The Application of a Hypothesis-driven Strategy to the Sensitive Detection and Location of Acetylated Lysine Residues

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The application of a hypothesis-driven method for the sensitive determination of lysine acetylation sites on enzymatically digested proteins is described. Comparative sensitivity tests were carried out using serial dilution of an acetylated bovine serum albumin (AcBSA) digest to assess the performance of a multiple reaction monitoring (MRM)–based approach as compared to a more conventional precursor scanning (PS) method. Both methods were capable of selectively detecting an acetylated peptide at the low femtomole level when spiked into a background of 500 fmol six-protein tryptic digest. The MRM approach was roughly tenfold more sensitive than precursor scanning with one acetylated peptide detected and sequenced at the level of 2 fmol on-column. The technique was subsequently applied to a gel-derived sample of cytokeratin-8 (CK8) shown to contain acetylated lysine residues by Western blot analysis. The strategy applied herein, termed MRM-initiated detection and sequencing (MIDAS), resulted in the facile identification of novel sites of acetylation on this protein. (J Am Soc Mass Spectrom 2007, 18, 1423–1428) © 2007 American Society for Mass Spectrometry

Acetylation is an important posttranslational modification (PTM) involved in the regulation of a number of key cellular processes including transcription [1], protein–protein interactions [2], and protein stability [3]. The importance of this biologically relevant modification has recently been compared to that of phosphorylation [4], which is a key regulator of biological processes, and thus there is a growing need for the sensitive detection and location of sites of acetylation on proteins. A number of mass spectrometry (MS)–based methods have been used for the determination of protein acetylation [5, 6]. One such approach relies on the detection of a characteristic reporter ion at 126.1 Th, corresponding to the immonium ion of acetyl lysine $-NH_3$, upon collisionally activated dissociation (CAD) [6]. Although this is indeed a sensitive approach, and has the added advantage of being compatible with additional protein/peptide modifications, a more promising strategy with regard to sensitivity would be to use a multiple reaction monitoring (MRM) transition as the trigger for an information-dependent acquisition (IDA) experiment and generate sequence information from a subsequent product ion scan. This technique, termed MIDAS—for MRM-initiated detection and sequencing—has been previously applied to phosphorylation studies and was shown to be on the order of ten times more sensitive than the commonly used −79 precursor ion scan [7]. In this work, for the first time, we demonstrate a similar improvement in sensitivity of a MIDAS-based acetylation method over an existing precursor scanning approach.

Experimental

Sensitivity Comparison

Acetylated bovine serum albumin (AcBSA, Invitrogen, Paisley, UK) was diluted to 15 pmol μL^{-1} in 100 μL of 25 mM ammonium bicarbonate and digested at 37 °C overnight by addition of modified porcine trypsin (Promega, Southampton, UK) at a 50:1 total protein:enzyme ratio. The digest was dried and resuspended in 150 μL of 2% (vol/vol) acetonitrile/0.1% (vol/vol) formic acid, providing a stock concentration of 10 pmol μL^{-1}. The stock sample was further diluted in the same solvent containing a six-protein mix tryptic digest, to generate standard solutions. A single preparation of this AcBSA

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1044-0305/07/$32.00
doi:10.1016/j.jasms.2007.04.021

Published online May 8, 2007
Received January 17, 2007
Revised April 19, 2007
Accepted April 27, 2007

digest was used in all experiments. The six-protein mix sample, consisting of trypsin-digested BSA, alcohol dehydrogenase, apo-transferrin, β-galactosidase, lysozyme, and cytochrome-C, was diluted from a 100 pmol lyophilized sample (LC Packings, Amsterdam, The Netherlands) using 2% (vol/vol) acetonitrile/0.1% (vol/vol) formic acid and constituted the background matrix (500 fmol on-column loading).

Preparation of CK8 Sample

HCT116 colon carcinoma cells were cultured in Dulbecco's modified Eagle medium (Invitrogen), 10% (vol/vol) fetal calf serum (Harlan Sera Labs, Loughborough, UK), and penicillin/streptomycin (Invitrogen). Cells were treated with sodium butyrate (Calbiochem, Nottingham, UK) as indicated.

Cytoskeletal isolations were performed mainly as described by Herrmann et al. [8]. Briefly, HCT116 cells were cultured with sodium butyrate (5 mM for 17 h). Low-detergent buffer (low buffer) and high-detergent buffer (high buffer) were prepared as detailed previously [8]. A pellet containing cytoskeleton was used for further analysis.

Samples were prepared for two-dimensional (2-D) gels using the Ettan 2-D Clean-up kit, followed by protein assay using the Ettan™ 2-D Quant kit (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions. Protein samples were resuspended in 125 μL of isoelectric focusing buffer [9 M urea, 2 M thiourea, 4% (wt/vol) CHAPS, 65 mM dithiothreitol, 0.5% (vol/vol) immobilized pH gradient (IPG) buffer (Amersham Biosciences, Little Chalfont, UK)] and loaded onto 7-cm immobilized pH gradient strips (pH 4–7). Isoelectric focusing was performed using the IPGphor™ (Amersham Biosciences) at 20 °C. The second dimension was a standard SDS–PAGE protocol using 10% mini format gels. Strips were equilibrated for 10 min in equilibration buffer [50 mM Tris-HCl, pH 6.8, 6 M urea, 30% (vol/vol) glycerol, 2% (vol/vol) SDS] containing 65 mM dithiothreitol and then for 10 min in the same buffer containing 240 mM iodoacetamide before running the second dimension. Gels were stained using Coomassie blue (Sigma, Poole, UK). In-gel tryptic digestions were performed as described previously [9].

Liquid Chromatography and Mass Spectrometry

For each experiment, 5 μL of digested sample was loaded onto a 15 cm × 75 μm inner diameter (ID) PepMap, C18, 3 μm column (LC Packings), using a standard LC Packings UltiMate pump and FAMOS autosampler. HPLC buffers A and B consisted of 2% (vol/vol) acetonitrile:0.1% (vol/vol) formic acid, and 80% (vol/vol) acetonitrile:0.1% (vol/vol) formic acid, respectively. Samples were desalted on-line before separation using a micro precolumn (5 mm × 300 μm ID) cartridge. The washing solvent was 0.1% formic acid delivered at a flow rate of 30 μL min^{-1} for 4 min. Peptides were separated over a gradient from 8% Buffer B to 40% Buffer B over 40 min at a flow rate of 300 nL min^{-1}. Chromatography was performed on-line to a 4000 QTRAP mass spectrometer (Applied Biosystems, Framingham, MA, USA).

The instrument settings on the mass spectrometer were kept constant for each scan mode. An ion spraying voltage of +2300 V was used with a source gas setting of 3. The interface temperature was held at 155 °C.

MRM-initiated Detection and Sequencing (MIDAS)

The MIDAS protocol, which takes full advantage of the combined functionality of the 4000 QTRAP, is described in detail elsewhere [7]. Briefly, a triple quadrupole MRM scan is used to sensitively select for postulated acetylated peptides. Under low-energy collision conditions, acetyl lysine residues have been shown to fragment, producing a diagnostic ion at *m/z* 126.1 corresponding to the immonium−NH_3 ion [6]. A table of potential acetylated peptides for the protein under study is first generated using a software script developed by Applied Biosystems. This represents the set of precursor masses to be sequentially transmitted by Q1, held at low resolution, while Q3 was held static at 126.1 *m/z* (unit resolution). Limited studies in our laboratory suggest that optimal collision energies to facilitate these transitions approximate to the precursor *m/z* × 0.1 irrespective of charge state. Collision energies between 50 and 80 eV (laboratory frame of reference) were assigned to each transition depending on the precursor *m/z* such that *m/z* of about 400–600, CE = 50 eV; about 600–700, CE = 60 eV; about 700–800, CE = 70 eV; >800, CE = 80 eV.

Each step in the MRM list has an associated dwell time. Should a signal exceed a user-defined threshold (200 cps) following this period, then once all MRMs have been completed the instrument is instructed to switch modes (enhanced product ion, with Q3 now functioning as a linear ion trap) to perform three MS/MS scans before once again repeating the full list of MRM transitions. The total cycle time for the sensitivity comparison, including MS/MS switching, was 7.7 s, which is equivalent to a 40-ms dwell time for each of the 72 MRM transitions.

Precursor Scanning at m/z 126.1

Precursor scanning is a selective and sensitive scanning technique used on triple quadrupole mass spectrometers [10, 11]. Based on previous reports the immonium−NH_3 ion of acetyl lysine (*m/z* 126.1) is a more sensitive and selective reporter ion for precursor scanning analyses [6] than the immonium ion itself (*m/z* 143.1) [12]. Q3 was therefore held static at 126.1 Th with resolution set to unit, whereas Q1 was scanned over the

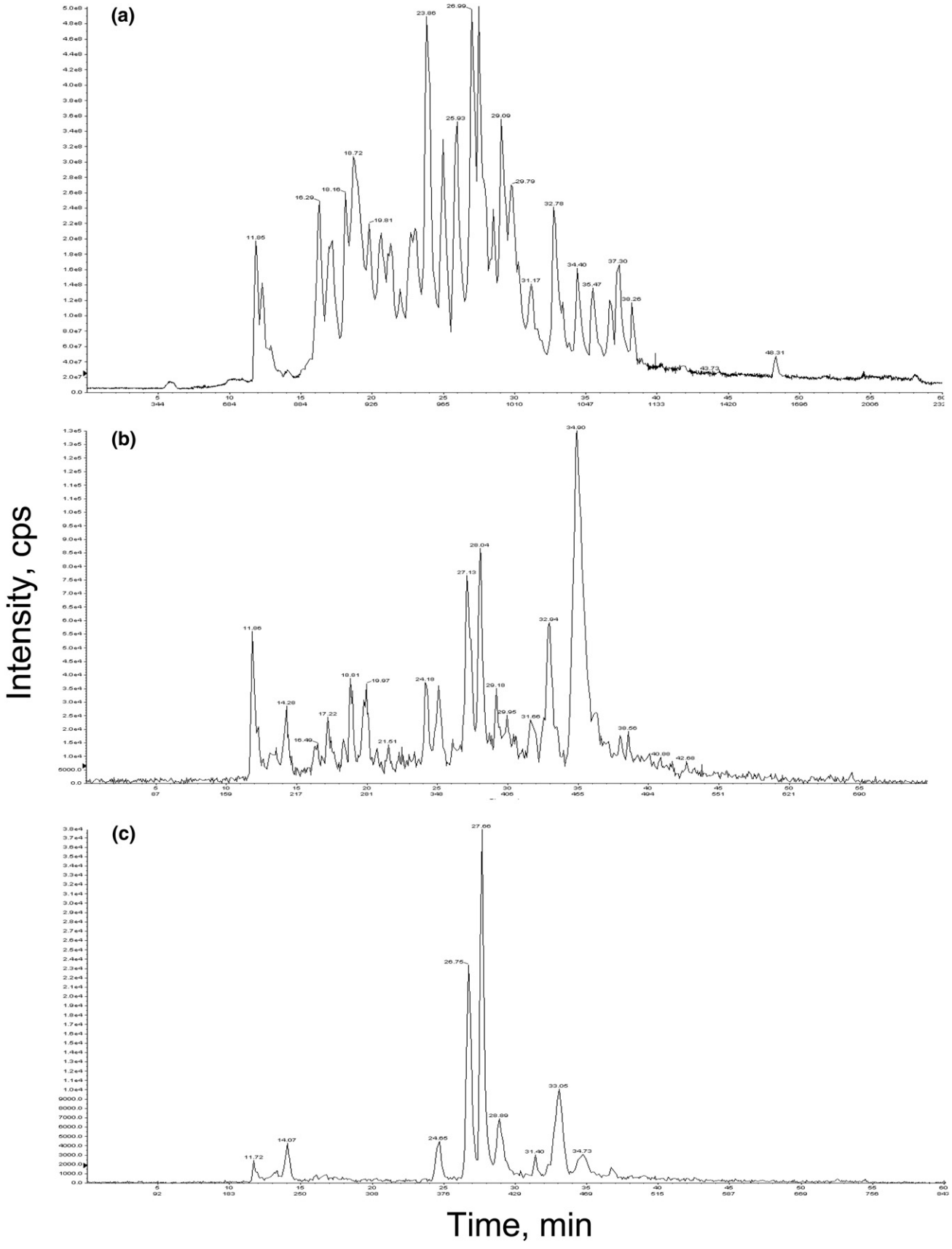

Figure 1. Total ion chromatogram (TIC) of 10 fmol acetylated BSA in 500 fmol six-protein mix using (**a**) information-dependent acquisition (TIC of EMS), (**b**) precursor ion scanning at m/z = 126.1 (TIC of precursors), and (**c**) MIDAS (TIC of MRM) analysis.

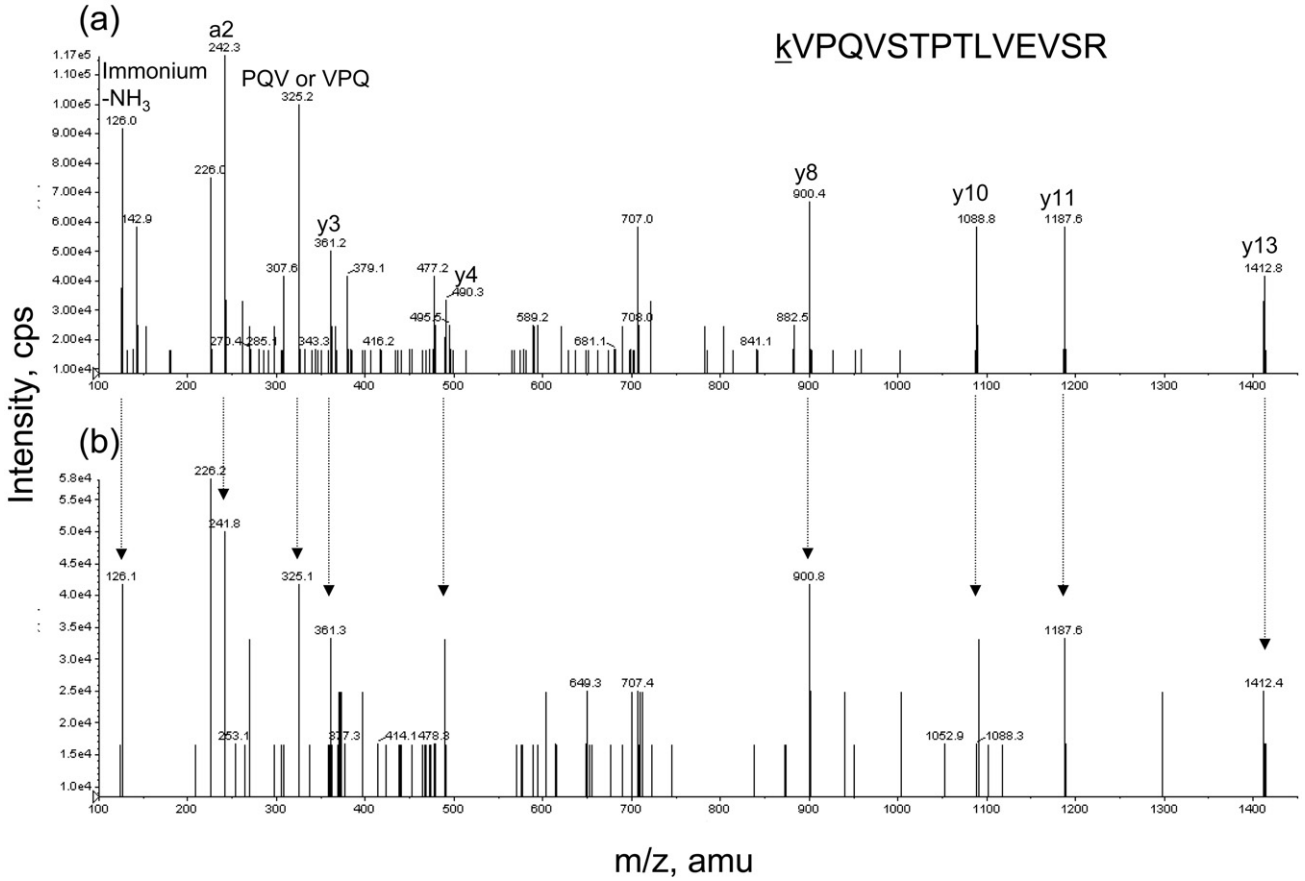

Figure 2. MS/MS spectra for the acetylated BSA peptide *k*VPQVSTPTLVEVSR upon MIDAS analysis of (**a**) 5 fmol and (**b**) 2 fmol acetylated BSA in 500 fmol six-protein digest on-column loading. *k* denotes an acetylated lysine residue.

mass range 450–1200 Th over a period of 3 s with a 1 amu step size (resolution set to low). The collision cell was ramped over 50–80 eV (laboratory frame of reference) during each scan. An IDA trigger threshold of 1000 cps was set, and subsequent MS/MS data were acquired from 100 to 1500 Th. The total cycle time including MS/MS switching was 8.2 s.

Results and Discussion

Sensitivity Evaluation

Acetylated BSA (AcBSA) was used to assess the sensitivity and suitability of the MIDAS method compared to precursor scanning for this application. A dilution series of trypsin-digested AcBSA in the range 50 to 1 fmol (on-column loading) was prepared, spiked into a 500 fmol six-protein digest and analyzed in triplicate.

First, an MS-driven IDA experiment was carried out with 10 fmol AcBSA in a 500 fmol six-protein digest injected on-column. The background proteins were easily identified, although the level of acetylation was below the threshold detectable for all peptides using this approach (data not shown). A more focused strategy was therefore required to identify lower levels of acetylation. Both MIDAS and the precursor scanning methods offer an improvement in sensitivity and selectivity. Figure 1 shows the total ion chromatogram (TIC) for MS-driven IDA (Figure 1a), precursor scanning at 126.1 Th (Figure 1b), and MIDAS (Figure 1c) of 10 fmol AcBSA in a 500 fmol six-protein digest. This figure demonstrates how the MS-driven IDA experiment spends the majority of the duty cycle switching to MS/MS mode on prominent nonacetylated peptides at the expense of the less abundant acetylated peptides. In contrast, both precursor ion scanning and MIDAS are tuned to select for only those peptides that are likely to be acetylated (i.e., release an ion of *m/z* 126.1 upon fragmentation) and therefore suffer from much less interference in the respective TIC. This figure also demonstrates how relatively uncluttered the MIDAS scan is (Figure 1c) compared to the precursor scan (Figure 1b).

To ensure a valid comparison between the two methods, it was necessary to keep both cycle times approximately the same. Thus, a cycle time, including any MS/MS scans, was set to about 8 s, with the initial triggering scan (MRM or precursor scan) having a cycle time of about 3 s. The consequence of this was a dwell

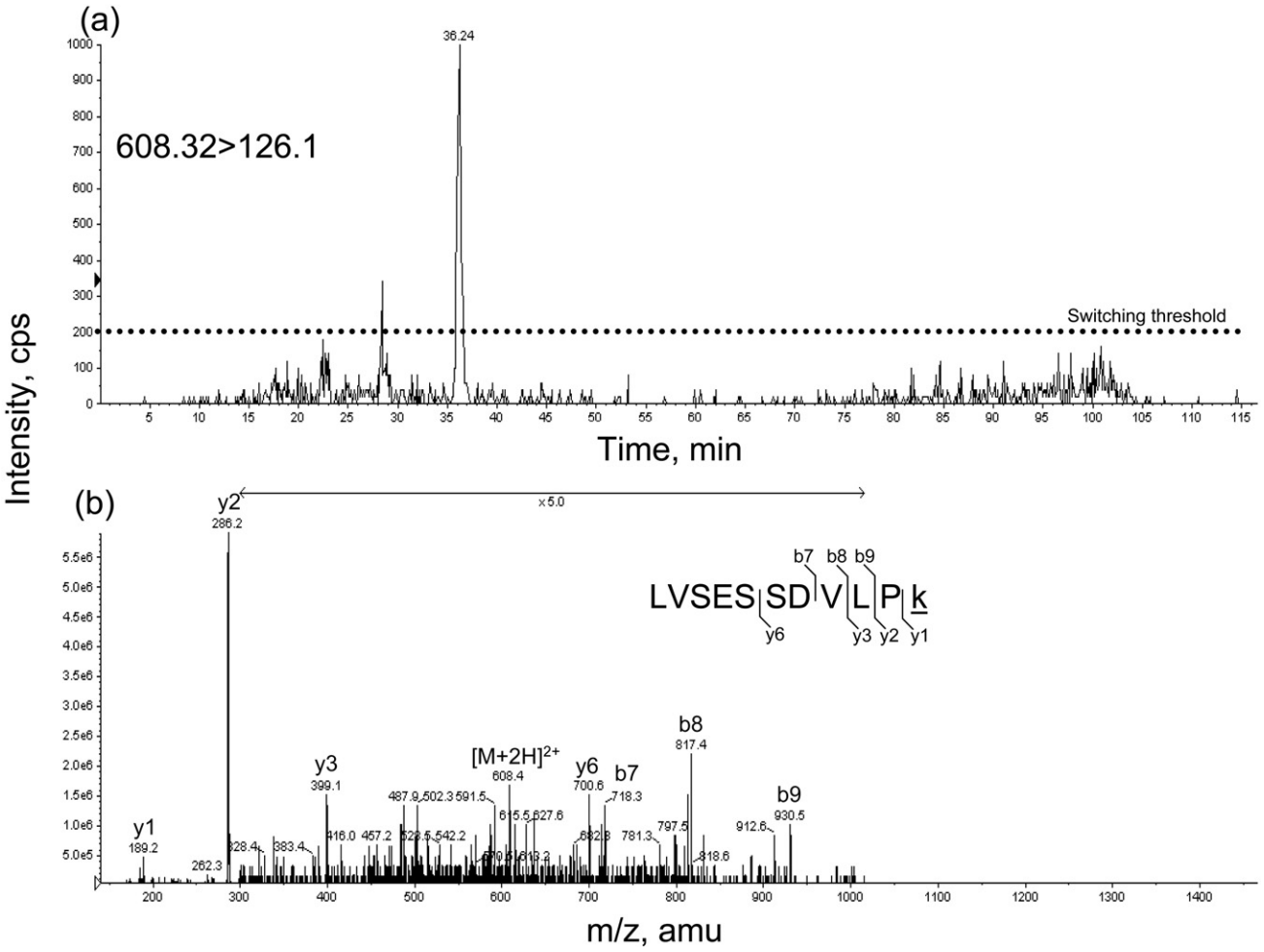

Figure 3. MIDAS experiment for the acetylation of CK8 showing (**a**) extracted ion chromatogram (XIC) for the MRM transition 608.32 → 126.1 with a switching threshold of 200 counts per second (cps), and (**b**) the annotated MS/MS spectrum generated for the acetylated peptide LVSESSDVLP*k*, where *k* denotes an acetylated lysine residue.

time of 40 ms for each MRM transition (72 transitions with 5-ms pause between each) and a corresponding "dwell time" for the PS of 4 ms (450–1200 amu in 3 s with 1 amu step size). From this alone, one would expect the MIDAS method to be on the order of tenfold greater sensitivity than the PS method for this protein. This was indeed found to be the case. Figure 2 shows the MS/MS spectrum for the acetylated peptide, *k*VPQVSTPTLVEVSR (where *k* denotes an acetylated lysine residue), detected at 5 fmol AcBSA in a 500 fmol six-protein digest (Figure 2a) and 2 fmol AcBSA (Figure 2b) using MIDAS. This peptide was clearly detected in all three replicates at the 5 fmol level but in only one of the 2 fmol samples, indicating that range for reliable level of detection. In contrast, 50 fmol of AcBSA was required for similar identification using the precursor scan approach (data not shown).

It is also worth noting that the MIDAS method detected an acetylated peptide (AL*k*AWSVAR) in the six-protein digest when no AcBSA was added. Using precursor scanning this peptide was detected with only 50 fmol AcBSA present.

Analysis of Cytokeratin 8

Having confirmed that MIDAS was superior to both precursor scanning and IDA analysis on our instrument for identification of acetylation sites on a simple peptide mixture, we next demonstrated the application of this technique to a biological system by probing lysine acetylation on the intermediate filament protein, cytokeratin 8. Intermediate filaments (IFs) are cytoskeletal structures that are crucial for maintaining the structural and mechanical integrity of cells and tissues [13]. Cytokeratin 8 was partially purified from cells by enrichment of the cytoskeletal fractions using differential detergent solubility [8]. Proteins were resolved using gel electrophoresis. Western immunoblot analysis with anti cytokeratin 8 and anti-acetyl lysine antibodies detected the presence of cytokeratin 8 and an acetylated protein of similar molecular weight in the cytoskeletal-enriched fractions (data not shown). The acetylation status of cytokeratin 8 was analyzed by mass spectrometry using the MIDAS approach based on detection of the

126.1 *m/z* diagnostic ion because this was determined to be the most sensitive approach.

A 5-μL aliquot of the cytokeratin 8 digest reconstituted in Buffer A was injected on column and analyzed using a MIDAS protocol. Three MRM transitions relating to previously determined nonacetylated peptides of cytokeratin 8 (LSELEAALQR, ASLEAAIADAEQR, and ELQSQISDTSVVLSNDNSR) were included in the list of 84 MRM triggers to confirm the protein identity and to check successful enzymatic digestion. These transitions had a shorter dwell time (20 ms) than the predicted acetylated peptides (50 ms) and the three peptides were identified by the analysis confirming the identity of CK8. In addition, five novel acetylation sites were identified (unpublished observations). Typical data for the acetylated peptide LVSESSDVLP*k* (AcK482) are presented in Figure 3. Figure 3a shows the extracted ion chromatogram for the transition 608.32 → 126.1. A prominent peak at a retention time of 36.24 min with a signal of about 1000 cps is shown. Because this signal breached the preset switching threshold of 200 cps, the instrument switched modes and acquired MS/MS data enabling unambiguous confirmation of the acetylation (Figure 3b).

Conclusion

The application of a hypothesis-driven analysis (MIDAS) for the determination of acetylated lysine residues in a mixture of tryptic peptides has been demonstrated for the first time. Initial experiments using acetylated BSA as a model compound show an approximately 10-fold improvement in sensitivity over the 126.1 *m/z* precursor scanning approach on a 4000 QTrap. When applied to the analysis of a set of Coomassie blue–stained 2-D gel spots, containing cytokeratin 8, five peptides incorporating acetylated lysine residues were detected and sequenced, leading to their unambiguous assignment. This application note demonstrates that the MIDAS approach may be applied to posttranslational modifications other than phosphorylation; indeed, any modification that generates a specific reporter ion, tag, or neutral loss may be amenable to investigation using this technique.

Acknowledgments

This work was supported by grants awarded by the Biotechnology and Biological Sciences Research Council and the Leukaemia Research Fund.

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