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FOCUS: EMERGING INVESTIGATORS: APPLICATION NOTE

Gas-Phase Fragmentation Behavior of Oxidized Prenyl Peptides by CID and ETD Tandem Mass Spectrometry

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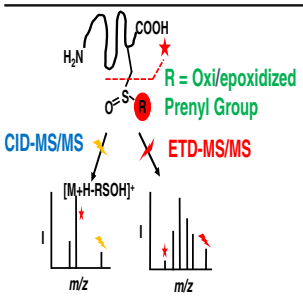

Abstract. Farnesylation and geranylgeranylation are the two types of prenyl modification of proteins. Prenylated peptides are highly hydrophobic and their abundances in biological samples are low. In this report, we studied the oxidized prenylated peptides by electrospray ionization mass spectrometry and identified them by collision-induced dissociation (CID) and electron-transfer dissociation (ETD) tandem mass spectrometry. Modified prenyl peptides were generated utilizing strong and low strength oxidizing agents to selectively oxidize and epoxidize cysteine sulfur and prenyl side chain. We selected three peptides with prenyl motifs and synthesized their prenylated versions. The detailed characteristic fragmentations of oxidized and epoxidized farnesylated and geranylgeranylated peptides were studied side by side with two popular fragmentation techniques. CID and ETD mass spectrometry clearly distinguished the modified version of these peptides. ETD mass spectrometry provided sequence information of the highly labile modified prenyl peptides and showed different characteristic fragmentations compared with CID. A detailed fragmentation of modified geranylgeranylated peptides was compared by CID and ETD mass spectrometry for the first time.

Keywords: Prenylation, Oxidized prenyl peptides, Farnesylated peptides, Geranylgeranylated peptides, CID-MS/MS, ETD-MS/MS

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Introduction

Prenylation is a type of post-translational lipid modification, which generally occurs at the cysteine residues situated at the carboxyl terminal of the protein. Farnesylation and geranylgeranylation are the two types of prenylation [1]. Farnesylation is a type of post-translational lipid modification where a 15-carbon prenyl group (~204 Da) is attached to a cysteine residue of the carboxyl terminal, whereas geranylgeranylation is a 20-carbon prenyl group (~272 Da) attached to the cysteine residue [2]. These modifications of proteins are connected with several human cancers, such as pancreatic, colon, and acute myeloid leukemia. They are also found to be involved with other diseases such as progeria, aging, parasitic, bacterial, and viral infections [1, 3]. A few mass spectrometric studies have been performed on farnesylated peptides by several researchers [4–6]. There are several challenges to detect the prenylated peptides in a large pool of non-prenylated peptides. The ionization efficiency of prenylated peptides in positive ion mode MS is typically lower compared with non-prenylated peptides because of the attachment of a long hydrophobic lipid side-chain group [6]. Loss of farnesyl group was observed by Hoffmann and Kast by MALDI-TOF/TOF and ESI-QqTOF-MS but the fragment was not that very distinct in the spectrum [5]. Overall, this signature fragmentation was not consistent in tandem MS; therefore, this method has never been used for the identification of farnesylated peptides and proteins [4, 6]. It is also noteworthy that although few studies can be found for farnesylated peptides, almost no studies in the literature are available on the fragmentation behavior of geranylgeranyl peptides, a major type of prenylation in proteins.

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Collision-induced dissociation (CID) and electron transfer dissociation (ETD) are two major fragmentation techniques in mass spectrometry [7, 8]. Owing to the fragmentation in amide bonds, ETD has become popular for sequencing peptides with labile postttranslational modifications (PTMs) [9–11]. A combination of these two fragmentations provides more information about peptide sequence and, at present, combinations are being widely used for sequencing peptides.

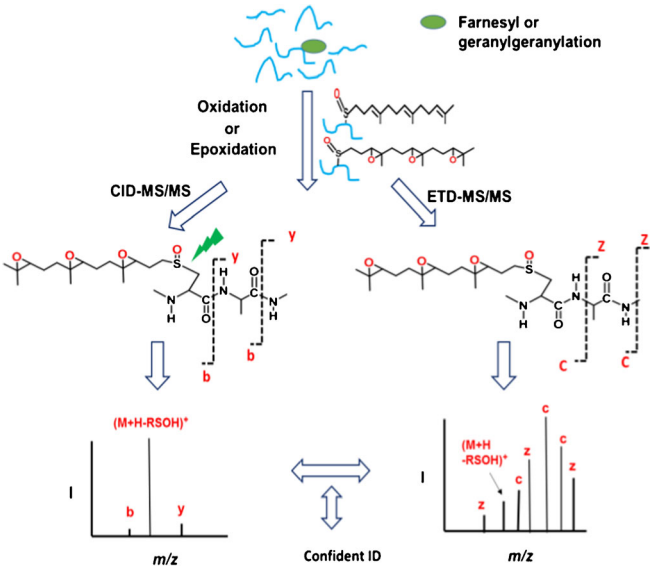
In this report, we are presenting comprehensive fragmentation studies of oxidized prenyl peptides with electrospray ionization and CID and ETD tandem mass spectrometry. We synthesized several prenyl peptides and studied their fragmentation behavior in gas phase utilizing CID and ETD mass spectrometry side by side. We oxidized these peptides to improve their hydrophilicity and in addition to CID, for the first time, we are providing the concurrent ETD mass spectrometry of these oxidized prenyl peptides [12]. For the first time, we are adding to the mass spectrometry body of literature by providing this detailed study on the mass spectrometric fragmentation of oxidized geranylgeranylated peptides with ETD-MS/MS. These two kinds of prenyl peptides (oxi- and epoxidized) and their combined CID and ETD mass spectrometry will provide the best identification route for these lipid PTMs and their types.

Materials and Methods

Peptide with sequence REKKFFCAIL was custom synthesized by Genscript Corp. (Piscataway Township, NJ, USA), and peptides KHSSGCAFL and DAEFRHDSGYEVC were obtained from AnaSpec Inc. (Fremont, CA, USA). The reagents, synthesis of prenyl peptides, and their sources are provided in the supplementary data. Mass spectrometry data was obtained by a LC-ESI-IT-TOF (Shimadzu, Japan) and LC-ESI-LIT Thermo Velos Pro mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). For details, please see the supplementary texts.

Results and Discussion

A general scheme was presented to show how CID and ETD-MS/MS of oxidized/epoxidized peptides will help in the identification of prenyl peptide from a large-scale experiment (Scheme 1). These modifications will increase hydrophilicity and will help establish efficient identification by LC-MS/MS. Prenyl proteins after enzymatic digestion will be subjected to oxidation and epoxidation and further CID-MS/MS and ETD-MS/MS will provide confidence on locations and types as well as sequence information of prenyl peptides. CID-MS/MS of oxi/epoxy modified peptides will provide signature fragments (loss of RSOH, where R = prenyl side chain) [12–14], and these will distinguish their types of prenylation, whereas ETD-MS/MS of modified peptides can provide their full sequence information.



Scheme 1. Scheme to identify oxidized and epoxidized prenyl peptides by CID and ETD-MS/MS

Fragmentation Studies on the Oxidized Farnesylated and Geranylgeranylated Peptides

CID Fragmentation Studies For mass spectrometric fragmentation studies, we have taken three peptides having the sequence KHSSGCAFL (N-terminal basic group and prenyl motif CaaX at carboxyl end), and DAEFRHDSGYEVC (N-terminal acidic group and C at the carboxyl end) and REKKFFCAIL (N-terminal basic group and having CaaX prenyl motif at the carboxyl end) [15]. The fragmentation study of DAEFRHDSGYEVC(oxyfar) of *m/z* 874.20 was carried out using CID in ESI-MS. The oxidized modified version efficiently generated a signature peak (–RSOH) due to the cleavage of a labile side chain. Few fragments from the peptide backbone (b and y ions) were also observed during ESI-CID-MS/MS experiment. Several doubly charged b and y ion were observed along with their singly charged ions at very low intensity. A doubly charged peak at *m/z* 747.84 was observed, which corresponded to the loss of the RSOH group (R = far) from the precursor (Figure 1a). CID-MS/MS of DAEFRHDSGYEVC(oxyger) generated similar –RSOH (R = ger) loss from the precursor peptides (Figure 1b). The peak is the most distinct [$(M + 2H - RSOH)^{+2}$] in the mass spectrum. The CID fragmentation of REKKFFC (oxyfar/ger)AIL and KHSSGC(oxyfar/ger)AFL was carried out in ESI-IT-TOF-MS (Figure S1A, B, C, D). The same expected peak with the loss (RSOH) was also formed at very high intensity compared with other peaks in the case of CID fragmentation of these peptides. The formation of signature fragments from each of the oxyfarnesylated and oxygeranylgeranylated peptides are very distinct in the CID-MS/MS (Figure 1a, b). It is clear from the spectra that oxidized-modified peptides appeared as very distinct due to the loss of RSOH (R = loss of farnesyl and geranylgeranyl group).

ETD Fragmentation Studies The fragmentation of these oxidized prenylated peptides was carried out using the

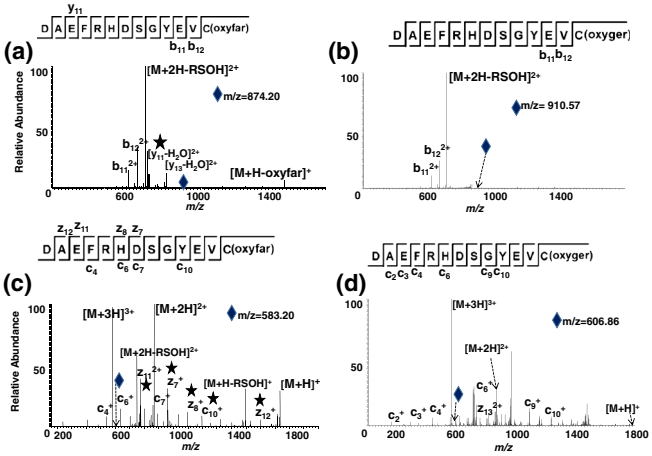


Figure 1. CID/ETD-MS/MS fragmentation of oxyfarnesylated/geranylgeranylated peptides. (**a**) and (**b**) CID - product ion mass spectra of oxyfar/oxyger modified peptides. (**c**) and (**d**) ETD - product ion mass spectra of oxyfar/ger modified peptides

ETD method. ETD fragmentation of triply charged DAEFRHDSGYEVC(oxyfar) at *m/z* 583.20 (3+) resulted in the efficient cleavage of peptide backbone. Various c and z ions were formed (Figure 1c). Similar types of fragmentation were observed with the formation of c and z ions for the peptide DAEFRHDSGYEVC(oxyger) at *m/z* 606.86 on ETD fragmentation (Figure 1d). Unlike the fragmentation of these oxidized farnesylated products in CID, the loss of RSOH from the precursor ions was not visible. Various c and z ions and charge-reduced precursor ions were observed in the spectrum as expected (please see the Excel file in the Supplementary Data for the *m/z* of the peaks). ETD fragmentation of triply charged REKKFFC(oxyfar/oxyger)AIL peptides was studied in a Thermo Velos Pro ESI-LIT using a direct infusion method. A similar fragmentation pattern was observed (Figure S2A, B). For the oxyfarnesylated (*m/z* 390.26, 3+) and oxygeranylgeranylated KHSSGCAFL (*m/z* 408.15, 3+) peptides, ETD fragmentation studies were also carried out and it was found that charge reduction of the triply charged precursor ions to its doubly and singly charged ions are more prevalent than the backbone fragmentations (Figure S2C, D). It is also important to mention that the unmodified version of these peptides fragmented very well in CID (data not shown), but efficient fragmentation in the backbone was also observed under ETD fragmentations with the modified peptides. The signature fragments were observed at very low intensities in a few of the peptides but not all of them. It is clear from these CID and ETD-MS/MS studies of the oxi-modified peptides that this combination will detect the prenylation sites and types in the peptides (Scheme 1) unambiguously.

Epoxidized-Farnesylated and Geranylgeranylated Peptide Fragmentation Studies

CID Fragmentation Studies Epoxidation reaction of prenyl peptides convert isoprenoid groups to epoxides along with the oxidation of the thio-ether bond (S=O) in the prenylated peptides [12]. The number of oxygen molecules incorporated in the isoprenoid side chain is directly related to the reaction time and concentration of mCPBA. This resulted in the increase of hydrophilicity of the prenylated peptides and also helped to design an enrichment strategy using the reactivity of the epoxy group for these low abundance modifications.

The epoxidized farnesylated peptides KHSSGC(epoxyfar)AFL were eluted by the C-18 column, and the retention time was between 42 and 47 min in a 70-min-long run gradient from 0–80% organic phase in nanoLC-ESI-IT-TOF-MS. During LC-ESI-MS/MS studies, doubly charged epoxidized peaks were seen at *m/z* 593.32 $(M + 2H + Far + 2O)^{2+}$, 602.32 $(M + 2H + Far + 3O)^{2+}$, and 610.32 $(M + 2H + Far + 4O)^{2+}$, respectively. A CID-MS/MS of a doubly charged epoxidized product of KHSSGC(epoxyfar)AFL peptide at *m/z* 602.32 $(M + 2H + far + 3O)^{2+}$ is shown in Figure 2a. CID fragmentations of all the peaks with a collision energy of 35% resulted in the formation of doubly charged marker ions at *m/z* 458.23 (loss of RSOH, where R = farnesyl + nO, n = number of epoxy group) (Figure 2a, and Figure S3A, B). Several other peaks were observed along with loss of water as well as the farnesyl group but the RSOH loss was the significant peak we expected.

Different epoxidized products of doubly charged geranylgeranylated peptides DAEFRHDSGYVEC were observed at *m/z* 916.30 $(M + 2H + Ger + 2O)^{2+}$, 924.29 $(M + 2H + Ger + 3O)^{2+}$, and 932.28 $(M + 2H + Ger + 4O)^{2+}$ in the mass spectra. The CID fragmentation of the peaks were also done at 35% collision energy, and it was found that in the case of *m/z* 932.28 $(M + 2H + Ger + 4O)^{2+}$, a very high intensity peak is obtained at *m/z* 747.48. This is the doubly charged peak with the predicted mass loss of RSOH group where, R = geranylgeranyl + nO (Figure 2b). Similar fragmentations were observed for other epoxy geranylgeranylated peaks at *m/z* 916.30 $(M + 2H + Ger + 2O)^{2+}$ and *m/z*

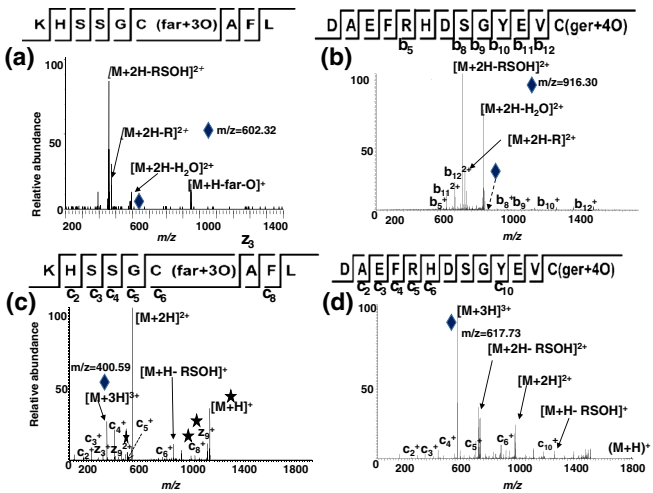


Figure 2. CID/ETD-MS/MS spectra of epoxyprenylated peptides. (**a**) and (**b**) - CID/ETD-MS/MS fragmentation of KHSSGC(epoxyfar)AFL. (**c**) and (**d**) - CID/ETD-MS/MS fragmentation of DAEFRHDSGYVEC(epoxyger)

924.29 (M + 2H + Ger + 3O)$^{2+}$ (Figure S4A, B). It is clear that RSOH losses are consistent and significant in all epoxidized products. The epoxidized products formations in REKKFFC(far)AIL peptide were further confirmed by mass analysis in ESI-IT-TOF and efficient cleavages of epoxidized side chains (RSOH losses) were observed in CID-MS/MS (Figure S5A, B, C).

ETD Fragmentation Studies To evaluate the fragmentation behavior of these epoxidized peptides in ETD, we studied the fragmentation pattern of the three epoxyfarnesylated products of peptide KHSSGCAFL. They were isolated in the MS spectrum at *m/z* 395.51 (M + 3H + Far + 2O)$^{3+}$ (Figure S6A), 400.59 (M + 3H + Far + 3O)$^{3+}$ (Figure 2c), and at *m/z* 406.17 (M + 3H + Far + 4O)$^{3+}$ (Figure S6B). The ETD fragmentations of these three epoxidized products were obtained. It was found that several c and z ions (see Excel file in the supplementary data) were formed and in each case along with the charge-reduced molecular ion species (Figure 2c and Figure S6A, B). ETD fragmentation studies on epoxyfarnesylated and epoxy-geranylgeranylated DAEFRHDSGYVEC were studied in detail. It was observed that the ETD fragmentation of epoxy-geranylgeranylated products of *m/z* 606.83 (M + 3H + ger + 2O)$^{3+}$, 611.71 (M + 3H + ger + 3O)$^{3+}$, and 617.73 (M + 3H + ger + 4O)$^{3+}$ (Figure 2d and Figure S7A, B), resulted in the formation of mostly c fragments along with the formation of charge-neutralized doubly and singly charged molecular ion peaks. Fragmentation of epoxyfarnesylated products of peptide DAEFRHDSGYVEC [at *m/z* 587.43 (M + 3H + far + 2O)$^{3+}$, 593.51 (M + 3H + far +3O)$^{3+}$, and 599.43 (M + 3H + far + 4O)$^{3+}$] showed mostly the c and z ions but of much lesser intensity as compared to that of epoxy-geranylgeranylated ones (Figure S8A, B, C). The ETD fragmentation studies were also performed for other triply charged epoxyfarnesylated peptides of REKKFFCAIL [at *m/z* 503.22 (M + 3H + Far + 3O)$^{3+}$ and 508.47 (M + 3H + Far + 4O)$^{3+}$], and it showed a similar fragmentation pattern that we observed for other epoxyfarnesylated and epoxygeranylgeranylated peptides (Figure S9A, B, C). We did find a loss of signature mass (loss of RSOH) in the mass spectra with small intensities. The ETD fragmentation of epoxy farnesylated/geranylgeranylated peptides showed efficient backbone cleavage in different epoxy prenylated products, whereas CID showed efficient gas-phase cleavage on the mono-oxidized thio–ether bonds on the prenyl side-chains.

Conclusions

We have demonstrated the fragmentation behavior of oxi/epoxy-farnesylated and geranylgeranylated peptides in both low energy CID and ETD fragmentation. The labile nature of modified peptides was clearly demonstrated in ESI-CID-MS/MS whereas ETD showed mostly peptides backbone cleavage. Moreover, with these studies, for the first time, we demonstrated the combined CID and ETD fragmentation of oxidized geranylgeranylated peptides. We believe this study will open new avenues for a more efficient analysis of these peptides by mass spectrometry.

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