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Liquid chromatographic–high-resolution mass spectrometric and tandem mass spectrometric identification of synthetic peptides using electrospray ionization

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Abstract

Liquid chromatography–high-resolution electrospray mass spectrometry (LC–ESI-MS) was investigated for the identification of known and unknown synthetic peptides in a research effort designed to evaluate the applicability of this and complementary MS techniques for peptide characterization and identification. The monoisotopic molecular masses of five related peptides with molecular masses between 2000 and 2500 u were acquired with a resolution of 3000 (10% valley). Under narrow and wide mass range magnetic sector scanning conditions monoisotopic molecular mass errors were typically in the 10–20 and 30–40 ppm range, respectively. Tryptic maps were generated for each peptide following LC–ESI-MS analysis and collisionally activated dissociation (CAD) in the ESI interface resulted in the production of characteristic product ions that enabled amino acid sequencing of the tryptic fragments. Unknown identification was demonstrated during analysis of an incomplete synthetic peptide reaction mixture. The synthesis of an 18 amino acid peptide, LTTAVKKVLTTGLPALIS, was not successful. In its place were six unknown peptides that were identified on the basis of monoisotopic molecular mass and amino acid sequence data. The monoisotopic molecular masses of these unknowns were determined to within 10–20 ppm with a resolution of 3500 (10% valley). Amino acid sequences for the six peptides were generated during ESI–MS–MS analysis. Finally two synthetic peptides differing only by the incorporation of a ^{13}C at leucine were analysed with a resolution of 6000 (10% valley) to confirm that the isotopic distributions were consistent with theoretical expectations.

Keywords: Liquid chromatography–mass spectrometry; Electrospray ionization; Peptides

1. Introduction

Mass spectrometry (MS) combines exceptional sensitivity, specificity and speed for the analysis of synthetic peptides, providing access to molecular mass and amino acid sequence information. Barber et al. [1] demonstrated the use of fast atom bombardment (FAB) MS for the analysis of peptides. Electrospray ionization (ESI) MS [2,3] extended the mass range of mass spectrometry for biomolecule applications and has become a commonly used technique for peptide characterization [4–7].

ESI was initially interfaced to a quadrupole mass spectrometer [2,3], however many analysts conduct research with higher-resolution instruments, in large part because of the improved mass accuracy. Di- or tri-focusing magnetic sector mass spectrometers have been used extensively for the acquisition of high-

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resolution data. Use of high resolution enables the assignment of charge state to multiply charged isotope clusters and aids in the interpretation of amino acid sequence data during collisionally activated dissociation (CAD) in the region between the capillary exit and skimmer in the electrospray interface [8–17]. Particularly valuable is the ability to determine monoisotopic molecular mass, as these values are independent of $^{12}C/^{13}C$ variations.

Starrett and DiDonato [15], working at a resolution of 5000 under voltage scanning conditions over a narrow mass range reported 5 ppm differences between the theoretical and observed product ion for several bioactive peptides. Larsen and McEwen [10] employed resolutions of 5000 and 10 000, with internal calibration, for accurate molecular mass determination and found that errors seldom exceeded 25 ppm. High-resolution separation of a $(M+9H)^{9+}$ isotopic cluster was demonstrated by Cody et al. [13] for lysozyme at a resolution of 10 000 (10% valley) during ESI-MS analysis. Errors associated with these measurements were in the 5 to 20 ppm range with internal calibration and the 5 to 90 ppm range with external calibration. Errors of similar magnitude were found during ESI-MS of synthetic peptides with a tri-focusing magnetic sector instrument [16].

The application of di- or tri-focusing magnetic sector instruments for accurate mass measurement of peptide ions following chromatographic separation is limited [6]. Perkins and Tomer [18] reported mass measurement data following capillary electrophoretic separation with a resolution of 1000, and accurate mass data were recently demonstrated for bioactive peptides at 3000 resolution following packed capillary liquid chromatographic separation [17].

Liquid chromatography–high-resolution electrospray mass spectrometry (LC–ESI-MS) was investigated for the identification of known and unknown synthetic peptides in a research effort designed to extend the applicability of this and complementary MS techniques for peptide characterization and identification. High-resolution mass measurement enabled accurate determination of molecular and product ion masses of peptides and their tryptic fragments and was required to assess ^{13}C incorporation at leucine in a synthetic peptide.

2. Experimental

2.1. Samples

All the synthetic peptide samples were provided by Dr. R. Hodges' Group (Protein Engineering Network of Centres of Excellence, University of Alberta, Edmonton, Canada). The five similar synthetic peptides (numbered 11, 12, 15, 16 and 18 in Table 1) were shipped and received at Defense Research Establishment Suffield as frozen aqueous solutions (0.5 ml) in the 0.4 to 1.1 mg/ml range. The incomplete peptide reaction mixture, resulting from incomplete coupling of amino acids residues at various steps in the synthesis, was dissolved in distilled water at 1 mg/ml. Unlabelled and ^{13}C-labelled Ac-KVSALKERVSALKEKVSALKEK-VSALKEKVSALKE-NH_2 were received as lyophilized powders and were dissolved in distilled water at 0.1 mg/ml. All solutions were kept frozen at −18°C until just prior to LC–ESI-MS analysis.

Tryptic digests were performed by dissolving

Table 1
LC–ESI-MS analysis of synthetic peptides with a resolution of 3000 (10% valley) and a mass range of 1400 to 400 u

No.	Peptide sequence	Theoretical monoisotopic molecular mass[a]	Calculated monoisotopic molecular mass[a]	Error (ppm)
11	Ac-ELEKLLKELEKLLKEKEK-NH_2	2280.3668	2280.30±0.02	29
12	Ac-ELEKLLKEQEKLLKELEK-NH_2	2266.3511	2266.27±0.05	36
15	Ac-ELEKLLKECEKLLKELEK-NH_2	2255.2810	2255.21±0.03	31
16	Ac-ELEKLLKEYEKLLKELEK-NH_2	2315.3351	2315.26±0.02	32
18	Ac-ELEKLLKEWEKLLKELEK-NH_2	2238.3511	2338.28±0.02	32

[a] Monoisotopic molecular masses represent the mean±S.D. of four LC–ESI-MS analyses. During each analysis the mean monoisotopic molecular mass was calculated using $(M+nH)^{n+}$ ($n=2$, 3 and 4 for a sampling cone voltage of 75 V; $n=2$ and 3 for a sampling cone voltage of 150 V) isotopic clusters.

modified sequencing grade trypsin (Boehringer Mannheim, Mannheim, Germany) in 0.1 *M* ammonium bicarbonate and adding 50 μl of this trypsin solution (0.01 mg/ml) to 50 μl of the peptide (1 mg/ml), giving an enzyme–substrate ratio of 1:40. Digestions were carried out for about 18 h at 37°C and were quenched with 10 μl of 10% trifluoroacetic acid (TFA) (to pH 2–3) prior to LC–ESI-MS analysis.

2.2. Instrumental

All electrospray mass spectra were acquired using a Micromass Autospec-Q hybrid tandem mass spectrometer (Manchester, UK) equipped with the Mark II electrospray interface. The electrospray needle was operated at 7.6 kV and ions were accelerated into the mass spectrometer at 4 kV. Sampling cone voltages of 75 to 150 V were utilized. Nitrogen (Very Dry, Liquid Carbonic, Scarborough, Canada) bath gas was introduced into the interface (80°C) at a flow-rate of 400 l/h. Nitrogen nebulizer gas was introduced at a flow-rate of 14 l/h. The electrospray interface was pumped with both a rotary and a turbomolecular pump, which enabled maintenance of a $4 \cdot 10^{-4}$ and $7 \cdot 10^{-6}$ Pa pressures within the source and analyzer regions of the instrument, respectively.

LC–ESI-MS data for the five related synthetic peptides (numbered 11, 12, 15, 16 and 18 in Table 1) and their tryptic fragments were acquired in the continuum mode by scanning the magnetic sector from 1400 to 400 u (12 s/decade) or 670 to 230 u (15 s/decade). Monoisotopic molecular mass data for the unknown components in the incomplete synthetic peptide reaction mixture were acquired during LC–ESI-MS by magnetic sector scanning over 1900 to 400 u (10 s/decade) or 910 to 575 u (30 s/decade) in the continuum mode. ESI–MS data for Ac-KVSALKERVSALKEKVSA**L**KEKVSALKEKVSALKE-NH_2 (either ^{12}C or ^{13}C at **L**) were acquired from 825 to 700 u (125 s/decade). In all cases, five to ten scans were typically averaged to enhance the signal-to-noise ratio. Resolutions of 2600 to 6000 (10% valley) were employed during magnetic sector scanning to facilitate accurate mass measurement of the ions. External calibrations were performed with aqueous solutions of polyethylene glycol (PEG) 200, 600 and 1540.

ESI-MS–MS data for the incomplete synthetic peptide reaction mixture were acquired following loop injection with a quadrupole CAD cell energy of 75 or 100 V and an argon pressure of approximately $1.3 \cdot 10^{-2}$ Pa near the CAD cell. The preferred conditions attenuated the precursor ions by about 60% to 70% (CAD cell energy of 100 V) and represented a good compromise between sensitivity and spectral content for these analyses. Product spectra were obtained at unit resolution by scanning the quadrupole from 800 to 80 u (10 s/scan). The instrument resolution was set at 1000 (10% valley) for these analyses.

All peptide separations were performed with an Applied Biosystems Model 140B dual syringe pump (Foster City, CA, USA) equipped with a 15 cm× 0.53 mm I.D. C_{18} (5 μm) packed J&W DB-1 fused-silica capillary column (Courtesy of Mr. L. Hogge and Mr. D. Olson, NRC, Saskatoon, Canada) and a Rheodyne 8125 (Cotati, CA, USA) injector with a 5-μl sample loop. The following solvent compositions were prepared for sample introduction: solvent A (0.05% TFA in water) and solvent B (0.05% TFA in acetonitrile–water, 80:20). Chromatographic separations were performed using a 5% to 75% B gradient over 35 min. In order to minimize dead volume effects and ensure reproducible mixing, the mobile phase was delivered at 200 μl/min and split prior to the injector such that the flow through the column was 20 μl/min. Loop injections (20 μl) were made with a second Rheodyne 8125 located after the column using 50% B at 20 μl/min.

3. Results and discussion

3.1. LC–ESI-MS analysis of five related peptides

Five related peptides (numbered 11, 12, 15, 16 and 18 in Table 1) were selected from a set of synthetic peptides used in research studies involving synthetic vaccines, antibacterial peptides or the de novo design of helical peptides and proteins [16], and the intact peptides and their tryptic fragments were analysed by LC–ESI-MS. Monoisotopic molecular masses were measured for the peptides and amino acid sequence data were determined following LC–ESI-MS analysis of the tryptic fragments under high-resolution

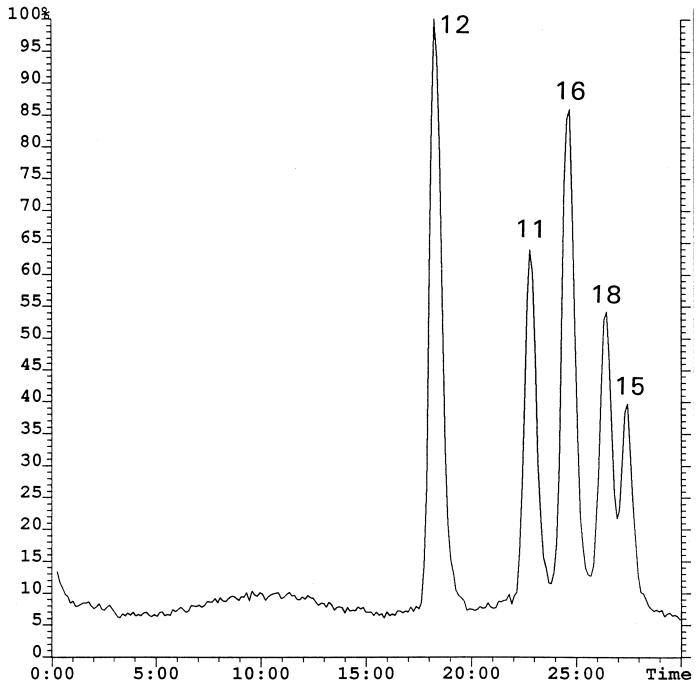

Fig. 1. LC–ESI-MS total ion-current (1400 to 400 u) chromatogram for a mixture of synthetic peptides 11, 12, 15, 16 and 18 with a resolution of 3000 (10% valley) and a sampling cone voltage of 75 V (time in min).

CAD-MS conditions. Fig. 1 illustrates the LC–ESI-MS total ion-current chromatogram obtained for a mixture of the synthetic peptides with a resolution of 3000. All five peptides were resolved in under 30 min, with a gradient of 2% B/min. Isotopic clusters of the general form, $(M+nH)^{n+}$, were observed using a sampling cone voltage of 75 V (where $n=2$, 3 and 4), while at a higher sampling cone voltage, 150 V, charge stripping associated with the CAD process eliminated the presence of the $n=4$ isotopic clusters. The mass spectra did not contain product ions indicative of the amino acid sequence, with the only product ions present being due to loss of water from the $(M+3H)^{3+}$ ion.

The monoisotopic molecular masses of each of the peptides were calculated from the ^{12}C only ion in the isotopic clusters and the calculated values were compared to theoretical values. Table 1 lists both the theoretical and calculated monoisotopic molecular masses for the five peptides. Errors associated with the monoisotopic molecular masses when scanning from 1400 to 400 u during LC–ESI-MS were in the 30–40 ppm range, comparable to the 5 to 60 ppm range found during loop ESI-MS [16].

Tryptic digestions, where the peptide chain cleaves at the C-terminal of lysine or arginine amino acid residues, have been used frequently to produce lower mass peptide fragments that aid in identification. Low-resolution LC–MS and LC–MS–MS data for peptides and their tryptic fragments have been reported frequently using FAB [19], ion-spray [20] and ESI [21] interfaces. The molecular masses of the observed tryptic fragments forms a tryptic map that further characterizes the intact peptide or protein [19–24]. The peptide mass and tryptic map may be used for identification of peptides in databases, with the number of database matches being reduced with increased mass accuracy [25].

The peptides were each enzymatically digested using trypsin and the digests analysed by LC–ESI-MS with a resolution of 3000. Tryptic maps based on cleavage on the C-side of lysine were generated for the five peptides and all the expected tryptic fragments were detected with the exception of EQEK for

peptide 12. Fig. 2 illustrates typical tryptic map data obtained for synthetic peptide 18 during LC–ESI-MS analysis. Chromatographic peak widths at the base were typically 90 s. Subsequent experience has indicated that increasing the TFA concentration to 0.1% results in a 30 to 45 s peak width improvement with no improvement in mass accuracy. Tryptic map data, listed in Table 2, were acquired over a narrower mass range (670 to 230 u) and errors between observed and theoretical monoisotopic molecular mass were typically in the 10–20 ppm range. Several minor additional tryptic fragments that had not undergone complete cleavage at the C-side of every lysine were also observed, as well as evidence of deamidation at the C-terminal.

The similarity in peptide structures resulted in the formation of a number of common tryptic fragments. These tryptic fragments were not always resolved chromatographically for each peptide, but it was possible to select representative CAD-MS spectra from the data. A higher voltage sampling cone voltage, 150 V, resulted in the acquisition of CAD-MS data that contained product ions indicative of the amino acid sequence of these tryptic fragments (Table 3). The tryptic fragment identified as ELEK contained product ions in the yn-series, corresponding to cleavage of E (y_3) and L (y_2) amino acid residues from the $(M+H)^+$ ion at m/z 389.2413 and m/z 276.1521, respectively. Leucine was not differentiated from isoleucine during CAD-MS analysis and in all cases the isomer that would be present in the tryptic fragment has been assumed in the structures presented. The mass of the remaining amino acid residues would be 257.1337 u (based loss of H_3O from the y_2 ion at m/z 276.1521) or 257.1426 u [based on loss of H_2O and the mass of the b_2 ion at m/z 243.1272 from the $(M+H)^+$ ion]. Possible two amino acid residue combination with a

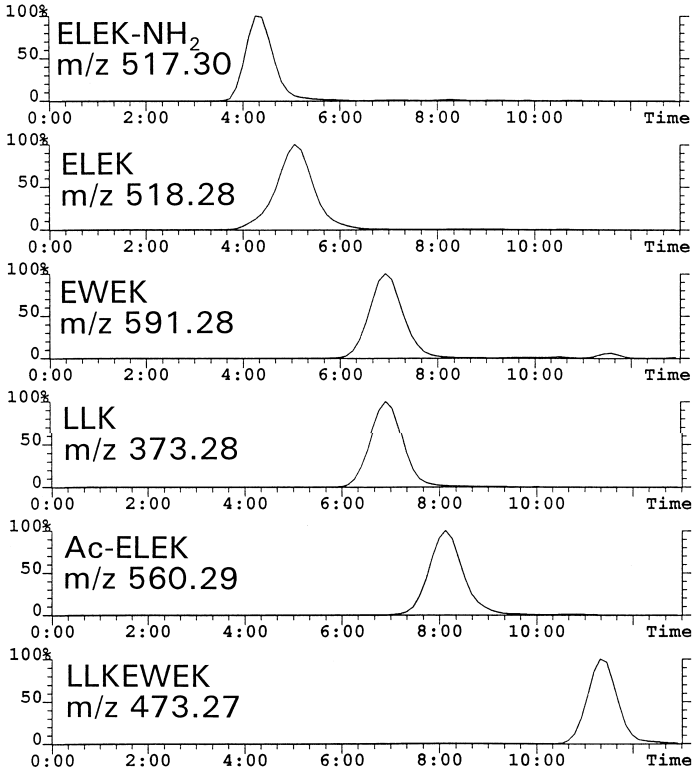

Fig. 2. Reconstructed ion-current chromatograms for the $(M+H)^+$ ions of tryptic fragments of synthetic peptide 18 obtained during LC–ESI-MS analysis with a resolution of 3000 (10% valley) and a sampling cone voltage of 75 V (time in min).

Table 2
Tryptic fragments observed during LC–ESI-MS analysis of tryptic digests of peptides 11, 12, 15, 16 and 18

Tryptic fragment (theoretical monoisotopic molecular mass)	Observed monoisotopic molecular mass [Resolution: 3000 (10% valley), mass range 670 to 230 u] (ppm error)				
	Peptide 11	Peptide 12	Peptide 15	Peptide 16	Peptide 18
Ac-ELEK (559.2853)	559.2882 (5)	559.2883 (5)	559.2806 (8)	559.2903 (9)	559.2903 (9)
LLK (372.2736)	372.2735 (0.3)	372.2755 (5)	372.2691 (12)	372.2693 (12)	372.2710 (7)
ELEK (517.2748)	517.2701 (9)	517.2747 (0.2)	517.2657 (18)	517.2686 (12)	517.2762 (3)
EK (275.1481)	275.1508 (10)				
EK-NH_2 (274.1641)	274.1724 (30)				
EQEK (532.2493)		Not detected			
ELEK-NH_2 (516.2908)		516.2869 (8)	516.2780 (25)	516.2896 (2)	516.2886 (4)
ECEK (507.1999)			507.1886 (22)		
EYEK (567.2540)				567.2470 (12)	
EWEK (590.2700)					590.2752 (9)

nominal mass of 257 u are E and Q (257.1012 u), W and A (257.1164 u), E and K (257.1376 u) and R and T (257.1488 u). The last two combinations were closest to measured values. An intense loss of H_2O from the y_2 ion was observed and would be favoured for a tryptic fragment with an N-terminal E (giving rise to pEK where E cyclizes to pE, pyroglutamic acid). This evidence would suggest an amino acid sequence of ELEK.

The tryptic fragment, identified as LLK, exhibited a single y_2 product ion at m/z 260.1940, which indicated the presence of an N-terminal leucine (Table 3). The observed amino acid residue mass for the remainder of the peptide was 241.1756 u based on the y_2 ion (based loss of H_3O from the y_2 ion at m/z 260.1940). Two possible amino acid residue combinations, Q and L (241.1426 u) and K and L (241.1790 u), have a nominal mass of 241 u. In this case the amino acid sequence could was assigned as LLK.

Table 3
Sequence information for common tryptic fragments following LC–ESI-MS analysis with a resolution of 3000 (10% valley) and a sampling cone voltage of 150 V

Tryptic fragment	Ion identity	Theoretical mass (u)	Observed mass (u)	Error (u)
(a) ELEK	$(M+H)^+$	518.2826	518.2804	0.0022
	y_3	389.2400	389.2413	0.0013
	y_2	276.1559	276.1521	0.0038
	y_2-H_2O	258.1454	258.1462	0.0008
	b_2	243.1345	243.1272	0.0073
(b) LLK	$(M+H)^+$	373.2815	373.2792	0.0023
	y_2	260.1974	260.1940	0.0034
(c) Ac-ELEK	$(M+H)^+$	560.2932	560.2884	0.0048
	y_3	389.2400	389.2422	0.0022
	y_2	276.1559	276.1500	0.0059
	y_2-H_2O	258.1454	258.1391	0.0063
	b_2	285.1451	285.1379	0.0072
(d) LLKEWEK	$(M+2H)^{2+}$	473.2744	473.2802	0.0058
	y_6^{2+}	416.7323	416.7337	0.0014
	y_4	591.2778	591.2747	0.0031
	y_3	462.2353	462.2397	0.0044

The tryptic fragment from the N-terminal, identified as Ac-ELEK, exhibited y_n-series ions at m/z 389.2422 (y_3) and m/z 276.1500 (y_2) due to cleavage of Ac-E and L, respectively (Table 3). The mass of the remaining amino acid residues would be 257.1316 u (based on loss of H_3O from the y_2 ion at m/z 276.1500) or 257.1399 u [based on loss of H_2O and the mass of the b_2 ion at m/z 285.1379 from the $(M+H)^+$ ion]. Possible two amino acid residue combinations with a nominal mass of 257 u are E and Q (257.1012 u), W and A (257.1164 u), E and K (257.1376 u) and R and T (257.1488 u). The last two combinations were closest to the observed value. However once again an intense loss of H_2O from the y_2 ion would be favoured for a tryptic fragment with an N-terminal E (giving rise to pEK). This evidence would suggest an amino acid sequence of Ac-ELEK.

An unexpected tryptic fragment for peptide 18 was believed to have an amino acid sequence of LLKEWEK or EWEKLLK, the result of incomplete cleavage of a peptide bond on the C-side of lysine. A y_6^{2+} ion at 416.7337 indicated loss of L from the N-terminal and suggested LLKEWEK as opposed to EWEKLLK. This was further supported by the y_4 and y_3 ions at m/z 591.2747 and 462.2397, respectively.

3.2. Analysis of an incomplete synthetic peptide reaction mixture

Not every synthesis will be successful and analytical methods will be required to establish the identity of the principal sample components. The identity of the reaction products could give the researcher an indication of difficult coupling problems due to steric hindrance, incomplete deprotection or synthesizer failure. An incomplete synthetic peptide reaction mixture for LTTAVKKVLTTGLPALIS was obtained to evaluate the amount of information that could be extracted during ESI-MS analysis. The amino acid sequence of the desired product was known but the identity of the peptides in the reaction mixture was unknown. The identification of these peptides was based solely on the acquired ESI-MS and MS–MS data.

Preliminary LC–ESI-MS of the sample indicated that the synthesis failed to produce the desired product, but a number of other unknown peptides, presumably related to the desired peptide product were observed. Fig. 3 illustrates the LC–ESI-MS total ion-current chromatogram obtained under a fairly rapid gradient of 2% B/min. Most of the total ion-current was due to six peptides, with three of the components coeluting under the LC conditions employed. They were however resolved by mass under lower sampling cone voltage conditions (75 V) that did not favour product ion production (inset in Fig. 3). Monoisotopic molecular masses were calculated for each of the six unknown peptides and compared to theoretical data obtained following ESI-MS–MS amino acid sequencing of the six peptides (Table 4). Monoisotopic molecular mass errors under wide and narrow mass range magnetic scanning conditions were in the 35 to 60 ppm and 5 to 20 ppm range, respectively. It was not possible to distinguish between leucine and isoleucine and assignment of these amino acid residues was assumed to be in the same order as the desired amino acid sequence, LTTAVKKVLTTGLPALIS.

In general CAD-MS amino acid sequence information was preferred over MS–MS data in this study as CAD-MS data may be acquired under high-resolution conditions. The major advantage of MS–MS lies in the its ability to differentiate sample components on the basis of mass, thereby reducing the need for chromatographic separation. In the case of the incomplete synthetic peptide reaction mixture, chromatographic resolution of all the sample components in a reasonable timeframe would be unlikely. As a result CAD-MS data would contain product and precursor ions for all coeluting sample components, thus complicating interpretation. ESI-MS–MS was therefore used for the determination of amino acid sequence since this technique allows mass selection of the precursor ion prior to CAD in the quadrupole cell.

The observed peptides yielded y_n-, b_n- and a_n-series product ions as well as a number of internal fragment ions. The formation of internal fragments requires the cleavage of two peptide bonds and charge retention by the resulting fragment ion [26]. In order to describe the internal fragment ion, the nomenclature suggested by Roepstorff and Fohlman [27] was used with slight modification. The presence of internal fragments can be useful in reconstructing internal amino acid sequence for unknown peptides.

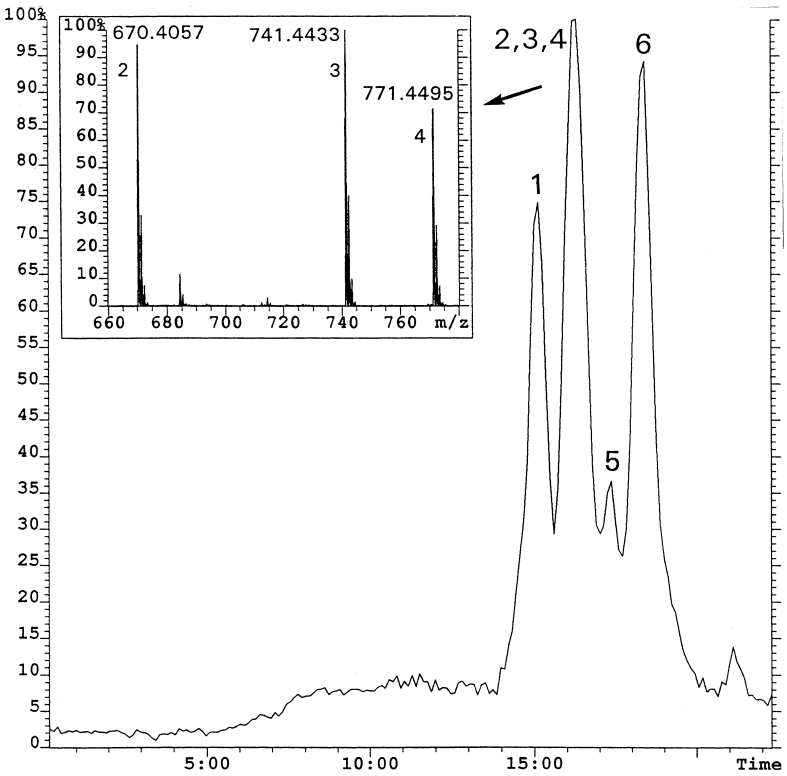

Fig. 3. LC–ESI-MS total ion-current (910 to 575 u) chromatogram of the peptides in an incomplete synthetic reaction mixture obtained with a resolution of 3500 (10% valley) and a sampling cone voltage of 75 V. [Inset: $(M+H)^+$ isotopic clusters for peptides 2, 3 and 4] (time in min).

[The general form for internal fragment ions is $(b_r y_s)_{(r+s-t)}$ where r indicates the bond cleaved counting from the N-terminal, s indicates the bond cleaved counting from the C-terminal and *t* indicated the total number of amino acid residues in the peptide. The subscript $(r+s-t)$ therefore indicates the total number of amino acid residues in the internal fragment. For example, a peptide with the

Table 4
LC–ESI-MS determination of monoisotopic molecular masses for peptides in an incomplete synthetic reaction mixture

Sample component (peak No.)	Theoretical monoisotopic molecular mass	Observed monoisotopic molecular mass	
		[Resolution: 3500 (10% valley), mass range: 910 to 575 u] (ppm error)	[Resolution: 2600 (10% valley), mass range: 1900 to 400 u] (ppm error)
LPALIS (1)	613.3925	613.3883 (7)	613.3703 (36)
GLPALIS (2)	670.4139	670.4057 (12)	670.3854 (43)
AGLPALIS (3)	741.4510	741.4433 (10)	741.4231 (38)
TGLPALIS (4)	771.4616	771.4495 (16)	771.4194 (55)
VGLPALIS (5)	769.4823	769.4708 (15)	769.4360 (60)
LGLPALIS (6)	783.4980	783.4841 (18)	783.4575 (52)
	Average ppm error	13±4 (n=6)	47±10 (n=6)

sequence LPALIS with an internal fragment PAL, has $r=4$, $s=5$, $t=6$ would be described as an $(b_4y_5)_3$ ion].

The product spectrum of m/z 613.4, the $(M+H)^+$ ion of the first unknown peptide, contained an intense series of b_5 to b_2 ions, the corresponding a_n-series ions and y_5, y_3 and y_2 ions (Table 5). The presence of these series was sufficient to fully sequence this peptide as LPALIS. The product spectrum of m/z 670.4, the $(M+H)^+$ ion for the second peptide, was dominated by b_6 to b_2 series ions. Several a_n- and y_n-series ions were also detected but neither a b_1 nor a y_6 ion, necessary to establish the N-terminal was observed (Table 5). Two possibilities exist that would account for a two amino acid residue mass of 170 u. G and I/L or V and A would both be possible with GL being more probable given the fact that the desired peptide contained GLPALIS on the C-terminal. This assumption was used for the remaining peptides where multiple possibilities for the two amino acid residues nearest the N-terminal were possible.

The product spectra of the $(M+H)^+$ ions for the peptides AGLPALIS and TGLPALIS, illustrated in Fig. 4, were typical of those obtained for all six peptides. Again the spectra were dominated by b_n-series ions which allowed most of the amino acid sequence of these peptides to be determined. VGLPALIS and LGLPALIS also exhibited bn-series ions which provided most of the amino acid sequence necessary for identification.

Table 5 lists the product ions and internal fragment ions observed under ESI-MS–MS conditions for all six peptides identified in the incomplete

Table 5
ESI-MS–MS product ions for the peptides in an incomplete synthetic reaction mixture

Possible ion identity	Peptide amino acid sequence m/z (% relative intensity)					
	LPALIS	GLPALIS	AGLPALIS	TGLPALIS	VGLPALIS	LGLPALIS
Series ions						
b_7			636 (8)	666 (11)	664 (18)	678 (4)
b_6		565 (18)	523 (13)	553 (18)	551 (13)	565 (30)
b_5	508 (5)	452 (44)	410 (12)	440 (16)	438 (11)	452 (18)
b_4	395 (21)	339 (51)	339 (6)	369 (5)	367 (5)	381 (2)
b_3	282 (75)	268 (14)	242 (8)	272 (10)	270 (6)	284 (5)
b_2	211 (32)	171 (13)	129 (2)	159 (3)		171 (3)
a_7					636 (1)	650 (1)
a_6		537 (5)	495 (5)	525 (3)	523 (4)	537 (3)
a_5	480 (3)	424 (12)	382 (5)	412 (12)	410 (11)	424 (5)
a_4	367 (13)	311 (8)	311 (1)		339 (4)	353 (3)
a_3	254 (3)			244 (2)		
a_2	183 (13)	143 (11)				
y_6			613 (1)			613 (2)
y_5	500 (4)	500 (45)	500 (30)	500 (27)	500 (34)	500 (22)
y_3	332 (3)	332 (11)	332 (1)			
y_2	219 (6)	219 (9)	219 (1)			
Internal fragments						
PALI or LPAL	395 (21)	395 (11)	395 (8)	395 (13)	395 (4)	395 (14)
	$(b_5y_5)_4$	$(b_6y_5)_4/(b_5y_6)_4$	$(b_7y_5)_4/(b_6y_6)_4$	$(b_7y_5)_4/(b_6y_6)_4$	$(b_7y_5)_4/(b_6y_6)_4$	$(b_7y_5)_4/(b_6y_6)_4$
PAL or LPA	282 (75)	282 (63)	282 (36)	282 (50)	282 (35)	282 (47)
	$(b_4y_5)_3$	$(b_5y_5)_3/(b_4y_6)_3$	$(b_6y_5)_3/(b_5y_6)_3$	$(b_6y_5)_3/(b_5y_6)_3$	$(b_6y_5)_3/(b_5y_6)_3$	$(b_6y_5)_3/(b_5y_6)_3$
LI	227 (7)	227 (11)	227 (4)	227 (18)	227 (5)	227 (7)
	$(b_5y_3)_2$	$(b_6y_3)_2$	$(b_7y_3)_2$	$(b_7y_3)_2$	$(b_7y_3)_2$	$(b_7y_3)_2$
PA	169 (34)	169 (55)	169 (25)	169 (23)	169 (14)	169 (20)
	$(b_3y_5)_2$	$(b_4y_5)_2$	$(b_5y_5)_2$	$(b_5y_5)_2$	$(b_5y_5)_2$	$(b_5y_5)_2$

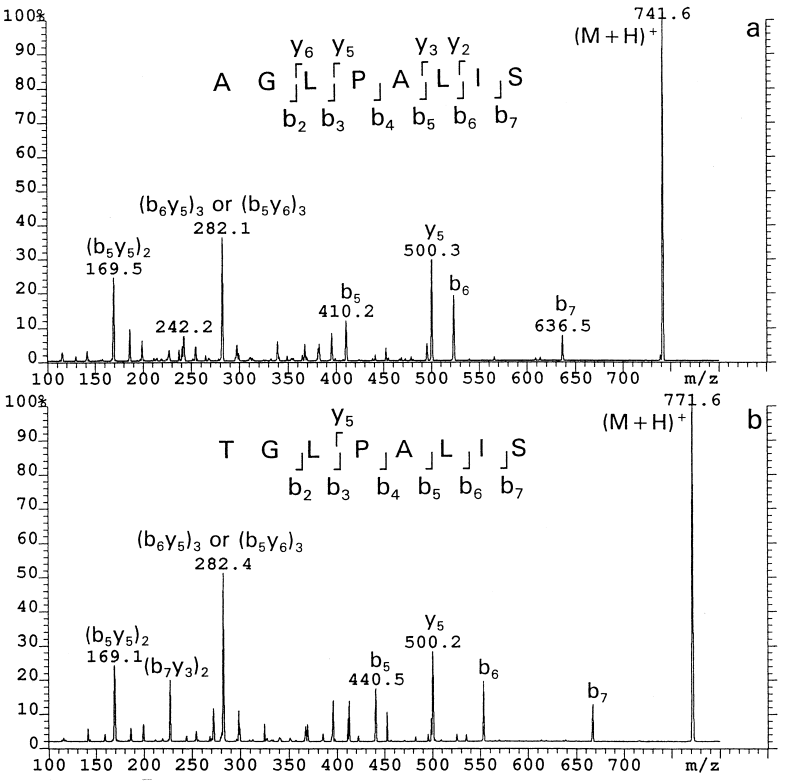

Fig. 4. Product ion mass spectra for peptides (a) AGLPALIS and (b) TGLPALIS obtained during loop injection of the incomplete synthetic reaction mixture. (Quadrupole CAD cell: 100 V, $1.3 \cdot 10^{-2}$ Pa argon) (*y*-axis: % relative intensity).

synthetic reaction mixture. In general the product ion spectra contained sufficient b-series ions that most of the primary sequence for the peptides could be assigned. In addition to b_n-series ions the corresponding a_n-series ions (at a mass 28 u less than the corresponding b_n-series ion) were also observed for most of the product ions. Few y_n-series ions were observed, but a significant y_5 ion at m/z 500 was present for all six peptides. Internal fragments were observed with the most intense being related to the fragments PA, LI, PAL and PALI.

3.3. Resolution of $(M+5H)^{5+}$ isotopic clusters

Two synthetic peptides differing in mass by the incorporation of a single ^{13}C at the indicated leucine residue in Ac-KVSALKERVSALKEKVSA**L**KEK-VSALKEKVSALKE-NH_2 were analysed by ESI-MS with a resolution of 6000 to determine if the isotopic distributions were consistent with products that contained ^{12}C and ^{13}C at one of the leucine carbons. The $(M+5H)^{5+}$ isotopic clusters were fully resolved at a resolution of 6000 and the charge state could be immediately assigned by the 0.2 u spacings as +5. Fig. 5 illustrates the theoretical isotopic distribution expected for ^{12}C, the observed ^{12}C isotopic distribution and the observed isotopic distribution for the successful incorporation of ^{13}C. The isotopic cluster for the 12C synthetic peptide was consistent with theory and the ^{13}C product distribution approximated the ^{12}C theoretical cluster, with a corresponding upward shift in mass due to the presence of ^{13}C.

Monoisotopic molecular masses for both synthetic peptides were calculated from the narrow mass range scanning data by averaging about eight scans. The calculated monoisotopic molecular mass for Ac-KVSALKERVSALKEKVSA**L**KEKVSALKEKVS-

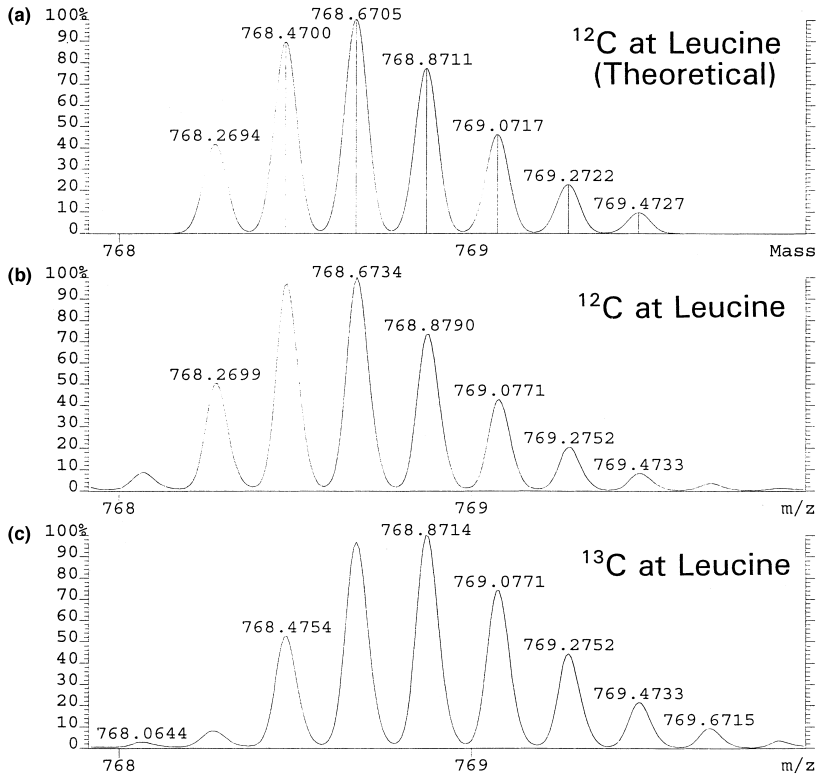

Fig. 5. (a) Theoretical isotopic distribution expected for $(M+5H)^+$ cluster of Ac-KVSALKERVSALKEKVSA**L**KEKVSALKEKVSALKE-NH_2 (^{12}C at **L**). Isotopic distribution obtained at a resolution of 6000 (10% valley) for the $(M+5H)^+$ cluster of (b) Ac-KVSALKERVSAL-KEKVSA**L**KEKVSALKEKVSALKE-NH_2 (^{12}C at **L**), calculated monoisotopic molecular mass 3836.3104 (Error, 1 ppm) and (c) Ac-KVSALKERVSALKEKVSA**L**KEKVSALKEKVSALKE-NH_2 (^{13}C at **L**), calculated monoisotopic molecular mass 3837.3379 (error, 7 ppm) (*y*-axis: % relative intensity).

ALKE-NH_2 (^{12}C at **L**) was 3836.3104 u, only 1 ppm from the theoretical value of 3836.307813 u. The calculated monoisotopic molecular mass for Ac-KVSALKERVSALKEKVSA**L**KEKVSALKEKVS-ALKE-NH_2 (^{13}C at **L**), was 3837.3379 u, only 7 ppm from the theoretical value, 3837.311168 u.

4. Conclusions

LC–ESI-MS under high-resolution conditions enables mass measurement accuracies for synthetic peptides and their tryptic fragments to within 10 to 40 ppm depending on the mass range scanned. Tryptic maps were generated for each peptide during high-resolution LC–ESI-MS analysis and CAD in the ESI interface resulted in the production of characteristic product ions that enabled amino acid sequencing of the tryptic fragments. The value of high-resolution was demonstrated during analysis of unknowns in an incomplete synthetic peptide reaction mixture. Six unknown peptides were identified on the basis of monoisotopic molecular mass and amino acid sequence data, with the monoisotopic molecular masses of these unknowns being determined to within 10–20 ppm. High-resolution analysis was required for the assessment of ^{13}C incorporation at leucine in two synthetic peptides. Use of this technique enabled complete resolution of the $(M+5H)^{5+}$ isotopic clusters and confirmed that the isotopic distributions were consistent with theoretical expectations.

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