

Chapter 2

Routine Identity Confirmation of Recombinant Proteins by MALDI-TOF Mass Spectrometry

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Abstract

Peptide mass fingerprinting (PMF) by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) provides a simple and direct means to unequivocally confirm identity of recombinant proteins based on predicted peptide profiles. Many universities or research institutions now carry mass spectrometry instrumentation as part of their core bioanalytical facilities or provide public service to outside investigators. This chapter provides methods we have used to generate routinely high quality samples for MALDI-TOF MS analysis. Following resolution of protein preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), we easily process sets of 12 samples manually for MS analysis. Target bands are alkylated and digested in-gel with trypsin, followed by extraction of peptides and desalting with a C18 adsorbent resin (e.g., a “ZipTips”). Acquisition of PMF data on MALDI-TOF mass spectrometers is fast, and with on-site instrumentation, the entire process can be completed within 2 days.

Key words: Peptide mass fingerprint, Protein identification, MALDI-TOF MS, Trypsin digestion, SDS-PAGE

1. Introduction

Preparation of a recombinant protein generally involves separating the protein of interest from the bulk of host cell proteins, purification by means of sequential chromatography (typically at least three steps), followed by biochemical and structural analysis. If the recombinant protein is a fusion protein containing an affinity tag such as a C-terminal poly-His sequence, it may be directly purified by one-step metal chelation affinity chromatography to get milligram quantities of pure proteins. The purity of the recombinant protein is usually evaluated by SDS-PAGE, which also provides critical information

for structural analysis and identification (1). The identity of putative recombinant protein band(s) may be confirmed by electroblotting to a suitable membrane, then probing by immunodetection (2) or by N-terminal amino acid sequence analysis (3). Immunodetection requires a suitably selective antiserum be available for the target protein. Matching the N-terminal amino acid sequence requires the N-terminal amine not be chemically modified, which blocks the Edman sequencing chemistry and results in no sequence information. In cases like this, more complex processing of samples is required to obtain internal peptide sequences for matching (4). This can take considerable time and expense. With the proliferation of high resolution biomolecule mass spectrometers over the past 10 years or so, a relatively simple and direct means for identifying proteins is available. This is based on the highly reproducible digestion of proteins with trypsin and acquiring highly accurate mass spectra of peptides (5), a process termed as peptide mass fingerprinting (PMF) (6). PMF by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) provides direct structural identification by matching highly accurate masses determined from peptide ions predicted peptide profiles determined from the known amino acid sequence. Mass spectrometry is now the method of choice for direct and unequivocal identification of a protein and it can readily distinguish between closely related isoforms (7).

Universities and research institutions now commonly carry a MALDI-TOF MS instrument as part of proteomic or core analytical facilities. Many also provide public service to outside investigators for routine protein identification (e.g., see Association of Biomolecular Resource Facilities, <http://www.abrf.org/index.cfm/dir.yip>). Commercial bioanalytical service companies can also be readily located via an internet search. MALDI-TOF mass spectrometers are highly accurate (10–50 ppm) with resolution (FWHM, full width half maximum) better than 12,000 in reflectron mode with sensitivity for detecting sub-femtomole levels of peptides. Owing to this high sensitivity, it is critical that high quality (clean) samples be prepared to enable ready detection and matching of protein-specific peptides – i.e., samples must be free of exogenously introduced proteins. Particularly, contaminating peptides from proteins such as keratins (from skin) introduced by direct contact or dust particles, or from microbial contamination in old buffer solutions, can overwhelm the MS spectrum, obscuring the peptides from the target protein.

This chapter describes procedures we have used routinely to generate high quality samples for MALDI-TOF MS identification of recombinant proteins (see Chapter 21). Trichloroacetic acid (TCA) precipitation of soluble protein in extracts (e.g., from plant tissues, yeast cells, or culture media) is used to remove salts and other interfering substances prior to separation by SDS-PAGE. Sample loading on PAGE is calibrated to provide ca. 1 µg of a target

protein resolved as a gel band. Individual bands are excised from a PAGE gel and then transferred to a microcentrifuge tube for alkylation, digestion with trypsin, and recovery of the resultant peptides. Extracted peptides may be immediately desalted and spotted with matrix solution onto target plates for immediate on-site MALDI-TOF MS analysis, or the extracted peptides may be stored in dry form until sending to a bioanalytical facility for final processing and analysis. We routinely process (manually) up to 12 protein samples at a time from a standard SDS-PAGE gel, and the sample processing and analysis can be completed in 2 days with a local instrument. The chapter concludes with a description for using PMF data to confirm identification of a protein.

2. Materials

2.1. General Considerations

Minimally detectable protein bands (at least 0.1 µg) taken from SDS-PAGE gels stained with (any) Coomassie Blue stain generally provide good yields for strong peptide ion signals by MALDI-TOF MS. We describe here our use of Invitrogen's NuPAGE gels with Bis-Tris buffer system, but any manufacturer's system for SDS-PAGE (or original Laemmli buffer system) can perform equally well. Similarly, proteins spots from two-dimensional PAGE gels can be used. What is critical for MS identification is to use ultraclean water in preparing solutions, prevent inadvertent dust or protein contamination, or to introduce chemicals that suppress peptide ionization (detergents, non-volatile buffers, plasticizers, salts) (see Note 1).

2.2. General

1. 0.15% Sodium deoxycholate: 10 mL working solution.
2. 75% TCA: 10 mL working solution from 100% reagent; (e.g., Sigma-Aldrich).
3. Acetone: pre-chilled at -20°C .
4. SpeedVac[®] or similar vacuum concentrator.
5. Sonication bath (optimal, but not essential).
6. Water bath (temperature at 70°C or 37°C).
7. NuPAGE Novex BisTris gel: 12% monomer (Invitrogen).
8. 4× NuPAGE LDS sample loading buffer (Invitrogen).
9. 10× Reducing agent (Invitrogen).
10. Antioxidant (Invitrogen).
11. 20× NuPAGE MOPS-SDS running buffer (Invitrogen).
12. XCell Surelock mini electrophoresis cell (Invitrogen).
13. Gel loading tips.
14. Electrophoresis power pack.

15. Coomassie-Blue G-250 stain: a pre-mixed colloidal dye solution such as SimplyBlue™SafeStain (Invitrogen).
16. Acetonitrile (ACN), LC grade or better.
17. 50% ACN with 0.1% trifluoroacetic acid (TFA): 100 mL ACN, 0.2 mL TFA, and 99.8 mL water.
18. 0.1% TFA: 0.1 mL TFA and 99.9 mL water.
19. 1 M ammonium bicarbonate (NH_4HCO_3) stock: 0.7906 g NH_4HCO_3 dissolved in 10 mL, pH 7.8.
20. 100 mM NH_4HCO_3 in 50% ACN: 1.0 mL 1 M stock, 5 mL ACN, and 4 mL water.
21. 40 mM NH_4HCO_3 in 10% ACN: 0.40 mL 1 M stock, 1 mL ACN, and 8.6 mL water.
22. 5% TFA in 50% ACN: 0.50 mL TFA, 5 mL ACN, and 4.5 mL water.
23. Trypsin Gold (Promega) or other mass spectrometry grade TCKP-treated trypsin.
24. 50 mM acetic acid: 0.0287 mL glacial acetic acid into 9.97 mL water.
25. 10 mM dithiothreitol (DTT) in 25 mM NH_4HCO_3 : 15.4 mg/10 mL, prepared ahead and stored frozen in 0.5 mL aliquots.
26. 25 mM NH_4HCO_3 : 19.8 mg NH_4HCO_3 in 10 mL water. Important: always make up fresh.
27. 30 mM iodoacetamide (IAA) in 25 mM NH_4HCO_3 : 11.1 mg in 2.0 mL (always prepare fresh).
28. C_{18} ZipTips (Millipore, ZTC18S096).
29. Stainless steel MALDI target plate (matches local instrument).

2.3. Gel Electrophoresis Buffer Preparation

1. 1× NuPAGE® SDS Running Buffer: prepare 800 mL by adding 40 mL of 20× MOPS NuPAGE® SDS Running Buffer to 760 mL of deionized water (dH_2O). Mix thoroughly and set aside 600 mL of this solution for use in the lower (*outer*) buffer chamber of the XCell SureLock Mini-Cell.
2. Immediately, prior to electrophoresis, mix 500 μL of NuPAGE® Antioxidant into the remaining 200 mL of 1× Running Buffer for use in the upper (*inner*) buffer chamber of the XCell SureLock Mini-Cell.

2.4. Matrix and Calibration Standards Preparation for MALDI-TOF MS Analysis

1. CHCA (matrix) solution: dissolve 10 mg of recrystallized α -cyano-4-hydroxy-cinnamic acid (CHCA, Sigma Aldrich) into 1 mL of solution containing 49.5% ethanol, 49.5% ACN, and 1% aqueous 0.1% TFA. Prepare and use as freshly made solution.

2. Sodiated PEG (polyethylene glycol) for instrument mass calibration standards: Prepare 10 mg/mL stock solutions for each PEG in 50% ACN, then for final working solution, mix 1 μ L PEG 1000, 2 μ L PEG 2000, and 6 μ L PEG 3000 with 9 μ L of CHCA solution, then add 3 μ L of 2 mg/mL of sodium iodide.
3. Adrenocorticotrophic hormone (ACTH; 18–39 clip, 2,465.1989 Da, Sigma-Aldrich) for instrument resolution and sensitivity testing: Mix 1 μ L 1 mg/mL ACTH with 39.5 μ L of 0.1% TFA to give 10 pmol/ μ L. Mix 1 μ L of 10 pmol/ μ L solution with 9 μ L of 0.1% TFA to give 1 pmol/ μ L. Mix 1:1 with CHCA solution to give 500 fmol/ μ L.
4. GluFib peptide B (GFPB; 1,570.6774 Da, Sigma-Aldrich) for instrument lock mass: Mix 1 μ L of 1 mg/mL GFPB with 62.7 μ L of 0.1% TFA to give 10 pmol/ μ L. Mix 1 μ L of 10 pmol/ μ L solution with 9 μ L of 0.1% TFA to give 1 pmol/ μ L. Mix 1:1 with CHCA solution to give 500 fmol/ μ L.

3. Methods

Protein samples are initially prepared for SDS-PAGE by TCA precipitation in the presence of sodium dioxicholate (see Subheading 3.1). This step is used to stabilize and concentrate proteins in samples extracted from cells and tissues. Recombinant enzymes secreted into culture media, as described in Chapter 21 of this book, were prepared and analyzed by the methods provided here. Your target protein(s) is assumed to be expressed in a soluble form and in sufficient yield that it is readily observable by SDS-PAGE (see Note 2). If SDS-PAGE methods (Subheading 3.2) are routinely used in your laboratory, and your protein of interest is observable as a band on a Commassie Blue stained gel, then, proceed to Subheading 3.3 for trypsin digestion. The dried peptides recovered from the trypsin digestion can be submitted directly to a MALDI-TOF MS facility, or if you have access to a local instrument, Subheading 3.4 describes sample clean up. The process for sample preparation is summarized in Fig. 1. Subheading 3.5 introduces use of the Mascot search engine with PMF data acquired from your samples to established protein identity.

3.1. Trichloroacetic Acid Precipitation of Protein Samples (8)

1. Pipet a volume from samples determined to contain 10–100 μ g of total protein into a 0.5-mL microcentrifuge tube and add water or extraction buffer to provide a final volume of 100 μ L (see Note 3).
2. Add 10 μ L of 0.15% sodium deoxycholate, mix well, and place on ice for 10 min.
3. Add 10 μ L of 75% TCA, mix well, and place on ice for 20 min.

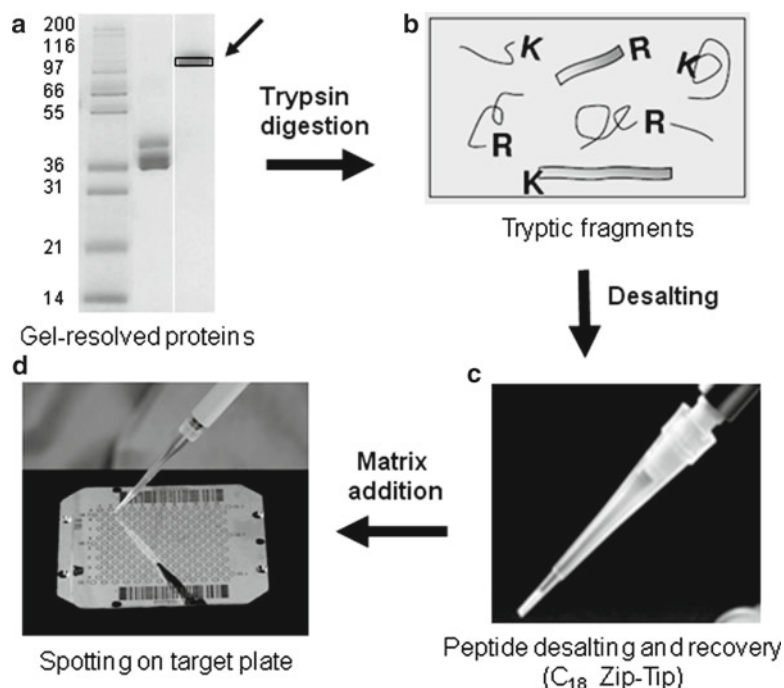


Fig. 1. Sample preparation for MALDI-TOF MS analysis. The selected protein band (*indicated by small arrow*) is excised from the SDS-PAGE gel (a) and transferred to a microcentrifuge tube to be destained and digested with trypsin. The digested fragments (b) are desalted using a ZipTip (c) and are directly eluted into the CHCA matrix solution, which is then spotted onto the target plate. (d) Lanes in the SDS-PAGE gel are molecular weight markers and two recombinant xylanolytic enzymes (β -xylanase C, AN1818.2; and β -xylosidase, AN2359.2; which are described in Chapter 21).

4. Centrifuge in a microcentrifuge at ca. $17,000 \times g$ for 10 min and immediately decant or siphon off the supernatant (carefully draw from the side opposite of the pellet). The pellet will appear as a slight white residue along the outer side of the microcentrifuge tube.
5. Wash pellet with 0.25 mL of cold acetone. Incubate at -20°C for 20 min (see Note 4).
6. Centrifuge again for 10 min and aspirate acetone from the pellet. Apply vacuum in a SpeedVac briefly to remove traces of solvent, but do not dry excessively. At this stage, you can cap and store samples at -20°C until ready to run electrophoresis.
7. Dissolve the dried pellet in 50 μL of $1\times$ LDS sample loading buffer using vigorous vortexing or sonication – the buffer solution should be blue in color (see Note 5).
8. Heat samples at 70°C for 10 min, then place on ice. Centrifuge again before loading on to an electrophoresis gel.

3.2. SDS-PAGE for Separating Proteins (9)

1. Prepare precast BisTris page gel and assemble electrophoresis cell (see Note 6).
2. Fill fully the upper (inner) buffer chamber with 1× running buffer containing antioxidant. Confirm there is a good seal – no immediate leakage is observed from the chamber.
3. Fill the lower (outer) buffer chamber with 600 mL of 1× running buffer.
4. Using a pipet with gel loading tips, load protein samples, control proteins, and molecular mass markers (see Note 7).
5. Run power with 200 V constant for 55 min.
6. Rinse the gel three times for 5 min each with 100 mL of dH₂O (discard each rinse). Be sure gloves are on and rinsed before handling gel!
7. Stain the gel with *ca.* 25 mL with Coomassie Brilliant Blue for up to 1 h at room temperature (RT) with gentle mixing.
8. Discard the staining solution, rinse the gel briefly in dH₂O and then continue destaining for 1 h with gentle mixing. Repeat with a second 100 mL volume to further clear gel background.
9. Transfer gel to a Zip-lock bag and obtain a gel image, such as by flatbed scanner (600 dpi) (Fig. 1). Gel can be stored in temporarily at 4°C (1 or 2 days) before further processing by trypsin digestion in the next section.

3.3. In Gel Trypsin Digestion of Protein Samples (10)

1. Place the gel on a clean glass cutting surface. Cut out the target protein bands using a new razor blade. Transfer the gel slices (see Note 8) to a prewashed 0.5-mL microcentrifuge tubes (see Note 9).
2. Destain gel pieces by adding 200 µL of 100 mM NH₄HCO₃ in 50% ACN, shake well, cap the tubes, and incubate at 37°C for 45 min. Spin down briefly; then pipet off (or use vacuum siphon) supernatant and discard. Repeat this step once.
3. Add 100 µL of 10 mM DTT in 25 mM NH₄HCO₃ at 37°C for 30 min to reduce proteins in gel pieces. Remove the supernatant.
4. Add 100 µL of 30 mM IAA in 25 mM NH₄HCO₃ and incubate *in the dark* at 37°C for 30 min to alkylate cysteine residues. Remove the supernatant.
5. Add 200 µL of 25 mM NH₄HCO₃ for 15 min at RT with occasional mixing to wash gel pieces. Spin down the gel slices and discard the supernatant. Repeat this twice.
6. Add 100 µL of 100% ACN for 10 min at RT to dehydrate gel pieces. The gel pieces will shrink and become “whitish.” Briefly spin down and remove the solvent, then dry gel pieces in the SpeedVac (for 10–15 min – no heat). Samples can be stored temporarily in this dried form (longer term storage with desiccant in the freezer).

7. Add 25 μL of trypsin working solution (or about 1.5 \times the original gel slice volume) to each tube, cap tightly, and incubate for 1 h at RT (see Note 10). The slices will rehydrate during this time period promoting trypsin diffusion into the gel slice. If the gel slices appear white or opaque after 1 h, add an additional 10 μL of trypsin working solution and further incubate 30 min.
8. Remove excess trypsin solution that is not absorbed into the gel piece, then add 50 μL of 40 mM NH_4HCO_3 in 10% ACN. Cap the tubes tightly and incubate overnight at 37°C.
9. The next day, add 150 μL of ultrapure water to the gel slice containing tryptic digests and incubate for 15 min with frequent vortex mixing (use sonication bath, if available). Spin down briefly and transfer the supernatant containing the extracted peptides to a new pre-washed and labeled microcentrifuge tube.
10. Add 50 μL of 5% TFA in 50% ACN to the gel pieces and incubate at 37°C for 30 min (vortex or sonicate intermittently) to further extract peptide products. Spin down the gel slices and pool with the supernatant from step 9. Repeat this step once.
11. Dry the pooled supernatants in the SpeedVac. Do not use heat. Samples can be stored in this dry form until ready for desalting and MS data acquisition (see Note 11).

**3.4. C_{18} ZipTip
Treatment of Samples
and Spotting on the
MALDI Target Plate
(see Note 12)**

1. Reconstitute dried peptide samples by adding 10 μL of 0.1% TFA – vortex or sonicate to dissolve. Briefly centrifuge to bring fluid to bottom of the tubes and set aside.
2. Place *ca.* 1.0 mL each of 100% ACN, 50% ACN containing 0.1% TFA, and 0.1% TFA into cleaned microcentrifuge tubes. These are working solutions for ZipTip activation.
3. Prepare a C_{18} ZipTip by pipetting in 10 μL of 100% ACN and expelling it to waste, three times, then repeating with three times with 10 μL of 50% ACN containing 0.1% TFA, then finally treating three times with 10 μL of 0.1% TFA. Do not draw air into the resin bed between volumes.
4. Bind the peptides to the ZipTip resin by pipetting in and out the 10 μL peptide sample five times.
5. Wash the ZipTip by pipetting in 10 μL of fresh 0.1% TFA and discharging the solution to waste. Repeat this at least three times.
6. Elute peptides by pipetting in 10 μL of freshly prepared CHCA solution, then expelling into a pre-washed and labeled microcentrifuge tube. Re-pipet the sample volume in and out three cycles, being careful to not introduce air into the resin packing. Expel the last volume of solvent from the tip by raising the tip just above the solution in the tube and pushing out with air.

7. Pipet 1 μL of each target protein sample and control standards (gel blank and BSA or ADH digestions) onto wells of a MALDI target plate (see Note 13). Make sure you record on a log sheet the well positions of all samples and standards.
8. Pipet 1 μL each of a resolution check standard working solution (e.g., ACTH) and calibration standard working solution (e.g., sodiated PEG) onto wells of the target plate and finally 1 μL of GFPB solution onto calibration (a.k.a. lock mass) wells of the target plate.
9. Place target plate in a covered desiccator for 20 min at RT. Do not warm the plate during solvent evaporation. The MALDI target plate is now spotted and ready for loading into the MALDI-TOF MS instrument for data acquisition (see Note 14).

3.5. Identification of Recombinant Proteins with Mascot (see Note 15)

1. Go to <http://www.matrixscience.com> to open *Mascot* search engine. Click on *Peptide Mass Fingerprinting* to open the program for information entry (see Note 16).
2. Enter the all necessary information and search parameters. Provide *Name*, *Email*, and *Search Title*. Select: *Database(s)* (*SwissProt and/or NCBIInr*), *Enzyme* (*Trypsin*), and *Allow up to* (1) missed cleavages. For *Taxonomy*, select appropriate classification to limit the taxonomic search to related groups, e.g., (*Viridiplantae*) for all green plants. For *Fixed modifications*, select (*carbamidomethyl*) for alkylated cysteine residues and for *Variable modification*, select (*Oxidation – M*) for variable oxidation of methionine residues. The field *Protein mass* is left blank and set *Peptide tol. \pm* to (*50 ppm*), and finally select for *Mass values* (*M + H⁺*) and *Monoisotopic* ions.
3. Select *Browse* to activate window to select file path to the saved MS data file, launch by activating *Start Search* button.
4. Your search result will be displayed with a histogram indicating best “hits” (protein scores with significance $p < 0.05$) (Fig. 2b).
5. If your protein is not indicated in the Protein Summary Report, manually inspect the MS spectrum using the list of peptide ions generated by in silico trypsin digestion of your recombinant protein (see Note 16).

4. Notes

1. Use powder-free nitrile gloves (rinsed with dH_2O) at all times for handling gels, tips, vials, etc. Use “low-protein-binding” polypropylene tubes that are cleaned of plasticizers, as well as new vials, disposable plasticware, and new razor blades. We recommend using high quality bottled water, such as a Spectro- or

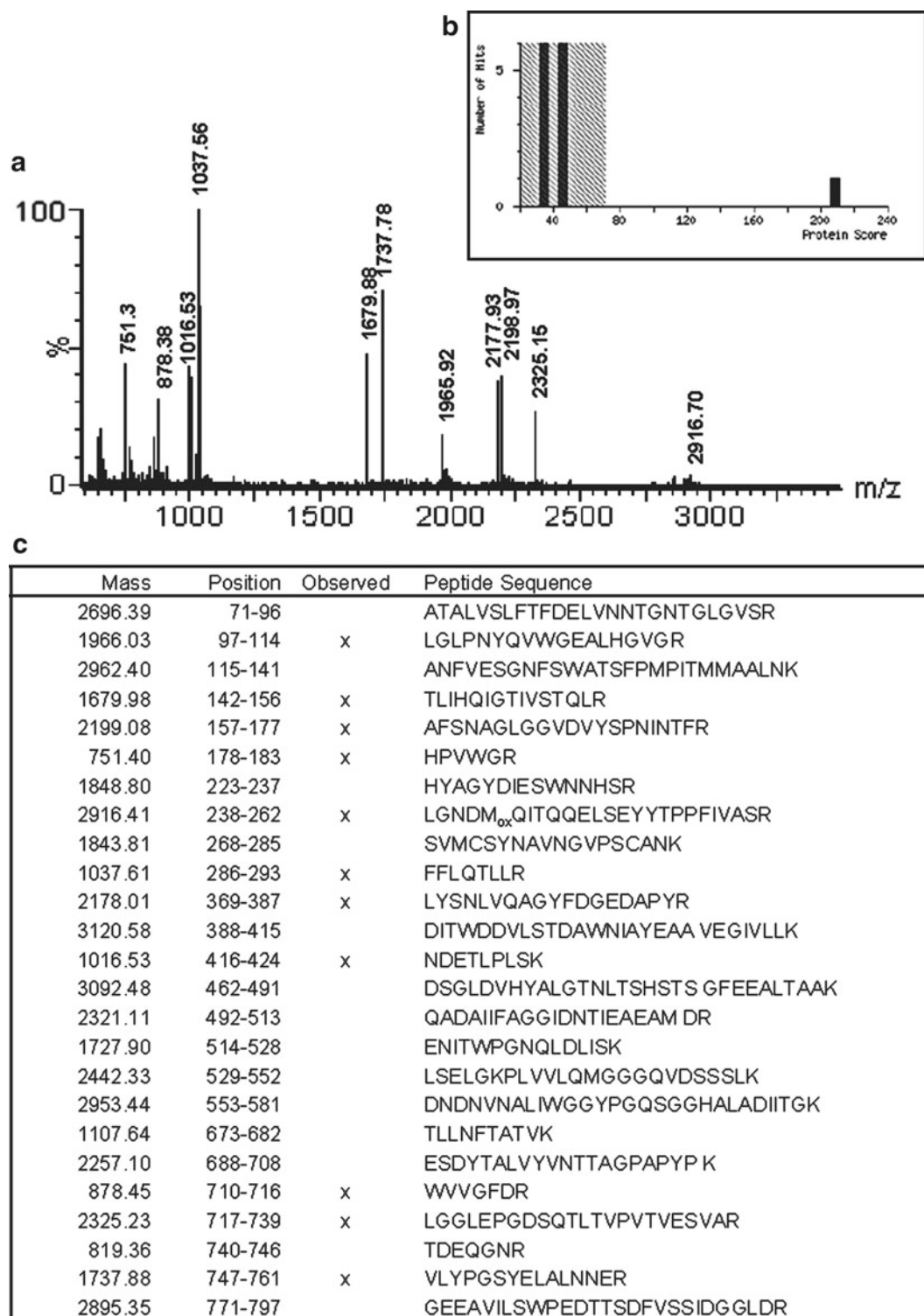


Fig. 2. The MALDI-TOF mass spectrum ("peptide mass fingerprint") (a) from a trypsin digest of *Aspergillus nidulans* β -xylosidase, AN2359.2 (see Chapter 21). The inserted histogram (b) shows results from a protein database search with the MS spectrum peak list data using Mascot. The top score (209) matches the correct *A. nidulans* accession. Theoretical peptide ion list from the protein sequence (c) (range for 750–3,500 m/z) is used for direct manual inspection of MS data. Peptides observed in the spectrum are indicated. Sequence of coverage is commonly 30–60%.

LC-grade water for preparing MS reagent solutions. Laboratory water deionization systems (e.g., Nanopure or MilliQ) that are regularly sanitized may provide suitable in place of bottle ultrapure water. Always use fresh solutions, including electrophoresis solutions and reagent buffers. Minimize introduction of dust by storing materials in sealed dust-free containers. If available, use a laminar-flow transfer hood during all steps for processing samples following the electrophoresis stage. Clean staining containers and all other equipment that comes into contact with the gel or gel slices with laboratory detergent (e.g., Alconox), rinse thoroughly with dH₂O, and finally wipe with alcohol or 50% aqueous acetonitrile immediately prior to use.

2. In depth discussion and details for extraction, solubilization, and quantification of protein preparations are readily found in manuals and reviews (11–13). For concentration of dilute proteins (such as from culture medium), microcentrifuge ultrafiltration devices can also be used (e.g., Millipore Microcon-3).
3. Approximate protein determination is needed to calibrate sample protein loadings on SDS-PAGE gels. We use Coomassie Brilliant Blue G-250 dye (“Bradford’s reagent”) for routine protein determination. Prepared solutions are available from vendors such as Pierce Chemical or Bio-Rad.
4. If a large amount of protein is precipitated resulting in a thick pellet, vigorously vortex or sonicate it to break it up, then incubate at –20°C, centrifuge, and repeat pellet washing with cold acetone.
5. A greenish-yellow sample indicates presence of residual TCA. This may be corrected with 1 µL aliquots of 1 M Tris solution. If the sample does not then readily turn blue, you should repeat the precipitation step by adding 4 volumes of cold acetone, incubate, and centrifuge as before.
6. The assembly of electrophoresis cells and running gels are taken from the Invitrogen instruction guide (9). Be sure to rinse out the samples wells in the BisTris precast gel with 1× NuPAGE running buffer and to remove the sealing tape on the bottom. Clamp the gel into XCell SureLock Mini-Cell according, using the plastic buffer dam if you are using only one gel.
7. Generally, load a volume of protein samples containing between 2.5 and 25 µg of total protein, depending on anticipated relative purity of the target protein(s). Ideally, minimal bands (at least 0.5 µg) stained by Coomassie Brilliant Blue are suited for processing for MALDI-TOF MS analysis. Loading samples at multiple volumes (e.g., 5, 10, and 20 µL) allow for variance from estimated protein contents. Be sure to use at least one known protein such as BSA or ADH loaded in separate lanes. These are used as positive controls to assess the effectiveness of the trypsin

digestion, peptide recovery, and peptide ion signal intensity. Leave at least one lane empty to use for negative control gel pieces in Subheading 3.3, step 1.

8. Gel bands should be cut at *ca.* 0.5–0.8 mm wide, eliminating as much unstained polyacrylamide as possible. For heavy protein bands, cut gel piece from the upper edge of the band (multiple bands can be excised and treated from large bands). From empty gel lanes, cut similarly sized gel pieces for use in negative control digestions – these samples are used to assess for background peptide ions introduced by systemic contamination.
9. Plasticizer residues present in microcentrifuge tube will interfere with MALDI-TOF MS analysis by contributing polymer peaks and suppressing peptide ionization by affecting matrix crystallization. Microcentrifuge tubes must be prewashed thoroughly with a solvent solution such as 50% ACN in 0.1% TFA to remove these residues. Fill microcentrifuge tubes with solution for *ca.* 10 min and then rinse with an alcohol or ACN. Store washed dry microcentrifuge tubes in a sealed container.
10. Trypsin Gold (V5280) from Promega (10) is prepared by adding 100 μ L of 50 mM acetic acid directly to the vial containing 100 μ g trypsin. After dissolving completely, dispense 5 μ L (5 μ g) aliquots into microcentrifuge tube (0.5 mL, prewashed) and store at -20°C freezer. To prepare working trypsin solution, add 45 μ L of 50 mM acetic acid to an aliquot, then dilute with 200 μ L of 40 mM NH_4HCO_3 in 10% ACN. This provides a final working solution with concentration of 20 $\mu\text{g/mL}$, which is sufficient for performing for 10–12 digestions. Unused trypsin working solution can be stored at -20°C ; discard after five freeze–thaw cycles. Trypsin digestion of proteins can be accelerated and peptide recovery improved with 0.1% w/v 1-*O*-*n*-octyl- β -d-glucopyranoside or the acid-labile surfactant RapiGest SF (0.05%) added to the working trypsin solution (14). These are particularly useful for hydrophobic proteins. Trypsin digestion is complete in 2 h, providing an alternative to overnight digestion.
11. Dried peptide samples are stable for short-term storage (refrigerated or in freezer), and you should submit your samples to a biomolecule mass spectrometry facility in this form. Contact the facility you will be sending your samples to for specific shipping instructions. To support effective analysis of a sample, send an image of the SDS-PAGE gel along with the samples and the expected amino acid sequence (best as a FASTA file). Include contiguous sequence(s) for any inserted epitope or other affinity tag (e.g., poly-His peptides). The facility should be able to do basic matching of peptide ions from the sequence with the MS spectrum and provide a summary of results. If you have direct local access to a MALDI-TOF MS instrument, proceed with ZipTip sample cleanup as described in Subheading 3.4.

12. This section describes final treatment to samples for analysis with a local MS instrument. C₁₈ ZipTips (Millipore; 15) are micropipet tips packed with 0.6 µL of C₁₈ reversed-phase resin. They are used to bind peptides from aqueous solution and wash them free of salts and contaminants introduced during gel digestion and extraction, resulting in improved spectral data from MALDI-TOF MS. ZipTip treatment is best done immediately before acquisition of MS data, because the CHCA used in the ZipTip elution solution will start to decompose once samples are spotted onto a target plate, resulting in degraded spectral data.
13. Stainless steel MALDI targets plates must match the instrument. Samples may be further diluted with matrix solution at 1:3 and 1:10 (v/v) before spotting, which sometimes improves signal intensities.
14. An experienced user must perform operation of the local mass spectrometer. Skill in instrument tuning and calibration is as critical as high quality sample preparation. MALDI-TOF MS spectrum peak list data files acquired from protein digests (i.e., the PMF) will be evaluated with integrated software for search and matching, or the data files are saved for export and/or later analysis. Be sure to obtain peak list data files for samples submitted to a core or commercial facility. How to use the Mascot search engine to identify a protein from MS spectrum peak list data file is described in the next section.
15. The high mass accuracy of current generation MALDI-TOF mass spectrometers enables confident identification of proteins from PMFs using a search engine such as Mascot (16). However, identification of an unknown protein requires that the sequence for it already exist in a protein database (e.g., NCBItr or SwissProt). A recombinant protein whose sequence that may not be publically disclosed can be identified simply from manual inspection of the MS spectrum for predicted peptide ions. There will be multiple peptide ions present that match masses calculated from a theoretical peptide list (Fig. 2). Software programs and related tools for protein identification and characterization are accessed through the EXPASy Proteomics Server (<http://expasy.org>). Links to various search engines are found there as well as prediction tools such as PeptideMass for in silico trypsin digestion.
16. To generate a list of theoretical peptide ions for manual inspection of an MS spectrum (Fig. 2) open PeptideMass at <http://www.expasy.ch/tools/peptide-mass.html> and paste in the sequence. The sequence of any signal peptide should be removed first using the SignalP 3.0 program found in the ExPASy Proteomic Tools. Select for peptide masses: cysteines treated with (*Iodoacetamide*) and check or activate “with methionine oxidized,” “[M+H]⁺,” and “monoisotopic.” Select an enzyme

(*Trypsin*) and set for display mass bigger than (750) and smaller than (3,000) Da. You can select to “sort by” peptide masses or in chronological (sequential) order in the protein. Click on *Perform* button to obtain the peak list.

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