

Fast LC-MS using Ettan LC-MS for peptide mass fingerprinting and identification of post-translational modifications

Key words:

Fast chromatography • confirmation of protein identity • identification of post-translational modifications • high mass accuracy • peptide mass fingerprinting (PMF)

Summary

Liquid chromatography-mass spectrometry (LC-MS) using Ettan™ LC-MS allows rapid peptide mass fingerprinting and identification of post-translational modifications in a variety of different high molecular weight proteins. Reversed phase chromatography of the protein samples was performed on Ettan microLC comparing non-porous and conventional porous silica C18 columns. Protein identification was performed by mass spectrometry on Ettan ESI-ToF. Results show that the non-porous silica C18 column facilitates very fast peptide mass fingerprinting. Ettan LC-MS is capable of extremely fast LC-MS generating peak durations as low as 1 sec making it ideal for rapid peptide mapping. Ettan ESI-ToF acquires high quality mass spectra, which allows easy identification of proteins from natural sources—identification of several fragments carrying post-translational modifications was also achieved.

Introduction

Mass spectrometry is an essential tool for protein identification and has contributed to speeding up the proteomics research phase in drug discovery. High-performance LC-MS equipment, with peptide mapping capability, is ideal for the analysis of post-translational modifications (PTM), drug candidates, micro-heterogeneities in recombinant proteins, and confirmation of the identity of proteins purified from natural sources.

We report results on the rapid purification and precise identification of various proteins using Ettan LC-MS. The mass spectrometer is equipped with dual ionization probes, facilitating internal calibration and high mass accuracy by the option of simultaneous spraying of sample and calibrants.

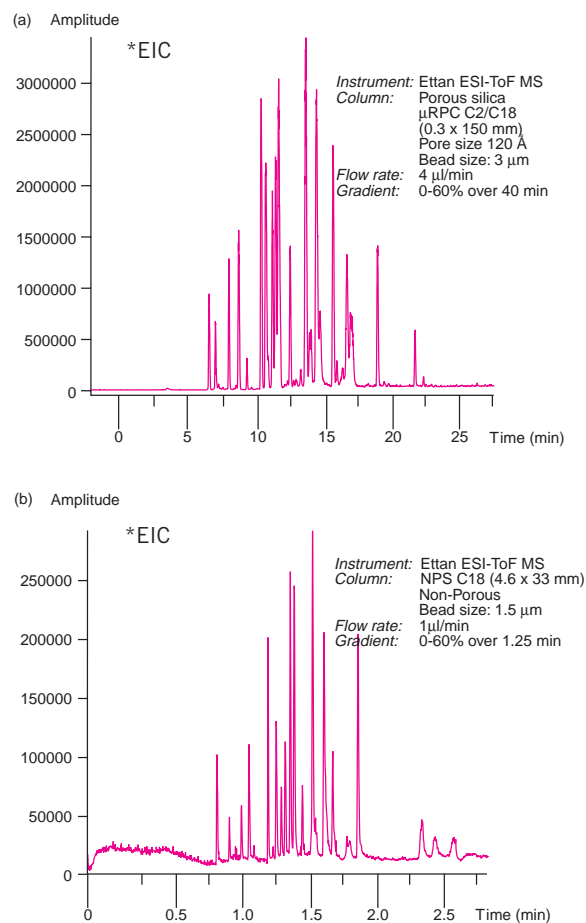


Fig 1. The chromatographic performance when separating a tryptic digest of β -lactoglobulin on (a) porous silica C18 (μ RPC C2/C18 column; 0.3 x 150 mm), gradient 0–60% B over 40 min compared with (b) non-porous silica C18 (NPS C18 column; 4.6 x 33 mm), gradient 0–60% B over 1.25 min. *Extracted Ion Chromatogram (EIC) for the m/z range 485–2000.

Ettan ESI-ToF can be operated at a spectral rate of 100 spectra/sec and is thus suited for extremely fast LC-MS runs, generating peak durations as low as 1 sec.

The performance of non-porous silica, as well as conventional, porous silica reversed phase chromatography columns was compared and evaluated.

Products used

Amersham Pharmacia Biotech products used in this study:

Ettan LC-MS system	Inquire
μ RPC C2/C18 ST (300 μ m \times 150 mm)	17-6002-89
NPS 4.6 \times 14 mm, 1.5 μ m	17-6002-87

Methods and Instrumentation

Purified proteins— β -lactoglobulin (Sigma), bovine serum albumin (BSA, Sigma), bovine β -casein (1), acyl-CoA-binding protein (ACBP, [2]), cholecystokinin-39 (CCK-39) purified from porcine intestines essentially as described in (3, 4), and myelin basic protein (MBP) isolated from pig brains (5)—were digested by means of modified trypsin. Peptide mass fingerprinting was performed on Ettan LC-MS using a small particle, non-porous silica (NPS) C18 column (4.6 \times 33 mm, 1.5 μ m beads) connected to the Ettan ESI-ToF mass spectrometer. Eluents were (A) 0.065% trifluoroacetic acid (TFA) in water and (B) 0.05% TFA in acetonitrile. The flow rate was 1 ml/min and the gradient was 0–60% B over 1.25, 2.5, or 5 min. The mass spectrometer was operated in positive mode at a spectral rate of 10 spectra/sec and a capillary exit voltage of 150 V.

The chromatographic performance of the NPS column was compared to that of the conventional reversed phase chromatography (RPC) column, μ RPC C2/C18 (0.3 \times 150 mm, 3 μ m porous silica beads, 120 Å pore size).

Results and Discussion

Performance of non-porous and porous silica RPC media

1. Beta-lactoglobulin and bovine serum albumin

The comparison between the NPS column and the μ RPC C2/C18 column is shown in Figure 1. The separated sample was a tryptic digest of β -lactoglobulin and gradients were 0–60% B over 1.25 min using the NPS column and 0–60% B over 40 min using the μ RPC C2/C18 (0.3 \times 150 mm). As seen in Figure 1, the chromatographic performance achieved by the two columns is similar both regarding separation selectivity and chromatographic resolution. In the NPS separation the main portion of the peptides eluted within 50 sec and peak durations were 1 sec or less.

For tryptic digests of larger proteins separated on the NPS column, slightly longer gradients were used; 2.5 or 5 min. Figure 2 shows the separation of a tryptic digest of BSA.

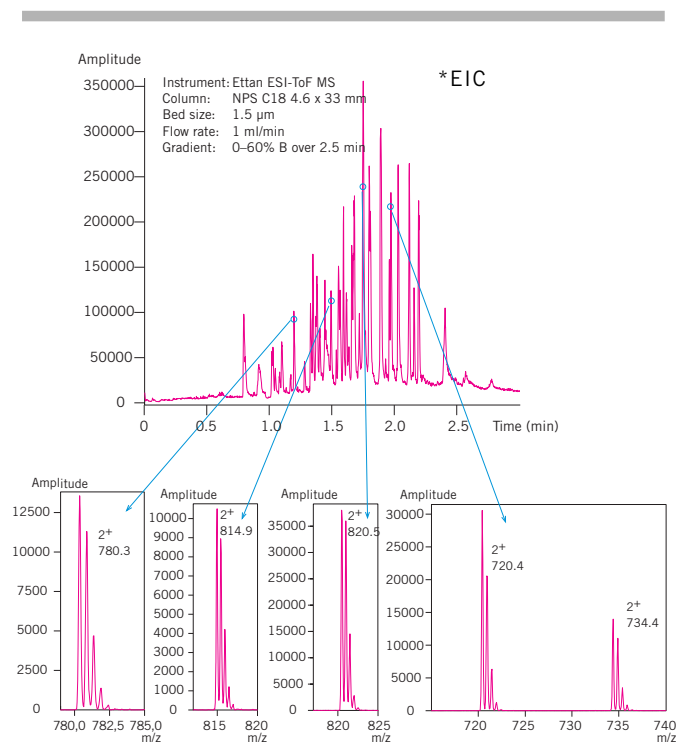


Fig 2. A tryptic digest of bovine serum albumin was performed on non-porous silica C18. The gradient was 0–60% B over 2.5 min. The spectra acquisition rate was 10 spectra/sec. *Extracted Ion Chromatogram (EIC) of the m/z range 485–2000.

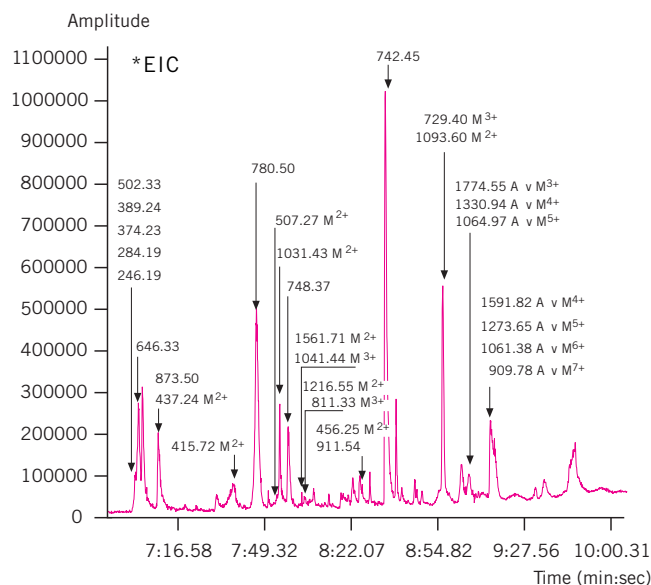


Fig 3. Chromatogram from the separation of the tryptic digest of bovine β -casein sample. The gradient was 0–60% B over 2.5 min. *Extracted Ion Chromatogram (EIC) of the m/z range 100–2000. The mass values (m/z) indicated for the peaks are related to the structure of the β -casein molecule.

As seen, despite the very fast elution of the peptides, mass spectra of very good quality were achieved allowing a database search for protein identification.

2. Bovine β -Casein

The milk protein β -casein plays an important role in determination of the surface properties of casein micelles. It is also the source of β -casomorphins, a group of opioid peptides with potent biological activity (1). The protein has five phosphorylated post-translational modifications.

The peptide mass fingerprint from the separation of the tryptic digest of β -casein is shown in Figure 3. Masses (m/z) related to the protein structure are indicated in the Figure. Protein database searches based on the peptide mass fingerprinting experiments revealed a 100% coverage of the molecule when compared to the theoretical tryptic cleavage of P02666 (CASB_BOVIN) and consequently all fragments carrying phosphorylations were identified (Fig 4).

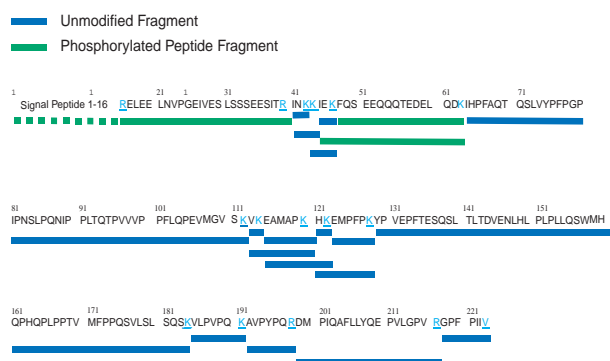


Fig 4. The experimental coverage map (protein amino acid sequence, one-letter code) from the peptide mass fingerprint of the bovine β -casein sample. Horizontal bars indicate the sections of the structure covered by identified protein fragments.

3. Porcine Acyl-CoA-Binding Protein (ACBP)

ACBP binds medium- and long-chain acyl-CoA esters with very high affinity and may function as an intracellular carrier of acyl-CoA esters. ACBP is also able to displace diazepam from the benzodiazepine recognition site located on the GABA type A receptor. It is therefore possible that the protein also acts as a neuropeptide to modulate the receptor. The protein is N-terminally acetylated.

The peptide mass fingerprinting of ACBP revealed 98% coverage of the molecule (Fig 5). The acetylated fragment of ACBP was identified and the mass of native ACBP (calculated mass 9807.18) was determined with a mass accuracy of 33 ppm (Fig 6).

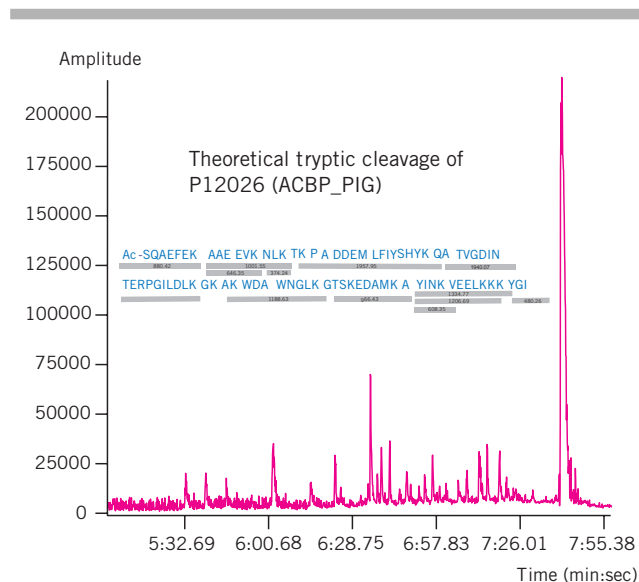


Fig 5. The peptide mass fingerprint and coverage map (98% coverage of the molecule when compared to the theoretical tryptic cleavage of P12026 (ACBP_PIG) from the analysis of the ACBP sample. Capital letters indicate in one letter code the amino acid sequences covered by the analysis.

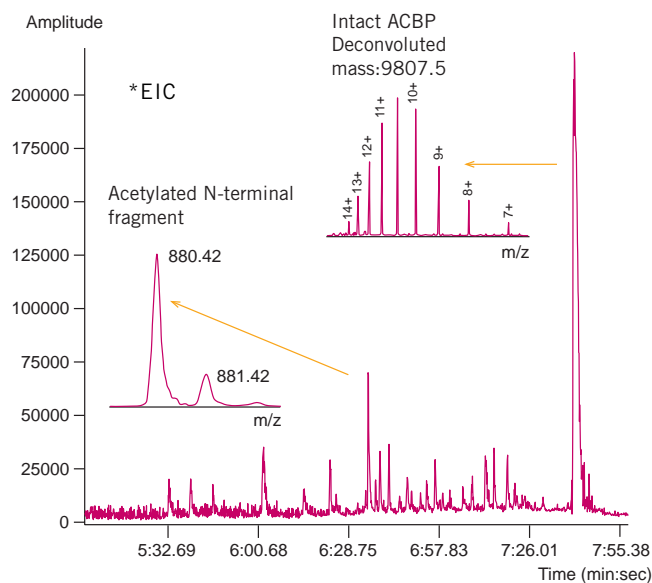


Fig 6. Peaks in the chromatogram from the analysis of ACBP corresponding to the acetylated N-terminal fragment and the intact molecule are indicated with arrows. (*Extracted Ion Chromatogram EIC of m/z range 100–2000)

4. Myelin Basic Protein – MBP

MBP protein may function to maintain a proper structure of myelin. Subcellularly it is located on the cytoplasmic side of myelin. It has one acetylation and one methylation site (potentially single and double methylation). Also, a fraction of the protein may carry no methylation at all.

The peptide mass fingerprinting of MBP revealed 86% coverage of the molecule (Fig 7) and the N-terminal acetylation of the protein was identified (Fig 8). Furthermore, the results presented in Figure 8 indicate that there are in fact three different populations of MBP, either with unmodified arginine-107 or modified at arginine-107 by single or double methylation. The identified masses 859.61 m/z and 873.59 m/z correspond to mono- and dimethylated GR(107)GLSLSR, respectively. The mass 632.42 reveals the existence of the fragment GLSLSR, which would be generated from the population of unmodified MBP. These results indicate that trypsin does not cleave peptide bonds involving a methylated arginine.

ASQK rpsqr hgsk YLASASTMDHAR HGFLPR hrdtgidslgr
 FFGADR GAPK RSGKDGHHAAARTTHYGSLPQK AQHGR
 PQDENPVVHFFK NIVTPR TPPPSQGK **GRGLSLSR**
 FSWGAEQK PGFGYGR APDYK PAHKGK
 GAQDAQGLSK IFK LGGR dsr GSGPMAR r

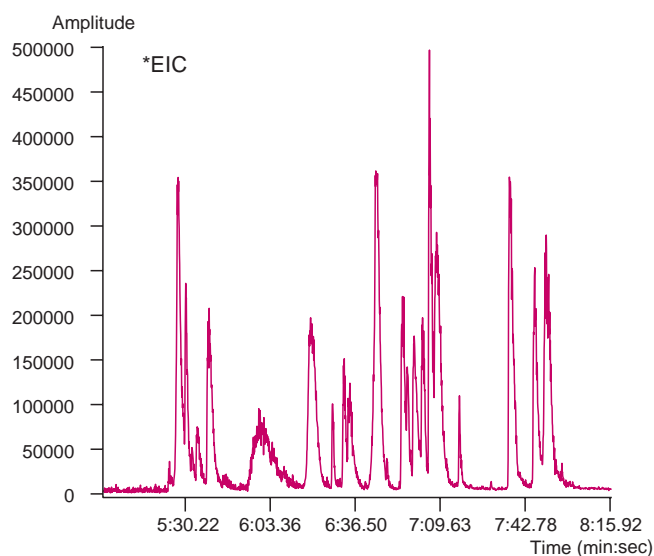


Fig 7. The peptide mass fingerprint and coverage map (86% coverage of the molecule when compared with the theoretical tryptic digest of P81558 (MBP_PIG) from the analysis of the MBP sample. Capital letters indicate in one letter code the amino acid sequences covered by the analysis. Lower case letters indicate parts of the sequence not covered. Tryptic fragments expected to carry PTMS are indicated in red. *Extracted Ion Chromatogram (EIC) of the m/z range 100–2000.

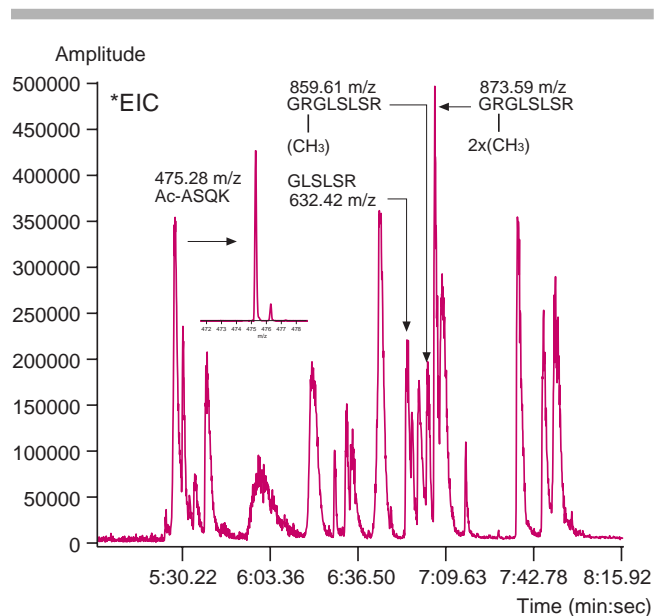


Fig 8. Analysis of the MBP sample revealed the N-terminally acetylated fragment, as well as fragments corresponding to mono-, di-, and non-methylated MBP. *Extracted Ion Chromatogram (EIC) of the m/z range 100–2000.

5. Cholecystokinin 39 (CCK-39)

CCK-39 induces gall bladder contractions and the release of pancreatic enzymes. It is also present in the brain, but its function there is not clear. The biological activity of CCK-39 is largely dependent on sulfation of tyrosine-33 close to the C-terminus of the molecule.

In the peptide mass fingerprinting experiment all tryptic fragments of the molecule were identified. The fragment having a mass corresponding to the sulfated CCK-39-fragment ISDRY(33)MGWMDF was identified as eluting late in the gradient (Fig 9).

The CHOLESYSTOKININ (CCK-39) Amino Acid Sequence
YIQQARKAPSGRVSMIKNLQSLDPSHRISDRDYMGWMDF

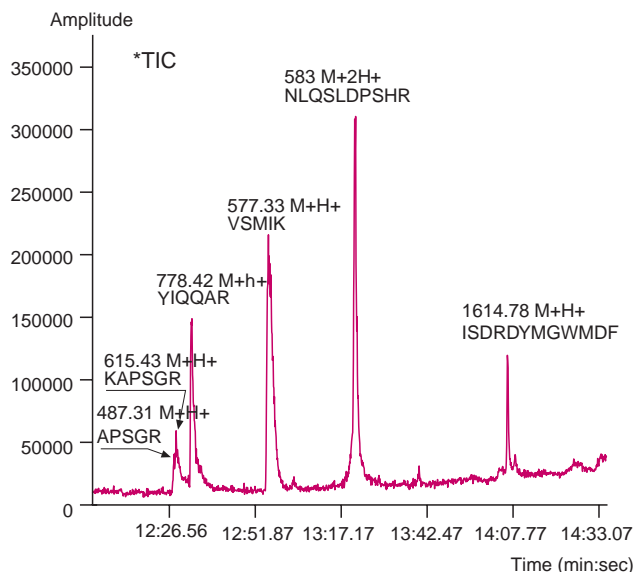


Fig 9. All tryptic fragments in the Cholecystokinin 39 (CCK-39) molecule were identified by peptide mass fingerprinting. Moreover, the C-terminal fragment carrying the sulfation PTM was identified. *Total Ion Chromatogram (TIC) of the entire m/z range.

Conclusions

The column packed with non-porous silica stationary phase facilitates very fast peptide mapping. Ettan ESI-ToF allowed acquisition of high quality mass spectra, which facilitates identification of proteins purified from natural sources. Furthermore, Ettan LC-MS allowed very rapid protein identification due to the high spectral rate and low peak durations provided by Ettan ESI-ToF.

The protein database search based on the peptide mass fingerprinting experiments revealed a coverage of 86–100% of the protein (average 96%) when compared to the theoretical tryptic cleavage of the proteins.

Several fragments carrying post-translational modifications such as acetylations, phosphorylations, methylations, and acetylations, were successfully identified using Ettan ESI-ToF.

Acknowledgements

Cholecystokinin 39, ACBP, and MBP were kindly supplied by Assoc. Prof. Rannar Sillard, Dep. of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden. This study was performed in collaboration with Assoc. Prof. R. Sillard.

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