

Differential Proteomics Based on Multidimensional Protein Identification Technology to Understand the Biogenesis of Outer Membrane of *Escherichia coli*

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Abstract

Cell envelope proteins in bacteria are typically difficult to characterize due to their low abundance, poor solubility, and the problematic isolation of pure surface fraction with only minimal contamination. Here we describe a method for cell membrane fractionation followed by mass spectrometry-based proteomics to analyze and determine protein abundance in bacterial membranes.

Key words MudPIT, Membrane proteins, Gel-free, Gram-negative cell envelope, Outer membrane, Cell fractionation

1 Introduction

The traditional proteomics approach is based on two-dimensional gel electrophoresis (2DG), where the protein spots of interest are isolated and identified by mass spectrometry (MS) via in-gel digestion. The 2DG approach has a relatively high resolution, which is limited however by the difficulty in detecting certain classes of proteins. These include membrane proteins due to their low solubility in gel electrophoresis buffer, proteins with either low (<10 kDa) or high (>200 kDa) molecular weight (MW), as well as those with an extreme isoelectric point (pI<4 or >9). Moreover, this approach has a limited dynamic range, which impairs the analysis of low-abundance proteins and it is tedious and time consuming.

These problems are overcome by “mass spectrometry-based proteomics,” using approaches such as Multidimensional Protein Identification Technology (MudPIT), which is a shotgun proteomics methodology [1]. MudPIT represents a fully automated technology based on two-dimensional capillary chromatography coupled to tandem mass spectrometry (2DC-MS/MS).

2DC-MS/MS combines ion exchange with reversed-phase separation of peptide mixtures obtained from direct digestion of total (or pre-fractionated) proteins by means of two micro- or nano-HPLC columns and direct analysis of eluted peaks by data-dependent fragmentation (MS/MS) [2]. This technology provides a significant improvement over gel-based proteomic analysis; in fact, MudPIT approach enables the quantitative determination of proteins [3] and the identification of the protein mixtures in wide pI and MW ranges. In addition, mass spectrometry-based proteomics approach allows the characterization of hydrophobic proteins making possible to analyze the proteome of membranes, from both eukaryotic [4] and prokaryotic cells [5].

The outer membrane (OM) is the hallmark of gram-negative bacteria. The cell envelope of these diderm organisms consists two membranes that delimit an aqueous space, the periplasm, containing a thin layer of peptidoglycan. The inner (cytoplasmic) membrane (IM) is composed by phospholipids whereas the OM is an atypical asymmetric membrane composed of phospholipids in the inner leaflet and a unique glycolipid, lipopolysaccharide (LPS), in the outer leaflet [6]. IM and OM contain many integral and peripheral proteins as well as lipoproteins to fulfil the numerous functions played by the bacterial envelope including metabolic activities, signal transduction, interaction with the environment, adhesion, and immune evasion [7–9]. Based on their different structure and composition, IM and OM possess different densities and these properties allow their separation onto a discontinuous sucrose gradient [10, 11].

Here, we combine cell fractionation procedures to separate IM and OM membranes and therefore their respective proteins, with the MudPIT technology to analyze and characterize the membrane of *Escherichia coli*.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents.

Diligently follow all waste disposal regulations when disposing waste materials.

Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Membrane Purification and Fractionation

1. LD medium: Dissolve 10 g bacto-tryptone, 5 g yeast extract, and 5 g NaCl in 900 mL deionized water. Adjust pH to 7.5 with NaOH. Make up to 1 L with deionized water. Sterilize by autoclaving.
2. Potassium phosphate buffer, pH 8: Prepare solution A dissolving 27.8 g $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ in 1 L deionized water, and solution

B dissolving 53.65 g $K_2HPO_4 \cdot 7H_2O$ in 1 L deionized water. To obtain potassium phosphate buffer 0.1 M mix 5.3 mL of solution A and 94.7 mL of solution B, and make up to 400 mL with deionized water.

3. Tris-HCl pH 8.0, 1 M: Dissolve 121.1 g of Tris base in 800 mL of water. Adjust pH 8 by adding concentrated HCl. Make up to 1 L with water and sterilize by autoclaving.
4. EDTA 0.5 M: Weigh 186.12 g EDTA, transfer it to 2 L beaker, and add 800 mL deionized water. While stirring vigorously on a magnetic stirrer, add NaOH pellet or 10 N NaOH to adjust the solution to pH 8.0 (*see Note 1*). Make up to 1 L with water. Filtration can be used to remove any undissolved material.
5. Phenylmethylsulfonyl fluoride (PMSF): 0.1 M solution. Store at 4 °C.
6. Lysozyme: Dissolve solid lysozyme at a concentration of 10 mg/mL in 10 mM Tris-HCl pH 8, immediately before use.
7. DNase: Dissolve solid DNase at 50 mg/mL in MilliQ water. Store at -20 °C.
8. Sucrose solution, 0.25 M: Weigh 85.57 g sucrose, transfer it to 2 L beaker, and add 800 mL of 10 mM Tris-HCl pH 8.0. Mix vigorously on a magnetic stirrer until the sucrose is dissolved. Make up to 1 L with 10 mM Tris-HCl pH 8.0.
9. Solutions of increasing sucrose concentration: Weigh 855.6 g sucrose, transfer it to 2 L beaker, add 460 mL of 10 mM Tris-HCl pH 8 and 5 mM EDTA, and mix vigorously on a magnetic stirrer until the sucrose is dissolved to obtain a 65% (w/w) sucrose solution. 55, 50, 45, 40, 35, 30, and 25% sucrose solutions are obtained by dilution in 10 mM Tris-HCl pH 8 and 5 mM EDTA. Store at 4 °C.

2.2 Resolution of Inner and Outer Membranes

1. β -Nicotinamide adenine dinucleotide (NADH), 50 mM solution: Weigh 35 mg of solid NADH and dissolve it in 1 mL of Tris-HCl 50 mM Tris-HCl pH 7.5. Make it fresh each time and keep on ice and away from light.
2. Pierce™ Coomassie Protein Assay: Store at 4 °C.
3. Albumin standard ampules (BSA), 2 mg/mL: Prepare by serial dilutions (2 \times) of BSA standards: 2, 1, 0.5, 0.25, and 0.125 mg/mL.
4. Loading buffer (5 \times): 0.12 M Tris-HCl (pH 6.8), 5.5% SDS, 9% β -mercaptoethanol, 0.01% bromophenol blue (BPB), 15% glycerol.
5. Acrylamide/Bis-acrylamide 37.5/1, 30% solution.
6. SDS-PAGE running buffer (10 \times): Weigh 30 g Trizma base, 144 g glycine, 10 g SDS. Transfer Trizma base and SDS to 2 L

beaker and add 800 mL deionized water. While stirring vigorously on a magnetic stirrer, add glycine gradually. Make up to 1 L with water.

7. Resolving gel buffer: 1.37 M Tris-HCl, pH 8.8, 0.4% SDS. Dissolve 187 g Trizma base in 800 mL water. Add 40 mL of 10% SDS solution and adjust solution pH 8.8 with HCl. Make up to 1 L with water.
8. Stacking gel buffer: 0.45 M Tris-HCl, pH 8.8, 0.4% SDS. Dissolve 60.6 g Trizma base in 800 mL water. Add 40 mL of 10% SDS solution and adjust solution pH 6.8 with HCl. Make up to 1 L with water.
9. Ammonium persulfate: 10% solution in water. Store at 4 °C.
10. *N,N,N',N'*-Tetramethylethylenediamine (TEMED): Store at 4 °C.
11. Prestained protein MW marker: Broad range 10–180 kDa. Coomassie blue staining solution: 0.15% w/v Coomassie Brilliant Blue R-250, 50% ethanol, 10% acetic acid solution in water.
12. Destaining solution: 30% ethanol, 10% acetic acid solution in water

2.3 MudPIT Analysis

1. Ultrapure water.
2. Ammonium bicarbonate (NH_4HCO_3) 0.1 M pH 8.0: Add about 50 mL water to a 250 mL graduated cylinder. Weigh 1.97 g ammonium bicarbonate and transfer to the 250 mL graduated cylinder. Add water to a volume of about 200 mL. Mix and adjust pH if necessary. Make up to 250 mL with water. Store at room temperature.
3. RapiGest SF (Waters Corporation, Milford, MA, USA): Store at 4 °C.
4. SPNTM—Protein Assay (G-Biosciences, St Louis, MO, USA).
5. Trypsin: Split into aliquots of 1 µg and store at -20 °C.
6. Acetonitrile (CH_3CN), HPLC gradient grade.
7. Trifluoroacetic acid (TFA) eluent additive for LC-MS (CF_3COOH).
8. PepCleanTM C-18 Spin Columns.
9. Vacuum system.
10. Formic acid (HCOOH), eluent additive for LC-MS, ~98%.
11. Evolution 60S, UV-visible spectrophotometer.

2.4 MS Analysis

1. 0.1% Formic acid in water (eluent A).
2. 0.1% Formic acid in acetonitrile (eluent B).
3. Ammonium chloride (NH_4Cl).

4. Solutions of increasing ammonium chloride concentration: 20, 40, 80, 120, 200, 400, and 700 mM. Prepare 1 mL for each concentration. In each 1 mL glass vial put 20, 40, 80, 120, 200, 400, and 700 μ L of ammonium chloride and add, respectively, 980, 960, 920, 880, 800, 600, and 300 μ L of 0.1% formic acid in water.
5. Surveyor AS (Thermo Finnigan Corp., San Jose, CA, USA).
6. Biobasic SCX column, 0.32 i.d. \times 100 mm, 5 μ m (Thermo Electron Corporation, Bellefonte, PA, USA).
7. Peptide trap (Zorbax 300 SB-C18, 0.3 mm \times 5 mm, 5 μ m) (Agilent Technologies, Santa Clara, CA, USA).
8. Biobasic C18 column (0.180 i.d. \times 100 mm, 5 μ m) (Thermo Electron Corporation, Bellefonte, PA, USA).
9. Nano-LC electrospray ionization source (nano-ESI) (Thermo Finnigan Corp., San Jose, CA, USA).
10. LCQ Deca XP plus or LTQ (Thermo Finnigan Corp., San Jose, CA, USA).

2.5 Computational Analysis

1. Cluster PC.
2. Non-redundant *Escherichia coli* protein sequence database downloaded from the NCBI website (<http://www.ncbi.nlm.nih.gov/>).
3. Bioworks version based on SEQUEST algorithm (University of Washington licensed to Thermo Fisher Scientific Inc., USA).
4. Proteome Discoverer version (Thermo Fisher Scientific Inc., USA).
5. MAProMa software (Multidimensional Algorithm Protein Map) [12].
6. EPPI (Experimental Proteotypic Peptide Investigator) [13].

3 Methods

Perform all procedures at room temperature unless otherwise specified.

The use of low-retention pipette tips and the use of glass vials or inserts are suggested in order to minimize sample loss, in particular for peptide mixtures.

The whole procedure is depicted in Fig. 1.

3.1 Bacterial Growth

Grow the *E. coli* bacterial strain static at 37 °C in 5 mL LD for 16–18 h. Set up the control and the treated cultures diluting the static pre-culture to an $OD_{600}=0.05$ in 500 mL of suitable LD medium. Grow at 37 °C with aeration (160–180 rpm) for 300–330 min and harvest 125 OD_{600} of each culture by centrifugation

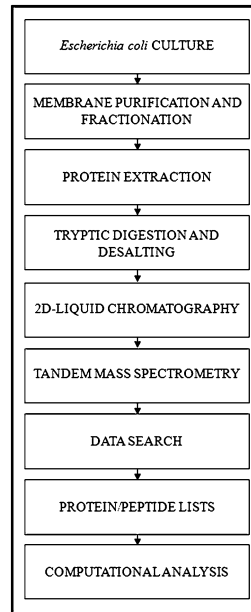


Fig. 1 Experimental procedure flow chart. General steps for the differential analysis of membrane proteome

at $3000 \times g$ at 4°C . Wash cells with 10 mL of 0.1 M potassium phosphate buffer pH 8.0 and collect cells by centrifugation at $3000 \times g$ at 4°C . Cell pellets can be stored at -20°C before further processing.

3.2 Whole Membrane Purification

1. Resuspend cell pellets (125 OD_{600}) in 3 mL of 10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM PMSE, and 0.2 mg/mL lysozyme and incubate on ice for 30 min. Add 0.2 mg/mL DNase and disrupt cells by a single passage through a cell disruptor at 25,000 psi (*see Note 2*).
2. Unbroken cells are removed by centrifugation at $3000 \times g$ for 20 min at 4°C . The cleared lysates were then subjected to ultracentrifugation at $100,000 \times g$ for 60 min at 4°C . Discard the supernatant, resuspend pellets that contain whole-cell membranes, in 0.4 mL of MilliQ water, and lyophilize for 16–18 h.
3. Lyophilized samples are then ready to be processed according to the method in Subheading 3.5 below.

3.3 Membrane Fractionation

1. Resuspend cell pellets (125 OD_{600}) in 12 mL of 10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM PMSE, and 0.2 mg/mL lysozyme and incubate on ice for 30 min. Add 0.2 mg/mL DNase and disrupt cells by a single passage through a cell disruptor at 25,000 psi (*see Note 3*).
2. Unbroken cells are removed by centrifugation at $3000 \times g$ for 20 min at 4°C . The cleared lysates are then subjected to ultracentrifugation at $100,000 \times g$ for 90 min in an ultracentrifuge.

Discard the supernatant and resuspend pellets that contain whole-cell membranes, in 9 mL of 0.25 M sucrose and 3.3 mM Tris pH 8.0.

3. Whole-cell membranes are ultracentrifuged at $100,000 \times g$ for 90 min in an ultracentrifuge. Discard supernatant and resuspend membranes in 0.8 mL 25 % sucrose. Save 50 μ L for total protein quantification (see below).
4. Prepare a discontinuous sucrose gradient in a 12 mL polyallomer tube by layering the following sucrose solutions: 0.5 mL 55 %, 2 mL 50 %, 2 mL 45 %, 2 mL 40 %, 2 mL 35 %, and 2 mL 30 % (see **Note 4**). Load 750 μ L of membranes in 25 % sucrose on the top of the sucrose gradient. Membranes are ultracentrifuged for 13 h at $240,000 \times g$ at 4 °C in a TH-641 swinging bucket rotor in an ultracentrifuge (see **Note 5**).
5. 0.3 mL Fractions (usually 36 fractions) are collected manually from the top of each tube and stored at -20 °C. Usually odd fractions are analyzed.

3.4 Resolution of Inner and Outer Membranes from Sucrose Gradient

The distribution of inner and outer membranes along the sucrose gradient is analyzed as follows: total protein profile along the gradient is assessed by the Bradford assay, the NADH activity is assayed as a marker of the inner membrane, and the profile of OmpC/F and OmpA porins, assessed by SDS-polyacrylamide gel electrophoresis, is used as a marker of outer membrane.

3.4.1 Total Protein Profile: Bradford Assay

Dilute the Bradford reagent twofold in deionized water (1 part Bradford:1 part water). Add 5–10 μ L of each fraction to 1 mL of the diluted reagent and mix. Incubate at room temperature for 5 min and measure the blue color formed at 595 nm. Prepare a standard curve with samples of known protein concentration using a serial dilution series (0.125–2.0 mg/mL) of BSA. Using the standard curve extrapolate the amount of BSA in a given fraction; determine protein concentration in the fraction by dividing the amount of protein by the sample volume.

3.4.2 Inner Membrane Profile: NADH Activity Assay

Incubate mixtures containing 50 mM Tris-HCl pH 7.5, 0.3 mM NADH, and 50 μ L of each fraction in a volume of 1 mL. The decrease in absorbance at 340 nm in 1 min is measured at room temperature in a JASCO V-550 spectrophotometer. The specific activity was calculated using an extinction coefficient of 6.22 mM/cm (see **Note 6**).

3.4.3 Outer Membrane Profile: SDS-Polyacrylamide Gel Electrophoresis and Coomassie Blue Staining

1. Prepare 12.5 % running gel by mixing 2.5 mL of resolving buffer, 4.16 mL of acrylamide solution, and 3.34 mL of water in a 50 mL conical flask. Add 50 μ L of 10 % ammonium persulfate and 10 μ L of TEMED, mix gently, and cast gels within a 1.0 mm spaced gel cassette (4.5 mL for each gel). Gently overlay with ethanol.

2. Prepare 4.5 stacking gel by mixing 2.5 mL of stacking buffer, 1.5 mL of acrylamide solution, and 6 mL water. Remove ethanol from the top of stacking gel with filter paper. Add 50 μ L of 10% ammonium persulfate and 15 μ L of TEMED to the mix and layer it on stacking gel. Insert a 10-well gel comb avoiding air bubble formation.
3. Add 5 μ L of loading buffer 5 \times to 20 μ L aliquot from odd fractions and heat in boiling water for 5 min. Centrifuge the heated samples for 0.5 min at max speed to remove insoluble material and load 20 μ L of each fraction on two gels. Prestained protein ladder is used as marker. Run the gels at 100 V until the BFB has reached the bottom.
4. At the end of the run, separate the gel plates and remove the stacking gel. Put the gel in a glass bowl.
5. Rinse the gel with water to remove traces of running buffer.
6. Stain the gel with Coomassie blue staining solution for 1 h.
7. Destain the gel by soaking for at least 2 h in destaining solution with at least two changes of this solvent. If the gel still has a Coomassie blue background then continue destaining until the background is nearly clear. Rinse the gel with water and store destained gel in MilliQ water. OmpC/F and OmpA are visible on SDS-PAGE gel as polypeptides migrating approximately at 40 and 37 kDa.
8. Fractions containing the inner membrane and those containing the outer membrane are separately pooled. Inner membrane and outer membrane pools are ultracentrifuged at 100,000 $\times g$ for 2 h in an ultracentrifuge at 4 °C, to remove the sucrose. The pellets are resuspended in 2 mL of water and lyophilized for 16–18 h.
9. Lyophilized samples are then ready to be processed according to the method in Subheading 3.5 below.

3.5 Sample Preparation for MudPIT Analysis

1. Resuspend the lyophilized sample in 100 μ L of 0.1 M ammonium bicarbonate, pH 8.0. Control pH and make sure that pH > 7 (*see Note 7*).
2. Reconstitute the 1 mg lyophilized RapiGestTM SF (Waters Corporation) in 100 μ L of ammonium bicarbonate 0.1 M pH 8.0 to reach the concentration of 1% w/v (*see Note 8*).
3. Add RapiGestTM SF to sample to give 0.2% (w/v) (*see Note 9*).
4. Boil the protein/RapiGestTM SF mixture at 100 °C for 15 min.
5. Cool the sample to room temperature for 5 min and then quantify protein content using SPNTM-Protein Assay (G-Biosciences) (*see Note 10*).
6. Put 50 μ g of sample in a glass insert and add acetonitrile (ACN) at the final concentration of 10% (w/v).

7. Digest protein sample adding sequencing-grade trypsin in a ratio of 1:50 (1 μg enzyme:50 μg substrate) and incubate at 37 °C overnight, at 300 rpm (*see Note 11*).
8. To improve the digestion efficiency, add a second aliquot of trypsin in a ratio of 1:100 (0.5 μg enzyme:50 μg substrate) and then incubate the sample at 37 °C for 4 h, at 300 rpm.
9. Stop the tryptic digestion adding trifluoroacetic acid (TFA); the final TFA concentration should be 0.5 % (w/v). Make sure that pH is 2 using a litmus paper.
10. Evaporate ACN in a vacuum system (60 °C for 5 min). Prevent loss of peptides, trying not to dry the sample.
11. Incubate peptide mixture at 37 °C for 45 min (300 rpm) and then centrifuge acid-treated sample at 13,000 $\times g$ for 10 min; a pellet may be observed.
12. Collect the supernatant and discard the pellet.
13. Desalt and concentrate the sample using PepClean™ C18 Spin Column (*see Note 12*).
14. Gently dry the sample in a vacuum system at 60 °C and wash it three times with 20 μL of 0.1 % formic acid in water.
15. Reconstitute sample in 50 μL of 0.1 % formic acid to obtain a final protein concentration of 1 $\mu\text{g}/\mu\text{L}$.

3.6 MS Analysis

Trypsin-digested peptides are analyzed by two-dimensional micro-liquid chromatography coupled to ion trap mass spectrometry (MudPIT).

1. By means of an autosampler, load 10 μL of the digested peptide mixtures onto a strong cation-exchange column and then elute using eight steps of increasing ammonium chloride concentration (0, 20, 40, 80, 120, 200, 400, and 700 mM). Eluted peptides, obtained by each salt step, are at first captured in turn onto two peptide traps mounted on a 10-port valve, for concentration and desalting, and subsequently loaded on a reversed-phase C-18 column for separation with an acetonitrile gradient. Set the gradient profile as shown: 5–10 % eluent B in 5 min, 10–40 % eluent B in 40 min, 40–80 % eluent B in 8 min, 80–95 % eluent B in 3 min, 95 % eluent B for 10 min, 95–5 % eluent B in 4 min, and 5 % eluent B for 15 min (eluent A, 0.1 % formic acid in water; eluent B, 0.1 % formic acid in acetonitrile). The flow rate is 100 $\mu\text{L}/\text{min}$ split in order to achieve a final flux of 1.5 $\mu\text{L}^{-1} \text{min}^{-1}$ (*see Note 13*).
2. The peptides eluted from the C-18 column are directly analyzed with an ion trap mass spectrometer equipped with a nano electrospray ionization source (nano-ESI). Nanospray is achieved with an un-coated fused silica emitter held to 1.5 kV and the heated capillary is held at 185 °C (*see Note 14*).

3. Acquire full mass spectra in positive mode and over a 400–2000 m/z range, followed by three MS/MS events sequentially generated in a data-dependent manner on the first, second, and third most intense ions selected from the full MS spectrum, using dynamic exclusion for MS/MS analysis (collision energy 35%) (*see Note 15*). MudPIT analysis generates files in .raw format, containing the experimental mass spectra (full Ms and MS/MS); the number of files created is equivalent to the salt step number set in the analysis.

3.7 Computational Analysis

1. From National Central for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>) download the non-redundant (NR) protein sequence database in FASTA format.
2. From “NR” retrieve the *Escherichia coli* protein sequence database.
3. Save .raw files and protein sequence database into computer equipped with SEQUEST algorithm (*see Note 16*).
4. Using software based on SEQUEST algorithm (University of Washington), such as Bioworks (Thermo Fisher Scientific Inc.) or Proteome Discoverer (Thermo Fisher Scientific Inc.), correlate the experimental mass spectra to tryptic peptide sequences by comparing with theoretical mass spectra, obtained by in silico digestion of *Escherichia coli* protein database. Set the following parameters: no enzyme mode (*see Note 17*), tolerance on the mass measurements of 2.00 amu for precursor peptide, and 1.00 amu for fragment ions.
5. Combine the files generated by applying stringent filters: Xcorr scores greater than 1.5 for singly charged peptide ions and 2.0 and 2.5 for doubly and triply charged ions, respectively, peptide probability ≤ 0.001 , and protein consensus score value ≥ 10 .
6. Export and save in Excel format the protein list and the peptide list (*see Note 18*).
7. From the protein list generate the 2D map of the sample using software such as an in-house algorithm called MAProMa (Multidimensional Algorithm Protein Map) [12]. Proteins are plotted according to their theoretical pI and MW.
8. Compare the protein lists obtained from the analysis of all samples with MAProMa or similar software; the final file is a list of all the proteins identified in the samples and for each protein is reported the values of SEQUEST Spectral Count (SpC) and of SEQUEST score.
9. Verify the repeatability of the analysis by plotting SEQUEST scores of each identified protein in the first technical replicate versus the second technical replicate. Evaluate the linear correlation (R_2) and compare the slope value obtained with the theoretical value (1.00).

10. Identify proteins with significant differences in level by other two tools of MAProMA: DAve (Differential Average) and DCI (Differential Coefficient Index) (*see Note 19*).
11. Characterize proteotypic peptides by means of another in-house software called EPPI (Experimental Proteotypic Peptide Investigator) [13].

4 Notes

1. EDTA powder dissolves completely when solution reaches pH 8.0. About 20 g NaOH pellet is required to adjust to pH 8.0.
2. Alternatively, cell lysis could be performed by six cycles of sonication at 20% amplitude for 10 s.
3. Alternatively, membranes can be prepared by conversion of the cells to spheroplasts by the lysozyme–EDTA treatment and disruption of spheroplast by sonication. Add 100 $\mu\text{g}/\text{mL}$ lysozyme to the cell suspension and leave on ice for 2 min. Keep the sample in ice (put a small beaker in a bigger one filled with ice) and add 12 mL of 0.3 mM EDTA over a period of 10 min with a peristaltic pump by mixing gently with a magnetic stirrer. EDTA solution must be added dropwise by the side of the beaker. Add 10 μM PMSF and 0.2 mM DTT. Take an aliquot and read absorbance at 450 nm (A_{450}). Disrupt spheroplasts by sonication at 10% amplitude for 15 s. Repeat this step four times leaving the samples on ice for 1 min after every cycle of sonication. Read A_{450} of the spheroplast suspension and check whether it has decreased to approximately 5% of its original value; if not sonication should be continued for 1–2 cycles. Remove the unbroken cells by centrifugation at $3000\times g$ for 20 min at 4 °C and continue with the protocol.
4. Carefully layer the sucrose solutions 1 mL at the time. Once the sucrose gradient is poured discrete layers of sucrose should be visible in the tube.
5. Immediately after the run the tube should be removed from the rotor, taking great care not to disturb the layers of sucrose. Two discrete membrane layers should be visible.
6. The following formula can be used to calculate the enzyme activity: $(|\text{OD}_{430\text{f}} - \text{OD}_{430\text{i}}| / \text{time} \times \epsilon) \times \text{cuvette vol.}$; $\text{OD}_{430\text{f}}$, $\text{OD}_{430\text{i}}$: final and initial absorbance values at 430 nm; time: expressed in minutes; ϵ : $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$; cuvette vol 1000 μL . We find that storing fractions at -20 °C reduces NADH oxidase activity.
7. The lyophilized sample needs to be completely covered by ammonium bicarbonate. If 100 μL is not enough, keep on adding 10 μL until the sample is not completely resuspended. If the final volume exceeds 200 μL , concentrate the sample in a vacuum system to a final volume of 200 μL .

8. Rapigest is an enzyme-friendly and a mass spectrometry-compatible reagent. It is able to help the solubilization of proteins and thus the enzymatic digestion. The lyophilized powder is stable at room temperature until the expiration date written on package, but once reconstituted the solution is stable for 1 week at 2–8 °C.
9. The recommended concentration is 0.1% (w/v), but hydrophobic proteins, as membrane proteins, may require higher concentrations.
10. SPN™-Protein Assay is a protein estimation method which is fast, efficient, and compatible with laboratory agents and detergents, such as sodium dodecyl sulfate (up to 2%). The kit is stored at ambient temperature and requires only 0.5–10 g of proteins, polystyrene cuvettes, and deionized water as blank. Protein concentration is determined by comparing the optical density (OD₅₉₅) obtained from sample treated with the reference OD₅₉₅. The absorbance can be read either using spectrophotometer or using microplate reader.
11. Trypsin is the most common protease used for digestion due to its well-defined specificity. Many factors and parameters could affect the effectiveness of protein digestion, in particular pH of the reaction and temperature. A slightly alkaline environment (pH 8) is optimal and a temperature of 37 °C is recommended. The use of modified trypsin is also necessary to avoid autolysis.
12. PepClean™ C-18 Spin Column is useful for removing contaminants and for concentrating peptides, realizing them in MS-compatible solutions. Each column can bind up to 30 µg of total peptide from 10 to 150 µL of sample volume. Mix 3 parts sample to 1 part sample buffer (2% TFA in 20% ACN); prepare column adding 200 µL of activation solution (50% ACN), centrifuge at 1500×g for 1 min, discard flow-through, and repeat. Repeat this procedure using equilibration solution (0.5% TFA in 5% ACN) instead of activation solution. Load sample on column, and centrifuge at 1500×g for 1 min. To ensure complete binding, recover flow-through and reload sample on column. After centrifugation, add 200 µL wash solution (0.5% TFA in 5% ACN) and centrifuge (1.5 g for 1 min). Repeat this step three times. Elute sample by adding 20 µL of elution buffer (70% ACN) twice.
13. The flow is maintained constant and equal to that set by a splitter to pulse dampener. If the pressure and the flow increase, part of the flow is conveyed toward the waste in order to maintain the outlet flow predetermined.
14. Eluted peptides can be analyzed also using an LTQ mass spectrometer (Thermo Finnigan Corp.) equipped with a nano-LC electrospray ionization source. The values of the parameters set are the same between LCQ Deca XP plus and LTQ.

15. With LTQ, acquire full mass spectra in positive mode and over a 400–2000 m/z range, followed by five MS/MS events sequentially generated in a data-dependent manner on the five most intense ions selected from the full MS spectrum, using dynamic exclusion for MS/MS analysis (collision energy 35%).
16. Cluster PC or multi-processor is useful for reducing processing time.
17. “No enzyme mode” guarantees the identification of peptides generated by nonspecific cuts. This parameter ensures the identification of a greater/larger number of peptides.
18. Using the Autoformat tool of an in-house algorithm called MAProMa (Multidimensional Algorithm Protein Map) [12], arrange the raw protein list to have for each protein identified: reference, accession, spectral count (SpC), SEQUEST-based SCORE, isoelectric point (pI), and molecular weight (MW).
19. These two algorithms are based on score values assigned by SEQUEST software to each identified protein in two samples compared. Specifically, DAve is an index of the relative ratio between the two samples and DCI evaluates the confidence of DAve. Briefly, using MAProMA each identified protein in the two samples is aligned and then DAve and DCI indexes are calculated for all proteins. The threshold values imposed could be very stringent (DAve > 0.4 and DAve < -0.4, DCI > 400 and DCI < -400) or less stringent (DAve > 0.2 and DAve < -0.2, DCI > 200 and DCI < -200). It is necessary that both indexes, DAve and DCI, satisfy these thresholds [14].

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