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Mass spectrometry-based proteomics of oxidative stress: Identification of 4-hydroxy-2-nonenal (HNE) adducts of amino acids using lysozyme and bovine serum albumin as model proteins

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Abstract

Modification of proteins by 4-hydroxy-2-nonenal (HNE), a reactive by-product of $\omega 6$ polyunsaturated fatty acid oxidation, on specific amino acid residues is considered a biomarker for oxidative stress, as occurs in many metabolic, hereditary, and age-related diseases. HNE modification of amino acids can occur either via Michael addition or by formation of Schiff-base adducts. These modifications typically occur on cysteine (Cys), histidine (His) and/or lysine (Lys) residues, resulting in an increase of 156 Da (Michael addition) or 138 Da (Schiff-base adducts), respectively, in the mass of the residue. Here, we employed biochemical and mass spectrometry (MS) approaches to determine the MS “signatures” of HNE-modified amino acids, using lysozyme and bovine serum albumin (BSA) as model proteins. Using direct infusion of unmodified and HNE-modified lysozyme into an electrospray quadrupole time-of-flight mass spectrometer, we were able to detect up to seven HNE modifications per molecule of lysozyme. Using nanoLC-MS/MS, we found that, in addition to N-terminal amino acids, Cys, His, and Lys residues, HNE modification of arginine (Arg), threonine (Thr), tryptophan (Trp) and histidine (His) residues also can occur. These sensitive and specific methods can be applied to the study of oxidative stress to evaluate HNE modification of proteins in complex mixtures from cells and tissues under diseased vs. normal conditions.

Keywords

Proteomics; mass spectrometry; peptide modification; 4-hydroxy-2-nonenal; Michael addition; Schiff-base; pyrrole adducts

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Introduction

4-Hydroxy-2-nonenal (HNE) is a reactive aldehyde, formed as a by-product of lipid peroxidation (specifically, polyunsaturated ω 6 fatty acid peroxidation) [1]. Lipid peroxidation occurs during oxidative stress, a state in which the production of reactive oxygen species overwhelms the ability of a cell or tissue to remove them. Oxidative stress has been associated with a wide variety of diseases, including inflammatory, metabolic, neurological, and age-related diseases [1,2,3]. HNE can modify proteins by forming covalent adducts on specific amino acids residues; this typically results in reduced or altered protein function [4,5] and can serve as a biomarker for such diseases [1]. The formation of HNE-modified amino acid residues is due to the presence of an electrophilic double bond in HNE and the nucleophilic amino acid residues on proteins. Such adduct formation can occur by Michael addition, usually on Cys, His, and/or Lys residues, leading to an increase in the molecular masses of these amino acids by 156 Da (the molecular mass of HNE). Alternatively, Schiff bases also can be formed by HNE with ϵ -NH₂ groups of Lys residues, resulting in a mass increase of 138 Da (Schiff base formation with a net loss of water) [6,7]. These reactions are described in detail elsewhere [8,9]. Of the two types of reactions, Michael addition is more common and also more readily detected, since Schiff base formation is reversible [7].

Although the biological modification of Cys, His, and Lys residues of proteins by HNE is well-known [5,6,7,8,9,10,11,12,13,14,15], relatively little is known about what other amino acids undergo such modification and the mechanisms involved (Michael addition or Schiff base formation). For example, Zhao and colleagues [11] reported that, in addition to Cys, His and Lys, HNE modification of arginine (Arg), glutamic acid (Glu), and asparagine (Asn) occurs by Michael addition. Also, Doorn and colleagues [16] reported the covalent modification of Arg by 4-oxo-2-nonenal, another by-product of lipid peroxidation. Given the scarcity of studies concerning HNE modification of proteins on amino acid residues other than Cys, His, and Lys, and the potential importance of such reactions in the context of oxidative stress and disease, the present study was undertaken. Here we used lysozyme and bovine serum albumin (BSA) as model proteins to investigate the formation of HNE adducts of their amino acids, using a combination of electrospray ionization mass spectrometry (ESI-MS) and gel-based proteomics followed by nanoliquid chromatography/tandem mass spectrometry (nanoLC-MS/MS) analysis.

This model system was used for acquiring the knowledge of HNE-modification which can be further applied to proteins modified by HNE from cell lysates, tissues, organs, or organisms, either using the classical enrichment using anti-HNE antibodies [17], or even bypassing the enrichment entirely.

Materials & Methods

Unless otherwise stated, reagents and biochemicals were used as purchased from Sigma-Aldrich (St. Louis, MO). HNE (as the dimethyl acetal derivative, dissolved in hexane) was subjected to mild acid hydrolysis (1 mM HCl, 30 min at 4°C) to convert the acetal to the free HNE aldehyde. In a series of parallel reactions, 1.0 mg of each protein (lysozyme and BSA)

was treated with 0, 2, 5 and 10 mM HNE in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, and the samples were incubated in a 37°C water bath for 2 hours, as described elsewhere [10]. The reaction was stopped by addition of 100 µl of 1 M Tris, pH 8.0, followed by buffer exchange using a PD10 column (BioRad, Carlsbad, CA). To investigate HNE modification at the peptide level, direct infusion analysis of the untreated and HNE-treated lysozyme samples was performed. The protein samples (dissolved in water: acetonitrile: 0.1% formic acid, 49.9:50:0.1, by vol.) were analyzed by electrospray ionization mass spectrometry (ESI-MS) in positive ionization mode on a Quadrupole Time-of-Flight (QTOF) Micro mass spectrometer (Waters, Milford, MA), using the direct infusion through a syringe with a constant flow rate of 10 µl/min, as previously described [18,19]. Calibration was performed using an authentic standard of myoglobin (Waters, Milford, MA). HNE-treated lysozyme and BSA, as well as their untreated (control) counterparts, were subjected to SDS-PAGE, then stained with Coomassie Blue; the gel bands were excised, subjected to in-gel trypsin digestion, and the resultant peptides extracted and purified (C18 Ziptip™; Millipore, Billerica, MA), following by nanoLC-MS/MS analysis using a nanoACQUITY® UPLC coupled with a QTOF Ultima API mass spectrometer (Waters, Milford, MA), as previously described [20]. Figure 1 shows a schematic outline of the direct infusion experiment (Fig. 1A) and the nanoLC-MS/MS experiment (Fig. 1B), respectively. Data analysis was performed using ProteinLynx Global SERVER (PLGS version 2.4, Waters), with a custom-created database against unmodified and modified lysozyme and BSA, as well as using *de novo* sequencing. In addition, a Mascot database search was performed using free web-based software (Matrix Science; www.matrixscience.com).

Results and Discussion

Using direct infusion ESI-MS, analysis of HNE-treated lysozyme, in comparison with untreated lysozyme, we found that HNE can modify the intact protein with up to 7 HNEs per molecule, *i.e.*, there were lysozyme molecules with 1–7 amino acids modified by HNE (Figure 2A). This labeling was constant on all observable charge states of lysozyme (+8 to +15). A closer look at the 10(+) ion (Figure 2B) confirmed that there were up to 7 HNE molecules per molecule of lysozyme, as demonstrated by MS peak separations of 156 Da, the mass of HNE. Furthermore, comparison of the 10(+) ion peaks that corresponded to the unmodified and HNE-modified lysozyme (Figure 2B) suggests that each of the peaks corresponding to an HNE-modified *vs.* unmodified species split into two dominant peaks. The peak spacing between the HNE-modified sub-peaks was 138 Da and 156 Da, suggesting that lysozyme as a 10(+) species peak is modified by one molecule of HNE through formation of a Schiff base (from peak with *m/z* 1430.8778 (10+) to peak with *m/z* 1444.6575 (10+), a 138 Da difference) or through a Michael addition (from peak with *m/z* 1430.8778 (10+) to peak with *m/z* 1446.5391 (10+), a 156 Da difference). Therefore, mostly likely the first modification of lysozyme by HNE targeted two different amino acids: one through formation of a Schiff base and the other one through a Michael addition. Alternatively, but less likely, a Michael addition (a gain of 156 Da) followed by a neutral loss of a water molecule (18 Da) is also possible, depending on the nature of the amino acid modified. Similar differences were also observed for the 10(+) peak modified by 2, 3, 4, 5, 6 and 7 HNE molecules (Figure 2B), when HNE treatment was completed using either 2 mM

(middle panel, Fig. 2B) or 5 mM HNE (lower panel, Figure 2B). The ESI-MS experiments of untreated and HNE-treated BSA were only partially successful. Although we could observe the BSA protein envelope and HNE modifications, we could not definitely determine the exact number of HNE molecules per molecule of BSA, due to the size of the protein (~67 kDa) and insufficient resolution of the mass spectrometer employed,

To further confirm the HNE modifications observed by direct infusion ESI-MS and to determine the identity of the amino acids that are modified by HNE, we separated HNE-treated and untreated lysozyme by SDS-PAGE (Supplemental Figure 1). In parallel, we also treated BSA with HNE as an additional model protein for study and carried out SDS-PAGE on HNE-treated vs. untreated (control) BSA. Visual inspection of the Coomassie Blue-stained SDS-PAGE gel illustrates the electrophoretic mobility difference between HNE-treated and untreated proteins (red arrow, Supplemental Figure 1).

To identify the amino acids modified by HNE, we excised the gel bands, performed in-gel trypsin digestion on them, and the peptides were extracted and analyzed by nanoLC-MS/MS. We were able not only to confirm the HNE modifications in both lysozyme and BSA, but also to identify the specific modified amino acids, both at the peptide level and amino acid level. Specifically, we were able to identify Cys, His, and Lys modifications, as well as some other amino acid modifications and the N-terminal amino acid modifications of several peptides.

As an example of an HNE modification at both the peptide and amino acid level, we found a peptide that contained an HNE-modified Cys residue (156 Da increase in the peptide's mass) in lysozyme samples that were treated with 2, 5 or 10 mM HNE, but not in the untreated control (Supplemental Figure 2). Specifically, we found a peak with m/z 1303.99 (2+), which corresponded to a peptide with the amino acid sequence NLC(IAA)NIPC(HNE)SALLSSDITASVNC(IAA)AK (IAA being the iodoacetamide-induced carbamidomethyl modification of Cys). In the extracted ion chromatograms (XIC) for untreated lysozyme, we found no evidence for this peak (Supplemental Figure 2B); however, an m/z 1303.99 (2+) peak was present in the XICs of lysozyme treated with 2, 5 and 10 mM HNE (Supplemental Figure 2A). MS/MS fragmentation of the precursor ions from HNE-treated lysozyme with m/z 1303.99 (2+) produced a series of product ions, the analysis of which revealed the amino acid sequence NLC(IAA)NIPC(HNE)SALLSSDITASVNC(IAA)AK (Supplemental Figure 2C). Therefore, one of the Cys residues within this peptide was modified by HNE through a Michael addition (156 Da difference).

Arg has not been reported heretofore as being commonly modified by HNE; however, the modification is possible because of the electrophilic attack of HNE on the NH_2 group in the side chain of Arg. Our analysis of HNE-treated lysozyme led to identification of a peptide that had a 156 Da increase in the peptide's mass, relative to untreated lysozyme, in samples treated with 2, 5 or 10 mM HNE (Supplemental Figure 3). Specifically, we found a peak with m/z 760.6 (3+), which corresponded to a peptide with sequence HGLDNYR(HNE)GYSLGNWVCAAK. The XIC analysis showed this ion peak was present in all specimens of HNE-untreated lysozyme, but not in untreated lysozyme

(Supplemental Figure 3A). Summed spectra for these peaks in XIC revealed the MS precursor ion with m/z 760.6 (3+) in HNE-treated lysozyme, but not in the untreated one (Supplemental Figure 3B). In like manner, MS/MS of this precursor ion peak produced a series of fragment ions (Supplemental Figure 3C), the analysis of which led to identification of a peptide also having the amino acid sequence HGLDNYR(HNE)GYSLGNWVCAAK, with Arg (R) modified by HNE. Since the difference between unmodified and HNE-modified lysozyme was 156 Da, this suggests that Arg was modified by HNE through a Michael addition mechanism.

HNE adduction of Arg residues was also confirmed in HNE-treated BSA samples. The LC-MS chromatogram of HNE-treated BSA exhibited a peak with m/z 651.3 (2+) (156 Da increase in the peptide's mass, relative to untreated BSA), which corresponds to a peptide with the amino acid sequence AWSVAR(HNE)LSQK, with Arg modified by HNE (Figure 3). The XIC (Figure 3A) and summed spectra (Figure 3B) show the presence of the peptide in all HNE-treated samples, but not the untreated control sample. MS/MS of this peak produced a series of fragment ions (Figure 3C), the analysis of which also revealed a peptide with the amino acid sequence AWSVAR(HNE)LSQK, with Arg modified by HNE and with the difference between unmodified and HNE-modified BSA of 156 Da, suggesting that Arg also was modified by HNE through a Michael addition mechanism.

Another amino acid that was found to be modified by HNE in BSA samples was Thr (Figure 4), in the peptide with amino acid sequence of RPCFSALT(HNE)PDETYVPK and m/z 990.42 (2+) (156 Da increase in the peptide's mass, compared to untreated BSA). Thr has an OH group in its side chain, which could be a target of electrophilic attack by HNE. The peptide level (Figure 4A and 4B) and amino acid level (Figure 4C) analysis of the 990.42 (2+) peak provided compelling evidence for this HNE modification, which also occurred by a Michael addition.

Other HNE-modified peptides, containing HNE adducts either on Cys, His, or Lys residues (for lysozyme and BSA), also were identified. However, since these are common examples of HNE-modified amino acids, the data are not shown here.

We also observed modifications on the side chains of the amino acids that are downstream of positively-charged amino acids, such as Arg and Lys. These modifications were on the N-terminal amino acids of the peptides generated by trypsin digestion. Since excess HNE was removed after the initial reaction by size exclusion chromatography (using buffer exchange) and the HNE-modified proteins were subsequently separated by electrophoresis, artifactual HNE modification was obviated. Examples of HNE modifications of Arg and tryptophan (Trp) are shown in Supplemental Figures 4 and 5, respectively. Trp has the NH nucleophilic group in its side chain that could be attacked by HNE. The Arg adduct was a Michael type HNE modification: m/z 396.15 (3+) for the peptide sequence of (HNE)RHGLDNYR corresponded to a 156 Da increase in the peptide's mass (Supplemental Figure 4). However, the Trp (W) modification was via a Schiff base formation: m/z 537.66 (3+) for the peptide sequence of (HNE)WWCNDGR corresponded to a 138 Da increase in the peptide's mass (Supplemental Figure 5). Alternatively, this may have occurred by a Michael addition (+156 Da), followed by a neutral loss of water (−18 Da), leading to a net gain of 138 Da.

It has been reported previously that HNE can react with Lys to form stable pyrrole-type HNE–Lys adducts [11], resulting in a mass increase of 120 Da, relative to untreated Lys. In our experiments, we additionally found HNE-His pyrrole adducts. We identified two such modifications at the N-terminal His (H) residue of peptides generated from both BSA (Figure 5) and lysozyme (Supplemental Figure 6). For the BSA samples, the N-terminal His of a peptide with the amino acid sequence (HNE)HLVDEPQNLIK (a peak with m/z 713.26 (2+) corresponding to a 120 Da increase in the peptide's mass (Figure 5)) was found to be modified by HNE through pyrrole adduct formation. In the lysozyme samples also, the N-terminal His of a peptide with the amino acid sequence (HNE)HGLDNYR was modified through pyrrole adduct formation (a peak with m/z 497.67 (2+), corresponding to a 120 Da increase in the peptide's mass (Supplemental Figure 6)). Importantly, these results were unexpected, since such adducts heretofore have been reported only for HNE modification of Lys residues [11]. However, since the 120 Da adducts observed in the present study were both found at the N-terminus of the tryptic peptides, this suggests that the amino acids downstream of positively-charged Arg and Lys (the trypsin cleavage sites) are influenced by them and follow a more general mechanism of pyrrole type HNE modification. This finding is significant for proteomics-based experiments, as a similar mechanism occurs during IAA alkylation, where IAA almost exclusively modifies the amino acids downstream of Arg and Lys [21].

A wide range of proteins, including soluble and integral membrane (or membrane-associated) proteins, may become modified by HNE as a result of oxidative stress [1–4]. In order to obtain more detailed information regarding which amino acid residues are affected on specific proteins of interest, a number of separation strategies may be implemented. Ethen et al. [22] utilized 2-dimensional separation techniques to isolate proteins with unique migration patterns; MS analysis was used to correlate their position on gels with parallel immunodetection. However, these authors acknowledged that this methodology was not ideal for membrane-associated proteins or those with low abundance. An alternative approach would be to use immunoaffinity methods to selectively isolate proteins of interest from a cell or tissue lysate or homogenate. For example, an HNE-modified fraction may be isolated from a membrane pellet obtained after centrifugation of a crude native homogenate by means of immunoprecipitation with the aid of an anti-HNE antibody and protein A-agarose beads [23]. Then, further separation and complete analysis of selected proteins eluted from polyacrylamide gels could be accomplished by MS techniques outlined here, with confirmation by Western blot analysis. Indeed, proof-of-concept for this approach was demonstrated by Ethen et al. [22] for a high abundance cytoskeletal protein, β -actin. However, these approaches may not be quantitative; hence, some HNE-modified proteins may elude detection or quantification by these methods. Obviating gel-based methods by utilizing the mass spectrometry-based methods described herein will afford more reliable, sensitive, and quantitative detection of HNE-modified proteins as well as providing direct identification and quantification of the modified amino acids.

Conclusion

In summary, the combined use of direct infusion and nanoLC-MS/MS methods are capable of identifying the type (based on the mass shift), number, and location of HNE modifications

of amino acid residues in proteins. Overall, our data suggests that, in addition to Cys, His, and Lys residues, other amino acids— including Arg, Thr, and Trp— can be modified by HNE. These modifications can form either through a Michael addition, Schiff base formation, or pyrrole-type adduct formation on N-terminal amino acids (in the present instance, His). These studies of “model” proteins modified by HNE demonstrate the MS signatures of HNE-modified amino acids, which can be used to interpret the results of MS-based proteomic analyses of cells and tissues under physiological and pathological conditions. Such analyses also may have particular utility for future biomarker discovery to monitor oxidative stress in cells and tissues under a variety of disease and environmental conditions, and to assess efficacy of therapeutic interventions for reducing oxidative stress under such conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
IAA	iodoacetamide
HNE	4-hydroxy-2-nonenal
BSA	bovine serum albumin MS, mass spectrometry
ESI-MS	electrospray ionization MS
MS/MS	tandem mass spectrometry
nanoLC-MS/MS	nanoliquid chromatography tandem mass spectrometry
m/z	mass/charge

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Figure 1. Schematic outline for MS and LC-MS/MS experiments. **(A)** Direct infusion experiment using ESI-QTOF Micro MS. **(B)** NanoLC-MS/MS using nanoACQUITY UPLC coupled with QTOF Ultima API MS.

Figure 2.
 Direct infusion analysis by ESI-MS of untreated and HNE-treated lysozyme. **(A)** Different charge states of untreated and HNE-treated lysozyme. **(B)** Magnification of (10+) peak to determine the number of HNE molecules per protein and the type of HNE-induced modification.

Figure 3. Example of HNE modification of Arg residue in BSA, at the peptide and amino acid levels. **(A)** Extracted ion chromatograms (XIC) of the peak with m/z 651.31 (2+) in BSA sample treated with 0, 2, 5 and 10 mM HNE. **(B)** Summed spectra from the XIC of the peak with m/z 651.31 (2+). **(C)** Product ions (MS/MS fragmentation) of the precursor ion from HNE-treated BSA, with m/z 651.31 (2+).

Figure 4. Example of HNE modification of Thr residue in BSA, at the peptide and amino acid levels. (A) XIC of the peak with m/z 990.45 (2+) in BSA sample treated with 0, 2, 5 and 10 mM HNE. (B) Summed spectra from the XIC of the peak with m/z 990.45 (2+). (C) Product ions (MS/MS fragmentation) of the precursor ion from HNE-treated BSA, with m/z 990.45 (2+).

Figure 5.

Example of pyrrole-type His modification by HNE in BSA, at the peptide and amino acid levels. (A) XIC of peak with m/z 713.26 (2+) in BSA sample treated with 0, 2, 5 and 10 mM HNE. (B) Summed spectra from the XIC of the peak with m/z 713.26 (2+). (C) Product ions (MS/MS fragmentation) of the precursor ion from HNE-treated BSA, with m/z 713.26 (2+).