SHIMADZU
Excellence in Science

Application News

No. MO216

AXIMA-QIT MALDI-QIT-TOF

Characterization of Complex Protein Gel Bands by Offline LC-MALDI QIT TOF MS

- Combination of offline LC separation with MALDI-QIT-TOF
- Increased number of proteins identified from 1D gel bands
- MS/MS data used for database searching for confident protein identification
- Immobilized sample permits increased time for acquisition and reduction in signal supression

Characterization of Complex Protein Gel Bands by Offline LC-MALDI QIT TOF MS

Introduction

Traditionally, identification of complex protein mixtures has required a series of time-consuming protocols. Protein mixtures are separated using 1D or 2D gel electrophoresis, protein bands or spots excised and digested producing a peptide mass fingerprint (PMF) to identify the protein using MALDI mass spectrometry and database searching. Limitations can arise, including co-migration of more than one protein, suppression effects during ionization, or the PMF not correlating with any protein in the databases. In many cases, it is simply the complexity of the sample which may contain hundreds or thousands of tryptic peptides from a number of different proteins that proves to be the restrictive factor when attempting to assign putative protein identification.

It is clear that complicated samples require an added dimension of separation prior to MALDI analysis and the use of reverse phase liquid chromatography followed by deposition of the separated peptides directly onto a MALDI target has been recently reported. In this instance, the separated peptides are analyzed initially by MS techniques to provide a "candidate list" and subsequently by MS/MS which can provide sufficient information to generate useful sequence data and protein identification via database searching. Here, in an attempt to circumnavigate many of these issues we have analysed digested 1D gel samples using offline LC-MALDI QIT TOF MS.

Methods

Human proteins from BJAB (EBV-negative Burkitt's lymphoma) cell full lysate were grown in suspension in RPMI medium supplemented with 10% fetal calf serum, harvested and washed with PBS three times. The cell pellet was re-suspended in lysis buffer (1% CHAPS, 8.7% glycerol, 150 mM NaCl, 50mM Tris-HCl pH 7.4 and protease inhibitor cocktail). Following centrifugation, soluble proteins in the supernatant were recovered. The protein mixture was denatured (12.5 mM Tris-HCl pH 6.8, 4% glycerol, 0.4% SDS, 1% ∫-mercaptoethanol) and heated for 5 minutes at 95°C before loading on a 12% acrylamide 1-DE gel. After protein migration, the gel was stained with Coomassie brilliant blue G-250. From this gel, the 33-42 kDa region was excised and subjected to a tryptic digestion. Briefly, the gel slice was dried in neat acetonitrile before performing cysteine reduction and alkylation with iodoacetamide followed by trypsin digestion at 37°C overnight. After digestion, the supernatant was removed before a first extraction with 1% formic acid solution in water. This solution was pooled with the previous supernatant and a second extraction with 50% acetonitrile / 1% formic acid performed to increase peptide recovery.

Protein Identification - MS/MS Ions Mascot® Database Search
Phosphoglycerate kinase 1
Actin gamma
Aldolase A
SET protein (HLA-DR associated protein II)
Poly(rc) binding protein 1; heterogeneous nuclear ribonuclearprotein X
Laminin-binding protein
Translation elongation factor eEF-1 delta chain
Glyceraldehyde-3-phosphate dehydrogenase
HLA-A11K protein precursor
MHC class 1 histocompatibility antigen alpha chain
COP9 complex subunit 4
Heterogeneoous nuclear riboprotein A2/B1
Cysteine and histidine-rich domain (CHORD)-containing
HRS
Aspartate aminotransferase 2 precursor, glutamic-oxaloacetic transaminase 2
Ribosomal protein P0, 60S acidic ribosomal protein P0
Proteinx0005
Thioredoxin-like 2
Heterogeneous nuclear ribonuclearprotein AB isoform b
Unknown gene product
Complement component 1 , q subcomponent binding protein precursor
Phosphoserine aminotransferase, isoform 2
Isocitrate dehydrogenase 3 (NAD+) alpha precursor

Table 1

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Peptide
6	976.47	975.46	975.44	0.02	0	AGFAGDDAPR
10	1036.69	1035.68	1035.64	0.04	1	IKIIAPPER
20	1198.72	1197.71	1197.70	0.01	0	AVFPSIVGRPR
22	1203.63	1202.62	1202.55	0.07	0	HQGVMVGMGQK + 2 Oxidation (M)
36	1354.73	1353.72	1353.62	0.11	1	DSYVGDEAQSKR
75	1791.00	1789.99	1789.88	0.11	0	SYELPDGQVITIGNER
83	1954.08	1953.07	1953.06	0.02	0	VAPEEHPVLLTEAPLNPK
84	1954.12	1953.11	1953.06	0.06	0	VAPEEHPVLLTEAPLNPK
96	2231.21	2230.20	2230.06	0.15	0	DLYANTVLSGGTTMYPGIADR + Oxidation (M)

Figure 1. Peptides used to identify Actin gamma (human)

This complex mixture of tryptic peptides was subsequently separated by capillary liquid chromatography using a shallow 65 minute gradient (A = 0.05% TFA in water, B = acetonitrile with 0.05% TFA). The eluent was spotted directly onto a MALDI target. The spotting process was performed by an automated software-controlled system (AccuSpot, Shimadzu Corporation) which can accommodate up to nine 384 well MTP stainless steel MALDI targets and automatically change targets during a long LC run allowing multiple targets to be spotted without user intervention. The matrix (dihydroxybenzoic acid (DHB) at 10 mg/ml in 50:50 acetonitrile / 0.1% TFA) was coaxially introduced to the LC eluent allowing thorough mixing before deposition on the target. The system is also equipped with a camera allowing observation of the spotting process at close proximity. Following each run, the probe was automatically washed with acetone and blown dry using nitrogen gas to ensure no cross contamination between runs was observed.

The two targets produced were analyzed by a MALDI QIT TOF MS (Axima-QIT™, Shimadzu Biotech), initially in positive ion MS mode to provide confirmation of position of peptides on the target and their masses. From this information, a candidate list for MS/MS was compiled. In this instance, helium was used as the buffer gas in the quadrupole ion trap, where ions are trapped using a patented rapid RF start-up method, cooled and ejected into a floated TOF analyzer equipped with a two stage gridless reflectron.

Next, MS/MS was performed on the candidate peptides. Prior to MS/MS analysis, precursor ions were isolated using the filtered noise field (FNF) method which ejects all unwanted ions from the trap. Argon gas was used as the collision gas. The resultant fragments were ejected from the trap following cooling and analyzed in the reflectron TOF analyzer. Data obtained were collectively searched on completion of the total experiment using the Mascot® search engine (Matrix Science) in order to identify the proteins from which the peptides originated.

Results

The two microtitre plate format unmodified stainless steel MALDI targets were analyzed as described and a Mascot® database search performed. The proteins identified are listed below in Table 1. The vast majority of these proteins were assigned using multiple peptide ion MS/MS spectra lending increased confidence to the putative identification. The database search was initially limited to the Human species, however a secondary search was performed specifying Mammals which resulted in the inclusion of human keratin and porcine trypsin as additional proteins (data not shown). Also, a small number of possible homologous proteins were proposed - mostly from rat and mouse - that added to the number of proteins identified (Table 1).

Proteins were identified using a number of individual peptide ions observed at varying positions throughout the targets analyzed. In a combined ions search, these results were accumulated and used to provide a confident score. An example of this is demonstrated in Figure 1. Here, actin gamma is identified using the information obtained from nine discrete MS/MS experiments. It may be noted that the mass accuracy of the peptide hits is well within the specified search tolerance of 0.2 Da. Typical MS/MS spectra are shown in Figures 2 and 3.

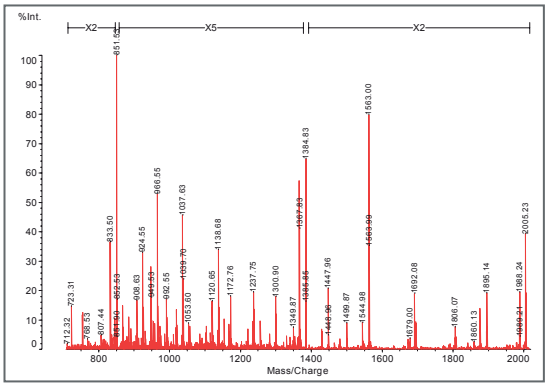

Figure 2. MS/MS of m/z 2023, identified as phosphoglycerate kinase 1

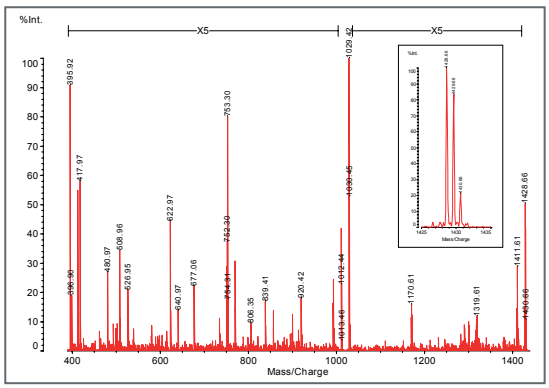

Figure 3. MS/MS of m/z 1446, identified as SET protein

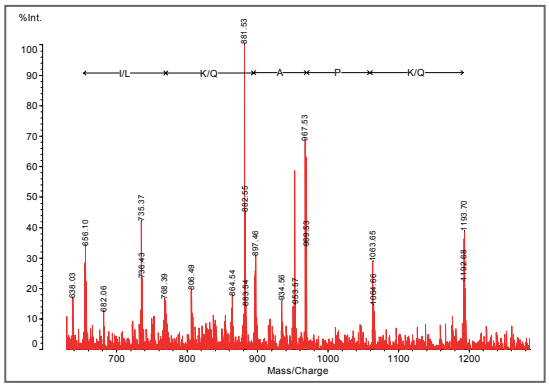

Figure 4. MS/MS of m/z 1848 with sequence tag annotated

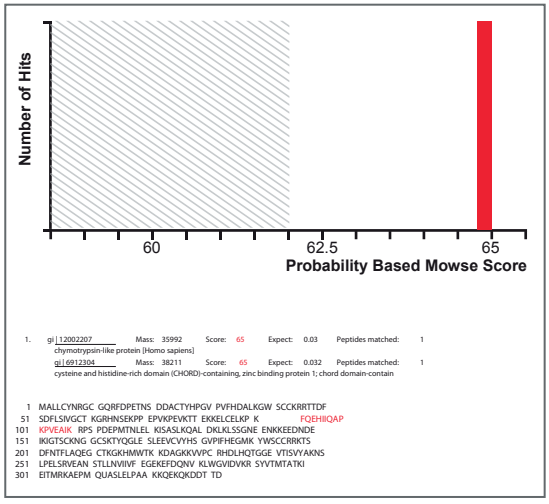

Figure 5. Mascot ® Sequence Query identification

In some instances, the search was unsuccessful and no confident positive identification was provided for the fragmentation data submitted. Here, as much de novo sequence information as possible was generated from the MS/MS spectrum and the resulting sequence tag searched using the Mascot® Sequence Query facility in order to attempt to identify the protein from which the peptide originated. An example of this type of data interrogation is displayed in Figure 4 where a sequence tag of I/LK/QAPK/Q was generated from an MS/MS spectrum of m/z 1848. The search result is shown in Figure 5. Chymotrypsin-like protein and cysteine and histidine-rich domain (CHORD)-containing, zinc binding protein are the top hits, which were also observed in the initial batch database search of the MS/MS spectra acquired.

Conclusion

- We have demonstrated the novel offline LC-MALDI QIT TOF MS approach for the characterization of complex protein samples allows confident identification of multiple proteins not possible by MALDI peptide mass fingerprinting alone
- The high resolution spectra obtained, demonstrating >8000 resolution in both MS and MS/MS modes, in combination with the impressive mass accuracy permit both database searching and sequence tag interrogation
- In direct comparison to more traditional LC/MS/MS methods, the immobilisation of the sample on a MALDI target allows the acquisition of data over prolonged periods, necessary when MS/MS is required on co-eluting peptide ions, and subsequent reanalysis of samples if required
- A significant reduction in signal suppression was observed in the LC separated MS peptide spectra in addition to the temporal separation of peptides of the same nominal mass that would overlap on a MALDI spectrum unless separated

Second Edition: August, 2013

SHIMADZU Corporation
www.shimadzu.com/an/

Related Solutions

- Price Inquiry
- Product Inquiry
- Technical Service / Support Inquiry
- Other Inquiry