Determination of Potential 4-hydroxy-2-nonenal Covalent Binding Sites on Electron Transfer Flavoprotein Using Liquid Chromatography-Mass Spectrometry

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ABSTRACT

Oxygen radicals and the reactive by-products they create have long been indicated in the disease pathways of metabolic disorders, given that they have the potential to react with the key components of proteins and thus disrupt their functioning. One of the most common by-products of oxygen radicals reacting with components of the cell is 4-hydroxy-2-nonenal (4HNE). Numerous studies have indicated that 4HNE is able to adduct to single amino acids, peptide sequences, as well as whole proteins. Electron transfer flavoprotein (ETF) is a highly active electron transferase of the fatty acid and amino acid oxidation pathways, and deficiency of this protein has been indicated in numerous disease pathways. ETF has demonstrated reduced activity when incubated with 4HNE, therefore this study attempted to determine possible binding sites of 4HNE on ETF by utilizing model peptides and LC-MS analysis. Distinct 4HNE adducts were formed with the peptide angiotensin II as well as a peptide from the alpha subunit of ETF. The groundwork has been laid for further research into the possibility of cross-linking occurring between ETF subunits as well as analysis of 4HNE adducts on ETF through tryptic digest.

INTRODUCTION

The mitochondria of the cell consist of proteins responsible for vital metabolic functions. Damage to these proteins is indicated in the pathology of numerous degenerative diseases, such as Alzheimer's disease, atherosclerosis, and various metabolic disorders.[1] Reactive oxygen species resulting from reactions occurring in the electron transport chain of the mitochondria initiate much of this damage through the creation of reactive by-products that cause damage to surrounding proteins.[2] These reactive by-products have the ability to manipulate the functioning of mitochondrial proteins by binding to enzyme active sites or altering allosteric conformation and thus inhibiting their enzymatic activity. Inhibition of mitochondrial protein activity by reactive by-products results in the accumulation of substrates in the cell negatively affecting metabolic processes.[1,2] By studying the specific interactions between reactive by-products and the mitochondrial proteins they affect, researchers will be able to create tailored treatments for the disease states caused by these interactions.

A key mitochondrial protein that is subject to the effects of reactive by-products is electron transfer flavoprotein (ETF). ETF is found in the mitochondrial matrix and assists with the movement of electrons as an electron transferase in the electron transport chain.[3] ETF is the specific electron acceptor of electrons from fatty acyl-CoA dehydrogenases, sarcosine and dimethyl glycine dehydrogenases, glutaryl-CoA dehydrogenase, and the branch chain acyl-CoA dehydrogenases in the fatty acid or amino acid oxidation pathways.[3,4,5,6,7] ETF acts as a one electron carrier to transfer electrons to electron transfer flavoprotein-ubiquinone oxidoreductase, allowing electron transfer flavoprotein-ubiquinone oxidoreductase to transfer electrons to the ubiquinone pool in the mitochondrial membrane.[8] Studies performed by McKean et al. indicate that ETF is heterodimeric, consisting of an alpha subunit (31,000 daltons) and a beta subunit (27,000 daltons).[9] A flavin adenine dinucleotide (FAD) cofactor lies between the two subunits, as seen in Figure 1.[10,11] The FAD cofactor is reduced and oxidized as it accepts and transfers electrons, while the surrounding subunits help to stabilize the active site.[11] In addition, the single

redox center of ETF in combination with the heterodimeric subunits indicates the possibility of separate electron acceptor and donor sites and of separate binding and electron transfer domains.[9]

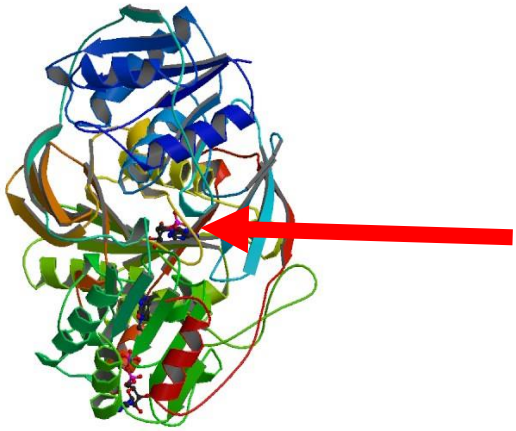


Figure 1. Crystal structure of electron transfer flavoprotein.[12] Flavin adenine dinucleotide cofactor is depicted by a red arrow.

The highly reactive aldehyde 4-hydroxy-2-nonenal (4HNE) is a by-product of the peroxidation of phospholipids in the cell membrane by reactive oxygen species (Figure 2). 4HNE is one of the best recognized and most studied lipid peroxidation by-products, despite being highly unstable ex vivo.[10] The organic molecule is capable of adducting to amino acids via Michael addition, most notably histidine, cysteine, and lysine residues, as seen in Figure 3.[11] Studies performed by Bolgar et al., 1996 and Uchida et al., 1992 suggested the formation of 4HNE adducts with low density lipoprotein and glyceraldehyde-3-phosphate dehydrogenase respectively most notably with histidine residues.[13,14] In addition, considerable evidence has been presented recently indicating 4HNE Schiff base cross-linking between low density lipoprotein and glyceraldehyde-3-phosphate dehydrogenase protein subunits, thus decreasing the activity of the protein (Figure 3).[14,15] Numerous studies have demonstrated a consistent decrease in activity when proteins are incubated with 4HNE, indicating an inhibitory nature of 4HNE, perhaps due to cross-linking or alteration of the active site.[13,14,15] A study recently performed by Sondalle et al., 2011 indicated that ETF demonstrates considerably reduced activity in the presence of 4HNE.[16] The reactive aldehyde may linking the two subunits in some fashion, thus decreasing the activity of the FAD cofactor between the

two components. This would decrease the overall reducing ability of ETF. Deficiency of functioning ETF would lead to the buildup of amino and fatty acids in the bloodstream and tissues causing metabolic acidosis and the possible initiation of a disease state.[17] Given the vital role ETF plays in the electron transport chain, fatty acid and amino acid oxidation, and metabolism as a whole, investigating the effects of reactive by-products on the heterodimeric protein could provide insight into the pathology for a multitude of diseases.

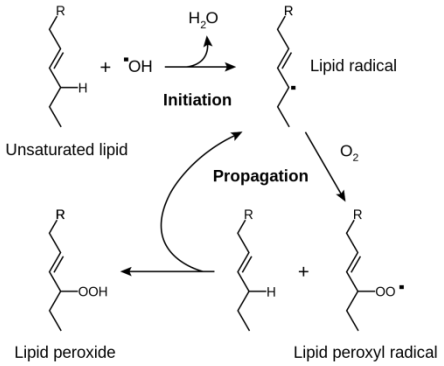


Figure 2. Peroxidation of lipids by reactive hydroxyl radicals.[18]

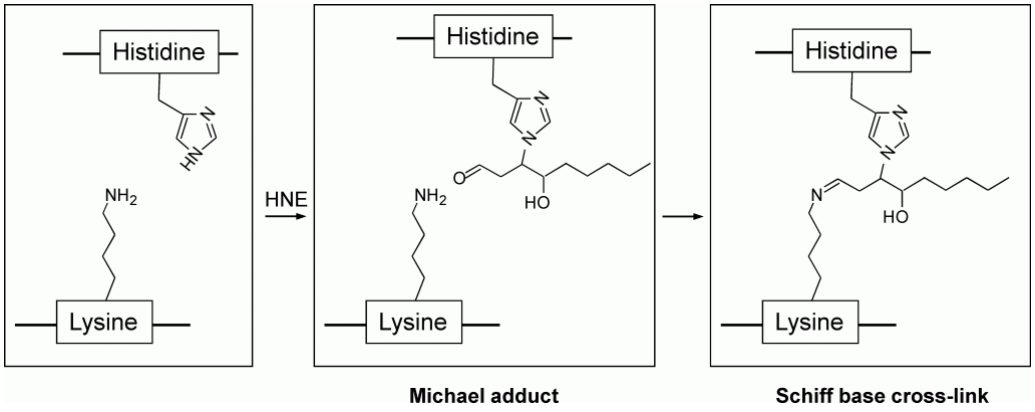


Figure 3. 4HNE Michael addition to histidine and Schiff base cross-linking by 4HNE.[18]

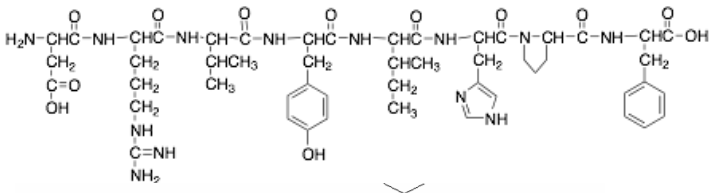
The goal of the present study is to determine if 4HNE adducts to ETF and, if bonds are detected, determine the location of these bonds. This study will focus on amino acid sequences found at the interface between the two subunits of ETF. The electron transferring site, or active site, of the protein is located in the groove between the alpha and beta subunits.[19,20] The formation of a 4HNE adduct on the alpha or beta subunit at the interface between the two subunits would greatly decrease the stability of the

two subunits of ETF, and thus the electron transferring ability of the protein. In addition, Western blot data suggests the formation of a cross link between the two subunits of ETF in the presence of 4HNE.[16] Therefore, this study will focus on a peptide sequence found on the alpha chain of ETF at the interface between the two subunits, sequence SGAIQHLAGMK. As 4HNE is able to form stable, easily isolated adducts to cysteine and histidine residues, this study will investigate adducts of 4HNE to these specific amino acid residues of the alpha and beta subunit, as well as examine the possibility of cross-linking.

Introduction to Peptide Analysis

In order to manageably investigate adducts of 4HNE to ETF, several model peptides containing key residues, peptides from a tryptic digest of ETF, and a synthesized peptide containing sequence SGAIQHLAGMK found on the alpha chain at the subunit interface of ETF were studied for 4HNE adduct formations. Angiotensin II, [Lys^3] bombesin, and glutathione were utilized as model peptides, depicted in Figure 4 A, B, and C. The synthesized peptide from the alpha subunit is pictured in Figure 4 D.

A



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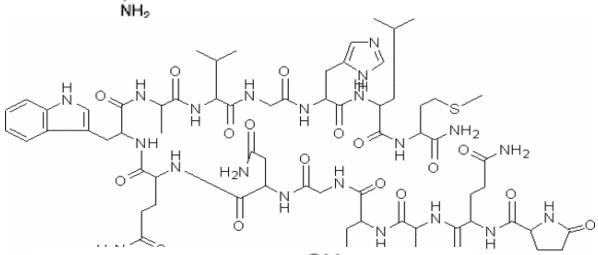

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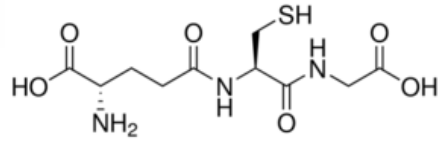


D

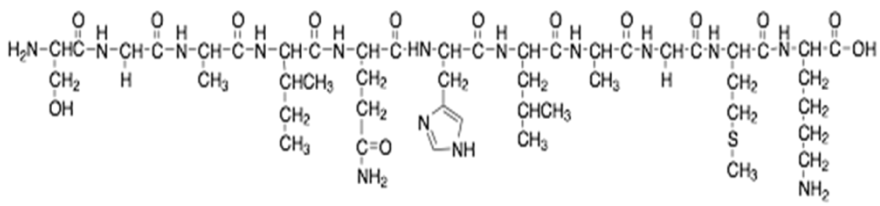


Figure 4. a) Angiotensin II b) [Lys^3] Bombesin c) Glutathione d) Alpha subunit peptide

These model peptides allow for identification of clear mass signals on electrospray ionization spectra in preparation for analysis of more complicated protein peptides. Ionization masses of the model peptides can be viewed in Table 1. Adducts of 4HNE are expected to form at histidine, cysteine, or lysine residues via Michael additions. Individual peptides will be tested for formation of 4HNE adducts, and combinations of peptides will tested for formation of 4HNE cross-linking via Schiff base addition. Theoretical masses of 4HNE adducts with the model peptides can be viewed in Table 2.

Table 1. Expected ionization masses of model peptides

Peptide	Nominal	Monosiotopic	$M+H^+$	$M+2H^+$	$M+3H^+$
Angiotensin II	1046.1750	1045.5230	1046.5418	523.7746	349.5188
$[Lys]^3$ Bombesin	1591.5230	1590.8089	1591.8162	796.4118	531.2769
Glutathione	308.4118	307.0838	308.0911	154.5492	103.3686
Alpha subunit peptide	1112.2980	1111.5700	1112.5773	556.7923	371.5306

Table 2. Expected 4HNE adduct masses of model peptides

Peptide	4HNE adduct $+H^+$	4HNE adduct $+2H^+$	4HNE adduct $+3H^+$
Angiotensin II	1202.6568	601.8321	401.5571
$[Lys]^3$ Bombesin	1747.9312	874.4693	583.3153
Glutathione	464.2061	232.6067	155.4069
Alpha subunit peptide	1268.6923	634.8498	423.5690

Analysis of model peptides allows for a targeted study of residues found at the interface of the two subunits of ETF. Liquid chromatography-mass spectrometry (LC-MS) analysis was utilized in the analysis of 4HNE adduct formations with model peptides, ETF tryptic digest peptides, and the synthesized peptide in order to pinpoint the charges as well as specific masses of the peptides with and

without adducts. An additional goal of the present study is to develop and optimize LC-MS methods utilizing Ripon College's newly acquired LC-MS.

EXPERIMENTAL

Materials

Acrolein, dichloromethane, Hoveyda-Grubbs catalyst 2nd Generation, octen-3-ol, silica gel, deuterated chloroform, dithiothreitol, iodoacetamide, urea, ammonium bicarbonate, acetonitrile, triflouroacetic acid, trypsin, formic acid, angiotensin II, [Lys^3] bombesin, glutathione, methanol and ethanol were purchased from Sigma Aldrich. The aldehyde 4-hydroxy-2-nonenal was purchased from Caymen Chemical Company. The peptide sequence from the alpha subunit of ETF was custom synthesized by GenScript Inc. Piscataway, New Jersey.

An Agilent Technologies 6230 Time-of-Flight Liquid Chromatography-Mass Spectrometer (LC-MS) was utilized for all LC-MS analyses. A reverse phase C18 column was utilized for LC analysis, and acetonitrile and water with 0.1% formic acid were utilized as solvents. Concentrations of acetonitrile and water varied for each substance analyzed. Electrospray ionization with in positive ion mode was utilized for mass spectrometry analysis. All samples were loaded into 1.5 mL LC-MS vials at either 1 ppm or 10 ppm concentrations.

4-hydroxy-2-nonenal Synthesis

The aldehyde 4-hydroxy-2-nonenal (4HNE) was synthesized utilizing a modified method from Soulère et al. 2007.[20] A stock solution of acrolein was created by mixing acrolein (4.195 g, 74 mmol, 0.00748 equiv.) with dichloromethane (6.65g, 78 mmol, 0.0783 equiv.). Into a 1 dram screw cap vial octen-3-ol (38 μL, 6.5E-9 mmol), acrolein (300 μL) stock solution, and ~0.001 g Hoveyda-Grubss catalyst 2nd Generation were added while a balloon blasted argon gas into the 1 dram vial. The 1 dram vial was capped and parafilm was wrapped around the cap. The reaction was allowed to spin for an hour while capped, after which 300 μL of acrolein and ~0.001 g Hoveyda-Grubbs catalyst 2nd Generation were added

again to the 1 dram vial under argon gas and allowed to spin for three to four more hr.[20] The reaction set-up can be viewed in Figure 5.

Figure 5. 4HNE reaction set-up.

The reaction solution was then evaporated utilizing a rotary evaporator (rotovap) in order to remove the solvent and purified for 4HNE using flash column chromatography. The sample was purified through a 1 L column, containing a stationary phase of silica gel and mobile phase. Fractions from the column were collected in 28 mL Pyrex test tubes in approximately 10 mL aliquots, assisted by a pressurized air hose. The mobile phase initially consisted of 1:1 hexane to ethyl acetate. Once the mobile phase became level with the silica gel in the column, pure ethyl acetate was added and run through the column until the silica gel became dry. Thin layer chromatography (TLC) was performed on the fractions in order to determine presence of the product, 4HNE. TLC plates were spotted with the solutions from the flash column chromatography fractions, placed in a sand bath saturated with 1:1 hexane to ethyl acetate, and allowed to run until the solution was approximately two centimeters from the top of a standard TLC plate. Fractions that were found to contain the product were then combined and rotovaped to remove solvent, and an NMR was obtained to confirm the presence of the product. The 4HNE was then diluted to 100 ppm and 10 ppm with methanol and stored in 1.5 mL Eppendorf tubes at -20℃.

Model Peptide LC-MS Analysis

Several studies that have performed similar analyses of model peptides angiotensin II, glutathione, and [Lys3] Bombesin have utilized peptide concentrations of approximately 10 ppm.[13,14,15] In order to optimize peak visibility, we initially utilized concentrations of 50 ppm and 10 ppm for peptide analysis. Given the high sensitivity of the instrument, 10 ppm concentrations were found to elicit highly visible peaks in ESI spectra for all model peptides. A standard peak for angiotensin II from a study performed by Shulz et al. in which angiotensin II was analyzed via ESI-MS at approximately 10 ppm concentration can be viewed in Figure 6.

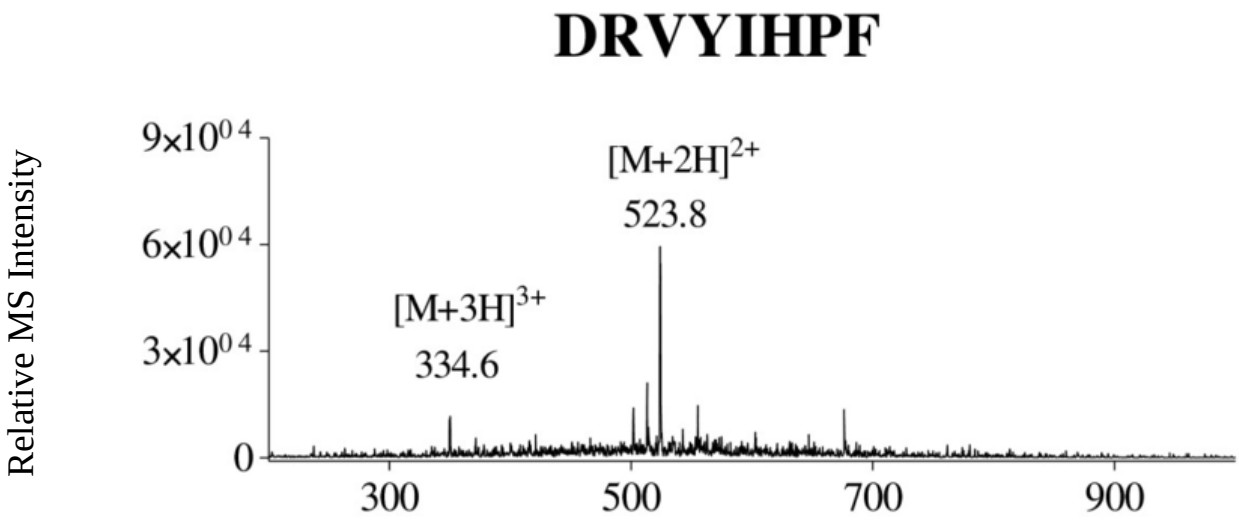


Figure 6. Standard ESI peaks for angiotensin II at +2 and +3 ionizations. Amino acid sequence of angiotensin II is pictured at the top of the figure.[21]

Two dilutions of glutathione, 50 ppm and 10 ppm, were created by diluting glutathione with pure deionized water. The angiotensin II and [Lys^3] bombesin obtained from Sigma Aldrich was diluted to 100 ppm and 10 ppm utilizing a 50% acetonitrile and 0.1% aqueous formic acid solution. The 10 ppm dilution was utilized for all LC-MS analyses. A 200:1 ratio of synthesized 4HNE to model peptide was prepared for angiotensin II, [Lys^3] bombesin, and glutathione, placed in a 1.5 mL LC-MS vial and analyzed on the LC-MS. The mixtures were analyzed at 30% acetonitrile/70% water and 70% acetonitrile/30% water mobile phase for five to ten min.

In order to analyze a possible cross link that could form between peptides, 10 ppm dilutions of angiotensin II and [Lys^3] bombesin and angiotensin II and glutathione were incubated together with 4HNE at a 200:1 ratio of 4HNE to peptide. This mixture was added to a 1.5 mL LC-MS vial and analyzed at a 30% acetonitrile mobile phase. All peptides and peptides in 4HNE solutions were stored in 1.5 mL Eppendorf tubes at -20℃.

ETF Tryptic Digest and Purification

Modifications were made to a tryptic digest method utilized by Sondalle et al.[21] Approximately 6.67 μL of 1.5 g/mL ETF in 20 mM Tris-buffer HCl was placed in a 1.5 Eppendorf tube and diluted to 100 μL with 8 M urea in 25 mM ammonium bicarbonate solution. The mixture was reduced with 5 μL of 200 mM dithiothreitol and alkylated with 20 μL 200 mM iodoacetamide and allowed to react in the dark for one hr. Then 20 μL dithiothreitol and 900 μL of 25 mM ammonium bicarbonate were added, along with trypsin at a 20:1 trypsin to protein ratio. The digest was placed in a 37° C oven to react in the dark overnight. The digest concentration was 10 mg/ 100 μL.

The digest was purified utilizing solid phase extraction chromatography. Solid phase extraction chromatography C10 cartridges were prepared by flushing through first 2 mL of acetonitrile and then 1.5 mL of water. The protein digest was then added to the cartridge and three different eluting solutions were flushed: 500 μL of a 0.1% triflouroacetic acid solution, 500 μL of a 0.1% formic acid solution, and 300 μL of a 75% acetonitrile 0.1% formic acid solution. Each elution was collected in a 1.5 mL Eppendorf tube. The final elution, containing 10 μg/300 μL or 3000 ppm ETF digest, was stored in a 1.5 mL Eppendorf tube. A 30 ppm and 3 ppm dilution was created from this stock sample utilizing a 75% acetonitrile 0.1% formic acid solvent.[21] Peptides resulting from ETF digest were stored in a 1.5 mL Eppendorf tube at -20℃.

ETF Tryptic Digest Peptides and Alpha Subunit of ETF Peptide LC-MS Analyses

The 3 ppm concentration of the ETF tryptic digest was utilized for all LC-MS analyses. In addition, a solution of the ETF digest was incubated with 4HNE, at a concentration of 200:1 peptide to 4HNE, was created and incubated for 24 hr. The ETF tryptic digest peptides and peptides incubated with 4HNE were analyzed utilizing the LC-MS and a gradient elution of acetonitrile in order to distinctly separate the multitude of masses associated with the varying peptides resulting from the digest. The gradient is pictured in Table 3.

Table 3. Mobile phase gradient of acetonitrile concentration utilized for LC-MS analyses of ETF tryptic digest peptides

Minute	% Acetonitrile
0	5
15	35
35	70
40	35
55	5
75	End

The 11 amino acid length peptide containing a sequence found near the ETF interface on the alpha subunit, SGAIQHLAGMK, was put into solution utilizing pure deionized water. Dilutions of 100 ppm, 10 ppm, and 1 ppm were created using pure deionized water. In addition, a solution of alpha subunit peptide incubated with 4HNE, at a 200:1 peptide to 4HNE concentration, was created as well and allowed to incubate for 24 hr. The alpha subunit peptide and alpha subunit peptide incubated with 4HNE were analyzed on the LC-MS utilizing a 30% acetonitrile solvent for approximately 5-10 minutes.

RESULTS

The optimal LC-MS results for model peptides, alpha subunit peptide, model peptides incubated with 4HNE, and alpha subunit incubated with 4HNE were achieved by utilizing an isocratic 30% acetonitrile mobile phase concentration. Optimal LC-MS results for ETF digest peptides and peptides incubated with 4HNE were obtained by utilizing the gradient mobile phase described in Table 3.

Synthesis of 4HNE Analysis

The synthesis of 4-hydroxy-2-nonenal (4HNE) was successful. ^{1}H NMR results are depicted in Figure 7.

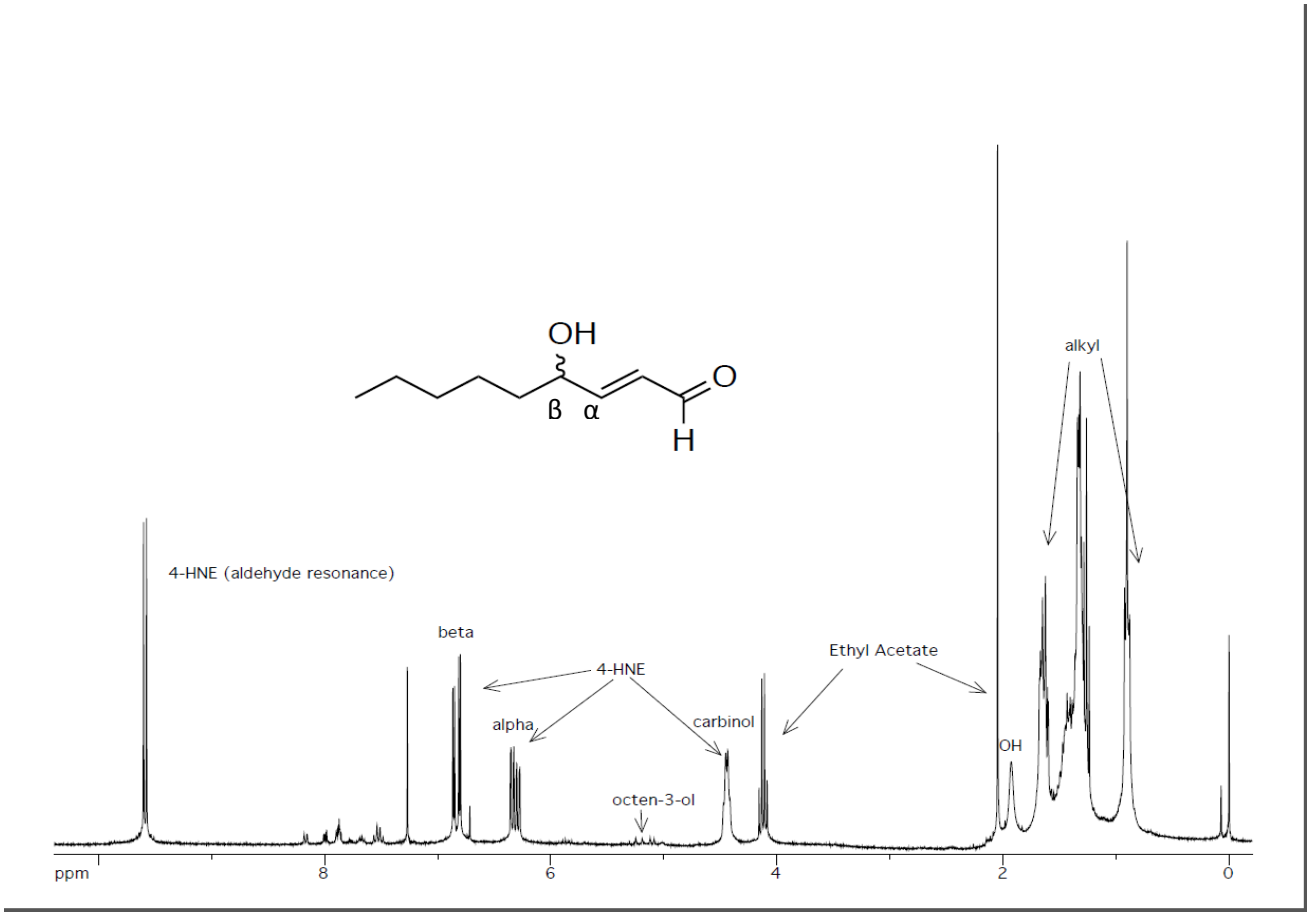


Figure 7. ^{1}H NMR spectrum of 4HNE in $CDCl_3$. Peaks of interest are labeled.

Model Peptides Analyses

Angiotensin II in 50% ACN and 0.1% formic acid when analyzed via LC-MS for five min demonstrated three distinct ionization states, +1,+2, and +3 under a mobile phase of 30% acetonitrile. Angiotensin II when incubated with 4HNE at 200:1 concentration was the only model peptide that demonstrated a clear 4HNE adduct formation at a +2 ionization. The electrospray ionization mass spectrum can be viewed in Figures 8 A, B.

A

B

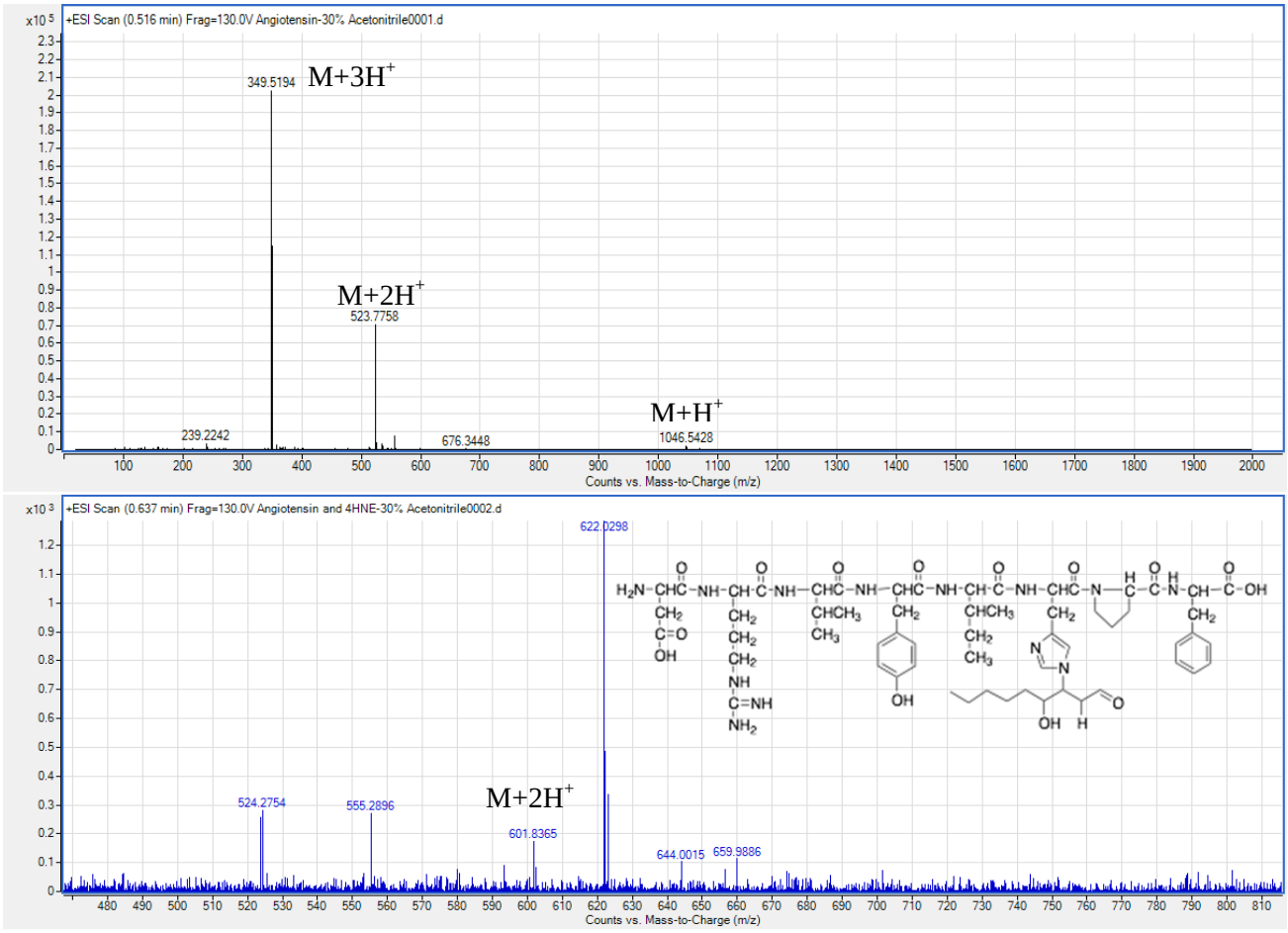


Figure 8. a) Mass spectrum of angiotensin II. Peak at 349.5194 denotes +3 ionization, peak at 523.7758 denotes +2 ionization, peak at 1046.5428 denotes +1 ionization. b) Mass spectrum of angiotensin II incubated with 4HNE. Peak at 601.8365 denotes adduct formation with 4HNE at a +2 ionization. Overlay contains theoretical structure of adduct.

Results Specific to LC-MS Studies

When analyzing [Lys^3] bombesin in 50% ACN 0.1% formic acid solution for approximately five min, a +2 ionization was successfully isolated. The peak appeared to be of relatively low concentration;

however this can be attributed to the incredible sensitivity of the instrument as well as the fact that a researcher for a separate project was utilizing the LC-MS at the same time as our study. An electrospray ionization spectrum can be viewed in Figure 9 on the following page.

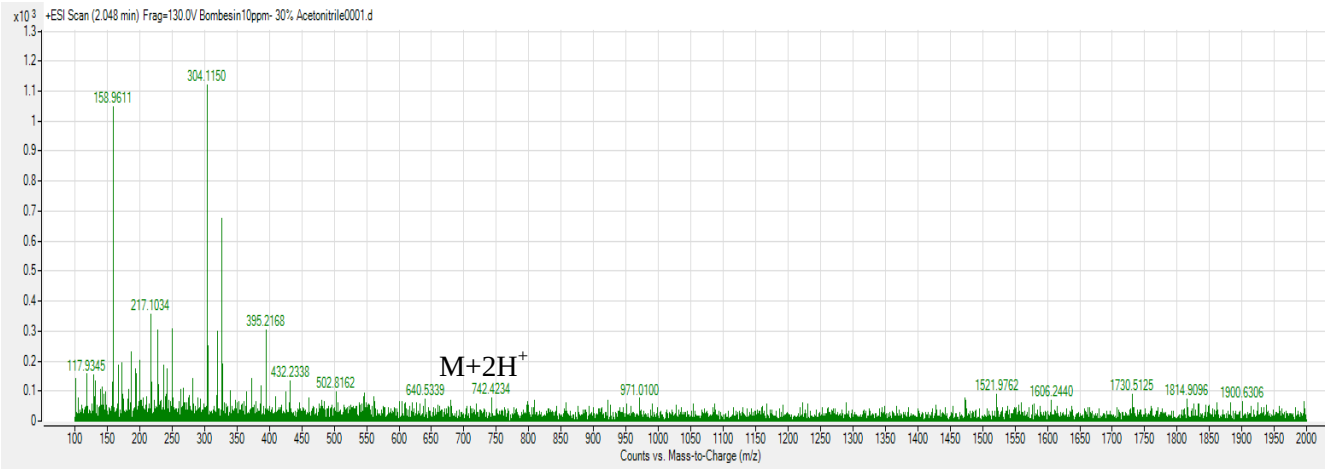


Figure 9. Electrospray ionization mass spectrum of [Lys^3] bombesin. Peak at 742.4234 denotes +2 ionization.

When testing glutathione in 50% ACN 0.1% formic acid for approximately five minutes, a +1 ionization state was successfully isolated. Electrospray ionization mass spectrum can be viewed in Figure 10.

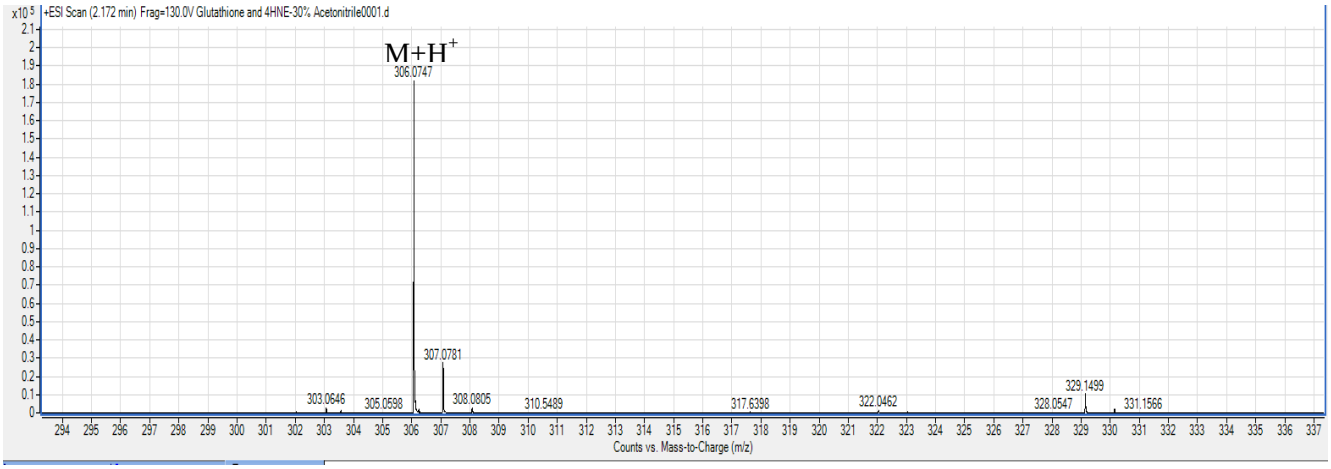


Figure 10. Electrospray ionization mass spectrum of glutathione. Peak at 306.0747 denotes +1 ionization.

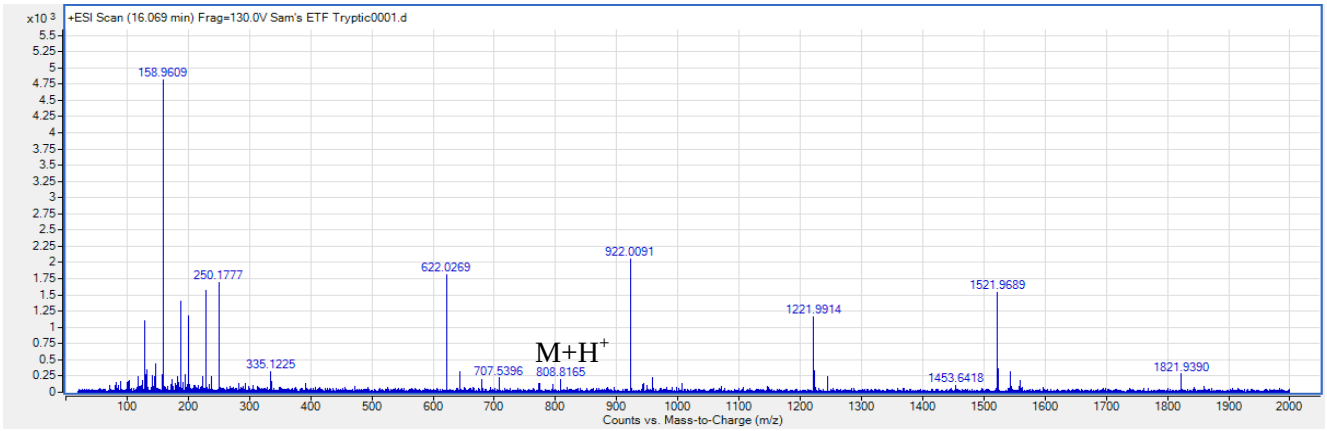
Tryptic Digest of ETF Peptides Analysis

Isolation and determination of mass spectral peaks correlating to ETF digest peptides proved incredibly difficult. In addition, several missed cleavages from the tryptic digest of ETF were noted as likely occurrences given the presence of peptides containing a missed cleavage site. Figure 11 A, B notes the electrospray ionization mass spectra of the ETF digest under a gradient acetonitrile mobile phase, as described in Table 3 previously. Table 4 details the expected mass results of the two ETF digest peptides found in the mass spectra. Range of accuracy was calculated up to 3 ppm, roughly +/- 0.001 for 10 m/z.

Table 4. Expected mass results of the two peptides from the ETF digest located in the ETF digest mixture analyzed via LC-MS. Expected range is included for identification accuracy. IEVIKPGDLGVDLTSK contains missed cleavage by trypsin.

ETF Peptide (Beta Subunit)	M+1H	M+2H
IEVIKPGDLGVDLTSK	1684.9600 ± 0.0168	842.9837 ± 0.0084
VIDYAVK	808.4683 ± 0.0081	404.7378 ± 0.0040

A



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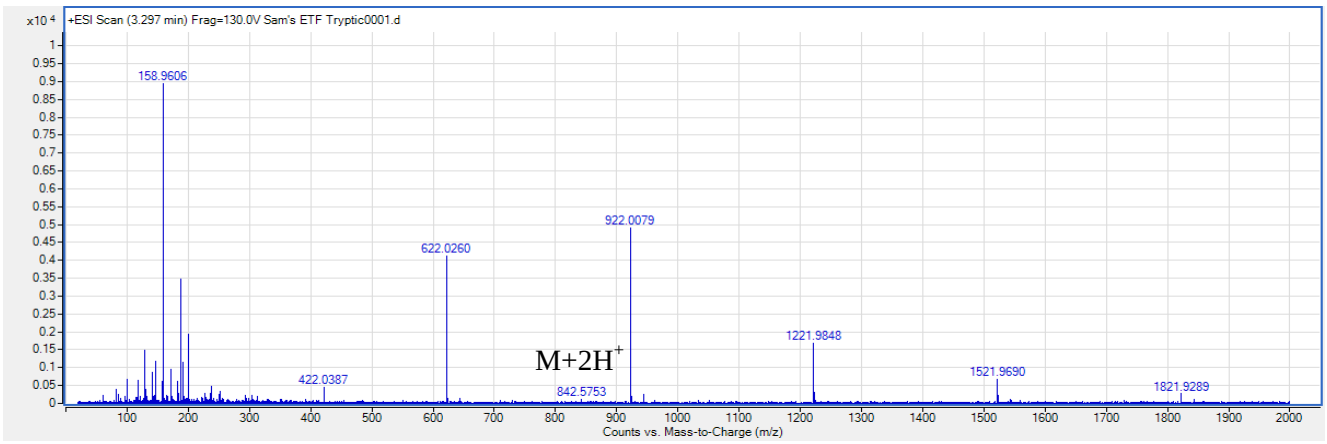

Figure 11. a) Mass spectrum containing peptide VIDYAVK. Peak at 808.8165 denotes +1 ionization. b) Mass spectrum containing peptide IEVIKPGDLGVDLTSK. Peak at 842.5753 denotes +2 ionization. This peptide contains a missed cleavage by trypsin, on the carboxyl side of the first lysine.

Attempts at isolating an adduct of 4HNE to ETF tryptic digest peptide have been unsuccessful thus far. Chromatograms of the ETF digest peptides incubated with 4HNE noted a decreased intensity in peak height. A chromatogram of the ETF digest peptides before and after 4HNE incubation, analyzed utilizing gradient mobile phase from Table 3, is pictured in Figure 12 A, B.

A

B

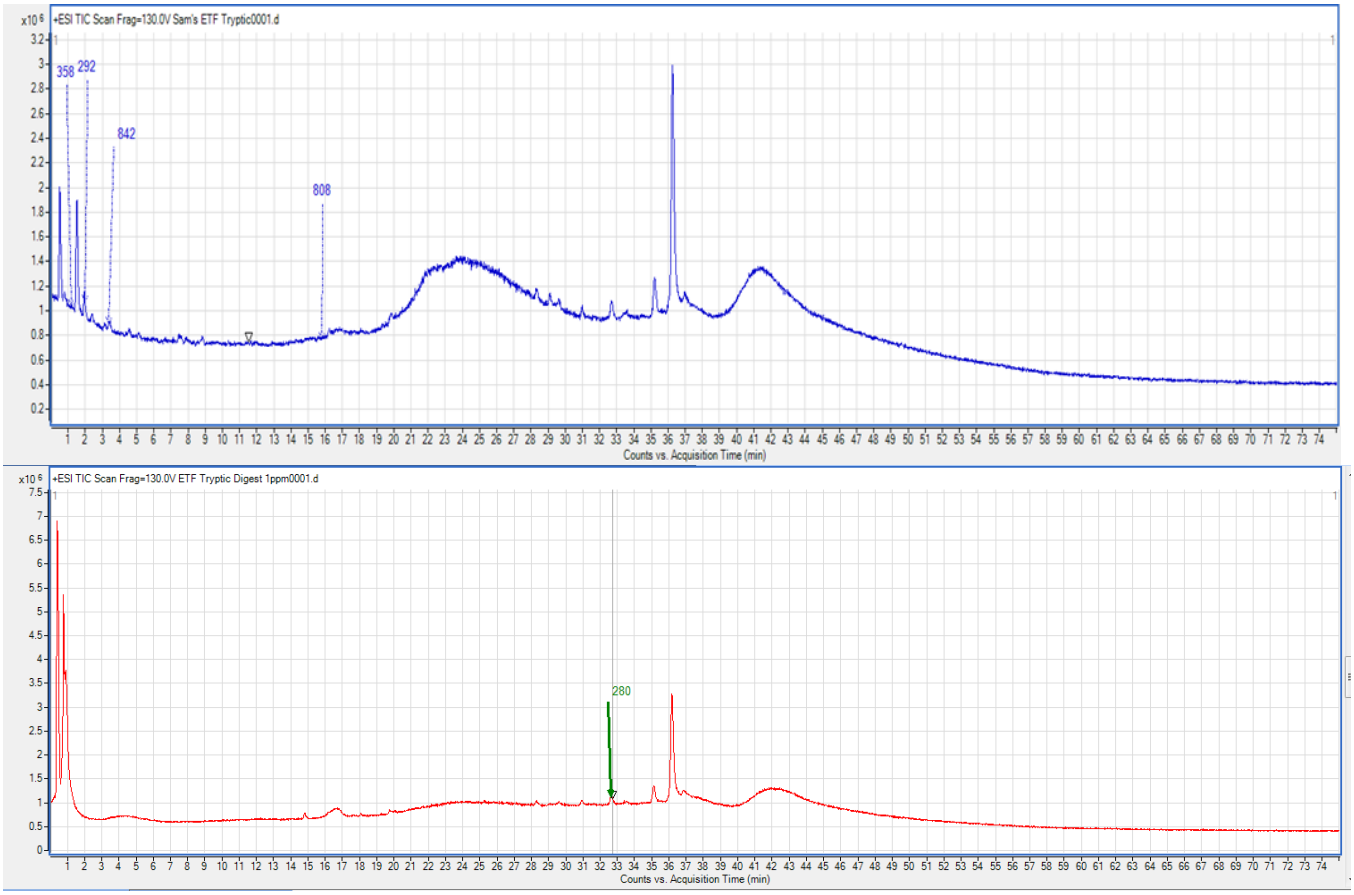

Figure 12. a) Chromatogram of ETF peptides prior to 4HNE incubation. Pictured numbers are part of the researchers notes and do not hold significance for this study. b) Chromatogram of ETF peptides after 4HNE incubation. Pictured number is part of the researchers notes and does not hold significance for this study.

Alpha Subunit Peptide Analysis

A 4HNE adduct to the alpha subunit peptide, with sequence SGAIQHLAGMK, was successfully isolated at a +4 ionization. The electrospray ionization mass spectrum can be viewed in Figure 13 on the following page. The peak appeared to be of relatively low concentration; however this can be attributed to

the incredible sensitivity of the instrument as well as the fact that a researcher for a separate project was utilizing the LC-MS at the same time as our study.

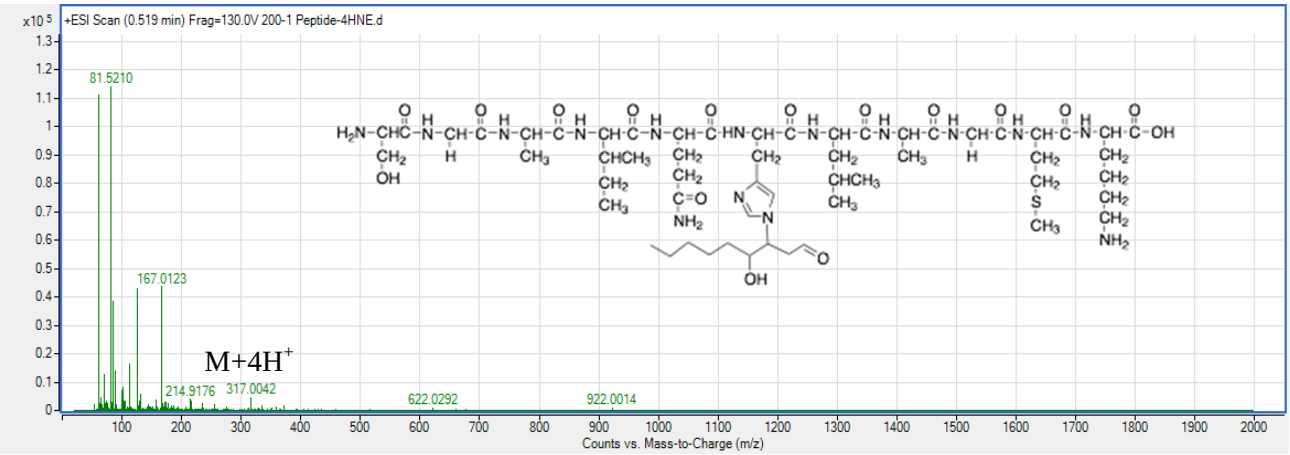

Figure 13. Mass spectrum of alpha subunit peptide incubated with 4HNE. Peak 317.0042 denotes an adduct with 4HNE at a +4 ionization.

Model Peptide 4HNE Linkage Analyses

When pairs of model peptides were incubated in 4HNE to be analyzed for cross-linking, no evidence of cross-linking was found. The peptides appeared to elute off the column separately from the 4HNE. The chromatogram of angiotensin II and [Lys^3] bombesin incubated with 4HNE can be viewed in Figure 14, and the chromatogram of angiotensin II and glutathione can be viewed in Figure 15 on the following page.

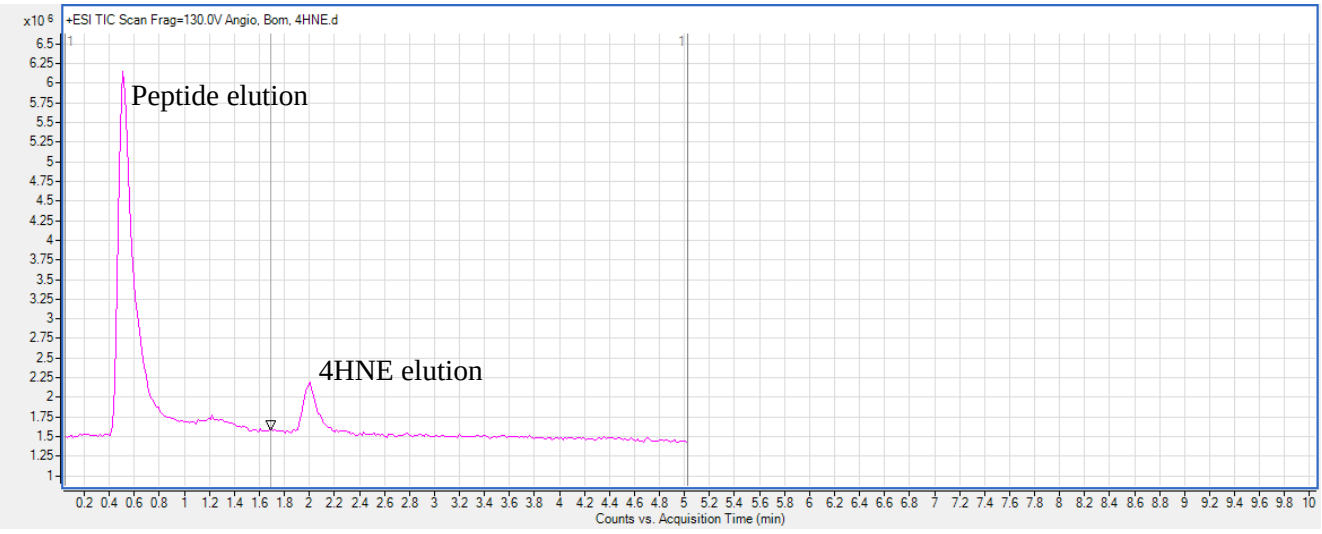

Figure 14. Chromatogram of angiotensin II and [Lys^3] bombesin incubated with 4HNE.

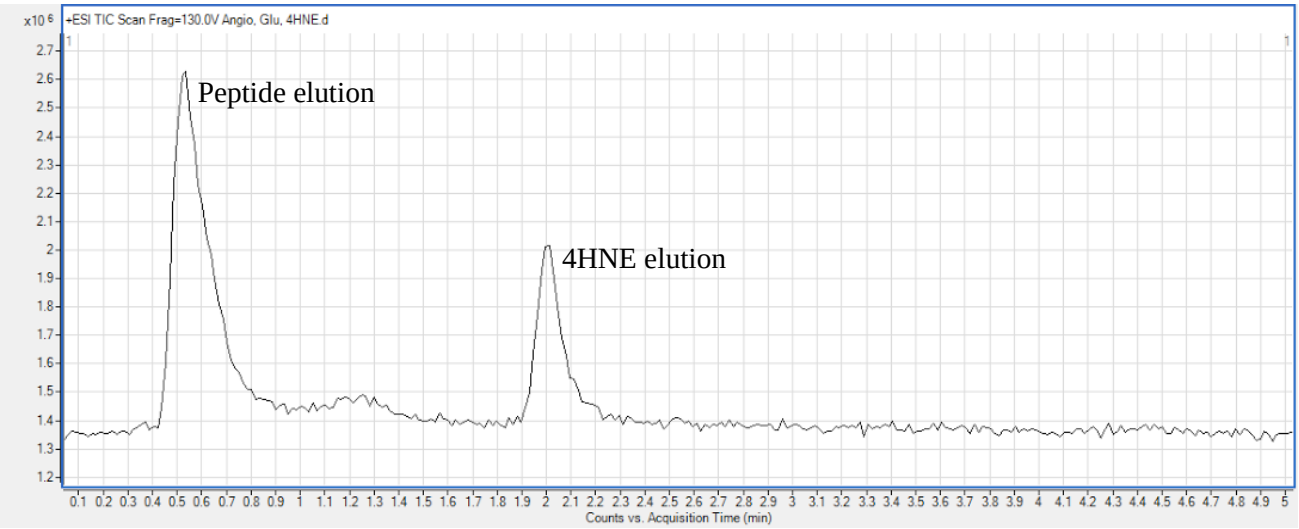

Figure 15. Chromatogram of angiotensin II and glutathione incubated with 4HNE.

DISCUSSION

The isolation of specific 4-hydroxy-2-nonenal (4HNE) adducts and their adduction sites on electron transfer flavoprotein (ETF) digest peptides was less successful than the isolation of 4HNE adducts to model peptides and the alpha subunit peptide. The model peptide angiotensin II contained residues from both the alpha and beta subunit of ETF, making it an excellent candidate for modeling possible 4HNE adduction sites. Furthermore, it contains two amino acids that could be ionized, along with the end amino and carboxyl group, allowing for a relatively simple analysis. The peptide contains a histidine in the middle of the eight amino acid sequence, allowing for optimal formation of a possible 4HNE adduct. A possible 4HNE adduct was noted at 601.8365 at a +2 ionization state. Potential 4HNE adductions were not noted for glutathione or [Lys^3] bombesin, in addition to difficulties incurred isolating specific protonation states. Angiotensin II contains a histidine and arginine, in addition to the end amino group, allowing for three possible ionization states. The unique structure of [Lys^3] bombesin may have contributed to difficulties incurred in isolating ionization states and possible 4HNE adducts. Glutathione only contains one ionization site, the end amino group, perhaps making it difficult to isolate the +1 ionization state. While glutathione does contain a cysteine residue, its relatively small size may have made it difficult for 4HNE to adduct to it in solution. Thus, angiotensin II proved the most successful in

adduct formations with 4HNE because it contains three different, easily accessible ionization sites and two different residues available for adduct formation with 4HNE. Furthermore, difficulties in isolation of ionization states as well as 4HNE adducts could be potentially attributed to the sensitivity of the instrument, given that numerous spectra indicated the presence of compounds being analyzed by another research group using the LC-MS.

In addition to the model peptide analyses, numerous difficulties were encountered when attempting to isolate specific peptides from the ETF tryptic digest. The exact number of missed cleavages that could have resulted from the tryptic digest were unable to be determined, thus several different possibilities for missed cleavages had to be taken into account. This further complicated the process of identifying definitive masses of peptides from the ETF tryptic digest. A study performed by Bolgar et al. encountered the same difficulties when attempting to standardize peptides resulting from a tryptic digest of low density lipoprotein.[13] When the peptides from the ETF tryptic digest were incubated with 4HNE there was a noted decrease of intensity in the peaks in the chromatogram. However, this study could not locate specific adducts of 4HNE to ETF tryptic digest peptides given the inability to confirm presence of specific ETF tryptic digest peptides during analyses of standards. This may be due to the large amount of peptides present in the sample, increasing the difficulty of isolating specific peptides in mass spectra.

A possible adduct of 4HNE to the alpha subunit peptide was successfully isolated, at a +4 ionization. Similar to angiotensin II, this adduct may have been easily isolated due to the numerous, easily accessible ionization sites on the alpha subunit peptide, sequence SGAIQHLAGMK, and the presence of a lysine and histidine in the 11 amino acid sequence. Both the alpha subunit peptide and angiotensin II were successful in forming an adduct of 4HNE. This could be attributed to the fact that both of the peptides contain a centrally located histidine residue, which is in literature the most common residue to form 4HNE adducts.[13,14] The study performed by Bolgar et al. that incurred difficulties in standardizing tryptic digest peptides was still able to isolate several adducts of 4HNE to histidines centrally located in a peptide chain.

Overall, there appeared to be difficulties in successfully isolating 4HNE adducts. These difficulties may be the result of differences in incubation times and the ratio of 4HNE to peptides. Numerous studies involving 4HNE adduction utilized a higher ratio of 4HNE to peptide than utilized in this study.[13,14,15] Thus, the ratio of 4HNE to peptide may not be high enough in order to encourage 4HNE adduct formations on the peptides. However, increasing the concentration of 4HNE to peptide so as to essentially saturate the peptide with the compound may not be realistically applicable to medical applications as the proteins in the body are not consistently saturated with 4HNE. Another possibility for the difficulties incurred in isolating adducts of 4HNE to peptides could be the acidity of the solution. Single adducts of 4HNE to peptide chains are, in theory, formed through Michael additions. Given that the solutions analyzed were rather acidic, this may have driven the product formation backwards thus reducing the amount of 4HNE adduct formation through Michael addition.[18] From this study, it can be concluded that 4HNE adduct formation is possible, however it is not optimized under these conditions and is so far difficult to isolate. Further research could include the standardization of the approximately 100 peptides resulting from the ETF digest, as well as further incubation of these peptides with 4HNE under low acidity and high 4HNE concentration conditions.

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REFERENCES

(1) Esterbauer, H., Schaur, R. J., Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med*. **1991**, *11*, 1, 81-128.

(2) Tang, X., Sayre, L. M., Tochtrop, G. P. A Mass Spectrometric Analysis of 4-hydroxy-2-(E)-nonenal Modification of Cytochrome c. *J. Mass. Spec*. **2011**, *46*, 3, 290-297.

(3) Crane, F. L., Mii, S., Hauge, J. G., Green, D. E., and Beinert, H. On the mechanism of dehydrogenation of fatty acyl derivatives of coenzyme A. I. The general fatty acyl coenzyme A dehydrogenase. *J. Biol. Chem.*. **1956**, *218*, 701-706.

(4) Frisell, W. R. and MacKenzie, C. G. Separation and purification of sarcosine dehydrogenase and dimethylglycine dehydrogenase. *J. Biol. Chem.*. **1962**, *237*, 94-98.

(5) Rhead, W., Mantangos, S., and Tanaka, K. Glutaric aciduria type II: in vitro studies on substrate oxidation, acyl-CoA dehydrogenases, and electron-transferring flavoprotein in cultured skin fibroblasts. *Ped. Res*. **1980**, *14*, 1339-1342.

(6) Noda, C., Rhead, W., and Tanaka, K. Isovaleryl-CoA dehydrogenase: demonstration in rat liver mitochondria by ion exchange chromatography and isoelectric focusing. *PNAS*. **1980**, *77*, 2646-2650.

(7) Ruzicka, F. J., and Beinert, H. A new iron-sulfur flavoprotein of the respiratory chain. A component of the fatty acid beta oxidation pathway. *J. Biol. Chem*. **1977**, *252*, 8440-8445.

(8) Watmough, N. J. and Frerman, F. E. The electron transfer flavoprotein: Ubiquinone oxidoreductases. *Biochim. Biophys. Acta*. **2010**, *1797*, 1910-1916.

(9) McKean, M. C., Beckmann, J. D., and Frerman, F. E. Subunit Structure of Electron Transfer Flavoprotein. *J. Biol. Chem.*. **1983**, *258*, 3, 1866-1870.

(10) Lin, D., Lee, H. G., Liu, Q., Perry, G., Smith, M. A., Sayre, L. M. 4-oxo-2-nonenal is both more neurotoxic and more protein reactive than 4-hydroxy-2-nonenal. *Chem. Res. Tox*. **2005**, *18*, 1219-1231.

(11) Roberts, D. T., Frerman, F. E., Kim, J. J. *Protein Database*. **1996**.

(12) Roberts, D. T., Frerman, F. E., Kim, J. J. Three-dimensional structure of human electron transfer flavoprotein to 2.1-A resolution. *Protein Database*. **1996**.

(13) Bolgar, M. S., Yang, C. Y., and Gaskell, S. J. First Direct Evidence for Lipid/Protein Conjugation in Oxidized Human Low Density Lipoprotein. *J. Biol. Chem.*. **1996**, *271*, 45, 27999-28001.

(14) Uchida, K. and Stadtman, E. R. Covalent Attachment of 4-Hydroxy-2-Nonenal to Glyceraldehyde-3-Phosphate Dehydrogenase. *J. Biol. Chem.*. **1993**, *268*, 9, 6388-6393.

(15) Roede, J.R., Carbone, D.L., Doorn, J.A., Kirichenko, O.V., Reigan, P., Petersen, D.R.. In vitro and in silico characterization of peroxiredoxin 6 modified by 4-hydroxynonenal and 4-oxononenal. *Chem. Res. Tox*. **2008**, *12*, 2289-2299.

(16) Sondalle, S. B., Byron, C. M., Nennig, H. M., Rindt, J. A., and Frerman, F. E. The Effect of 4-Hydroxy-2-Nonenal on the Activities of Electron Transfer Flavoprotein and Electron Transfer Flavoprotein-Ubiquinone Oxidoreductase. *Flavins and Flavoproteins*. **2011**, 417- 422.

(17) Roe, M. R., Xie, H., Bandhakavi, S., Griffin, T. J. Proteomic Mapping of 4-Hydroxynonenal Protein Modification Sites by Solid-Phase Hydrazide Chemistry and Mass Spectrometry. *Anal. Chem*. **2007**, *79*, 3737-3756.

(18) Vickers, T., Young, I. S., and McEneny, J. Lipoprotein oxidation and atherosclerosis. *Biochem. Soc. Trans*. **2001**, *29*, 358–62.

(19) Zhu, Xiaochun et al. Mass Spectrometric Characterization of Protein Modification by the Products of Non-Enzymatic Oxidation of Linoleic Acid. *Chem. Res. Tox.* **2009**, *22*, 8, 1386–1397.

(20) Soulère, L.; Queneau, Y.; Doutheau, A. An Expeditious Synthesis of 4-Hydroxy-2(E)-Nonenal (4-HNE), its Dimethyl Acetal and of Related Compounds. *Chem. Phys. Lip*. **2007,** *150*, 239-243.

(21) Shulz, A., Jankowski, J., Zidek, W., and Jankowski, V. Absolute quantification of endogenous angiotensin II levels in human plasma using ESI-LC-MS/MS. *Clinical Proteomics*. **2014**, *11*, 1, 37.

(22) Sondalle, S. B. Tryptic Digest Method. Personal communication.