AXIMA-TOF^{2}™

Protein Identification Using an Axima-TOF^{2}™

- Routine confident peptide mass fingerprinting with integrated Mascot® searching
- High energy CID MS/MS fragmentation for enhanced protein identification
- Novel ion gate design allowing precise precursor isolation with up to 500 resolution (FWHM)

SHIMADZU BIOTECH
bringing analysis to life

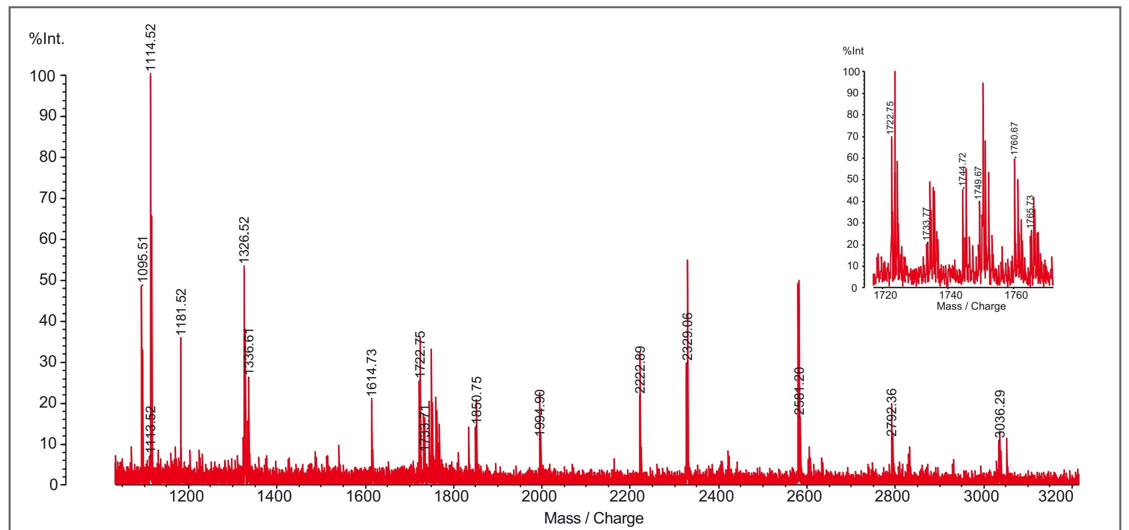
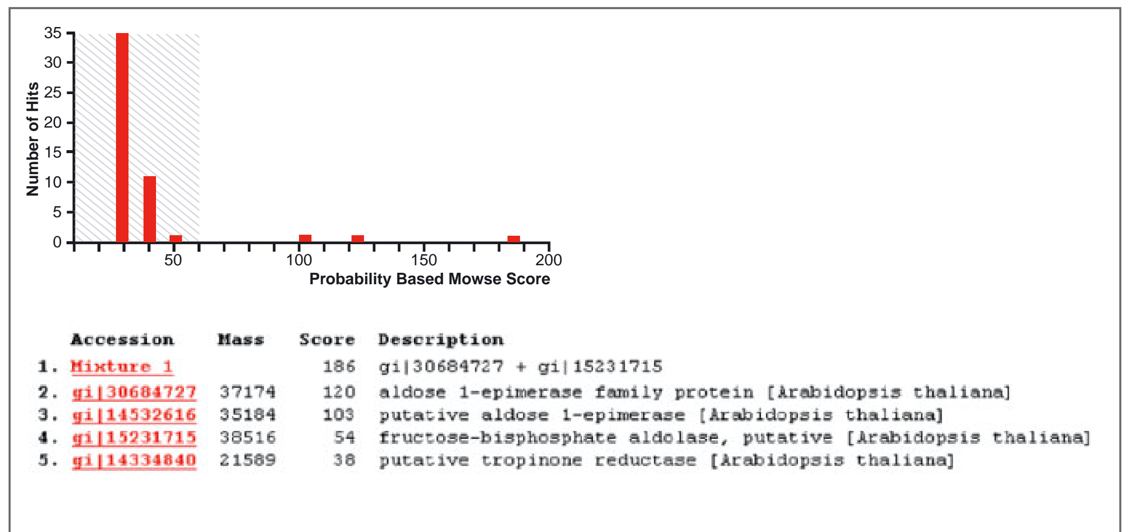


Figure 6. Peptide mass fingerprint, sample #12



	Accession	Mass	Score	Description
1.	Mixture 1		186	gi|30684727 + gi|15231715
2.	gi|30684727	37174	120	aldose 1-epimerase family protein [Arabidopsis thaliana]
3.	gi|14532616	35184	103	putative aldose 1-epimerase [Arabidopsis thaliana]
4.	gi|15231715	38516	54	fructose-bisphosphate aldolase, putative [Arabidopsis thaliana]
5.	gi|14334840	21589	38	putative tropinone reductase [Arabidopsis thaliana]

Figure 7. PMF Mascot® search result showing a mixture of proteins, sample #12

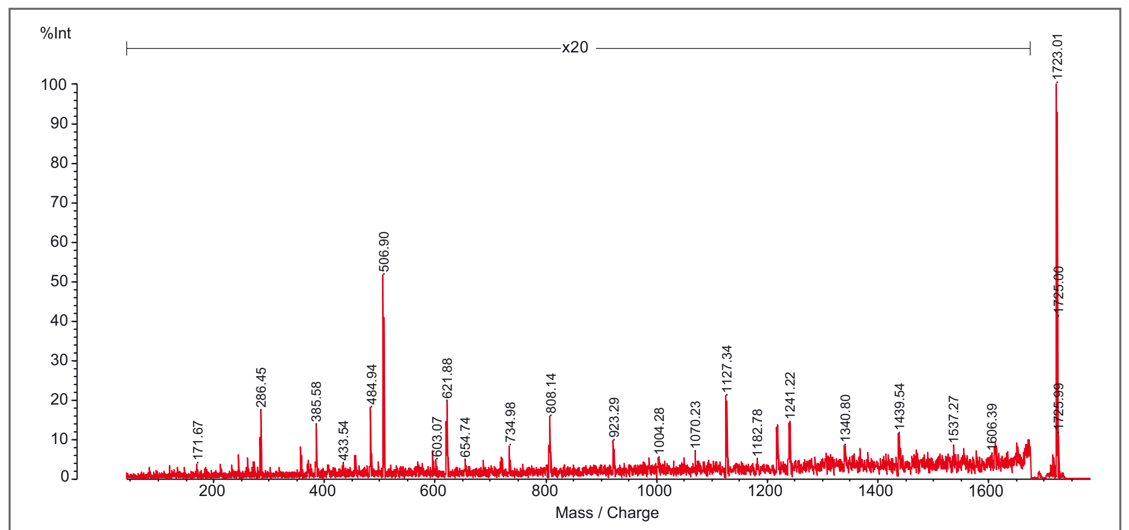


Figure 8. MS/MS spectrum of m/z 1722, sample #12

Protein Identification Using an Axima-TOF^{2}™

Introduction

In the field of proteomics, MALDI TOF MS technology has been used predominantly to perform peptide mass fingerprinting experiments followed by database searching to identify the protein by matching tryptic peptides to those generated *in silico*. Protein identification can be confirmed by seamless PSD (sPSD)[1,2], which examines the fragments produced when individual peptides are allowed to undergo metastable decay. The resultant fragments are again subjected to an independent database search and a protein identification suggested.

However, there are limitations to this conventional workflow. The resolution of the ion gate required for sPSD is relatively wide (+/-7 Da, a resolution of 70) and issues can arise when attempting to perform sPSD experiments on peptides with masses falling within these boundaries. In addition, peptides containing glutamic acid, aspartic acid and proline (D, E and P) can very often produce sPSD spectra containing only one or two major fragment ions due to selective fragmentation making database searching impossible.

In order to circumvent these potential problems, a new mass spectrometer[3] has recently been introduced, the Axima-TOF^{2}™. This system incorporates a MALDI ion source capable of accepting both standard microtitre format targets and alternative geometries. An innovative ion gate, the monoPULSE™, is also employed allowing the selection of precursor ions with an accuracy of +/- 1 Da, a resolution of 500 FWHM (at 1000 Da) eradicating the issue of precursor ions in close proximity. A CID (collisionally induced dissociation) cell is present utilizing helium as the collision gas, facilitating the fragmentation of precursor ions. A curved field reflectron is included allowing excellent sensitivity and mass accuracy of both MS and MS/MS derived ions.

Here, we investigate the value of this new mass spectrometer for protein identification by MS and MS/MS using a series of in-gel tryptic digests.

Methods

An Axima-TOF^{2}™ (Shimadzu Biotech, Manchester, UK) MALDI MS/MS system was used in positive ion reflectron mode for all experiments. Peptide mass fingerprints were generated and database searches performed using Mascot® (Matrix Science). MS/MS experiments were performed on individual peptide ions isolated from the tryptic digest using an innovative ion gate, the monoPULSE™. Fragmentation is performed by means of a CID cell positioned in the ion beam. Helium is used as the collision gas.

Coomassie stained protein 2D gel plugs were digested with trypsin and desalted using Ziptips (Millipore). CHCA, (alpha-cyano-4-hydroxycinnamic acid at 10 mg/ml in 50:50 0.1 % TFA: acetonitrile v/v) was utilized as the MALDI matrix. These analyses were performed using an unmodified 384 well microtitre plate format stainless steel MALDI target.

All samples were spotted directly onto the target using a volume of 0.5 µl and an equal volume of matrix added. All samples were allowed to air dry at ambient temperature. Samples were calibrated by close external calibration procedures using well defined peptide standards.

Results

In this series of experiments, peptide mass fingerprints (PMF) were generated for each sample. These subsequently underwent a database search in order to identify the protein. If the resultant Mascot® search score was found to be over 175, the protein was deemed be successfully identified. The Mascot® probability score for the search parameters employed in order to produce a significant hit was found to be >60 ($p<0.05$). However, if the score did not reach this limit of 175, MS/MS experiments were performed on peptide ions and the fragmentation data independently searched to provide an identity confirming the putative identity suggested by the PMF search. A summary of the protein identities obtained and the scores achieved is presented in Table 1.

Sample	Mascot® PMF Score	Mascot® MS/MS Score	Protein Identity
1	160	223	Expressed protein *(Arabidopsis thaliana)*
2	262	-	Methionine adenosyltransferase *(Arabidopsis thaliana)*
6	195	-	Heat shock protein 81-2 *(Arabidopsis thaliana)*
8	150	138	Lipoamide dehydrogenase precursor *(Arabidopsis thaliana)*
11	189	-	Keratin1, type II cytoskeletal (Human) Cytokeratin 1, K1 67kDA (Human)
12	186 (mixture)	263	Putative aldolase 1-epimerase *(Arabidopsis thaliana)*
		60	Fructose-bisphosphate aldolase *(Arabidopsis thaliana)*

Table 1. Summary of proteins identified from 2D gel by Axima-TOF^{2}™

An example of a typical peptide mass fingerprint is shown in Figure 1. Here, sample #2 was confidently identified as methionine adenosyltransferase with a Mascot® score of 262. As the PMF score is over 175, no confirmatory MS/MS was performed. An average mass accuracy of less than 20 ppm was observed with close external calibration, as demonstrated in Figure 2.

In some cases, a PMF Mascot® score of over 175 was not achieved. For example, sample #1 was identified as "expressed protein" from Arabidopsis thaliana by conventional peptide mass fingerprinting. This assignment was confirmed by MS/MS of 4 peptides from the PMF - the subsequent database search was carried out independently of the PMF search and the same protein identified with increased confidence. Two of the MS/MS spectra are shown in Figures 3 and 4. Figure 3 illustrates the MS/MS of m/z 1866. When interrogated via a database search, a comprehensive series of y-ions were determined, and a single ion search score of 107 obtained. Figure 4 shows the MS/MS spectrum for m/z 825. Again, a broad series of y-ions are observed, in addition to a significant number of b-ions allowing the full sequence identification - GVFFDIK. It should also be noted that immonium ions are clearly present in the spectrum and may be used to confirm the putative sequence. The combined search result for the 4 MS/MS spectra is shown in Figure 5. Thus, the protein identity provided by the PMF search is independently confirmed.

The average mass accuracy determined for the externally calibrated MS/MS ions was found to be 50 ppm. Mass accuracy for the MS spectrum was again less than 20 ppm.

The MS spectrum for sample #11 was searched using Mascot® and no significant matches were obtained when using the Arabidopsis definition. However, when searched with all species selected a mixture of two proteins was discovered - keratin 1, type II cytoskeletal (human) and keratin type II cytoskeletal 1 (Cytokeratin 1, K1 67 kDa, human). This indicates that a significant contamination had occurred at either the gel or the digestion level. However, a successful identification of the proteins within the gel spot was achieved.

The peptide mass fingerprint result, Figure 6, for sample #12 proved identification of a mixture of proteins, as demonstrated in Figure 7. In order to investigate whether this protein spot is indeed a mixture of two closely related proteins, MS/MS was performed on a selection of ions included in the MS spectrum - m/z 1118, 1326, 1336, 1614, 1722. A typical spectrum, MS/MS of m/z 1722, is shown in Figure 8.

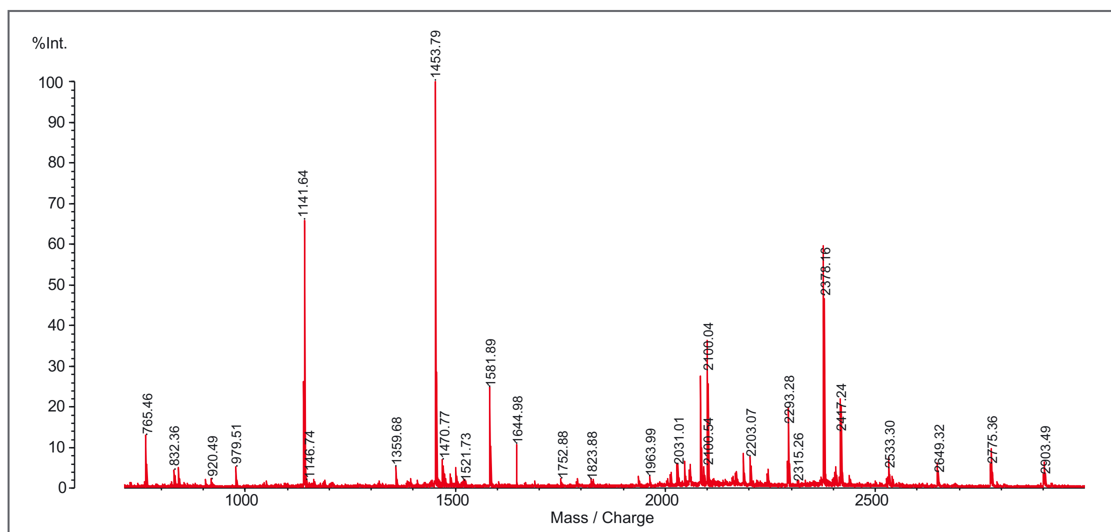
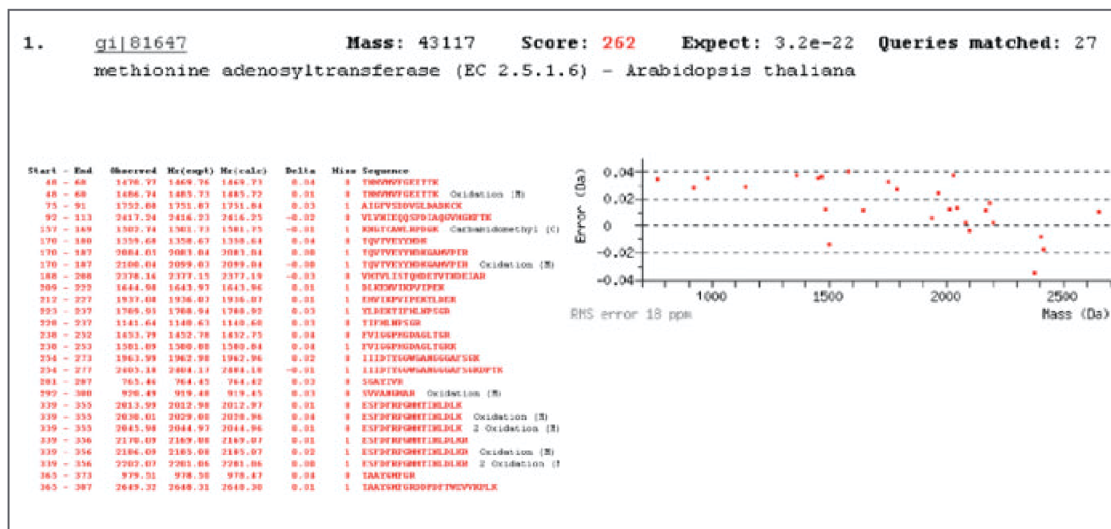


Figure 1. Typical peptide mass fingerprint, sample #2



1. gi|81647 **Mass:** 43117 **Score:** 262 **Expect:** 3.2e-22 **Queries matched:** 27
methionine adenosyltransferase (EC 2.5.1.6) - Arabidopsis thaliana

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
48 - 60	1470.77	1469.76	1469.73	0.04	0	TNMVMVFGEITTK
48 - 60	1486.74	1485.73	1485.72	0.01	0	TNMVMVFGEITTK Oxidation (M)
75 - 91	1752.88	1751.87	1751.84	0.03	1	AIGFVSDDVGLDADKCK
92 - 113	2417.24	2416.23	2416.25	-0.02	0	VLVNIEQQSPDIAQGVHGHFTK
157 - 169	1502.74	1501.73	1501.75	-0.01	1	KNGTCAWLRPDGK Carbamidomethyl (C)
170 - 180	1359.68	1358.67	1358.64	0.04	0	TQVTVEYYNDK
170 - 187	2084.05	2083.04	2083.04	0.00	1	TQVTVEYYNDKGAMVPIR
170 - 187	2100.04	2099.03	2099.04	-0.00	1	TQVTVEYYNDKGAMVPIR Oxidation (M)
188 - 208	2378.16	2377.15	2377.19	-0.03	0	VHTVLISTQHDETVTNDEIAR
209 - 222	1644.98	1643.97	1643.96	0.01	1	DLKEHVIKPVIPEK
212 - 227	1937.08	1936.07	1936.07	0.01	1	EHVIKPVIPEKYLDEK
223 - 237	1769.95	1768.94	1768.92	0.03	1	YLDEKTIFHLNPSGR
228 - 237	1141.64	1140.63	1140.60	0.03	0	TIFHLNPSGR
238 - 252	1453.79	1452.78	1452.75	0.04	0	FVIGGPHGDAGLTGR
238 - 253	1581.89	1580.88	1580.84	0.04	1	FVIGGPHGDAGLTGRK
254 - 273	1963.99	1962.98	1962.96	0.02	0	IIIDTYGGWGAHGGGAFSGK
254 - 277	2405.18	2404.17	2404.18	-0.01	1	IIIDTYGGWGAHGGGAFSGKDPTK
281 - 287	765.46	764.45	764.42	0.03	0	SGAYIVR
292 - 300	920.49	919.48	919.45	0.03	0	[illegible] Oxidation (M)
339 - 355	2013.99	2012.98	2012.97	0.01	0	ESFDFRPGMMTINLDLK
339 - 355	2030.01	2029.00	2028.96	0.04	0	ESFDFRPGMMTINLDLK Oxidation (M)
339 - 355	2045.98	2044.97	2044.96	0.01	0	ESFDFRPGMMTINLDLK 2 Oxidation (M)
339 - 356	2170.09	2169.08	2169.07	0.01	1	ESFDFRPGMMTINLDLKR
339 - 356	2186.09	2185.08	2185.07	0.02	1	ESFDFRPGMMTINLDLKR Oxidation (M)
339 - 356	2202.07	2201.06	2201.06	0.00	1	ESFDFRPGMMTINLDLKR 2 Oxidation (M)
363 - 373	979.51	978.50	978.47	0.04	0	TAAYGHFGR
363 - 387	2649.32	2648.31	2648.30	0.01	1	TAAYGHFGRDDPDFTWEVVKPLK

Figure 2. Mascot® search results for sample #2

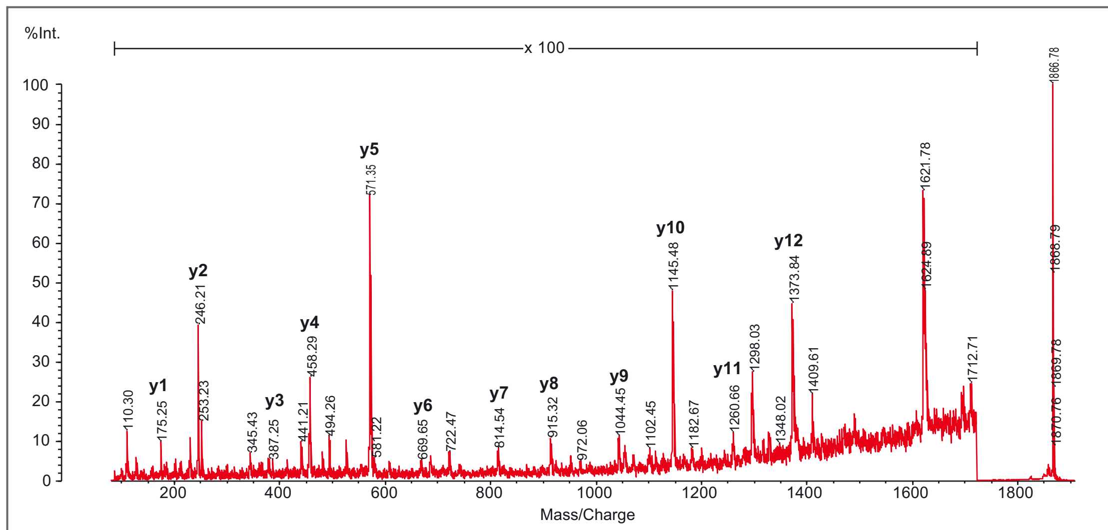


Figure 3. MS/MS of m/z 1866, sample #1

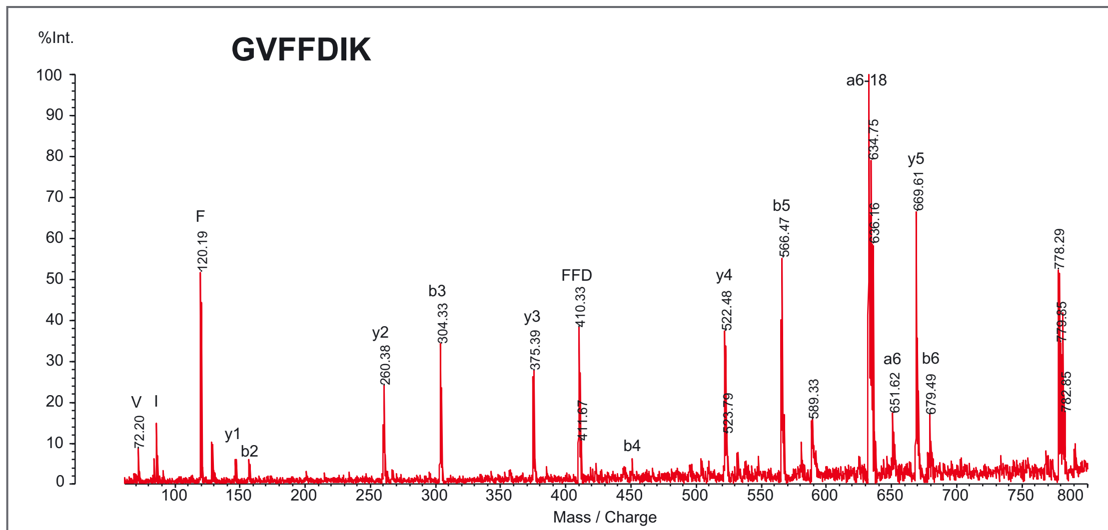
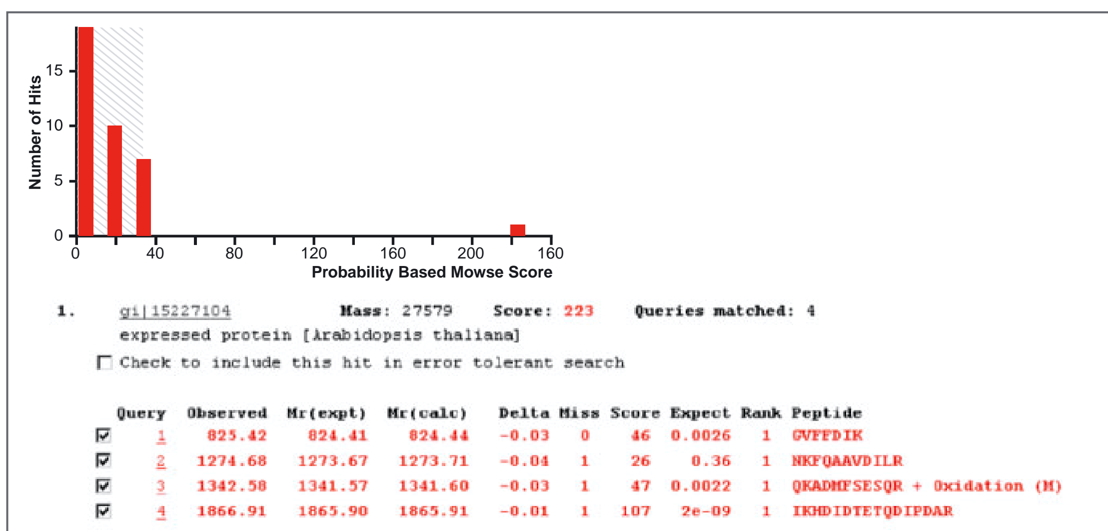


Figure 4. MS/MS of m/z 825, sample #1

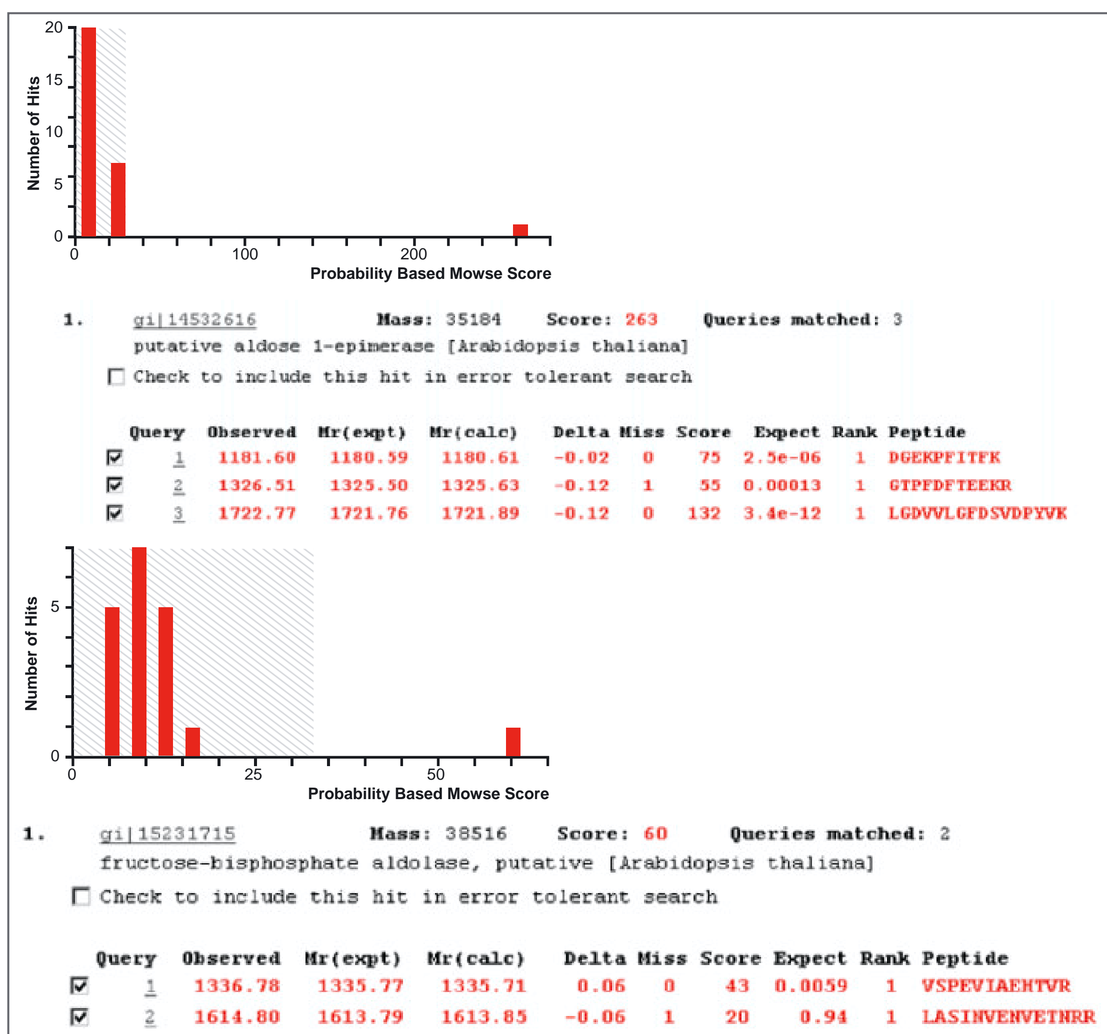


1. gi|15227104 **Mass:** 27579 **Score:** 223 **Queries matched:** 4
expressed protein [Arabidopsis thaliana]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
1	825.42	824.41	824.44	-0.03	0	46	0.0026	1	GVFFDIK
2	1274.68	1273.67	1273.71	-0.04	1	26	0.36	1	NKFQAAVDILR
3	1342.58	1341.57	1341.60	-0.03	1	47	0.0022	1	QKADMFSESQR + Oxidation (M)
4	1866.91	1865.90	1865.91	-0.01	1	107	2e-09	1	IKHDIDTETQDIPDAR

Figure 5. Mascot® combined MS/MS search result, sample #1

The data generated in these MS/MS spectra was subsequently searched using Mascot® MS/MS Ions Search and two independent proteins identified as shown in Figure 9 - **aldose 1-epimerase family protein** (molecular weight 37,174 Da) and **fructose-biphosphate aldolase** (molecular weight 38,516 Da). This is a credible result as the proteins have similar molecular weights and would be expected at a similar position on the 2D gel i.e. have co-migrated to the same excised gel spot.



1. gi|14532616 Mass: 35184 Score: 263 Queries matched: 3
putative aldose 1-epimerase [Arabidopsis thaliana]
☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	1	1181.60	1180.59	1180.61	-0.02	0	75	2.5e-06	1	DGEKPFITFK
☑	2	1326.51	1325.50	1325.63	-0.12	1	55	0.00013	1	GTPFDFTEEKR
☑	3	1722.77	1721.76	1721.89	-0.12	0	132	3.4e-12	1	LGDVVLGFDSVDPYVK

1. gi|15231715 Mass: 38516 Score: 60 Queries matched: 2
fructose-bisphosphate aldolase, putative [Arabidopsis thaliana]
☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	1	1336.78	1335.77	1335.71	0.06	0	43	0.0059	1	VSPEVIAEHTVR
☑	2	1614.80	1613.79	1613.85	-0.06	1	20	0.94	1	LASINVENVETNRR

Figure 9. Mascot® MS/MS search results, sample #12

Conclusion

- Peptide mass fingerprinting has been performed routinely on the Axima-TOF^{2}™ with an average mass accuracy of better than 20 ppm using external calibration
- MS/MS was successfully used to independently confirm any borderline identities suggested by the MS data
- High energy CID enhanced the fragment ion spectra generating immonium ions that may be used to help confirm the suggested database search score using amino acid information
- The monoPULSE™ ion gate allowed precursor ion selection with a window of +/- 1 Da (500 resolution) permitting precise isolation of peptides for sequence elucidation

REFERENCES

1. *T.J. Cornish et al, Rapid Commun. Mass Spectrom., 8, (1994), 781*
2. *M.M. Cordero et al, Rapid Commun. Mass Spectrom., 9, (1995), 1356*
3. *R.J. Cotter et al, Int J Mass Spectrom., 240, (2005), 169*

AXIMA-TOF^{2}™ and monoPULSE™ are trademarks of Kratos Analytical Ltd., Manchester, UK.

© June 2005 Shimadzu Biotech