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JMS Letters

Dear Sir,

Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry as a Tool to Probe the Reactions of *trans*-Hex-2-enal with Proteins

Since the 1970s, extensive study has been devoted to the reactions of proteins with carbonyl compounds.[1] Under conditions of oxidative stress, the presence of peroxides in the body results in lipid degradation, producing a large array of aldehyde byproducts.[1–3] These reactive aldehydes can modify proteins *in vivo*, thus compounding the damage from the original oxidative event. Recently, Novotny *et al.*[4] reported that diabetic rats and humans exhibit elevated levels of saturated and α,β-unsaturated aldehydes in their blood. They identified a possible correlation between the presence of these aldehydes and symptoms of 'accelerated aging' observed in diabetics. Furthermore, they learned that these aldehydes irreversibly inhibit several glycolytic enzymes, suggesting enzyme modification. Hence there is a clear need to understand the chemistry that occurs between aldehydes and proteins in biological systems.

Principally, aldehydes are believed to modify proteins by reacting with nucleophilic amino acid side-chains. α,β-Unsaturated aldehydes are particularly reactive owing to the presence of electrophilic centers at the β-carbon and the carbonyl carbon. These aldehydes have two likely reaction pathways: simple addition of a nucleophilic amino acid residue to the β-carbon (a Michael addition) and addition of a free amine to the aldehyde carbonyl carbon, followed by a condensation to form a Schiff base.

The recent establishment of mass spectrometry as a reliable bioanalytical technique has made it possible to probe these reaction products rapidly with high resolution. Mass spectrometry is well suited for determining the extent of protein modification by measuring the mass increase of the protein following exposure to small, reactive molecules. Furthermore, mass spectrometry can provide mechanistic information. Since Schiff base formation is a condensation process, the adduct mass will be the mass of the aldehyde minus that of water. On the other hand, the mass of a Michael adduct will simply be the mass of the aldehyde.

Most recent studies of protein–aldehyde interactions by mass spectrometry (MS) have been conducted via electrospray ionization (ESI).[5–9] However, ESI-MS produces a distribution of charge states for each analyte molecule. This leads to complicated spectra if the sample is heterogeneous. If this heterogeneity is extensive, the mass distribution may be difficult to distinguish from the charge envelope. Because matrix-assisted laser desorption/ionization time-of-flight (MALDI/TOF) mass spectrometry primarily generates singly charged ions, heterogeneous samples are not further complicated this distribution of charge states. Therefore, substantial simplification of the spectra should be possible through MALDI analysis. Additionally, recent advances by several groups utilizing pulsed extraction techniques have dramatically improved the resolution attainable in linear MALDI/TOF instruments.[10] The potential for considerable simplification of mass spectra, coupled with the improved resolution provided by delayed extraction, led us to utilize MALDI-MS for the exploration of protein–aldehyde reactions.

As in a recent study conducted by Zidek *et al.*,[5] our model aldehyde was *trans*-hex-2-enal, one of several toxic lipid peroxidation byproducts that have been identified in diabetics.[4,5] Model proteins and peptides were incubated with excess hex-2-enal according to a procedure outlined previously.[5] The standard incubation procedure involved dissolution of 1 mg of protein or peptide in 100 μl of 0.2% (v/v) aqueous hex-2-enal. No additional buffers were used as these could potentially complicate the resulting mass spectra. Samples were incubated at 37 °C in 1.5 ml Eppendorf tubes. A control sample of protein or peptide without aldehyde was incubated in all cases. Removal of free *trans*-hex-2-enal prior to MALDI analysis was accomplished by lyophilization and subsequent dissolution in doubly distilled water at a protein/peptide concentration of 1 mg ml^{-1}. Without this aldehyde removal step, MALDI samples do not crystallize properly, resulting in sample spots with an 'oily' appearance that do not yield ion signals on irradiation with the laser. A 7 μl volume of aqueous protein or peptide solution was mixed with 3 μl of a ferulic acid matrix solution in ethanol. The final mixture was 50 mM in matrix. Finally, 2 μl of this solution were deposited on a stainless-steel sample probe for MALDI analysis.

MALDI was performed on two linear, delayed-extraction TOF instruments constructed in-house. The frequency-tripled output of a Quanta-Ray DCR-2 Nd:YAG laser (355 nm) was used as the ionization source in these instruments. The two instruments differ primarily in that each is configured to analyze a different ion polarity. There were no discernible differences in quality between the positive- and negative-ion spectra, so data are reported from both instruments. The instrument used to record negative-ion spectra and the specifics of the data acquisition electronics have been discussed previously.[11] In all cases, the spectra reported represent an unsmoothed average of 200 individual laser shots. The data were calibrated by using two reference proteins or peptides on the sample probe. Calibrants were chosen such that they bracketed the mass range of interest. Times of flight were assigned by fitting each peak to a Gaussian function and determining the median.

Our study of protein interactions with *trans*-hex-2-enal began with cytochrome *c* (12 360 Da). We expected that the primary mode of chemical modification would be the formation of Michael adducts at lysine residues and at the polypeptide *N*-terminus.[5] Other researchers have observed exclusive formation of Michael addition products in ESI-MS experiments with another α,β-unsaturated aldehyde, 4-hydroxynonenal.[5–9] However, we wished to determine whether the Michael addition pathway is really favored over Schiff base formation. These two mechanistic possibilities should be easily determined by MALDI since the Schiff base adduct of hex-2-enal would result in a mass increase of 80 Da whereas the increase due to a Michael adduct is 98 Da.

Figure 1 presents mass spectra of a cytochrome *c* sample incubated with hex-2-enal for various periods of time. The data were calibrated from the protonated parent ions of cytochrome *c* and lysozyme that were deposited on the same sample probe with the modified cytochrome *c* samples. Figure 1(A) shows the cytochrome *c* mass spectrum recorded before addition of hexenal. Figure 1(B) shows that, after 10 min of incubation together, there are up to two molar equivalents of aldehyde attached to cytochrome *c*. The feature indicative of the first equivalent added is clearly a doublet, denoted as peaks a and b in Fig. 1(B). This doublet corresponds to Schiff base (+80.1 Da shift) and Michael (+95.5 Da shift) adducts. The other adduct peak, c, possesses two shoulder peaks (these were not sufficiently defined for a Gaussian fit) and a taller central peak corresponding to the mass of cytochrome *c* plus a Michael and a Schiff base adduct of hex-2-enal (+177.3 Da shift). After 1 h [Fig. 1(C)], there are up to six hexenal molecules attached to cytochrome *c*, although only four of these are resolved. At longer incubation times, the mass spectra

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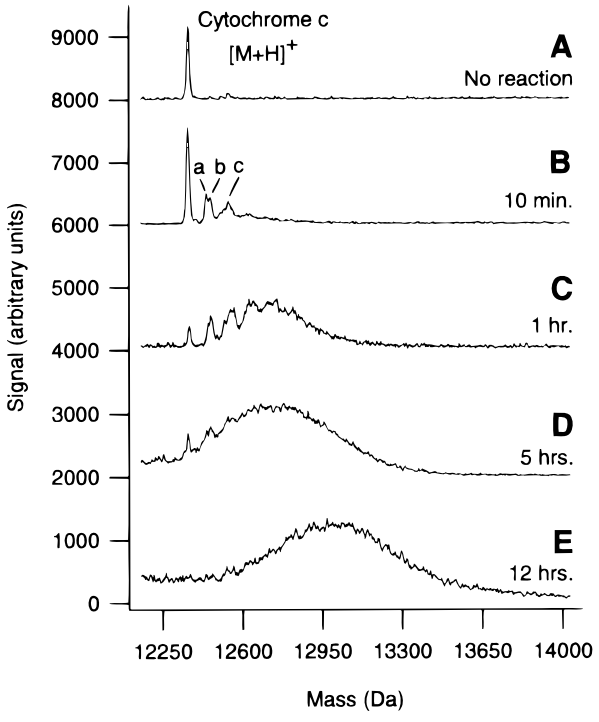

Figure 1. Positive-ion MALDI mass spectra of cytochrome *c* incubated with *trans*-hex-2-enal. A control sample (without hex-2-enal) is shown in (A). Peaks a, b and c correspond to Schiff base, Michael and Schiff base + Michael adducts, respectively. The control spectrum is followed by samples incubated for 10 min (B), 1 h (C), 5 h (D) and 12 h (E).

become progressively more complicated than we had originally anticipated, demonstrating an astounding heterogeneity of reaction products. With 5 h of incubation [Fig. 1(D)], The entire mass spectrum becomes an unresolved population of aldehyde adducts. This distribution indicates that there are between one and eight equivalents of hex-2-enal attached to the protein. After 12 h, an extensive amount of modification has occurred, resulting in the disappearance of unreacted cytochrome *c* [Fig. 1(E)]. The product distribution spans a mass range from one to twelve hexenal adducts. Further reaction times (up to 3 days) did not increase the median mass of this distribution significantly.

These data demonstrate that the chemistry between cytochrome *c* and hex-2-enal does not yield Michael adducts exclusively. When an identical sample of cytochrome *c* incubated with hex-2-enal for 2 days was analyzed via ESI-MS, the complexity of this reaction resulted in an uninterpretable spectrum. By removing the contribution from multiple charge states, the MALDI spectra clearly demonstrate the cause of this complexity in the ESI data. Owing to the occurrence of multiple reaction mechanisms, the mass distribution is simply too complicated to deconvolute from the charge distribution.

Bruenner *et al.*[7] have previously reported that, in their ESI experiments, they could not record intelligible spectra without extensive sample dialysis. Since Schiff base formation is reversible, we considered the possibility that some of the Schiff base linkages might dissociate after removal of free hex-2-enal from the incubation mixture by way of lyophilization. To test this, the sample whose spectrum appears in Fig. 1(E) was subjected to ultrafiltration using Nanospin 10 kDa molecular mass cut-off microconcentrators (Gelman Sciences, Ann Arbor, MI, USA) to remove any hex-2-enal that remained after lyophilization. This was done by adding 200 µl of modified cytochrome *c* solution to 300 µl of water in the microconcentrator and centrifuging for 10 min at 7000*g*. This process concentrated the sample volume to ~50 µl. The cytochrome *c* sample was then restored to its initial concentration by adding 150 µl of water. No decrease in the complexity of the MALDI spectra was observed after three repetitions of ultrafiltration, suggesting that the Schiff base adducts were not dissociating after the initial lyophilization step.

Thus, the cytochrome *c* data demonstrate that attachment of hex-2-enal can occur through both proposed mechanisms and that modification is extensive. By examining small peptides incubated with *trans*-hex-2-enal, the structure of this complexity becomes readily apparent. For example, modification of substance P (RPKPQQFFGLM-NH_2, 1347.6 Da) reveals a complicated pattern of aldehyde adducts as shown by the negative-ion MALDI spectrum in Fig. 2. The extent of heterogeneity observed was completely unexpected, but was found to be readily reproducible using different samples of substance P. Table 1 lists the adduct masses detected. The prominent fragment observed in the spectrum [Fig. 2(B), peak a] appears to be due to the loss of NH_2 from the deprotonated parent ion. Curiously, this fragment does not appear in the spectrum of unreacted substance P [Fig. 2(A)]. Peak b results from the oxidation of methionine to the sulfoxide and has been reported by other researchers.[12] Most of the hexenal adducts can be rationalized as the products of addition or condensation processes. Furthermore, recent data suggest that a double condensation of hexenal can occur upon a single primary amine, yielding pyridine derivatives. This reaction pathway produces 158 and 160 Da adducts, which are comparable to the attachment of two Schiff base adducts to different primary amines (+160 Da). Such a mechanism could, for example, explain the presence of peak g (+161 Da) observed in Fig. 2. The specifics of these structures and their elucidation will be the focus of a subsequent paper.[13] In all, up to four molar equivalents of hex-2-enal appear to be attached to substance P. This is noteworthy because there are only two obvious reaction sites (Lys and *N*-terminal amine). This sug-

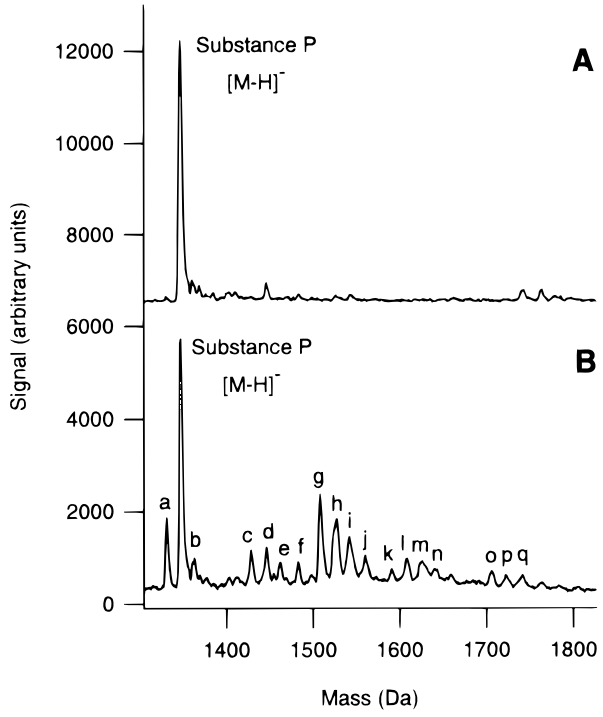

Figure 2. MALDI mass spectra of substance P. The spectrum in (B) demonstrates the effects of incubation with *trans*-hex-2-enal for 2 days (B). A control spectrum is provided for comparison (A). This data was acquired using negative-ion MALDI. Peak assignments are given in Table 1.

Table 1. Adducts of *trans*-hex-2-enal detected on substance P

Peak (Fig. 3)	Mass (Da)	Adduct mass (Da)	Possible adduct[a]	Theoretical mass shift[b]
a	1330.0	−16.3	NH_2	−16
b	1362.5	15.9	O	16
c	1427.6	81.0	SB	80
d	1445.2	98.6	MA	98
e	1460.6	114.0	Unidentified	—
f	1481.7	135.1	Unidentified	—
g	1507.5	160.9	2SB or PD	160 or 158
h	1525.3	178.7	SB + MA	178
i	1541.4	194.8	2MA	196
j	1558.8	212.2	Unidentified	—
k	1589.9	243.3	3SB or PD + SB	240 or 238
l	1607.3	260.7	2SB + MA or PD + MA	258 or 256
m	1626.3	279.7	SB + 2MA	276
n	1639.0	292.4	3MA	294
o	1705.0	358.4	2SB + 2MA or PD + 2MA	356 or 354
p	1721.5	374.9	SB + 3MA	374
q	1738.6	392.0	4MA	392

[a] Adduct types: SB, Schiff base; MA, Michael adduct; PD, pyridine derivative.
[b] Theoretical mass shifts in Da from *deprotonated* substance P ions (1346.6 Da).

gests that aldehyde reactions are more complex than commonly believed.

The substance P spectra confirm that the extensive heterogeneity observed in the cytochrome *c* data results from a complex adduct distribution created by multiple reaction pathways. Further evidence of this is reflected in the MALDI spectrum of bradykinin (RPPGFSPFR, 1060.2 DA) depicted in Fig. 3. Up to three molar equivalents of *trans*-hex-2-enal are attached to bradykinin here (see Table 2), even though we only expected reaction with the *N*-terminus of the peptide.

Also of interest is fragment peak a in Fig. 3(B), which seems to result from the loss of a neutral ammonia molecule, possibly arginine. MALDI analysis of hexenal-modified bradykinin fragment [2–7], which is free of Arg, does not reveal this fragment (data not shown). Although $[M - 17]^+$ ions are commonly reported for post-source decay spectra,[14] the appearance of this ion in our system seems unique. As observed with both substance P and bradykinin, the fragment only appears for hexenal-modified peptides. Thus, either the $[M - 17]^+$ ion is a byproduct of the reaction with hexenal, or the aldehyde modification makes this fragmentation more favorable during MALDI. If this ion is a result of fragmentation during the desorption/ionization process, then the fragmentation must be fairly rapid. Because the ions in our instruments are not further accelerated after extraction from the source, any fragmentation occurring after the ~ 2 μs extraction delay time will not be apparent in the mass spectra.

In conclusion, we have demonstrated that MALDI-MS is a viable method for exploring the extent of protein or peptide modification by small molecules such as *trans*-hex-2-enal. The technique is rapid, sensitive, requires little sample preparation, possesses higher resolution than gel techniques and generates simpler mass spectra than ESI. Measurement of the mass gained by a biological molecule gives a clear indication of the number of aldehyde molecules that are covalently attached. Unfortunately, elucidation of specific reactive sites in biomolecules is impossible without either a tandem experiment or the use of carefully designed peptides containing only a single nucleophile. The resolution provided by delayed extraction MALDI is sufficient to distinguish whether an aldehyde molecule has attached through a simple addition or by a condensation reaction. Moreover, the fact that both Michael and Schiff base adducts are detected by MALDI is encouraging. This indicates that introduction of a ferulic acid matrix during

Table 2. Adducts of *trans*-hex-2-enal detected on bradykinin

Peak (Fig. 4)	Mass (Da)	Adduct mass (Da)	Possible adduct[a]	Theoretical mass shift[b]
a	1044.5	−16.7	$-NH_3$	−17
b	1082.7	21.5	Na	22.0
c	1098.3	37.1	K	38.1
d	1140.8	79.6	SB	80
e	1157.9	96.7	MA	98
f	1193.9	132.7	Unidentified	—
g	1218.6	157.4	2SB or PD	160 or 158
h	1237.8	176.6	SB + MA	178
i	1318.2	257.0	2SB + MA or PD + MA	258 or 256
j	1337.0	275.8	SB + 2MA	276

[a] Adduct types: SB, Schiff base; MA, Michael adduct; PD, pyridine derivative.
[b] Theoretical mass shifts in Da from *protonated* bradykinin ions (1061.2 Da).

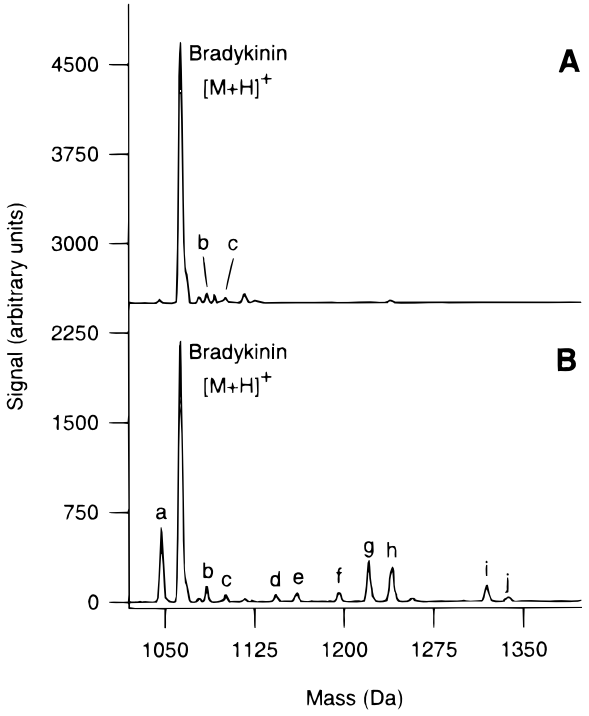

Figure 3. Positive-ion MALDI mass spectra of a bradykinin control sample (A) and a 2 day incubation of bradykinin with *trans*-hex-2-enal (B). Table 2 contains mass assignments for the peaks in this figure.

sample preparation does not lower the pH sufficiently to promote Schiff base hydrolysis. Nevertheless, we have noted that some matrices can reduce or remove peaks corresponding to Schiff base adducts, indicating that they lower the solution pH too far for a representative analysis of imine-linked reaction products. A more detailed study of this is under way.

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References

1. H. Esterbauer, R. J. Schaur and H. Zollner, *Free. Rad. Biol. Med.* **11**, 81 (1991).
2. H. Esterbauer, G. Jurgens, O. Quehenberger and E. Koller, *J. Lipid Res.* **28**, 495 (1987).
3. J. J. Hageman, A. Bast and N. P. E. Vermeulen, *Chem.-Biol. Interact.* **82**, 243 (1992).
4. M. V. Novotny, M. F. Yancey, R. Stuart, D. Wiesler and R. G. Peterson, *Biochim. Biophys. Acta* **1226**, 145 (1994).
5. L. Zidek, P. Dolezel, J. Chmelik, A. G. Baker and M. V. Novotny, *Chem. Res. Toxicol.* submitted for publication.
6. B. A. Bruenner, A. D. Jones and J. B. German, *Rapid Commun. Mass Spectrom.* **8**, 509 (1994).
7. B. A. Bruenner, A. D. Jones and J. B. German, *Chem. Res. Toxicol.* **8**, 552 (1995).
8. M. S. Bolgar and S. J. Gaskell, *Anal. Chem.* **68**, 2325 (1996).
9. M. Bolgar and S. Gaskell, *Biochem. Soc. Trans.* **23**, 907 (1995).
10. S. M. Colby and J. P. Reilly, *Anal. Chem.* **68**, 1419 (1996), and references cited therein.
11. N. P. Christian, S. M. Colby, L. Giver, C. T. Houston, R. J. Arnold, A. D. Ellington and J. P. Reilly, *Rapid Commun. Mass Spectrom.* **9**, 1061 (1995).
12. R. C. Beavis, T. Chaudhary and B. T. Chait, *Org. Mass Spectrom.* **27**, 156 (1992).
13. A. G. Baker, L. Zidek, P. Dolezel, J. Chmelik, D. Wiesler and M. V. Novotny, in preparation.
14. B. Spengler, D. Kirsch and R. Kaufmann, *Rapid Commun. Mass Spectrom.* **5**, 198 (1991).

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