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Application News

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AXIMA Confidence MALDI-TOF

A Simple Alternative Sample Preparation Method for Improved Protein Identification from 1D Gel Bands by MALDI-TOF MS

- Simple, fast and cost effective peptide preparation
- Increased numbers of proteins identified in complex mixtures
- Low abundance proteins more readily identified

A Simple Alternative Sample Preparation Method for Improved Protein Identification from 1D Gel Bands by MALDI TOF MS

Introduction

The analysis of proteins separated by 2D gel workflows by MALDI TOF MS has become routine over recent years. The protein sample is generally excised, destained and digested by trypsin prior to peptide mass fingerprinting. Often, a clean-up step is included, for example, ZipTips® packed with a C_{18} bed may be used to desalt the complex mixture of resultant peptides. 1D gel bands also contain a wealth of information and can be used to characterize a protein sample. The increased complexity of the sample, sometimes containing many different proteins, can complicate the analysis making it more difficult to identify proteins contained within the band. Often, only one or two proteins are identified when it is clear that many more proteins are present within the sample. This can be due to a number of factors, including ion suppression and competitive ionization when the sample is particularly complex. In addition, it is difficult for a database search to deconvolute the mixture of peptides and "pull out" the relevant protein identifications from MS data alone.

Simple separation of the peptide pool prior to MALDI mass spectrometric analysis can greatly increase the number of proteins identified from 1D gel bands. This can be achieved quickly and economically using stepped elution from a ZipTip® and requires no expertise or additional sample handling steps. The resultant peptides are analyzed by either seamless PSD (post source decay) or by MS/MS and their fragment ions subjected to a database search. Here, we present results obtained from a number of 1D gel bands from different sources demonstrating the increase in the number of proteins identified using this approach.

Methods

Coomassie stained bands of interest were cut from the 1D gel loaded with 20 µg per lane of whole rat bladder lysate or whole HEK cell lysate.

Rat bladders were excised from control (C) or diabetic (S) rats (induced by intra-peritoneal injection of streptozotocin 60 mg/kg). Whole rat bladder lysate was prepared by homogenization of whole bladder in extraction buffer (containing trizma base, sucrose, EDTA, EGTA, DTT, distilled water plus protease inhibitor cocktail) on ice using a rotor-stator. Samples were diluted with appropriate volumes of 5x sample buffer (containing trizma base, mercaptoethanol, glycerol, SDS and bromophenol blue). Lysates were heated at 95°C for 15 minutes before storage at -20°C. Samples were incubated at 95°C for 5 minutes before loading gels.

HEK-293 cell lysate was prepared by washing cultured cells in PBS and disrupting cell membranes using a P100 pipette. After 5 fold dilution with buffer, the lysate was heated at 95°C for 15 minutes before loading the gel.

Briefly, the specified whole cell lysate protein solution was loaded onto home made 10% polyacrylamide gels. The gels were run for 90 minutes at 120 V before being washed and Coomassie blue stained (Invitrogen, UK).

In-gel digestion: After washing of the gel and reduction (DTT - Sigma, Dorset, UK) and alkylation (iodoacetamide - Sigma, Dorset, UK) steps, proteins were digested overnight at 37°C using small aliquots of sequencing grade modified trypsin in ammonium bicarbonate (Sigma, Dorset, UK) at a concentration of 12 ng/µl.

ZipTip® method 1 (standard method):
Pre-wet: Aspirate and dispense to waste 5 times 10 µl with 50/50 acetonitrile /water 0.1% TFA.
Equilibrate: Aspirate and dispense to waste 5 times 10 µl with 0.1% TFA.
Bind peptides: Cycle the same sample solution through the tip 10 times - the sample must be prepared to 0.1%-1.0% TFA at a pH of <4
Wash peptides on tip: Aspirate and dispense to waste 5 times 10 µl with 0.1% TFA.
Elute peptides: cycle 2 µl of 80/20 acetonitrile/water 0.1% TFA 5 times through the tip and collect this eluant.

ZipTip® method 2 ('stepped' elution):
Pre-wet: equilibrate, bind peptides and wash peptides on tip as described in the standard method
Elute peptides: cycle 2 µl of 10/90 acetonitrile/water 0.1% TFA 5 times through the tip and collect this eluant. Repeat this procedure with the following solutions: 20/80 acetonitrile/water 0.1% TFA; 30/70 acetonitrile/water 0.1% TFA; 40/60 acetonitrile/water 0.1% TFA; 50/50 acetonitrile/water 0.1% TFA and finally 80/20 acetonitrile/water 0.1% TFA.

MALDI TOF: Peptide mass fingerprints and seamless post source decay (sPSD) spectra were acquired on an AXIMA Confidence™ MALDI mass spectrometer (Shimadzu, Manchester, UK).
All samples were analyzed using ∂-cyano 4-hydroxycinnamic acid (CHCA) matrix (10 mg/ml in 50/50 acetonitrile/water 0.1% TFA).

Mascot® database searching: Data sets obtained for the digested samples were searched against the NCBInr database using Mascot (www.matrixscience.com). The taxonomy was set as 'all entries' or 'Mammalia' and the enzyme specified as 'trypsin' (allowing for 1 missed cleavage). A search tolerance of ±0.2 Da was used to search the MS data and the $(M+H)^+$ values were searched as monoisotopic. Generally a search tolerance of ±0.5 Da was used to search the sPSD data (searched as average masses).

Results

The ~50 kDa 1D PAGE bands highlighted in Figure 1 were excised and subjected to in-gel tryptic digestion. The peptide pools were subjected to clean-up using the 'standard' ZipTip® protocol. Samples were analyzed in positive ion mode using an AXIMA Confidence™ MALDI TOF mass spectrometer. The MS spectra obtained are shown in Figure 2.

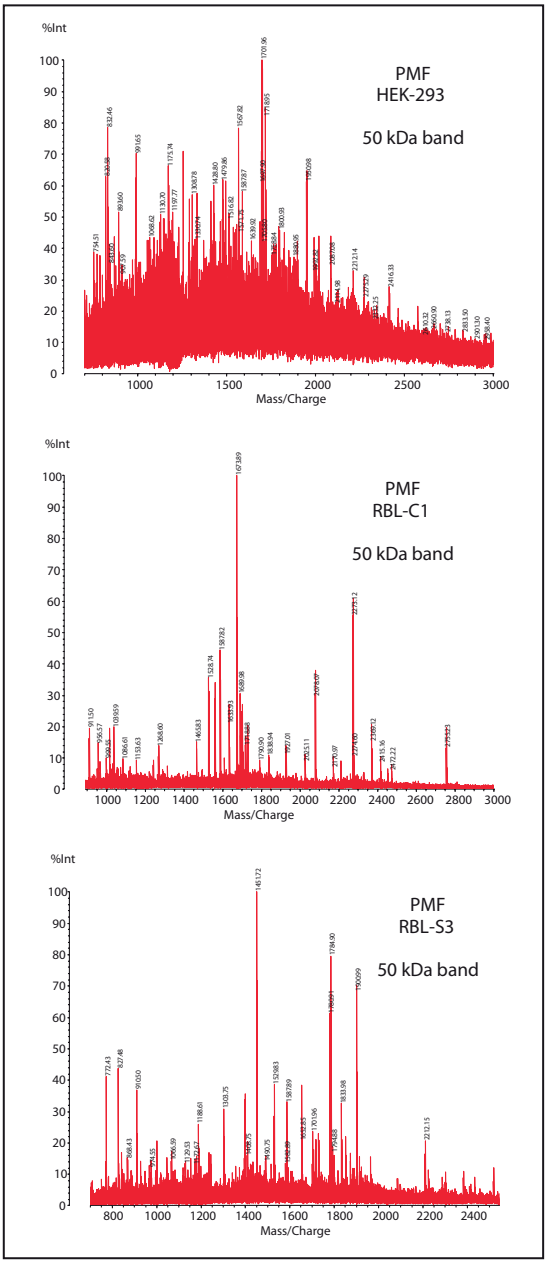

Figure 2. MALDI TOF MS spectra obtained from the in-gel digestion of ID PAGE bands highlighted in Figure 1

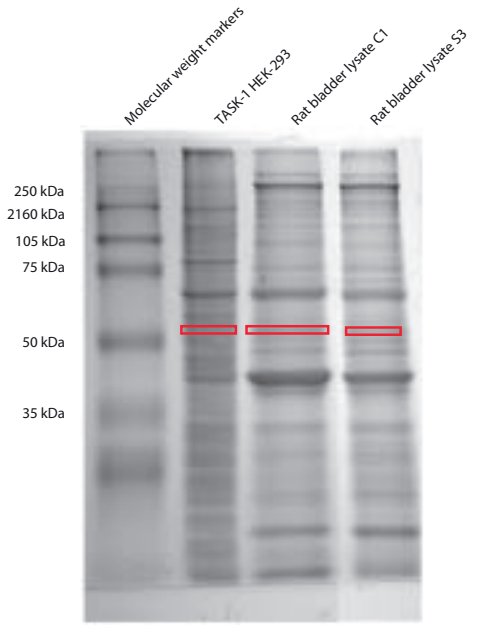

Figure 1. Coomassie stained 1D gel of HEK-293 cells and rat bladder lysates (RBL)

The peptide mass fingerprint spectra obtained from a standard clean-up procedure using ZipTips® generated very complex profiles indicating the predictable presence of more than one protein per band. Table 1 highlights the proteins identified from each of the in-gel digest samples using the standard ZipTip® clean-up procedure. The MS spectrum corresponding to the digestion of the whole rat bladder lysate C1 (RBL-C1) resulted in a confident match to the protein desmin. Desmin is a 53 kDa intermediate filament protein present in smooth muscle cells known to be present in bladder tissue. Neither of the in-gel digests (HEK-293 or RBL-S3) analyzed in the same manner produced a hit through PMF database searching.

Sample	HEK – 293 cell lysate 50 kDa	Whole rat bladder lysate 50 kDa - C1	Whole rat bladder lysate 50 kDa - S3
Number of proteins identified in protocol with 'standard' Zip-Tip® procedure	0	1	1
Proteins identified	-	Desmin (22 peptides PMF)	Calreticulin (1 peptide PSD)

Table 1. Proteins found in whole rat bladder or HEK cell lysates from 1D gel bands with 'standard' ZipTip® elution

MALDI TOF PSD analysis of the samples confirmed the identification of desmin in sample RBL-C1 and also identified calreticulin as a component of sample RBL-S3. Calreticulin is the major calcium binding protein found in smooth muscle.

Figure 3 shows the 6 PMF spectra obtained for each sample after 'stepped' elution of each in-gel digest. These correspond to separate 'stepped' elution of peptides from the ZipTip® using 10%, 20%, 30%, 40%, 50% and 80% acetonitrile.

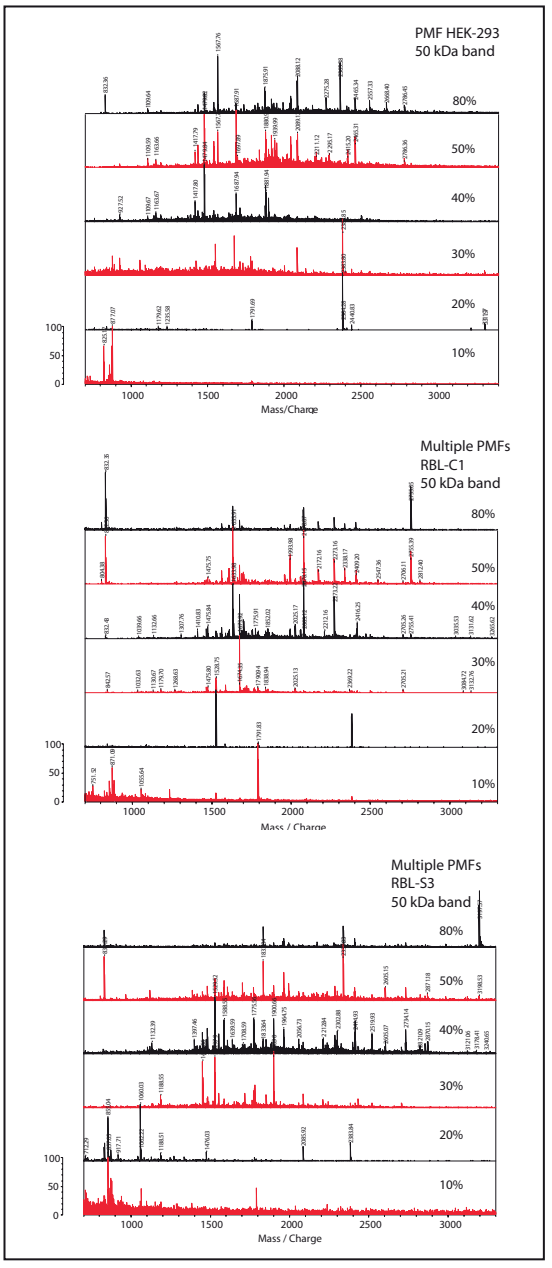

Figure 3. MALDI TOF MS spectra following in-gel digestion of 1D PAGE bands with 'stepped' ZipTip® elutions (10-80% acetonitrile)

Sample	HEK - 293 cell lysate 50 kDa	Whole rat bladder lysate 50 kDa - C1	Whole rat bladder lysate 50 kDa - S3
Number of proteins identified with 'stepped' Zip-Tip® procedure	4	3	4
Proteins identified	Heat shock protein 65 (1 peptide PSD) Heat shock protein 70 (1 peptide PSD) Albumin (2 peptides PSD) K+ voltage gated channel (1 peptide PSD)	Desmin (7 peptides PSD) Adenylate cylase associated protein ADH (2 peptides PSD) Keratin (2 peptides PSD)	Desmin (2 peptides PSD) Calreticulin (1peptide PSD) (1 peptide PSD) Disulfide isomerase (3 peptides PSD)

Table 2. Proteins found in whole rat bladder or HEK cell lysates from 1D gel bands with 'stepped' ZipTip® elutions

It can be seen that different peptides elute in different organic concentrations, with most peptides eluting between 40-50% acetonitrile. 'Stepped' ZipTip® elution may be performed in a few minutes, a significant improvement when compared with an HPLC separation, which would not only increase the time of the experiment but necessitates additional expensive equipment and chromatographic skill.

Selected peptides discovered in the various elutions were subjected to seamless PSD. Table 2 highlights the proteins identified following database searching of PSD data generated from peptides detected in all six PMF spectra originating from differing organic elutions. In all cases, an increased number of proteins were identified following 'stepped' ZipTip® elution and PSD analysis compared to the standard ZipTip® procedure.

Proteins identified using the alternative procedure ranged from high copy number proteins such as desmin to much lower copy number proteins such as a potassium channel protein demonstrating that an in-gel digest can be more effectively mined using the 'stepped' ZipTip® method.

Different proteins were identified from the various bladder samples of diabetic and control rats. A number of the proteins identified in diabetic rats have chaperone activity that is integral to protein folding. This includes calreticulin and protein disulfide isomerase. Recent research has hypothesized that these proteins may play a role in type 2 diabetes.

Conclusion

- In-gel digests from 1D gel bands are often too complex to identify their multiple protein components without prior peptide separation
- Increased numbers of proteins were identified from all analysed 1D PAGE bands following 'stepped' ZipTip® elution rather than the 'standard' single elution
- Peptide separation using 'stepped' elution is a very simple and cost efficient method when compared with an HPLC run
- 'Stepped' elution not only allowed identification of high copy number proteins but also less abundant proteins within the sample

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