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Mass Spectrometric Evidence for the Existence of Distinct Modifications of Different Proteins by 2(*E*), 4(*E*)-Decadienal

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Abstract

2(*E*), 4(*E*)-Decadienal (DDE), a lipid peroxidation product, was found to covalently modify Lys residues of different proteins by different reactions using mass spectrometry (MALDI-TOF-MS and LC-ESI-MS). DDE mainly formed Lys Schiff base adducts with cytochrome *c* and ribonuclease A at 10 min, but these reversibly-formed adducts almost disappeared after 24 h. In contrast, β -lactoglobulin (β -LG) was highly modified by DDE after 24 h. In addition to the Lys Schiff base adducts, DDE formed novel Lys pyridinium adducts as well as Cys Michael adducts with β -LG.

Introduction

Lipid peroxidation generates various aldehydes, including reactive α,β -unsaturated aldehydes. Characterization of protein modification by these aldehydes has been widely investigated using mass spectrometry in last two decades. Among them, 4-hydroxy-2(*E*)-nonenal (HNE), acrolein and malondialdehyde (MDA) are the most studied. HNE has been reported to form Cys/His/Lys Michael adduct, Lys Schiff base adduct, Lys pyrrole, Cys/His–Lys reversible cross-link adduct, and Lys–Lys fluorescent cross-link adduct (1-8). Acrolein has been reported to form the Cys/His Michael adduct, Lys Schiff base adduct, Lys 3-formyldihydropyridine adduct, Lys 3-methylpyridinium adduct, and His–Lys cross-link adduct (9-11). MDA has been reported to form the Lys–Lys bis-Schiff base cross-link adduct, Lys fluorescent adduct, and Arg–Lys cross-link adduct (12-16). More recently, 4-oxo-2(*E*)-nonenal (ONE) was discovered as a lipid peroxidation product (17,18), which has been found to be a more reactive electrophile with proteins and to exhibit greater neurotoxicity than HNE (3,19). ONE was reported to form Cys/His/Lys Michael adduct, Lys Schiff base adduct, Lys pyrrolinone adduct, Lys 4-ketoamide adduct, and His/Cys–Lys pyrrole cross-link adduct with proteins (3,4,7,19-21).

2(*E*), 4(*E*)-Decadienal (DDE) is a widespread dienaldehyde. It occurs in various foods and has been detected in fish (22,23), chicken (24), meat (25), bread (26,27) and heated oil (28). DDE has been shown to inhibit cell proliferation and induce the apoptosis of cells (29). It has also been reported to be carcinogenic and associated with lung adenocarcinoma in women (28). Additionally, DDE has been shown to covalently modify DNA (30,31). However, protein modification by DDE has been rarely studied, and the nature of chemistry between DDE and

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[#]This work was performed in Dr. Sayre's laboratory. Dr. Sayre was a respectable mentor and good friend. He passed away on May 8, 2009. This paper is dedicated to him.

Supporting Information **Available:** Figures S1–S3 and Scheme S1 are available free of charge via the Internet at <http://pubs.acs.org>.

protein nucleophiles is not completely clear. The Medeiros group has shown that Schiff base is the almost exclusive product found in DDE treated cytochrome *c* by mass spectrometry (32).

By incubating DDE with β -lactoglobulin (β -LG), cytochrome *c* and ribonuclease A (RNase A), this study shows that DDE has a unique modification on the Lys residue of β -LG, although the other two are also Lys-rich proteins. This finding of differential “selective” modification of specific proteins by DDE strongly suggests that lipid peroxidation products can and will differentially modify specific proteins. To determine why some proteins are especially sensitive to specific modifications can help identify and/or predict which proteins will be sensitive towards each type of modification *in vivo*. To our knowledge, this is the first report on the unusual phenomenon that distinct modifications of the same type of amino acid were observed in different proteins exposed to a lipid peroxidation-derived aldehyde.

Materials and Methods

General

Chymotrypsin, cytochrome *c*, β -LG and RNase A were purchased from Sigma. α -Cyano-4-hydroxycinnamic acid, DDE, iodoacetamide (IAM) and sinapinic acid were purchased from Acros Organics. Sequencing grade modified trypsin was received as a kit with buffer from Promega. PD-10 columns (Sephadex G-25) were obtained from GE Healthcare. ZipTips were purchased from Millipore. Dialysis tubing (Nominal MW 6000-8000) was purchased from Fisher Scientific. All other reagents were of the highest grade commercially available. The protein solution was concentrated through centrifugal evaporation under reduced pressure with a Savant Speed Vac SC110 system (Forma Scientific, Inc.). MALDI samples were spotted on the stainless steel target and analyzed with MALDI-TOF-MS as previously reported (33).

Proteolytic Digestion

Modified protein (1 mg) was dissolved in 100 μ L of pH 8.0 buffer containing 6 M guanidine hydrochloride and 50 mM Tris. After addition of dithiothreitol (200 mM, 2.5 μ L), the sample was vortexed and incubated at 37 °C for 1 h. Then IAM (400 mM, 5.0 μ L) was added, vortexed, and allowed to stand at 25 °C in a dark place for 1 h. Dithiothreitol (200 mM, 7.5 μ L) was added again, vortexed, and allowed to consume the unreacted IAM at 25 °C for another 1 h. 25 μ L of this denatured sample was added 50 μ L of trypsin (0.1 μ g/ μ L) suspension buffer or 10 μ L of α -chymotrypsin (2.0 μ g/ μ L) solution. Then the digestion mixture was diluted to 200 μ L with pH 7.8 NH_4HCO_3 solution (50 mM), and the mixture was incubated at 37 °C for 24 h. The solution was stored at -20 °C for LC-MS or MALDI-TOF-MS analysis.

HPLC-ESI-MS/MS

ESI-MS was performed with a Thermo LCQ DecaXP MAX or Advantage instrument in the positive mode using nitrogen as the sheath and auxiliary gas. The capillary temperature was 300 °C, the capillary voltage was 35.00 V, and the source voltage was 4.50 kV. Typically two scan events were used: (1) m/z 300–2000 full scan MS; (2) data dependent scan MS/MS on the most intense ion from event 1 or from the predefined parent mass list. The spectra were recorded using dynamic exclusion of previously analyzed ions for 0.5 min with three repeats and a repeat duration of 0.5 min. The MS/MS normalized collision energy was set to 35%. Reversed-phase HPLC was performed with a Surveyor LC system equipped with a 5 μ m 2.1 $\times$ 250 mm Grace Vydac C18 column with a gradient elution program at a flow rate of 200 μ L/min. Eluent A was a mixture of 95% H_2O , 5% MeOH and 0.1% formic acid. Eluent B was a mixture of 95% MeOH, 5% H_2O and 0.1% formic acid. The gradient program was: 0-70 min, 80% A to 20% A; 70-75 min, 20% A to 80% A; 75-80 min, 80% A.

Preparation of Cys121-Carbamidomethylated β -LG

GT buffer was prepared as a pH 8.0 mixture of 6.0 M guanidine hydrochloride and 50 mM Tris. A solution of β -LG (2.5 mM, 200 μ L) added to GT buffer (1000 μ L) and water (1284.4 μ L) was incubated with IAM (400 mM, 15.6 μ L) at 25 °C in the dark for 20 h. A 2 μ L aliquot was subjected to MALDI-TOF-MS. The remainder was dialyzed against pH 7.4 sodium phosphate buffer (50 mM, 500 mL) to remove excess IAM three times with 6 h/time.

Incubation of Proteins with DDE for MALDI-TOF-MS

A solution of β -LG, Cys121-carbamidomethylated β -LG, cytochrome *c* or RNase A (2.5 mM, 20 μ L) added to pH 7.4 phosphate buffer (50 mM, 160 μ L) was incubated with a solution of DDE in EtOH (50 mM; 20 μ L). The molar ratio of DDE to the protein was 20/1. After 10, 30 min, 1, 2, 6 and 24 h, 10 μ L of each sample was mixed with matrix and deposited onto the MALDI stainless steel target for analysis.

Preparation of DDE-Modified Proteins with or without NaBH₄ Quenching

A solution of β -LG or cytochrome *c* (2.5 mM, 100 μ L) added to pH 7.4 phosphate buffer (50 mM, 800 μ L) was incubated with a solution of DDE in EtOH (50 mM, 100 μ L) at 37 °C. The molar ratio of DDE to the protein was 20/1. After 24 h, a 500 μ L aliquot was diluted to 2.5 mL and subjected to a PD-10 column eluting with 3.5 mL water to remove the unbound DDE and the buffer salts. The eluted solution was concentrated by centrifugal evaporation prior to proteolytic digestion. Another 500 μ L aliquot was treated with NaBH₄ (3.8 mg) at 25 °C overnight prior to the PD-10 column elution.

Results and Discussion

Characterization of Intact DDE-Modified Protein by MALDI-TOF-MS

The Medeiros group reported that DDE modified cytochrome *c* mainly through Lys Schiff base formation with a mass increment of 134 Da (32), which was also observed in this study (Figure 1A). In agreement with this result, when RNase A was incubated with DDE, the major product was also Lys Schiff base adduct (Figure 1B). Moreover, the spectra indicate that DDE–Lys Schiff base formation is not stable during incubation under our conditions as the prominent +134 Da adduct gradually disappeared at longer incubation times, while apparently unmodified protein remained after 24 h.

However, when β -LG was incubated with DDE under the same conditions, the spectra observed revealed a totally different pattern of modification (Figure 2). The protein, composed of an A chain and B chain (33), was modified extensively by DDE over a period of 24 h. Figure 2 shows the formation of DDE–Lys Schiff base (M+134) and DDE Michael adduct (M+152) after 10 min. Under longer incubation times, the native β -LG peak decreases concomitant with an increase in the modified β -LG peaks. After 24 h, β -LG was heavily modified by DDE. In contrast, the spectra of DDE-modified cytochrome *c* and RNase A after 24 h are almost identical to those of their controls (Figure 1). Therefore, there are obvious distinctions in modification by DDE between β -LG and cytochrome *c* or RNase A.

β -LG contains a free Cys residue, i.e. Cys121, while neither cytochrome *c* nor RNase A has any free Cys. It is possible that the above difference observed is attributable to the reaction between DDE and Cys121 in β -LG. To investigate this possibility, Cys121 of β -LG is protected by carbamidomethylation after the reaction with IAM. MALDI mass spectra show that β -LG has a mass increment of 57 Da after the treatment of IAM in the guanidine hydrochloride and Tris buffer after 20 h (Figure S1, Supporting Information), suggesting that Cys121 is effectively carbamidomethylated (Scheme S1, Supporting Information). Figure 3 displays MALDI-TOF mass spectra of DDE-modified carbamidomethylated β -LG at various times. It indicates that

after 10 min some Schiff base adducts were formed on the protein ($M+n\times 134$), but Michael adducts were not observed ($M+152$), suggesting that Michael adducts formed on DDE-modified β -LG (Figure 2) were ascribed to Cys121 reaction. Longer incubation results in a more abundant native Cys121-carbamidomethylated β -LG (M) and less abundant Schiff base adducted peak ($M+134$), presumably due to the reversibility of Schiff base formation. However, other than this, carbamidomethylated β -LG is still intensely modified by DDE after 24 h. The most abundant modified protein has a mass shift of around 269 Da, which is apparently equal to the addition of two Schiff base adducts (2×134). If this peak does arise from the independent formation of two Schiff base adducts, the mono-Schiff base bound protein ($M+134$) should also be observed since the native protein (M) is as abundant as bis-Schiff base bound protein ($M+269$). However, the peak $M+134$ is not evident after 24 h. Therefore, the mass shift of 269 Da must be attributed to an alternative modification(s). As Cys121 was protected, this special modification was obviously not ascribed to the reaction of Cys121.

Characterization of DDE-Modified β -LG by Mass Spectrometry after Proteolytic Digestion

To characterize the unique adduct of β -LG, proteolytic digests of DDE-modified β -LG were analyzed by MALDI-TOF-MS and LC-ESI-MS/MS. MALDI-TOF mass spectra of chymotryptic digests of the control and DDE-modified β -LG are shown in Figure 4. After 10 min, the mass spectra of the modified and control digests showed minimal differences. However, after 24 h, the mass spectrum profile changes substantially in comparison to those of 10 min as well as the control, revealing that a large number of novel modified peptides were generated after the long-time incubation. This dramatic change in the peptide fingerprint was observed in the tryptic digest as well (Figure S2, Supporting Information).

In order to locate the modified residues as well as their mass shifts, all of these unique peaks were separated and subjected to collision-induced dissociation (CID) by the LC-ESI-MS/MS. The resulting tandem mass spectra indicate that all of the peptides marked with an asterisk in Figure 4 contain a modified Lys residue with a mass increment of 268 Da (Table 1). The addition of 268 Da suggests that the modification incorporates portions of at least two DDE molecules, whose molecular weight is 152. Arrows a, b and c in Figure 2 demonstrate that this adduct was gradually produced after 2 to 24 h on β -LG A chain. In accordance with this observation, none of this adduct was detected after 10 min incubation in Figure 4B.

All of the modified peptides ($M+268$) have unusually high intensities in MALDI-TOF mass spectra (Figures 4 and S2), which are comparable to or even higher than the most abundant native peptide. In other words, they are at least either more abundant in the solution or much more sensitive to MALDI-TOF-MS than native peptides. However, even if all of the protein modifications occur through this reaction with a mass shift of 268 Da, Figure 2 shows that at most 5 Lys residues are modified in a β -LG molecule after 24 h. Since a β -LG chain contains 15 Lys residues and most of the protein molecules have less than 5 modified Lys residues, native Lys containing peptides should be more abundant than the modified ones in the sample solution. Therefore, these Lys modified peptides must be more sensitive to MALDI-TOF-MS than normal peptides. It is well known that the peptides containing a fixed positive charge are extremely sensitive to MALDI-TOF-MS (34-36). Various Lys pyridinium adducts involving two or more alkenal molecules have been detected and characterized by MS and/or NMR upon incubation of alkenals with a model peptide or a protein (37-41). DDE belongs to the alkenal class, but with one more conjugated double bond. Hence it may also form a pyridinium adduct with Lys residues. The extraordinary sensitivity to MALDI-TOF-MS of these modified peptides implicates the formation of pyridinium derivative, which has a fixed positive charge. Based on the pyridinium product formed between alkenals and Lys (37, 38, 40), the proposed structures **1a** and **1b** of these DDE adducts as well as their formation mechanism are shown in Scheme 1. These two isomeric adducts were both detected in the LC-MS. Examples of selected

ion chromatograms of modified peptides KGL, KVL, DKAL and DIQKVAGTW are shown in Figure S3 (Supporting Information). Each of them has two peaks with different intensity and retention time. However, their tandem mass spectra are identical.

Additionally, two prominent characteristic fragment ions (Figure 5) of these modified peptides were always observed, which corroborated the presence of pyridinium moiety. One is m/z M-285 resulting from a neutral loss of the pyridine moiety from the precursor ion. The other product ion is the protonated pyridine moiety m/z 286 except for the peptide such as DIQKVAGTW whose tandem mass spectrum doesn't cover this ion because of the low mass cut-off rule for the ion trap. These two kinds of ions are known to be featured product ions of pyridinium cations from CID (39,42,43). Figure 6 shows four examples of tandem mass spectra of DDE-modified peptides, i.e. KVL, KIDAL, KGLDIQ and DIQKVAGTW. All of them have these characteristic product ions in the spectra.

Additional chemical evidence that suggests the presence of the pyridinium moiety came from reduction by sodium borohydride (40) which results in an additional mass increment of 3 Da (Table 2) and a dramatically reduced intensity in the MALDI-TOF mass spectra. The reduction of pyridinium **1a** (M+268) by NaBH₄ leads to the intermediate dihydropyridinium **2** (M+269) (Scheme 2). After protonation and rearrangement to iminium, dihydropyridinium is further reduced to produce tetrahydropyridinium **3** (M+271) (40). Compound **3** is a neutral amine, which requires protonation to be detected by mass spectrometry resulting in a m/z of M+272. Therefore, all of the reduced pyridinium adducts listed in Table 2 have an apparent mass shift of 272 Da instead of 271 Da. Since sodium borohydride was in large excess in our experiment, every pyridinium adduct should be converted to tetrahydropyridinium. As tetrahydropyridinium has lost the fixed positive charge, it is not highly sensitive to MALDI-TOF-MS anymore.

Other than pyridinium adducts, DDE-Lys Schiff base adducts were detected after borohydride treatment (Table 2). They are not observed without reduction because they are reversibly formed and hydrolyze after standard denaturation and 24 h digestion treatment. In addition, DDE-Cys Michael adducts were observed in the reduced sample after 10 min incubation. However, they were not observed after 24 h, suggesting that they were either reversible adducts or evolved into more advanced products. Considering that intensities of the native Cys containing peptides in LC-MS at 24 h decreased dramatically compared to that at 10 min (data not shown), it was possible that these Michael adducts were finally converted into other advanced products. Although both reduced DDE-Lys Schiff base adduct and DDE-Cys Michael adduct were observed, a reduced Lys-DDE-Cys Schiff base-Michael adduct cross-link was not detected with our conditions and analysis.

Modification of cytochrome *c* by DDE is significantly different from that of β -LG. As a control to β -LG, cytochrome *c* was incubated with DDE for 10 min and 24 h and subjected to proteolytic digestion and mass spectrometry. DDE-Lys Schiff base was observed at 10 min as expected (32). However, no pyridinium adduct was detected after 10 min or 24 h incubation by either MALDI-TOF-MS or LC-ESI-MS.

It is not clear what makes this difference in modifications between β -LG and cytochrome *c* by DDE. In fact, every Lys residue of β -LG was found to form the pyridinium adduct from combined results of both chymotryptic and tryptic digests. In contrast, none of the cytochrome *c* Lys residues were observed to form this adduct. The environment of Lys residues in X-ray structures of both proteins was investigated, but no obvious difference was observed. Further study is needed to answer this question.

Conclusions

DDE has been reported to exclusively form the Lys Schiff base adducts with cytochrome *c* (32), which to our knowledge is the first and only literature investigating the protein modification by DDE. In our study, we found that DDE not only forms the Lys Schiff base adduct with β -LG, but also forms other Lys product, pyridinium adduct, as well as DDE-Cys Michael adduct. After 24 h, β -LG was substantially modified by DDE while cytochrome *c* and RNase A were minimally modified. The pyridinium adduct was observed when the DDE concentration was as low as 500 μ M with a DDE/protein molar ratio of 2/1 (data not shown). At physiologic conditions, where the DDE concentration may be much lower, the formation of pyridinium adduct may be minimal. Further work is required to identify the conditions required to generate this pyridinium modification, as they may be physiologically relevant. Further, these results indicate that DDE displays different behaviors with different proteins, and suggest that different proteins may have different modifications by the same reactive aldehyde. MDA, acrolein, HNE and ONE etc. have been reported to modify proteins through various reactions. Although incubation of these aldehydes with different proteins has been investigated previously (44), none of them has been reported to modify different proteins in distinct reactions. Our results with DDE suggest that the surface nature of individual proteins can affect the observed products of modification by bifunctional lipid peroxidation products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

MALDI-TOF mass spectra of 0.25 mM cytochrome *c* (A) or RNase A (B) modified by 20 equivalents of DDE (5 mM) in pH 7.4 phosphate buffer at 37 °C for various times. The mass increment of 134 Da is due to the DDE–Lys Schiff base formation.

Figure 2.

MALDI-TOF mass spectra of 0.25 mM of β -LG modified by 20 equivalents of DDE (5 mM) in pH 7.4 phosphate buffer at 37 °C for various times. The mass increments of 134, 152 and 268 Da are due to the DDE-Lys Schiff base, DDE-Cys Michael adduct and DDE-Lys pyridinium adduct, respectively. Arrows a, b and c indicate that the pyridinium adduct was gradually formed after 2 to 24 h on β -LG A chain.

Figure 3.

MALDI-TOF mass spectra of 0.25 mM of Cys121-carbamidomethylated β -LG modified by 20 equivalents of DDE (5 mM) in pH 7.4 phosphate buffer at 37 °C at various times. The mass increments of 134 and 269 Da are attributed to the DDE-Lys Schiff base and DDE-Lys pyridinium adduct, respectively.

Figure 4. MALDI-TOF mass spectra of chymotryptic digests of 0.25 mM of β -LG control (A) and β -LG modified by 20 equivalents of DDE (5 mM) after 10 min (B) or 24 h (C). The peaks marked with an asterisk are peptides modified by DDE through Lys pyridinium formation.

Figure 5. Characteristic fragment ions Py^+ and M-285 of DDE-Lys pyridinium adducts.

Figure 6.

Tandem mass spectra of modified peptides KVL (m/z 627.32), KIDAL (m/z 827.34), KGLDIQ (m/z 941.41) and DIQKVAGTW (m/z 1285.49) through DDE-Lys pyridinium formation. Fragment ions Py^+ and $M-285$ are shown in Figure 5. Asterisks denote the modified residue.

Scheme 1. Proposed Reaction Mechanism for Formation of DDE-Lys Pyridinium Adducts 1a and 1b

Scheme 2. Reduction of DDE-Lys Pyridinium Adduct 1a by NaBH₄

Table 1
Modified Peptides Detected by LC-ESI-MS/MS from Chymotryptic Digest of DDE Treated β -LG at 10 min or 24 h

Peptides [§]	Position	m/z	Modified m/z	Assignment	Incubation time
LIVTQ	1-5	573.4	841.4	Pyridinium	24 h
KGL	8-10	317.2	585.2	Pyridinium	24 h
KGLDIQ	8-13	673.3	941.4	Pyridinium	24 h
DIQKVAAGTW	11-19	1017.5	1285.5	Pyridinium	24 h
VEELKPTPEGDLLEIL	43-57	1681.9	975.5 [#]	Pyridinium	24 h
LQKW	58-61	574.3	842.3	Pyridinium	24 h
KIPAVF	77-82	674.4	942.4	Pyridinium	24 h
KIDAL	83-87	559.3	827.3	Pyridinium	24 h
KIDALNENKVL	83-93	1256.7	763.1 [#]	Pyridinium	24 h
NENKVL	88-93	716.3	984.3	Pyridinium	24 h
KVL	91-93	359.3	627.3	Pyridinium	24 h
KKY	100-102	438.3	706.3	Pyridinium	24 h
VRTPEVDDALEK [¶] F	123-136	1647.8	958.7 [#]	Pyridinium	24 h
DKAL	137-141	446.3	714.3	Pyridinium	24 h
KALPM	141-145	559.3	827.3	Pyridinium	24 h

[§] Bold and underlined residue was modified.

[#] doubly charged peak.

Table 2
Modified Peptides Detected by LC-ESI-MS/MS from Chymotryptic Digest of DDE Treated β -LG at 10 min or 24 h after Reduction

Peptides ^{\$}	Position	m/z	Modified m/z	Assignment	Incubation time
<u>L</u> IVTQ	1-5	573.4	709.4	Redu Lys SB	10 min, 24 h
<u>K</u> GLDIQ	8-13	673.3	809.3	Redu Lys SB	10 min, 24 h
VEEL <u>K</u> PTPEGDLEIL	43-57	1681.9	909.8 [#]	Redu Lys SB	10 min, 24 h
LQKW	58-61	574.3	710.3	Redu Lys SB	10 min, 24 h
<u>K</u> PAVF	77-82	674.4	810.4	Redu Lys SB	10 min, 24 h
<u>K</u> DAL	83-87	559.3	695.3	Redu Lys SB	10 min, 24 h
KIDALNEN <u>K</u> VL	83-93	1256.7	1392.7	Redu Lys SB	10 min, 24 h
NEN <u>K</u> VL	88-93	716.3	852.3	Redu Lys SB	10 min, 24 h
VRTPEVDDEALE <u>K</u> VF	123-136	1647.8	893.0 [#]	Redu Lys SB	10 min, 24 h
DKAL	137-140	446.3	582.3	Redu Lys SB	10 min, 24 h
<u>K</u> ALPM	141-145	559.3	695.3	Redu Lys SB	10 min, 24 h
AC* QCL	118-122	594.3	748.3	Redu Cys MA	10 min
VC* QCL	118-122	622.3	776.3	Redu Cys MA	10 min
CL	121-122	235.1	389.1	Redu Cys MA	10 min
<u>K</u> GL	8-10	317.2	589.2	Redu Pyridinium	24 h
VEEL <u>K</u> PTPEGDLEIL	43-57	1681.9	978.0 [#]	Redu Pyridinium	24 h
LQKW	58-61	574.3	846.3	Redu Pyridinium	24 h
<u>K</u> DAL	83-87	559.3	831.3	Redu Pyridinium	24 h
KIDALNEN <u>K</u> VL	83-93	1256.7	1528.7	Redu Pyridinium	24 h
<u>K</u> VL	91-93	359.27	631.3	Redu Pyridinium	24 h
VRTPEVDDEALE <u>K</u> VF	123-136	1647.8	961.0 [#]	Redu Pyridinium	24 h
DKAL	137-140	446.3	718.3	Redu Pyridinium	24 h
<u>K</u> ALPM	141-145	559.3	831.3	Redu Pyridinium	24 h

^{\$} Bold and underlined residues were modified.

[#] doubly charged peak.

* Cys was S-carbamidomethylated with IAM.