

Chapter 4.4

PROCEDURES FOR MASS SPECTROMETRIC PROTEOME ANALYSIS

Gerhard Saalbach

*Plant Research Department; Risø National Laboratory; POB 49, Frederiksborgvej
399, 4000 Roskilde; DENMARK*

Email: G.Saalbach@risoe.dk

Phone +45 4677 4290 Fax +45 4677 4282

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INTRODUCTION

Any kind of protein or protein mixture can be analysed by mass spectrometry (MS) to identify proteins. The proteins are cleaved with an endopeptidase, like trypsin, and the peptides are analysed by MS. The data are used for database screening. The classical approach uses 2D gel-electrophoresis to separate protein mixtures. Individual spots can be cut out and analysed. Other approaches omit the 2D gels and use chromatographic procedures to separate the peptides before the mass spectrometric analysis. Here, we describe the procedures for the analysis of spots from 2D gels (or bands from 1D gels) with an LCQ mass spectrometer (Finnigan, San Jose, USA) using nanospray sample application. Work for the analysis of protein mixtures and use of 2D-chromatography is in progress.

The procedures described here have successfully been used for the analysis of proteins of the peribacteroid membrane (PBM) isolated from root nodules of *Lotus japonicus*. The procedure for PBM preparation is described in a separate protocol in this handbook. Such preparations can be dissolved in SDS-buffer and run on a normal 1D SDS-gel. Bands from such gels can be cut out and analysed according to the following protocols.

MATERIALS

The described procedures are for use with an LCQ mass spectrometer (Finnigan, San Jose, USA) using nanospray sample application. Consumable as well as chemicals, buffers, solution etc. are described in the protocols in detail.

The peribacteroid symbiosome membrane was isolated from *Lotus japonicus* root nodules as described in the accompanying protocol (S Wienkoop, Chapter 4.5). Aliquots of approximately 50 µg protein were separated by 1D gel electrophoresis. Bands for MS analysis were cut out after standard Coomassie Blue staining.

Abbreviations used are as follows: DTT: dithiothreitol; ACN: acetonitril; FA: formic acid.

PROCEDURES

In-gel trypsin digestion and peptide purification

This protocol is for samples treated with dithiothreitol (DTT) and iodoacetamide. This is normally the case with 2D-gel spots. Bands from 1D-gels have to be treated with DTT and iodoacetamide separately. Gels should preferably be stained with Coomassie brilliant blue. Throughout the staining procedure the concentration of ethanol and acetic acid should be reduced to 30% and 5%, respectively. The whole procedure should be kept as short as possible. The following protocols are based on published procedures (Shevchenko et al. 1996, Gobom et al. 1999).

Washing the gel pieces

- Cut the spots from the gel using a sharp scalpel, transfer the gel pieces to 1.5 ml tubes, can be kept at -20 °C
- Wash the gel pieces with 200 µl water, vortex 10 min, remove liquid
- Wash the gel pieces with 200 µl 100 mM NH₄HCO₃, vortex 10 min, do not remove liquid
- Add directly 200 µl acetonitrile (ACN), vortex 10-30 min, remove liquid
- Dark blue spots: wash again 10-30 min with 50 % ACN/ 50 mM NH₄HCO₃
- Repeat step 5 until the gel pieces are de-stained to a large extend, then remove all liquid
- Add pure acetonitril, vortex 10 min
- Remove all liquid, dry completely in speed vac.

In-gel trypsin digestion

- Make 10 ml buffer: 25 mM NH₄H₂CO₃, 10% ACN, 1 mM CaCl₂ (the CaCl₂ must be added last to avoid precipitation)
- Dissolve 1 vial of 25 µg Trypsin-Sequencing grade (Roche, Mannheim, Germany) with 50 µl 1 mM HCl, keep on ice

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- Take ca. 600 μ l from the buffer and add 15 μ l Trypsin, keep on ice, this gives 12.5 ng trypsin per μ l and is sufficient for ca. 30-40 spots
- Add this mix to the dry gel pieces: ca. 10 μ l to the small, 15 μ l to the medium, 20 μ l to the big spots
- Keep on ice for 45 min, but check the swelling from time to time and add more (5-10 μ l) trypsin solution, if necessary
- After ca. 45 min, wash the gel pieces with the trypsin-free buffer: add 100 μ l buffer to each piece and immediately remove all liquid again, then add 1 droplet of fresh buffer, just to cover the gel piece
- Close the lids of the tubes well, cover the whole rack of tubes in plastic wrap, incubate in an air incubator at 37°C overnight.

Extraction of the peptides

- To the trypsin treated gel pieces add 20 μ l 5% formic acid (FA), vortex 5 min, then transfer the liquid into a fresh siliconised tube, keep the pipette tip also in this fresh tube
- To the gel piece add 20 μ l of 1% FA, 5% ACN
- Vortex 15-30 min, remove liquid with the same pipette tip into the same fresh tube
- To the gel piece add 20 μ l of 1% FA, 50% ACN
- Vortex 15-30 min, remove liquid with the same pipette tip into the same fresh tube
- To the gel piece add 20 μ l of 1% FA, 90% ACN
- Vortex 15-30 min, remove liquid with the same pipette tip into the same fresh tube
- Discard the gel pieces.
- Dry down the collected extracts in a speed vac
- Re-dissolve in 15 μ l 5% FA.

Purification of the peptides for nanospray

- Make a micro-column from a 10 μ l Eppendorf GELoader Tip (0030 001.222), squeeze it at the very tip to close it (or narrow it), with another pipette tip fill it with 20 μ l of 50% methanol/5% formic acid (FA)
- Add a small volume of POROS R2 (PerSeptive Biosystems, Framingham, USA) suspended in methanol
- Press liquid through the tip using a 1 ml syringe adapted to the tip with suitable tubing, watch the growing column in the tip, stop if it has reached a length of ca. 5-7 mm (NOT MORE!), then remove all the remaining supernatant (especially excess POROS) from the tip

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- Wash this micro-column again with 20 µl of 50% methanol/5% FA
- Wash it with 2x 20 µl of 5% FA, finally leave a few µl of liquid in the column
- Add your sample into this purification tip; the whole amount can be applied, but this is mostly not necessary with Coomassie spots. It may be necessary with silver stained spots.
- After adding the sample press all liquid through with the syringe
- Wash 3x with 20 µl 5% FA, after last washing step press all liquid out
- Fix a nanospray capillary in the holder (see www.protana.com)
- Cut the purification tip 2 mm above the thin conical part (to make it fit into the centrifuge) and insert it into the nanospray capillary fixed in the holder
- Add 1-3 µl of 50% methanol/5% FA into the micropurification tip sitting in the nanospray capillary in the holder
- Spin the whole assembly in a suitable micro-centrifuge shortly at 5000 rpm
- Take out the micropurification tip and check whether all liquid has passed through, if not, repeat spin (depends on how tight you have squeezed the tip and on possible clogging)
- If the sample is eluted remove and discard the micro-purification tip.

Peptide analysis with LCQ-MS (ThermoFinnigan)

Mass spectrometry

- Take the capillary out of the holder, keep it always with the tip downwards, check that the eluate is sitting in the tip
- Insert the capillary into the holder which is already pre-mounted at the mass spec
- Connect high voltage cables and carefully adjust the tip to the heated capillary opening at the mass spec, apply some air pressure to the capillary with the syringe
- Break the tip by gently touching the heated capillary surface and by moving it up and down, breaking occurs mostly quickly and no big can be seen
- Check it by moving the needle back from the surface and applying some more pressure, then a small (!) droplet should be seen at the tip
- If this was successful, adjust the tip of the needle back to the inlet of the heated capillary
- Open a nanotune file where the capillary voltage is adjusted to 0.7 kV
- Start the LCQ (turn sheath gas off)
- Watch spectrum, stable peaks at intensities of 10^6 to 10^8 should appear

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- Do automatic tuning on base peak, check the result, if negative, repeat tuning on a different peak or discard it and restart the original file
- If the spectrum is finally OK and stable, save it
- Individual peptides can now be analysed with zoom scanning and tandem MS manually, or automatically by running an Xcalibur sequence.

Database search (Mascot)

- Copy the raw files from the nanospray experiment to the Xcalibur/data directory on the C: drive
- Start Sequest and set up a directory,
- Create *.DTA files for all your single raw files (stupid work), or just the one (or few) from the automatic recording (better)
- These will be saved in the directory you have generated before (under C:/Sequest)
- To the same directory copy a *.bat file made according to Mascot:

Here is a DOS batch file that does the job. It is very simple, with no error handling. The batch file and the *.DTA files to be merged must be in the same directory. The original files are not altered. The merged output is in a file called merge.txt. Just cut and paste the following lines into a text editor, (e.g. Notepad), and save as a file with the extension .bat (e.g. merge.bat):

```
ECHO OFF
ECHO. > blank.txt
ECHO. >> blank.txt
FOR %%i IN (*.dta) DO COPY /A %%i+blank.txt %%i.tmp
COPY /A *.dta.tmp merge.txt
DEL blank.txt
DEL *.dta.tmp
ECHO All done, output file is merge.txt
```

- Run this file; it generates a new file in this directory called merge.txt; this can be renamed according to the sample name; it contains all the data from the different DTA files in one file now (but a maximum of 300 DTA files can be sent to Mascot at one time.)
- Start the Mascot search at www.matrixscience.com/cgi/index.pl?page=../home.html, fill out the form, and select the NCBIInr database (EST can only be screened **after** this NCBIInr run, it takes much longer). The merge.txt file is the data file. Select the data format "Sequest(DTA)"!
- Start the search, and if it is running close this window (e.g. by going back to start the next search), the result will then be sent to your email address.

Here are examples of search results from two experiments:

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- www.matrixscience.com/cgi/master_results.pl?file=../data/20000726/FTgolre.dat
- www.matrixscience.com/cgi/master_results.pl?file=../data/20000726/FTgofrc.dat

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