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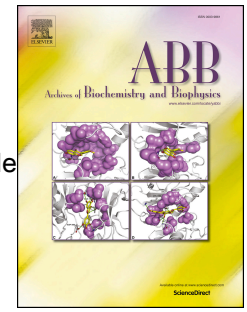

A mass spectrometry approach for the identification and localization of small aldehyde modifications of proteins

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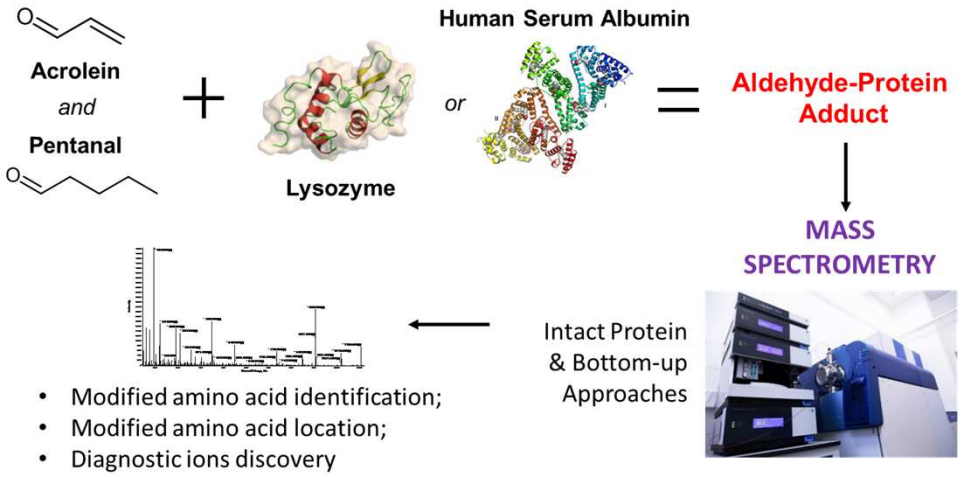

- Modified amino acid identification;
- Modified amino acid location;
- Diagnostic ions discovery

A mass spectrometry approach for the identification and localization of small aldehyde modifications of proteins.

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Abstract

Lipids containing polyunsaturated fatty acids are primary targets of oxidation, which produces reactive short-chain aldehydes that can covalently modify proteins, a process called lipoxidation. Improved mass spectrometry (MS) methods for the analysis of these adducts in complex biological systems are needed. Lysozyme and human serum albumin (HSA) were used as model proteins to investigate lipoxidation products formed by two short-chain aldehydes, acrolein and pentanal, which are unsaturated and saturated aldehydes respectively. The adducts formed were stabilized by $NaBH_4$ or $NaBH_3CN$ reduction and analysed by MS. Analysis of intact modified lysozyme showed a pentanal modification resulting from Schiff's base formation (+70 Da), and up to 8 acrolein adducts, all resulting from Michael addition (+58 Da). Analysis of tryptic digests identified specific histidine, cysteine and lysine residues modified in both lysozyme and HSA, and determined characteristic amino acid-specific fragmentations. Eight different internal fragment ions were found that could be used as general diagnostic ions for pentanal- and acrolein-modified amino acids. The combined use of intact protein analysis and LC-MS/MS methods provided a powerful tool for the identification and localization of aldehyde-protein adduct, and the diagnostic ions will facilitate the development of targeted MS methods for analysis of adducts in more complex samples.

Keywords

Acrolein; lipoxidation; pentanal; diagnostic ions; aldehydes; immonium ion

Introduction

Lipoxidation is the modification of proteins and peptides by reactive lipid oxidation products, involving reaction of the nucleophilic side chain of cysteine, histidine, arginine and lysine residues with electrophilic sites on the oxidized lipid products [1]. Reactive short-chain oxidation products from poly-unsaturated fatty acid (PUFAs) can be divided according to their chemical structure and reactivity into alkanals, alkenals and substituted alkenals [2,3]. While the alkanal has a saturated carbon chain containing an aldehyde group, alkenals additionally contain an α,β double bond on the carbon chain. The substituted alkenals are more complex due to the presence of different functional groups, for example hydroxyl or carbonyl groups, commonly on C4, which increase the susceptibility to nucleophilic attack and makes these aldehydes highly reactive. Typical reactions are formation of a Schiff's base between an amine and carbonyl group, a reversible covalent adduct resulting from the loss of a water molecule, or a Michael adduct, resulting from β-addition to an α,β-unsaturated carbonyl moiety by a nucleophilic amino acid side chain [4]. The chemistry and analysis of these protein modifications have been described in more detail in various reviews [1,4–7], and there is significant evidence for their occurrence in vivo (reviewed in Domingues 2013).

Pentanal and acrolein are two short aldehyde models of alkanals and alkenals respectively. Acrolein is the shortest alkenal identified as a lipid oxidation product, and is highly reactive [8]. Besides being a product of lipid peroxidation, it can also be formed during the combustion of organic matter, for example being present in tobacco smoke [9]. It has been linked with inhibition of cell proliferation, enhancement of apoptosis, and disruption of gene expression necessary to regulate inflammation and antioxidant defense [8,10]. Pentanal is a 5 carbon saturated aldehyde, and while it has not been studied as intensively as acrolein, it has been found in increased amounts in the exhaled breath of lung cancer patients [11] as well as in urine of patients with prostate cancer where it may help with stratification of disease severity [12].

Several physiological and pathological events have been linked with lipoxidation, and lipoxidation adducts have been found in several inflammatory diseases, including atherosclerosis [13] and Alzheimer's disease [14]. However, the detection and analysis of these adducts in biological samples is challenging, especially due to their low abundance [1,4]. Several studies were able to identify lipoxidation in biological samples using various techniques, including antibodies, chemical probes and mass spectrometry [1,4]. The latter is nowadays the technique of choice for the identification of protein-lipid adducts, since it can detect the mass shift caused by the adducts, and when used in tandem mode, enables localization of the modified amino acid within the protein sequence [15,16]. However, while in simple samples this technique can be straightforward, in more complex samples the data

output is much greater, potentially generating more false positive identifications, and the probability of failing to detect the lower abundance modified peptides in the presence of high abundance unmodified ones increases, making the identification of modifications difficult and time-consuming. To help overcome this, the information in the fragmentation spectra can be used to identify reporter ions, fragment ions characteristic and specific for each modification rather than the peptide sequence. These reporter ions can then be used to look specifically for the modification in more complex samples, using semi-targeted mass spectrometry approaches such as precursor ion scanning (PIS) or neutral loss scanning (NLS), that identify peptides which give rise to the diagnostic ions, simplifying the analysis [1,4–7,17,18]. Such an approach has shown promise previously for HNE-protein adducts, although mainly in simple biological samples [17,18].

In the study reported here, we used liquid chromatography coupled to tandem mass spectrometry to investigate protein modifications caused by acrolein and pentanal. Two proteins were used as models for the study of small aldehyde lipoxidation: lysozyme (14,306 Da), a small hydrolase, rich in lysine and cysteine, and human serum albumin (66,437 Da), the most abundant human plasma protein and one which is known to be modified in oxidative stress conditions. Non-physiologically high concentrations of aldehydes were used, as the aim was to generate abundant adducts in order to localize the amino acids modified by these aldehydes, and identify possible reporter ions for these modifications that would facilitate targeted identification of these adducts in biological or clinical samples in future studies.

Materials and Methods

Chemicals

All reagents were purchased from Sigma-Aldrich Chemical Co. (Dorset, UK) unless otherwise indicated. All solvents were of LCMS grade and Milli-Q water was used for the buffers and reactions. Formic acid and dithiothreitol (DTT) were purchased from Thermo Fisher (Runcorn, UK).

Synthesis of aldehyde-protein adducts

Lysozyme (1 mg/mL) was first reduced with 100 mM DTT. This step was omitted for HSA, which was used in its native form (1 mg/mL). Acrolein was added to the protein solution at 4, 8 or 14 mM and allowed to react for 2 hours at room temperature. Pentanal was added to the protein solution at 16.6 mM and allowed to react for 24h at 37°C. To stabilize adducts, 50 mM $NaBH_4$ was added to the reaction and left for 1 hour at room temperature, with the exception of the HSA-pentanal reaction, which was instead reduced with 50 mM $NaBH_3CN$. For direct infusion mass spectrometry analysis, excess DTT in the

reduced lysozyme samples was removed prior to the reaction with the aldehydes using Microcon Ultracel YM-10 10,000 MWCO centrifugal concentrators (Millipore, Massachusetts, USA) using the manufacturers protocol.

Direct infusion MS analysis of modified lysozyme

Modified lysozyme samples were dissolved in 50% acetonitrile, 0.5% formic acid in water and analysed by direct infusion into a 5600 TripleToF mass spectrometer (Sciex, Warrington, UK) using loop injection directly into a 2 µL/min flow rate of the same solvent and introduced into the source via either a 20 µm i.d. steel capillary mounted on a standard nanospray source with a spray voltage of 2.4 kV, a source temperature of 150°C, declustering potential of 100V and a curtain gas setting of 25, or a TurboIon source fitted with a 50 µm i.d. emitter with a spray voltage of 5.5 kV, a source temperature of 150°C, declustering potential of 100V, nebulizing gas flow of 15 and a curtain gas setting of 25. Data was summed for 3-5 minutes and deconvoluted using the Bio Tool Kit plugin and PeakView 2.2 software (Sciex, Warrington, UK) with a step size of 0.5 Da at high (30,000) resolution and Gaussian smoothed with a 3 point window.

Protein in-gel digestion

The reaction products from above were separated by 12.5 or 15 % SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie blue to visualize the bands prior to further processing. Bands present in the gel were then excised and tryptic digestion was performed according to Verrastro *et al*., 2016 [19]. The peptide extracts were dried for storage, and resuspended in H_2O/acetonitrile (98%/2%) with 0.1% formic acid prior to MS analysis.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis

Peptides were separated and analysed using an Ultimate 3000 system (Thermo Scientific, Hemel Hemstead, UK) coupled to a 5600 TripleTOF (ABSciex, Warrington, UK). The analysis was performed as previously described by Verrastro *et al*., 2016 [19]. Briefly, the peptide solution was loaded onto a C18 trap column (C_{18} PepMap^{TM}, 5 µm, 0.5 x 5mm, Thermo Scientific, Hemel Hemstead, UK) at 30 µL/min in 2% acetonitrile 0.5% formic acid followed by a 4 minute wash, before separation on a nano-HPLC column (C_{18} PepMap^{TM}, 5 µm, 0.075 x 150mm, Thermo Scientific, Hemel Hemstead, UK) at 300 nL/min using a gradient elution running from 2% to 45% aqueous acetonitrile, 0.1% formic acid over 45 minutes. Ionization of the peptides was achieved with spray voltage set at 2.4 kV, a source temperature of 150°C, declustering potential of 100V, nebulizing gas flow of 15 and a curtain

gas setting of 25. Survey scans were collected in positive mode from 350 to 2000 Da using high-sensitivity TOF-MS mode. Information-dependent acquisition (IDA) was used to collect MS/MS data using the following criteria: the 10 most intense with +2 to +5 charge states and a minimum intensity of 200 cps were chosen for analysis, using dynamic exclusion for 12 s and standard rolling collision energy settings.

Database Search

The Mascot® probability based search engine (Matrix Science, London, version 2.4.0) was used to interrogate the SwissProt 2017-07 primary database. LC-MS .wiff files of each sample were searched for protein identification and oxidative post-translational modifications (oxPTMs). For protein identification, variable modifications of methionine oxidation and carbamidomethyl cysteine were used. For the analysis of the lipoxidation products, the initial searches additionally used a variable modification list including pentanal and reduced pentanal at lysine and histidine and reduced and unreduced acrolein adducts at cysteine, lysine and histidine for pentanal and acrolein modified samples respectively. The data was then re-searched using the Mascot error tolerant search function. Other parameters for the searches were as follows: Enzyme: Trypsin; Peptide tolerance: ±0.6 Da; MS/MS tolerance: ±0.6 Da; Peptide charge state: +2, +3; Max Missed cleavages: 1; #13C: 0; Quantitation: None; Instrument: ESI-QUAD-TOF; Data format: Mascot Generic; Experimental mass values: Monoisotopic; Taxonomy: Chordata. All data identifying modifications were manually validated before inclusion.

Results

Direct infusion mass spectrometry was used to monitor the modification of lysozyme by either acrolein or pentanal. To increase the potential for reaction between the protein and the aldehyde, the disulfide bonds on the lysozyme were first reduced with DTT. The effect of reduction with DTT is shown in **Supplementary Figure 1**; a DTT concentration of 200 mM was required to reduce the DTT completely. After deconvolution of the charge state envelope, the lysozyme was observed at a mass of 14,314 Da when fully-reduced (4 disulfide bonds reduced, corresponding to a mass increase of 8 Da over the native form at 14,306 Da). Intermediate DTT treatments resulted in partial reduction of the protein with average masses of 14308, 14310 and 14312 Da corresponding to reduction of the equivalent of 1, 2 and 3 disulfides respectively. However, as the high DTT concentration required to achieve full reduction interfered with the aldehyde treatments, in subsequent experiments 100 mM DTT was used, which typically reduced 2-3 disulfide bonds.

Peaks corresponding to reduced acrolein adducts of intact lysozyme were observed at

mass increases of +58 Da, consistent with formation of a Michael adduct (+56 Da) and subsequent stabilization by borohydride reduction (+2 Da) (**Figure 1**). The optimal conditions for formation of acrolein-protein adducts were investigated. Reaction with acrolein for longer than 2h (data not shown) or increasing the aldehyde concentration above 8 mM resulted in more extensive modification but overall lower adduct peak intensities (Figure 1A-D). These conditions typically gave rise to a maximum of 6-8 adducts of acrolein linked to lysozyme. Based on this protocol, the pentanal-lysozyme reaction was also investigated; however, pentanal was much less reactive and only a single adduct was observed even at the highest treatment concentration (16.6 mM) and longest incubation time used (24 hr). For the reduced pentanal adducts, the observed mass difference was +70 Da, corresponding to a reduced Schiff's base on a lysine residue ($-H_2O + 2H$) (Figure 1 E).

To determine the specific amino acid residues modified, bottom up analysis was carried out by tryptic digestion of the modified protein and LC-MS/MS analysis of the peptides. Initially MASCOT software [20] was used to identify peptides modified with either acrolein or pentanal, and each potential modification was also confirmed by manual analysis of the MS/MS spectrum (examples in **Supplementary Figure 2**). The intensity of the MS/MS spectra for pentanal-modified peptides from lysozyme was low, and only two pentanal adducts could be identified on Lys13 and Lys116 (all numbering is based on the mature protein sequence). The MS/MS spectrum for the peptide with modification on Lys13 is shown in Supplementary Figure 2B. This bottom-up approach allowed the identification of several peptides from lysozyme modified by acrolein, based on the peptide molecular weight, mass/charge ratio and charge of the peptide ion, ion score, and LC retention time. The majority of acrolein adducts occurred on cysteine residues, although some lysine adducts were also identified (**Table 1**). One lysozyme peptide was found to contain 3 acrolein adducts, at Cys76, Cys80 and Lys96. Two diagnostic fragmentation products of acrolein-cysteine adducts resulting from internal fragmentation to give an immonium ion (breaking of two bonds in the peptide either side of the amino acid residue) were consistently observed at m/z 134.06 and 117.04 Da, while acrolein-lysine adducts gave a diagnostic fragment at m/z 159.15 Da (Table 1).

The same approach was applied to determine the sites of aldehyde modification in human serum albumin (HSA). **Figure 2** shows examples of HSA peptides modified by acrolein on Lys137 (A) and pentanal on Lys525 (B), where the peptides could be fully sequenced using the b and y fragment ions. In both cases the modified lysine immonium ions and other specific internal fragment ions could be detected, and are shown in the expanded low mass regions of the spectra. Pentanal-lysine internal fragment ions were observed at m/z 199.17, 171.18 and 154.15 Da (Figure 2C). For acrolein modification of lysine a diagnostic internal fragment ion was apparent at m/z 142.13 Da (Figure 2D). These

diagnostic ions occurred consistently in all the aldehyde-modified HSA peptides and are listed in **Table 2**. In total, eight different diagnostic fragment ions were identified, three for pentanal modification of lysine, and five for the acrolein modifications, specifically two for lysine, two for cysteine and one for histidine. The proposed structures of these diagnostic ions are shown in **Figure 3**.

Discussion

In this focused study, the modification of proteins by two model aldehydes, acrolein and pentanal, was evaluated as a model of lipoxidation adducts that may occur in inflammatory diseases. The aim was to map the sites of modification using LC-MS/MS and identify potential diagnostic for adducts with different amino acid residues. To ensure extensive adduct formation and to allow MS characterisation of the adducts formed, high concentrations of the aldehydes were used. While these do not correspond to the physiological levels of free aldehydes in plasma, it has been suggested previously that local levels of aldehydes are higher than plasma levels and in membranes may even reach low millimolar levels [21]. Pentanal modifications were identified in 2 lysozyme and 14 HSA peptides, all Schiff's base adducts at lysine residues stabilized by reduction, in agreement with the theoretical reactivity of this alkanal, which is limited to Schiff's base formation with amines. Acrolein modifications were found in 5 lysozyme and 11 HSA peptides, and were predominantly formed by Michael addition reactions with cysteine, histidine and lysine amino acid residues, based on the mass increases of 56 Da or 58 Da for unreduced or reduced forms respectively, despite the potential for this α,β-alkenal also to form Schiff's base adducts. Additionally, 3 diagnostic immonium ions for pentanal-lysine adducts and 5 diagnostic ions for acrolein adducts with cysteine, histidine and lysine were consistently observed in the spectra of modified peptides.

The bottom-up proteomic approach allowed identification of 8 lysozyme residues modified by acrolein: Cys6, Cys30, Cys64, Cys76, Cys80, Lys96, Cys155 and Lys116, which fitted well with the observation by intact protein analysis that up to 8 acrolein molecules were covalently bound per lysozyme. For HSA, mainly histidine and lysine residues were found to be modified: Cys 34, His67, Lys137, His146, Lys262, Lys276, His288, His338, Lys414, Lys525 and Lys574. The high number of cysteines modified in lysozyme is likely to result from the reduction prior to reaction with the aldehyde increasing the number of free cysteine residues, and confirms that the free cysteine residues are highly susceptible to acrolein modification. HSA was reacted in its physiologically relevant native state, where only a single cysteine (Cys34) is in the free thiol form, and consequently modifications of lysine and histidine were more prevalent. The tryptic peptide containing the HSA free cysteine (Cys34)

was challenging to detect, as it is a long peptide that gives low ion intensity, but it was identified in its carbamidomethylated and Michael adduct-modified form in a number of samples. Some of the peptides identified contained missed cleavages owing to modification of lysine residues, which could complicate relative quantification against control samples, but all showed the expected diagnostic ions. A number of the lysines and histidines observed to be modified were ones that have been reported previously to be susceptible to electrophilic attack, and the occurrence of acrolein-protein adducts has previously been reported both in vitro and in vivo [22,23], including studies of acrolein modification of albumin [24]. It is also worth noting that in a biological sample the product of a fully reduced malondialdehyde adduct, which could be formed from either lipoxidation and glucoxidation events, would be indistinguishable from the acrolein adduct.

In contrast, the alkanal pentanal has been much less studied, despite being known as a product of lipid peroxidation and detected as a volatile component of breath condensate and urine [11,12]. It was interesting that while there was overlap in the lysine residues of HSA that were modified by acrolein and pentanal, there were a significant number of residues that appeared to be susceptible to formation of pentanal adducts where no acrolein adducts were observed (Lys12, Lys73, K162, Lys199, Lys205, Lys212, Lys281, Lys351, Lys402, and Lys545).

A major aim of the study was to identify the diagnostic ions for the aldehyde adducts that could subsequently be used in targeted mass spectrometry approaches, such as precursor ion scanning or multiple reaction monitoring, or to confirm peptide identifications. The lower m/z range of the MS/MS spectra contains signals from immonium and related internal fragmentation ions specific to amino acids present in the peptides; these ions are potential MS/MS reporter ions for modified amino acid residues. From the MS/MS spectra of the modified tryptic peptides, several potential diagnostic ions for the pentanal and acrolein modifications were identified (Figure 3). While protein-acrolein adducts have been extensively studied previously [25–27], the focus was on the identification of the sites of adduction, and it appears that there aren't any specific studies of reporter ions that could be used for these modifications. In contrast, the predominant diagnostic fragment ions detected for pentanal-lysine adducts are in agreement with the diagnostic ions reported by Fenaille *et al.* for N^{α}-acetyl lysine and apomyoglobin adducts with hexanal and pentanal, specifically those at m/z 154 and 199 for pentanal [28]. Thus the present study not only confirmed the diagnostic ions of pentanal modification of lysine in modified proteins, but also uncovered a new, commonly formed diagnostic ion at m/z 171, which will help to increase specificity in the detection of this form of lipoxidation.

It is also important to consider potential physiological consequences of lipoxidation, as serum albumin has many biological functions that could be affected by modification. For

example, His67 was readily modified by both pentanal and acrolein, and is one of the residues contributing to the zinc binding site on albumin [29] [30], so its modification could disrupt zinc binding to and transport by albumin. As decreased binding of zinc can result in increased levels of fatty acids in the blood [31], acrolein modification of this site could contribute to plasma lipid changes in cardiovascular diseases and diabetes. Several of the lysine residues modified by pentanal have also been previously reported to undergo post-translational modifications and affect drug binding. K199, K205 and K281 are part of the albumin IIA subdomain and are near a major drug binding site in HSA, the Sudlow Site I, which binds drugs such as warfarin. K199, K205 and K281 were found to be modified in minimally glycated HSA, forming Nε-carboxymethyl-lysine (CML) and this protein was shown to have different binding behaviour depending on the glycation levels [32]. It is conceivable that pentanal modification of this residue could have similar physiologically relevant effects. Nε-carboxymethyl-lysine (CML) and Amadori-modified lysine (AML) formation on K351 and K525 have been found in HSA glycated peptides from clinical diabetic plasma, and N(ε)-(carboxyethyl)lysine (CEL) modification on lysine K525 [33,34]; Korwar et al. approached this by constructing a library of fragment ions from modified HSA peptides using SWATH, an untargeted MS technique [33].

While acrolein and pentanal were found to modify many relevant residues, under physiological conditions the profile of the modifications could be different, and only the most susceptible sites might be significantly modified. The data presented here and previous literature suggest that the cysteine residues would be major sites of modification [4,35]. Furthermore, the sequence coverage obtained was incomplete and varied depending on the treatment (typically 55-85%), and some potential modification sites, such as the N-terminal, were not covered; hence no conclusions can be drawn about whether modifications occurred at these sites. Nevertheless, these examples show how important this approach is to identify the location of protein modifications correctly and understand the relevance to disease.

Conclusion

In summary, this study has provided new data on the sites of modification in two model proteins by the aldehydes pentanal and acrolein, and has identified novel diagnostic fragment ions for pentanal adducts of lysine, and acrolein adducts of cysteine, lysine and histidine. This shows the power of combined use of direct infusion and LC-MS/MS to identify the type, the number and the location of protein adducts. The diagnostic ions can be used in targeted mass spectrometry approaches, such as precursor ion scanning or multiple reaction monitoring, to identify and quantify aldehyde adducts, and further research is needed to test this in complex biological or clinical samples. The MS approaches described here could also

be applied to study other types of adducts as biomarkers of lipoxidation in cells and tissues under pathophysiological conditions.

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Abbreviations

AML, Amadori-modified lysine; CML, Nε-carboxymethyl-lysine; DTT, dithiothreitol; ESI, electrospray ionisation; HSA, human serum albumin; HPLC, high-performance liquid chromatography; IDA, Information-dependent acquisition; LC, liquid chromatography; MS, mass spectrometry; NLS, neutral loss scan; oxPTM, oxidative post translational modification; PIS, precursor ion scan; PUFA, polyunsaturated fatty acid; QUAD, quadrupole; Rt, Retention time; SWATH, sequential window acquisition of all theoretical spectra; TOF, time of flight.

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Figure Legends

Figure 1. Deconvoluted ESI-MS spectra of lysozyme obtained after reaction with acrolein or pentanal. (A) DTT-reduced lysozyme untreated with aldehyde showing partial reduction of disulphide bonds (2-3 disulphides reduced). (B) Treatment with 4 mM acrolein for 2 h showing the formation of several acrolein Michael adducts, each adding +58 Da. (C) Treatment with 8 mM acrolein for 2 h. (D) Treatment with 14 mM acrolein for 2 h. (E) Treatment of partially-reduced lysozyme (1-2 disulfides reduced) with 16.6 mM pentanal for 24 h showing formation of a single adduct at +70 Da. Note that the x-axis scale has been expanded in this spectrum.

Figure 2. MS/MS spectra of different human serum albumin tryptic peptides (KYLYEIAR and KQTALVELVK) modified on a lysine residue by pentanal (A) and acrolein (B). The y and b ions indicated by the arrows confirm the peptide sequence and the modification on the lysine residue. Additionally, the ions at m/z 154.15, m/z 171.18 and 199.17, diagnostic of pentanal-modified lysine (C) and the ion at m/z 142.13, diagnostic of acrolein-modified lysine (D) are highlighted.

Figure 3. Proposed structures of the diagnostic ions found for the reduced pentanal modifications on lysine residues (1-3) and for the reduced acrolein modifications on lysine (4,5), cysteine (6,7) and histidine residues (8).

Table 1. Modifications of lysozyme by reactive aldehydes

Lysozyme modified peptide sequence ($_{a}$ [b])	Theoretical mass of modified peptide	Observed mass of modified peptide	m/z (charge state)	Ion score	Rt (min)	Diagnostic Ions [c]
Pentanal						
C_{6}ELAAAM**K**$^{+70}$$R_{14}$	1118.59	1118.62	373.9 (3+)	41	30.95	-
C_{115}**K**$^{+70}$GTDVQAWIR$_{125}$	1402.74	1402.79	368.6 (4+)	37	36.36	-
Acrolein						
C$_{6}^{+56}$ELAAAMK$_{13}$	891.07	891.42	446.5 (2+)	18	36.19	-
C$_{6}^{+58}$ELAAAMK$_{13}$	893.12	893.44	447.5 (2+)	79	27.57	6,7
G_{22}YSLGNWV**C**$^{+58}$AAK$_{33}$	1325.16	1325.64	663.6 (2+)	80	38.35	-
W_{62}W**C**$^{+58}$NDGR$_{68}$	993.06	993.41	497.5 (2+)	35	32.31	6
N_{74}L**C**$^{+56}$NIPCSALLSSDITASVNCAK$_{96}$	2506.26	2506.19	836.4 (3+)	77	42.39	-
N_{74}L**C**$^{+58}$NIPCSALLSSDITASVNCAK$_{96}$	2508.31	2508.20	837.1 (3+)	50	43.84	6,7
N_{74}LCNIP**C**$^{+58}$SALLSSDITASVNCA**K**$_{96}^{+58}$	2509.33	2509.22	837.4 (3+)	92	44.29	6
N_{74}L**C**$^{+58}$NIP**C**$^{+58}$SALLSSDITASVNCA**K**$_{96}^{+58}$	2510.33	2510.24	838.1 (3+)	60	44.84	6
C$_{115}^{+58}$KGTDVQAWIR$_{125}$	1333.21	1333.68	445.4 (3+)	17	31.19	-
C$_{115}^{+56}$**K**$^{+58}$GTDVQAWIR$_{125}$	1389.19	1389.71	464.1 (3+)	28	30.24	5
C_{115}**K**$^{+58}$GTDVQAWIR$_{125}$	1390.21	1390.70	464.4 (3+)	30	31.08	5

a (subscript) – amino acid position in the mature protein for the start and end residues

b (superscript) - mass difference corresponding to the modification on the affected residue (shown in bold red)

c (superscript) – numbers refer to the structures shown in Figure 3.

Table 2. Modifications of human serum albumin (HSA) by reactive aldehydes

HSA modified peptide sequence ($_{a}$ [b])	Theoretical mass of modified peptide	Observed mass of modified peptide	m/z (charge)	Ion score	Rt (min)	Diagnostic Ions [c]
Pentanal						
F_{11}**K**$^{+70}$DLGEENFK$_{20}$	1295.69	1295.68	648.9 (2+)	58	35.30	1, 3
S_{65}LHTLFGD**K**$^{+70}$LC*TVATLR$_{81}$	2001.11	2001.12	668.1 (3+)	76	47.16	1, 3
K$_{137}$$^{+70}$YLYEIAR$_{144}$	1124.66	1124.69	563.3 (2+)	39	37.19	1, 2, 3
Y_{161}**K**$^{+70}$AAFTEC*C*QAADK$_{174}$	1731.79	1731.81	578.3 (3+)	58	28.88	1, 3
L_{198}**K**$^{+70}$C*ASLQK$_{205}$	1016.61	1016.62	509.3 (2+)	37	25.15	1, 3
C_{200}*ASLQ**K**$^{+70}$FGER$_{209}$	1264.66	1264.68	633.3 (2+)	42	30.91	1, 2, 3
A_{210}F**K**$^{+70}$AWAVAR$_{218}$	1088.65	1088.68	363.9 (3+)	51	37.12	1, 2, 3
Y_{263}ICENQDSISS**K**$^{+70}$LK$_{276}$	1753.89	1753.91	585.6 (3+)	78	34.16	1, 3
L_{275}KEC*C*E**K**$^{+70}$PLLEK$_{286}$	1615.87	1615.88	539.6 (3+)	34	28.84	1, 3
L_{349}A**K**$^{+70}$TYETTLEK$_{359}$	1365.77	1365.78	456.3 (3+)	35	30.00	1, 3
Q_{390}NC*ELFEQLGEY**K**$^{+70}$FQNALLVR$_{410}$	2668.37	2668.43	890.5 (3+)	130	49.68	1, 2, 3
K$_{414}$$^{+70}$VPQVSTPTLVEVSR$_{428}$	1709.01	1709.03	855.5 (2+)	77	36.55	1, 2, 3
K$_{525}$$^{+70}$QTALVELVK$_{534}$	1197.76	1197.77	599.9 (2+)	55	37.32	1, 2, 3
E_{542}QL**K**$^{+70}$AVMDDFAAFVEK$_{557}$	1909.99	1910.01	637.7 (3+)	65	37.48	1, 3
Acrolein						
S_{65}L**H**$^{+58}$TLFGDK$_{73}$	1074.59	1074.57	359.2 (3+)	43	28.51	8
K$_{137}$$^{+58}$YLYEIAR$_{144}$	1112.63	1112.62	557.3 (2+)	16	27.69	4,5
R_{145}**H**$^{+58}$PYFYAPELLFFAK$_{159}$	1956.03	1956.03	653.0 (3+)	66	41.35	8
A_{258}DLA**K**$^{+58}$YICENQDSISSK$_{274}$	1998.95	1998.96	667.3 (3+)	73	31.29	5
Y_{263}ICENQDSISSKL**K**$_{276}$$^{+58}$	1741.86	1741.86	581.6 (3+)	109	26.71	4
S_{287}**H**$^{+58}$CIAEVENDEMPADLPSLAADFVESK$_{313}$	3031.34	3031.38	1011.5 (3+)	61	42.41	8
R_{337}**H**$^{+58}$PDYSVVLLLR$_{348}$	1524.89	1524.88	509.3 (3+)	83	34.59	8
Q_{390}N**C**$^{+56}$ELFEQLGEYK$_{402}$	1655.72	1655.75	829.4 (2+)	42	41.14	6
K$_{414}$$^{+58}$VPQVSTPTLVEVSR$_{428}$	1696.97	1696.97	566.3 (3+)	59	30.89	4
K$_{525}$$^{+58}$QTALVELVK$_{534}$	1185.76	1185.73	396.3 (3+)	52	28.84	4
K$_{574}$$^{+58}$LVAASQAALG$_{584}$	1198.73	1198.73	600.4 (2+)	62	33.67	4

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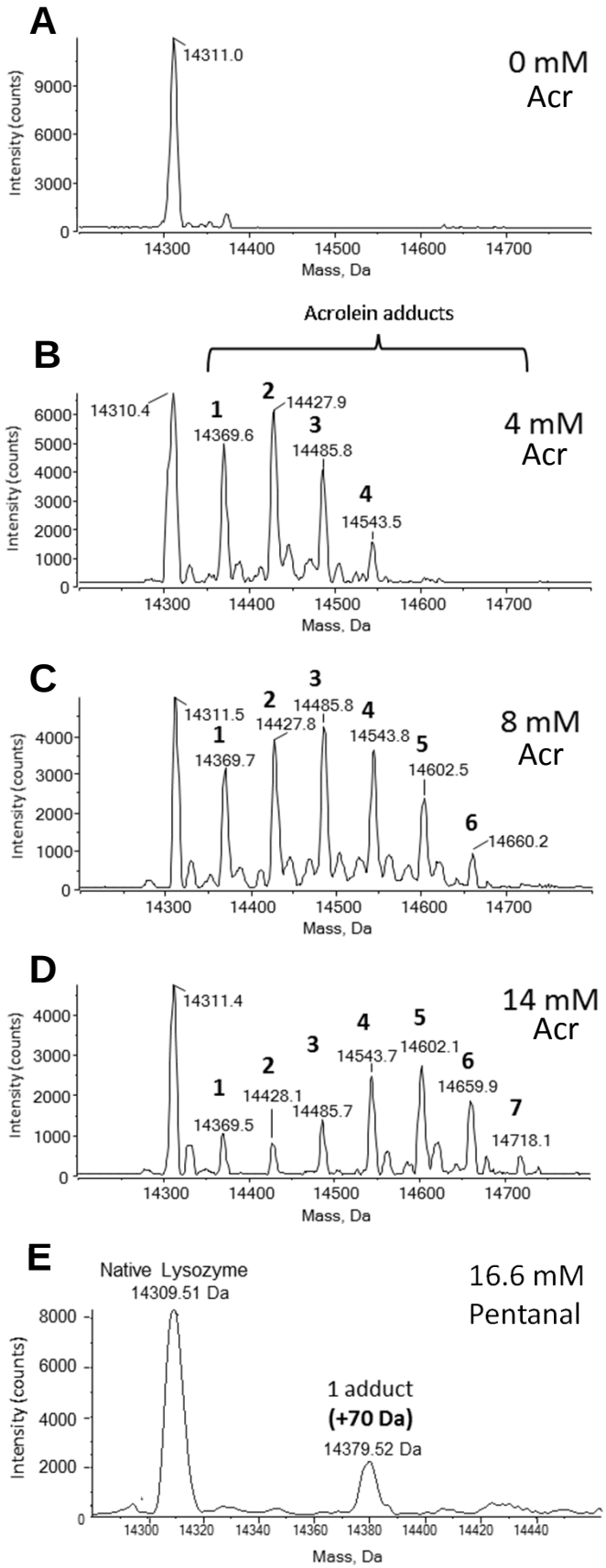

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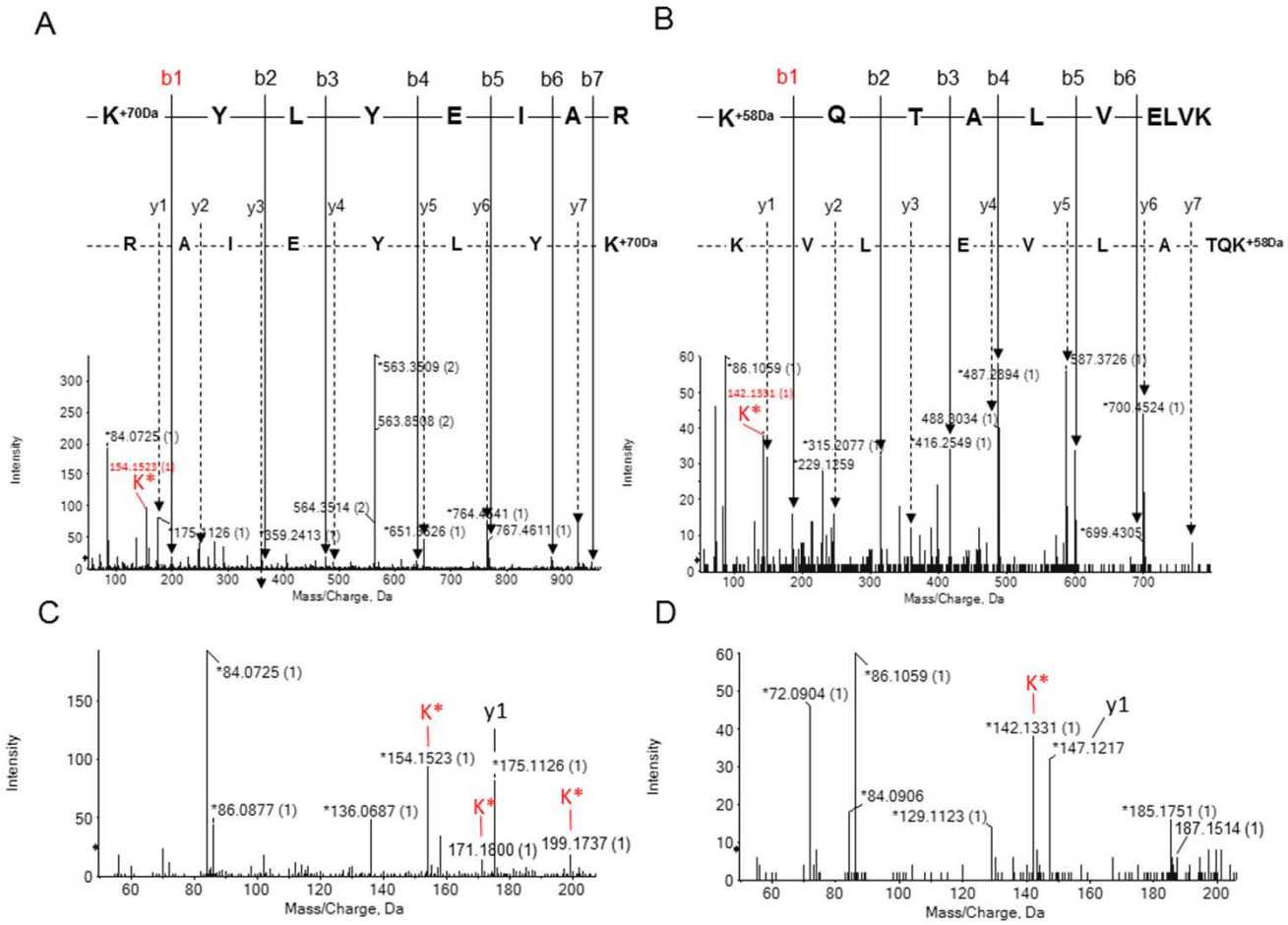

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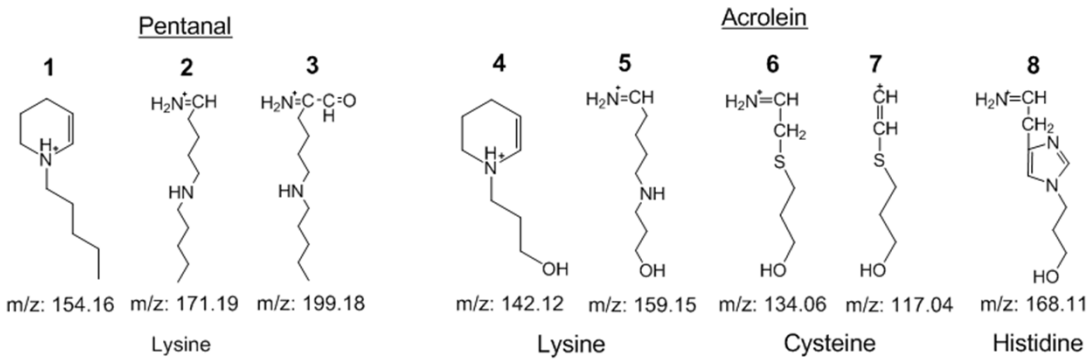

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A mass spectrometry approach for the identification and localization of small aldehyde modifications of proteins.

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Highlights

- 5 lysozyme and 11 HSA peptides were modified with acrolein
- 2 lysozyme and 14 HSA peptides modified with pentanal were identified
- 8 diagnostic ions for pentanal and acrolein modifications were identified
- Acrolein and pentanal modifications could have physiological effects
- MS is a valuable technique for identifying protein modifications