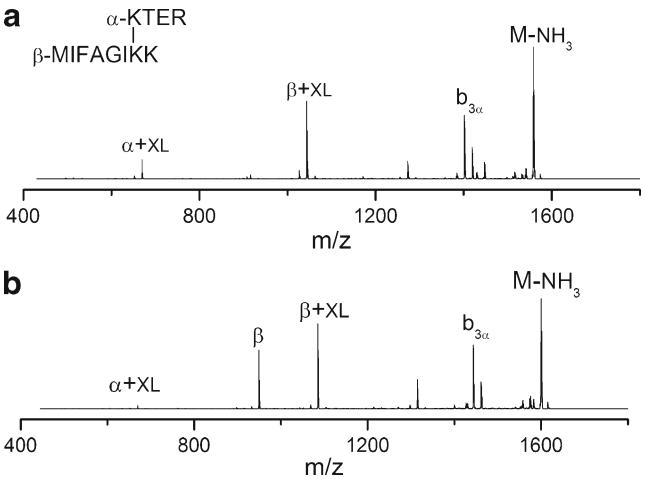


Figure 5. MALDI-LTQ MS/MS spectrum of [88]**K**TER[91] cross-linked to [80]MIFAGI**K**K[87] **(a)** before and **(b)** after guanidination

yielded a $b_{2\alpha}$ ion. It is formed by the cleavage of the peptide back bone at the position next to the modified lysine.

Peptide ions (α or β) are sometimes observed when arginine is present in the peptide sequence, since in this case the peptide has a basicity comparable to that of the amidinated group. Figure 4b is an MS/MS spectrum of [87]**K**KTER [91] cross-linked to [1]AcGDVE**K**GK[7]. In this case, [87]**K**KTER[91] is quite basic with several basic sites (one arginine, two lysines, and one N-terminus). Therefore, α and α+XL were observed with strong intensity. On the other hand, [1]AcGDVE**K**GK[7] contains only two lysines, and it is the least basic of four fragments formed by cleavages at the linkage sites. As a result, this peptide is not observed in the spectrum. There are also some fragments ($b_{4\alpha}$, $y_{3\beta}$, and $y_{5\beta}$) formed by the enhanced cleavage of the peptide backbone at the C-terminal side of an acidic residue [31–34].

The homoarginine formed by a guanidination reaction can also increase the basicity of the peptide chain and the signal of the modified peptide is enhanced. Figure 5a displays the MS-MS spectrum of [88]**K**TER[91] cross-linked to [80]MIFAGI**K**K[87]. As expected, α+XL and β+XL are observed in the spectrum with high intensities. The MS-MS spectrum of this same cross-link after being guanidinated is shown in Figure 5b. The C-terminal lysine in [80]MIFAGI**K**K[87] was now converted to homoarginine. The spectrum is similar to the previous one (Figure 5a), except that the peak intensity for the β peptide ion is greatly increased.

Application

The above results have shown that the amidine linkages of singly-charged DEST cross-linked peptide ions are preferentially cleaved during CID yielding simple fragmentation spectra. This unique feature can be utilized for identifying cross-linked peptides. Most MALDI MS-MS spectra contain peaks associated with α+XL and β+XL. The difference between the sum of these two and the precursor mass is equal to the mass of the linker plus a proton (137.10733 Da). This unique mass relation is summarized by Equation (1),

$$m_1 + m_2 - M(\text{precursor}) = 137.10733 \qquad (1)$$

where m_1 and m_2 represent masses of each peptide chain with a linker (α+XL and β+XL). Secondly, as discussed above, sometimes fragments from only one peptide chain are observed in the spectrum. For example, only the β chain is observed in the MS-MS spectrum of [6]G**K**K[8] cross-linked to [26]H**K**TGPNLHGLFGR[38] (Figure 4a). However, in this case, both the peptide and the peptide+linker peaks are significant. The difference between these two is the mass of a linker (136.10005 Da) as shown in Equation (2).

$$m_1 - m_2 = 136.10005 \qquad (2)$$

Here m_1 represents the peptide+linker and m_2 represents the peptide peak. Unique mass differences in these equations can be utilized to distinguish DEST cross-links from the reaction mixture.

An algorithm based on these two equations can easily search MS-MS spectra for evidence of cross-links. It should be able to provide the mass of the cross-linked precursor ion as well as masses of each peptide chain. This information shall be most helpful for identifying cross-linked peptides.

Conclusions

Singly charged DEST cross-linked peptide ions were found to undergo preferential cleavage at their linkage sites following collisional activation. Intense peaks for α+XL and β+XL product ions were observed. The relative intensities of α+XL and β+XL are mainly dependent on the basicities of the individual peptide chains. Two simple equations can be used to help integrate MALDI MS-MS spectra and identify DEST cross-linked peptides.

Acknowledgments

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