Comparative proteomics using 2-D gel electrophoresis and mass spectrometry as tools to dissect stimulons and regulons in bacteria with sequenced or partially sequenced genomes

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ABSTRACT

We propose two-dimensional gel electrophoresis (2-DE) and mass spectrometry to define the protein components of regulons and stimulons in bacteria, including those organisms where genome sequencing is still in progress. The basic 2-DE protocol allows high resolution and reproducibility and enables the direct comparison of hundreds or even thousands of proteins simultaneously. To identify proteins that comprise stimulons and regulons, peptide mass fingerprint (PMF) with matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF-MS) analysis is the first option and, if results from this tool are insufficient, complementary data obtained with electrospray ionization tandem-MS (ESI-MS/MS) may permit successful protein identification. ESI-MS/MS and MALDI-TOF-MS provide complementary data sets, and so a more comprehensive coverage of a proteome can be obtained using both techniques with the same sample, especially when few sequenced proteins of a particular organism exist or genome sequencing is still in progress.

INTRODUCTION

Some years ago, the basic protocol for 2-DE was described (1). This protocol is still valid today: isoelectric focusing (IEF) is performed and after equilibration with SDS buffer in the presence of DTT, the IEF gel is applied to a SDS gel which allows the resolution of hundreds or even thousands of proteins present in a particular cell, while allowing synthesis rates to be determined quantitatively, all in one experiment. The procedure has become the core technology to visualize the global changes in protein expression for proteome analysis (2-7), permitting higher resolution (8), improved reproducibility (9, 10), higher loading capacity (11), and simplicity for preparative 2-DE with subsequent spot identification by mass spectrometry (7, 12).

An even more impressive recent development in proteomics is the use of tandem mass spectrometry for the rapid and sensitive identification of gel-separated proteins. Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography electrospray ionization tandem-MS (LC-ESI-MS) are the keys to high-fidelity, high-throughput proteomics, and the two techniques have become the preferred methods for ionization of peptides and proteins due to their effective application on a wide range of proteins and peptides (13-18).
Bacterial genes encoding products with a common adaptational function are frequently coregulated. A stimulon is a set of proteins whose amount or synthesis rate changes in response to a single stimulus (6). Several stimulons have been described and range in size from a handful of proteins to nearly half of the proteins found in a cell (6, 19-21). A stimulon may consist of several different regulons (4, 19, 21), and a regulon is a protein group under the control of a specific regulatory protein. This organization ensures a balanced production of all proteins necessary for adaptation to a change in the environment which modify fundamental metabolic conditions, such as translation capacity, energy sufficiency, redox state, envelope integrity, building block supply, and protonmotive force (21).

We used 2-DE in combination with mass spectrometry (MS) to reveal protein synthesis patterns belonging to specific stimulons and regulons in R. etli (4, 6). Stimulons are studied comparing the protein expression profile in a control condition versus a test condition (6), while regulons are usually identified using strains in which the gene for the regulatory protein is mutated, and examining its response to specific conditions (4).

The current protocol of 2-DE, MS methodology, as well as recent modifications with respect to sample preparation, running conditions and staining techniques, will be critically discussed in this paper.

MATERIALS AND METHODS

Cell growth conditions and protein extraction

R. etli CE3 is a spontaneous streptomycin-resistant derivative of strain CFN42 (4). Cells were grown under aerobic (minimal medium (MM) plus biotin), and fermentative conditions (MM without biotin) as described previously ((22) and Fig. 1A). Additional details on growth conditions and metabolic characteristics of aerobic and fermentative cultures are given in references 4 and 6.

Apparatus and chemicals for electrophoresis

For IEF and SDS electrophoresis the Millipore Investigator 2-D electrophoresis system was used (Genomics Solutions, Ann Arbor, MI.) Ammonium persulfate, 2-mercaptoethanol (2-ME) and sodium dodecyl sulfate (SDS) were purchased from BioRad (Richmond, CA). Acrylamide, bisacrylamide, CHAPS, Coomassie brilliant blue G-250, silver nitrate, urea, thiourea, TEMED, and all other chemicals (analytical grade) for electrophoresis and staining were obtained from Research Organics. DTT, Formaldehyde, IGEPAL CA-630, bromophenol blue, Trizma base and Tris hydrochloride were from Sigma (St. Louis, MO). Acetone, acetic acid, methanol, glycerol, and EDTA were from J.T. Baker (Ecatepec, Mexico state), and protease inhibitor was protease inhibitor cocktail tablets from Roche (Germany). Ampholytes, NaOH and phosphoric acid were from Genomic Solutions (Ann Arbor, Michigan, USA).

Fig. 1: The proteomics approach suggested in this study, to identify the protein components of stimulons and regulons. Samples to be compared are grown (A), after harvesting the proteins are solubilized (B). The crude protein mixture is then applied to a first dimension tube gel that separate the proteins based on their isoelectric points (C). After this step, the gel is applied to a second dimension SDS-PAGE gel where proteins are denatured and separated on the basis of size (D). Gels are fixed and stained by coomassie or silver staining (E). The resulting protein spots are recorded and quantified (F). The spots of interest are then excised (G) and subjected to mass spectrometric analysis.

Sample preparation

In general, proteins were solubilized by sonication in 50mM Tris-HCl pH 6.8, with protease inhibitors, precipitated in cold acetone and resuspended in lysis buffer (see protocols for more detail) (Fig. 1B). The IEF sample solubilization buffer is based on O’Farrell’s (1) and is composed of 9.5 M urea, 2% Igepal CA-630, 5% (v/v) 2-mercaptoethanol and 5% v/v carrier ampholytes, the samples were stored in aliquots at -80°C until
analyzed (this strategy has been found very useful for the inactivation of proteases to minimize protein degradation).

**Protein electrophoresis and protein visualization**

Electrophoresis was performed as described in protocols and in the 2D Investigator instruction manual (Genomic Solutions, Ann Arbor, MI, USA) (Fig. 1C and 1D). In the first dimension, pH 4 to 8 ampholytes were used.

For analytical 2-D electrophoresis, we used 100 µg of protein from a total cell lysate in the first dimension. The optimum sample volume for loading is 15 µL – 45 µL. Volumes less than 15 µL are not recommended because of the increased risk of protein precipitation at the application point.

For preparative 2-DE, which is used to obtain proteins for MS (MALDI-TOF-MS or LC-ESI-MS) identification, 0.5 mg of protein (sample volume: 100 µL) was applied using a pH range 4 - 8 (4, 6).

After electrophoresis, the gels were fixed and stained with colloidal Coomassie brilliant blue (preparative gels) or silver staining (analytical gels) as described in protocols and Fig. 1E. Silver and Coomassie brilliant blue stained gels were digitized at 127 × 127 µm resolution using a PDI image analysis system and PD-Quest software (Protein Databases, Inc., Huntington Station, N.Y.) ((4) and Fig. 1F). IP and MW were determined by using a 2D SDS-PAGE standard (Bio-Rad, Hercules, CA).

**Mass spectrometry protein-identification**

**Procedure for in-gel digestion**

Protein spots from Coomassie stained preparative 2-D gels were selected and excised manually using a clean scalpel or razor blade (Fig. 1G), put into 1.5 ml Eppendorf tubes, covered with water and store in the freezer until digestion. The samples were prepared for MALDI-TOF-MS analysis using a slightly modified published procedure (6, 23). In brief, protein spots were destained (Fig. 2A), reduced, alkylated (Fig. 2B) and digested in the gel with trypsin (Promega, Madison, USA) (Fig. 2C) and extracted (Fig. 2D). Before obtain the mass spectra of the peptide mixture, the peptides were desalted using a C18 ZipTip (Millipore, Bedford, MA. USA) according to the manufacturer’s instructions (see protocols for more details).

All analyses were performed using a Bruker Daltonics Autoflex (Bruker Daltonics Bellerica, Mass. USA) operated in the delayed extraction and reflectron mode. Spectra were externally calibrated using a peptide calibration standard (Bruker Daltonics 206095). Peptide mixture was analysed using a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile/0.1 % trifluoroacetic acid. Peaks lists of the tryptic peptide masses were generated and searched against the Rhizobase (6) and NCBInr databases (Fig. 2F) using the search program Mascot, (Matrix Science, Ltd., London, United Kingdom; http://www.matrixscience.com). The *R. etli* genome sequencing is still in progress, and for that reason a database was created using sequences deposited as a result of the *Sinorhizobium meliloti* (24), *Mesorhizobium loti* (25), *Agrobacterium tumefaciens* (26) and *R.etli* symbiotic plasmid (27) sequence projects, named Rhizobase database (6).
Unknown samples, not identified by MALDI-TOF-MS were selected to be injected into a CapLC (Agilent 1100, Hewlett-Packard, Germany) System. Ten µl was injected and desalted on-line thorough a 100mm long, 360 OD, 75µm ID x 15 µm tip Aquasil C18 column (PicoFrit, USA) prior to introduction into the mass spectrometer. A typical reversed-phase gradient was used from low to high organic solvent over about 60 min. Mobile phase A was 0.1% formic acid and B was 100% MeCN, 0.1% formic acid. The system utilized a small split flow resulting in a column flow rate of approximately 300 nl/min.

As peptides elute into the mass spectrometer data-dependent data was performed. In this experiment, the mass spectrometer was set to acquired a conventional scan over the m/z range 400-1600. The most intense peak was selected for MS/MS experiments, which were performed on a ThermoFinnigan LCQ (ThermoQuest/Finnigan, San Jose, CA. USA) and the mass spectra were searched against databases using the Sequest search algorithm (http://www.thermoquest.com).

**Northern hybridization**

Northern hybridization was performed as described previously (6, 28). The qualitative examination of the Northern hybridization data was validated by scanning the blots with a densitometer and determining the relative amounts of each specific transcript with a Quantity one software package (Bio-Rad Laboratories, Hercules, Calif.).

**RESULTS AND DISCUSSION**

Whole-cell protein extracts of cells grown in MM supplemented with biotin (aerobic conditions) and MM without supplements (fermentative conditions) were analysed in at least three independent experiments, with duplicate analytical-gels being run for each extract as reported previously by us (6). Gels were qualitatively and quantitatively analysed with PD-Quest software. More than 900 protein spots were revealed on analytical-silver-stained 2-DE gels from 0.1 mg of protein samples prepared from cells from MM-biotin (Fig. 3). The protein spot pattern of the proteome obtained from biotin stimulon cultures was used as a reference standard for spot matching and also to describe relative changes in spot intensities during growth under other culture conditions (Fig. 3).

The biotin stimulon was revealed after comparison of protein patterns obtained by 2-DE from cells grown in MM-biotin versus cells grown in MM-without supplements, and constitutes a set of proteins whose amount or synthesis rate changes in response to a single stimulus (the presence of biotin) (Fig. 3A and 3B) and (6) or by a regulator protein as AniA regulon (4). The proteome data indicate that hundreds of specific genes may be induced or repressed during specific growth conditions (6). Based on these synthesis patterns, we identified distinct protein classes: (i) proteins unique to condition A (proteins unique to MM or MM with biotin) or B, (ii) proteins downregulated in condition A and
upregulated in condition B or conversely (proteins downregulated in MM and upregulated in MM with biotin or conversely), (iii) proteins present in comparable amounts under all conditions tested (Fig. 3).

**Protein separation using 2-D electrophoresis**

Currently, 2-DE is a widely used method for proteomic analysis enabling researchers to investigate and analyze levels of protein expression under a variety of protein expression conditions, however, some common problems are the presence of a few proteins visible on the SDS gel, probably caused by inappropriate sample extraction procedure and, in the 2nd dimension, poor protein transfer from the gel-tube to the SDS gel. Occasionally, low or high Mr proteins are missing on the SDS gels, caused by low Mr proteins not being adequately fixed after SDS polyacrylamide gel electrophoresis (SDS-PAGE). To avoid this, we suggest the use of 20% TCA or glutaraldehyde as a fixative, instead of 40% alcohol and 10% acetic acid and, when high Mr proteins are missing due to proteolytic endogenous degradation, proteases in the sample should be inactivated. Further problems include protein precipitation in the sample application zone, possibly caused by loading a very concentrated sample. Highly concentrated samples can be diluted with lysis buffer, while poorly soluble proteins can be resolved by adding high amounts of urea (>8 M), and/or a proper detergent (>1%) to the lysis buffer or a combination of urea-thiourea-chaps (7 M, 2 M and 4% respectively) (29). To avoid solubilization problems, we suggested in Material and Methods 9.5 M as the urea concentration (and not 8 M as used some groups, specifically users of IPG-strips (30, 31)). Urea at 9.5 M is the concentration reported originally by O’Farrell in 1975 in his paper about 2D electrophoresis, additionally this concentration is recommended by the manufacturers of several 2D electrophoresis systems.

Additionally, in the gels, a diffuse background smear is occasionally observed, probable due to the presence of endogenous proteases in the sample which were not inactivated, poor quality of chemicals (use analytical grade) or water (use 18 meg Ohm water), insufficient washing during silver staining or complex formation between carrier ampholytes and SDS and/or other detergents. A possible solution could be fixing the gel >3h or overnight and intensive shaking to remove SDS-carrier ampholyte complexes.

Indeed, 2-DE is regarded as the most powerful separation method for resolving complex mixtures of proteins. However, classical 2-DE for successful proteomics, now requires increased resolving power, the ability to analyze low (<8 kDa) and high Mr proteins (over 100 kDa); the capacity to resolve very alkaline, hydrophobic, or membrane proteins; and capability to detect minor components in the presence of large quantities of house-keeping proteins. In addition, simplification and automation of the laborious 2-DE procedure would be helpful to resolve some of the points mentioned, immobilized pH gradients (IPG-methodology) (9) is constantly being refined by, for example, the development of basic IPGs up to pH 12 for the analysis of very alkaline proteins, or the introduction of overlapping narrow IPGs (30) to stretch the first dimension for higher resolution and the analysis of minor components. Using the range covering pH 3-10, only 20-25% of the proteins in the theoretical proteome are predicted to be observable in the resulting 2D-PAGE gel. However, good separation of higher protein loads was achieved using the narrow-range pH 4.5-5.5 and 5.5-6.7, significantly increasing the proportion of theoretical proteins detected to over 50% (32). In conclusion, 2-DE is a technique that fulfils the basic requirements of proteome research and which is being continuously improved to meet the demands of the future has been established.

**Protein visualization**

Today, researchers have a multitude of protein stains to choose from according to their project requirements. Most commonly, samples are visualized by silver staining, Coomassie brilliant blue or Sypro fluorescent dyes (33-35). As the sensitivities of protein stains are improved, the challenge to acquire good mass spectra from lower abundance proteins is heightened. It is imperative that the dye does not interfere with MS methods of analysis.

After the proteins were separated by 2-DE and visualized with the staining method selected, silver staining for analytical gels and Coomassie brilliant blue for preparative-gels, as described in protocols. The
protein spot profiles were analysed using image analysis software (Fig. 3 and as described in Material and Methods and Protocols), and from there some proteins were selected for MS analysis (Figs. 4-6). These spots were excised from preparative-gels, and subjected to enzymatic digestion (as indicated in Material and Methods).

Additional a new generation of SYPRO dyes (40) and protein stains with attached fluorochromes (41) could be more sensitive (detection limit of these fluorescent dyes is in the range of 1-2 ng of protein per spot), and compatible with mass spectrometry, unfortunately they require expensive equipment for visualization, which is not available in all proteome laboratories.

**Protein identification**

Recent developments in technology and instrumentation have made mass spectrometry the method of choice for the identification of gel-separated proteins, and make use of the rapidly growing sequence databases (42). Proteins selected were subjected to enzymatic digestion (as indicated in material and methods), the resulting peptide fragments were analyzed using the complementary mass spectrometry methods MALDI-TOF-MS and LC-ESI-MS/MS. Each of these MS methods can be used independently for proteomic analysis, but when the high-throughput MALDI-TOF-MS analysis is combined with the sequence specificity of LC-ESI-MS/MS analysis, identification of unknown proteins from database searching is greatly facilitated, especially when the genome sequence from the bacteria studied is unknown or still in progress (18, 43, 44).

Proteins with a full-length sequence present in databases can be identified with high certainty and high throughput using the accurate masses obtained by MALDI-TOF MS peptide mapping (45). Sample protein mixtures can also be deciphered by peptide mapping (46) and the entire identification process, starting from in gel digestion and finishing with the acquisition of mass spectra and database searching, can be automated and are much easier to process than LC-ESI-MS/MS. Consequently, MALDI-TOF-MS was the first pass analysis. A representative spectra for spot 1 precursor is shown in Fig. 4. The peak list generated from MALDI-TOF-MS data (Autoflex, Bruker Daltonics, Billerica, MA) was used to search existing protein databases to identify the protein. In this example the protein was successfully identified because it belonged to the GroEL family, which is a group of proteins with very high similarity, even between prokaryotes and eukaryotes.
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Fig. 5: Peptide mass fingerprint of gel spot 2. In this case the mass information was not sufficient for searching in the database and the identification was not possible, and subsequent LC-MS/MS was required. Inset shown the spot analyzed from the Colloidal blue stained 2-D gel.

When the MALDI-TOF-MS information is insufficient for protein identification, amino acid sequencing is necessary (47). Only 1-3% of a total digest is consumed by MALDI-TOF MS analysis (48), even if the protein of interest is present on a gel in a subpicomolar amount, and the remaining protein digest can be analyzed by electrospray tandem mass spectrometry (LC-ESI-MS/MS) (14), producing data that allow highly specific database searches, specifically when the genome sequence from the bacteria studied is unknown. This is the case of spot 2, which demonstrated good MALDI-TOF-MS spectra (Fig. 5). Nevertheless, the protein was still not identified, and then LC-ESI-MS/MS was utilized. The masses of the peptide fragments are measured and fragmentation of the peptide is generated by collisionally activated dissociation (Fig. 6A). Then the proteins can be identified because the sequence of the unknown peptide can be determined from the resulting fragmentation patterns. A representative example are the spectra presented in Fig. 6B and 6C which demonstrate the additional data which may be acquired from LC-ESI-MS/MS analysis, the sequence obtained from fragmentation patterns obtained of MS/MS spectra was useful to identify the protein by homology as a trigger factor reported in *S.meliloti*. In *R. etli*, no sequences for trigger factors are present in the databases, but the additional information obtained from LC-ESI-MS/MS made possible its identification using databases from related organisms.

PMF using MALDI-TOF-MS data cannot be used to match homologous proteins unless the two sequences have very high identity. This shows the utility of the LC-ESI-MS/MS approach for identifying homologous proteins if the two sequences have very high identity, and showed that the additional information provided by LC-ESI-MS/MS was essential for identification of the unknown protein. These kinds of sequence data are necessary for protein identification when the protein of interest does not appear in available databases, which limits the identification of proteins using PMF.

Mass spectrometry has been widely recognized as a cornerstone of proteomic research. Its high sensitivity (down to the femtomole level) and throughput are the main characteristics that make MS the best methodology to identify proteins. Another key feature of MS analysis of gel-separated proteins and peptides is the ability to generate different types of structural information about a particular peptide of interest. For example, the mass spectrometer can directly provide information on the mass of a particular peptide and can also generate *de novo* amino acid sequence information from tandem mass spectra obtained either by post-source decay or collision induced dissociation (as described in (49) and
Additionally, Mass spectrometry can be used in studies to evaluate protein complexes and post-translational modifications (16).

Transcription analysis

Results of genome expression using proteome analysis require some kind of confirmation, but, remarkably, current technology expends nearly as much effort in confirming a suspected gene expression identity. There is clearly a need for technologies to simplify confirmation, and we used methods such as Northern blotting (6, 51-53). Using proteome analysis we identified a group of proteins present only in biotin stimulon, and from this set of proteins we selected the protein identified as catalase-peroxidase KatG (28) to investigate whether expression of this protein correlated at transcriptional level. Northern hybridization analysis was performed using a specific probe (6). The results clearly demonstrated that the gene encoding this protein was positively regulated at the transcriptional level, confirming the results of the proteome analysis, and also showing the utility of this methodology to confirm proteome results (6).

CONCLUDING REMARKS

A fundamental concept called proteome (PROTEin complement of a genOME) has recently emerged that should help to unravel biochemical and physiological mechanisms at the functional molecular level (54). Proteome analysis methodologies separate proteins by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) followed by powerful peptide mapping approaches in which gel-embedded proteins are enzymatically cleaved, peptide products are eluted, and identity determined by database searching of the masses or sequences derived from mass spectrometry (MS) analysis of those products. All these represent a set of methodologies with great capacity to analyze protein components of regulons and stimulons in bacteria. Additional, the present paper demonstrates that proteins of organisms with unknown genomes can be separated by 2-D electrophoresis, digested in-gel and rapidly identified at the femtomole level by peptide mass fingerprinting using MALDI-TOF and LC-ESI-MS/MS protocols. In general, MALDI-TOF MS is more effective for the analysis of a greater number of proteins, whereas LC-ESI-MS/MS offers better results to identify proteins for which the gene is not yet sequenced.

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PROTOCOLS

Protein extraction (sample preparation)

_**Lysis buffer**_

5.7 g Urea  
2.0 ml Igepal CA-630 10% (v/v)  
0.5 ml 2-mercaptoethanol  
0.125 ml Ampholytes 3-10  
0.375 ml Ampholytes 4-8  
2.4ml 18 meg Ohm water

1. Cells were washed, pelleted collected and sonicated in 1 ml of 50 mM Tris-HCl, pH 6.8 with protease inhibitors (complete tablets ROCHE).
2. The extract was precipitated over night in 5 volumes of acetone at -20°C.
3. Centrifuged at 16,000 g. 10 min at 4°C.
4. The pellet was resuspended in lysis solution and centrifuged at 16,000 10 min at 4°C (pellet was discard).
5. The protein concentration was determined to supernatant by the Bradford method.
6. The proteins extracts were aliquoted and stored at -70°C until analyzed.

_Electrophoresis_

2D electrophoresis was done according to the manufacturer’s instructions, with some modifications. Unless otherwise indicated, the water used for all solutions is 18meg Ohm purified, filtered through a 0.45 µm filter. To dissolve the urea, solutions were warmed to 37°C (do not heat above 37°C or the urea can carbamylate proteins in the sample causing charge heterogeneity). To thaw the urea solutions after storage, let stand at room temperature or place in a 37°C in water bath. All reagents are electrophoresis grade or the best quality as possible.

_1D buffers and solution_

_1D IEF gel casting solution_

57 g urea  
2.0 ml Igepal CA-630  
13 ml acrylamide solution  
0.3 g CHAPS  
Water to 95 ml. Aliquot 5.65 ml portions in analytical snap caps vials. Store at 70°C.

_Sample overlay buffer_

3.0 Urea  
0.2 ml Igepal CA-630  
0.25 ml Ampholytes (40% stock)  
0.77 g DTT  
Water to 100 ml. Aliquot into 0.5 ml portions. Store at 70°C.

_Cathode (upper) solution_
10 mL 10N NaOH
990 ml of water
Use freshly prepared. Do not filter.

Anode (lower) solution

1.4 ml 85% phosphoric acid
2 lt of water
Prepared freshly. Do not filter.

1D equilibration buffer

36.33 g Tris base
11.82 g Tris HCl
30 g SDS
Water to 1 lt. Store at room temperature

1D IEF gel storage buffer

10 ml glycerol
Water to 100 ml

Acrylamide Solution

300 g acrylamide
8 g bis acrylamide
Water to 1 lt. Store at 4°C. Do not store for more than 1 month.

Preparation of 1D gels

1. Fill the 1D-casting cylinder with 1100 ml of 18meg Ohm water.
2. Before running, add to each aliquot 87.5 µl of ampholyte stock solution pH 3-10 and 262.5 µl of ampholyte stock solution pH 4-8.
3. Add 40 µl of 10% ammonium persulfate. TEMED is not required due to the presence of ampholytes.
4. Mix the solution completely by inverting the vial several times. Try to minimize detergent bubbles when mixing.
5. Place the threaded glass capillary tubes in to the vial, guide the small end of the 1D casting funnel over the tops of the tubes and press the snap cap vial onto the assembly.
6. Slowly lower the snap cap vial, tubes and funnel combination into the casting cylinder so that the water entering through the lower hole in the funnel lays over the gel solution. Avoid mixing solution with the water.
7. Wait about 1 hour for the gel to polymerize. The length of gels is 18 cm.
8. Carefully remove the tubes and cast each tube gel into the 1D chamber.

Prefocusing 1D gels

9. Fill the 1D-running tank with anode solution, and upper reservoir with 800 ml of degassed cathode solution.
10. Fill a 1 ml syringe with cathode solution introduce into the gel tube and carefully expel cathode solution to remove the air bubbles.
11. Repeat until all tubes are completely debubbled.
12. Load approximately 5 µl of the sample overlay on the top of each gel.
13. Install the cover on the 1D running system.
14. Plug in the 1D chamber to programmable power supply, and start prefocus program following the manufacturer’s instructions.

*Isoelectric focusing 1D gels*

15. When the prefocus program is finished, stop the program and load the sample in the gels. We use 100 µg for analytical running and 0.5 mg or 1.0 mg for preparative running.
16. Carefully load on top of the gel the sample using a Hamilton syringe. Rinse the syringe and needle thoroughly each sample.
17. Install the cover on the 1D running system. Plug in to the programmable power supply.
18. Program the power supply for the isoelectric focusing. We follow the manufacturer standard conditions, except for the Volt-hours, which we programmed at 24 000 volt-hours.
19. While isoelectric focusing is running, cast the 2D slab gels.

*2D buffers and solutions*

*Tris Slab gel buffer*

130.8 g Tris Base  
66.3 g Tris HCl  
Water to 1.0 lt. Store solution at 4°C.

*2D gel running buffer (10X solution)*

60.55 g Tris Base  
288.27 g glycine  
20 g SDS  
Water to 2.0 lt. Store at room temperature.

*10% SDS stock solution*

10 g SDS  
Water to 100 ml  
Store at room temperature.

*10% Ammonium Persulfate (APS)*

1.0g APS  
Water to 10 ml  
Store at 4°C  
Prepare just before to use. The IEF gels polymerize poorly if the solution is more than 24 hours old. Don’t filter.

*Cleaning solution*

50 ml ammonium hydroxide  
100 ml alkaline detergent  
Tap water to 10 lt.

*2D slab gel fixation solution*

400 ml Methanol
Cleaning glass plates

The cleanliness of glass plates is very important. These were washed with soap to remove all waste of acrylamide. They were soaked in cleaning solution overnight, washed with hot tap water and let dry. All chambers and casting systems are washed with tap water and handed with clean gloves, to avoid contamination with other proteins and keratins.

Preparing 2D slab gels

Follow the manufacturer instructions for assembling the single glass plates on the 2D casting system for slab gels. When casting box is assembled prepare the Acrylamide casting solution, following the later instructions. Amounts must be sufficient for 5 single gels and are slightly in excess.

20. Mix 211 ml of acrylamide solution, 130.5 ml of Tris slab gel buffer and 180.5 ml of water.
21. De-gas the mixture for ten minutes at 25 mm of Hg. Add 11.7 ml of 10% SDS.
22. Initiate the polymerization by adding 2.9 ml of 10% APS and 0.376 ml of TEMED. Mix gently, and do not shake.
23. Pour the Acrylamide solution into the casting cylinder.
24. Then fill the casting box at adequate level, 0.5 cm below the bottom of the bevel.
25. Close the pinch clamp and wait 30 seconds for the acrylamide level to settle.
26. Carefully layer 1ml of water-saturated butanol gel overlay into the beveled V-shaped trough and onto the top of the Acrylamide solution in each plate assembly.
27. Wait for complete polymerization, at least one hour.
28. If you wish, the casting unit could be stored overnight before using. Pour out the butanol, rinse the top of gels and cover with a 1:4 dilution of the 2D slab gel buffer.

Second dimension gel run

29. Prepare 1D equilibration buffer. Just before using, add 0.77 g DTT and 0.01 g Bromophenol Blue to 100 ml 1D equilibration buffer.
30. When the Focusing program was finished, turn off and remove the tubes from the 1D running system and place on ice, for approximately 5 to 10 min.
   NOTE: If you don’t run 2D electrophoresis, you can store the 1D gels at -70°C in 1D IEF gel storage buffer.
31. Fill a holding tray with 1D equilibration buffer.
32. Carefully expel the gel using the gel extrusion adapter into a holding tray.
33. Incubate in 1D equilibration buffer for 2 min at room temperature.
34. Then load it onto the slab gel.
35. Place the cover to the 2D chamber and plug in to the programmable power supply.
36. Program according to the manufacturer’s instructions. Our run was made at 1000 mW constant. Longer times of running result in sharper spots. The run is stopped when dye front is one centimeter from the bottom gel.
37. Turn off the power supply and carefully open the sandwich gel, and place it into the fixation solution, at least 1 hour.

Preparative 2D gel electrophoresis

Follow the same procedure for analytical running, but use the preparative gel tubes of 2 mm 1D. In addition the 1D gel must be glued to 2D slab gel with agarose low melt (1% in 2D running buffer 1X with 0.01% bromophenol brilliant
blue). The running conditions for the focus gel is in accord to the manufacturer instructions, except for the Volt-hours which we set at 24 000 Volts-hour.

Note: If you observe vertical streaks on the SDS gel, the possible reasons could be: a) proteins insufficiently loaded with SDS; possible presence of glycoproteins in which case we suggest the use of Borate buffer instead of Tris buffer in SDS gel; use steep pore gradient to deglycosylate proteins, ii) Partial re-oxidation of free SH-groups in which case, add sufficient DTT to break the disulfide bonded aggregates, since equilibration buffer, alkylate proteins; use tributylphosphine instead of DTT and iodoacetamide, iii) Carbamylation trains, we suggest never heating urea-containing solutions at > 37°C; deionize urea prior to use, iv) endogeneous proteolytic enzymes have not been inactivated during sample preparation, in this last case try to inactivate proteases by TCA-acetone treatment, boiling with SDS and/or adding protease inhibitors.

Note: It is important that the quality of chemicals should be at least analytical grade; double-distilled or deionized water (conductivity 18meg Ohm) should be used in solution preparation.

**Visualization of proteins**

**Silver stain**

The times indicated here should be observed exactly in order to ensure reproducible image development. All steps were carried out on a shaker at room temperature (20-25°C).

**Washing solution**

500 ml ethanol
500 ml water

**Pretreatment solution**

0.2 g Na₂S₂O₃ • 5H₂O
Water to 1 lt

**Impregnation solution**

2 g AgNO₃
0.75 ml 37% formaldehyde
Water to 1 lt.

**Development solution**

60g Na₂CO₃
0.5 ml 37% HCOH
4 mg Na₂S₂O₃ • 5H₂O
Water to 1 lt

**Stop solution**

10 ml acetic acid
990 ml water
1. Fix gels for at least one hour. Wash in washing solution, three times for 20 minutes.
2. Incubate 1 minute in pretreatment solution. Rinse with water three times for 20 seconds.
3. Add impregnation solution and incubate for 20 minutes.
4. Rinse with water three times for 20 seconds.
5. Immerse in development solution for visualization of spots of proteins. Change development solution when is brown. Rinse with water two times for 2 minutes.
6. Immerse gels in stop solution. It can be stored in this solution at 4°C.
7. The gel is ready for analysis.

Suggestions: utilize pure chemicals (analytical grade) exclusively, highly purified deionized water (conductivity < 2 μS), meticulously cleaned and dust-free vessels only, never touch the gel with your fingers, always wear gloves, use fresh formaldehyde, check pH of developer, and the SDS gel has to be completely covered with buffer solutions during the silver staining procedure.

Coomassie stain

Water used in all solutions and in all rinses is 18meg Ohm purified. A fresh staining solution should be used.

Colloidal Coomassie brilliant blue

0.4 g CBB G-250
4.8 ml H₃PO₄ 85%
40 ml saturated ammonium sulfate
275 ml water
At the moment to use add: 80 ml methanol.

Saturated ammonium sulfate

767 g of (NH₄)₂SO₄ in 1 liter of water stir and gently warm. When the solution is still warm should be filtered by 0.45 μM filter.

1. After fixing the gels, wash 10 min in water. Then incubate in colloidal stain.
2. Gentle rock during 1 h. Stain only one gel at a time.
3. After spots are visible, store gels in water. Gels are ready for image analysis and cutting spots.

Sample digestion by mass spectrometry

All reagents are prepared immediately prior to use. The water used in all components of the procedure should be 18meg Ohm purified. The acetonitrile and methanol are HPLC grade. All other reagents are the highest possible commercial grade available. The trypsin used is sequencing-grade enzyme that has been modified to inhibit autolysis and to minimize non-tryptic protease activities.

100 mM ammonium bicarbonate

0.2 g ammonium bicarbonate
20 ml of water

50 mM ammonium bicarbonate

2 ml of 100 mM ammonium bicarbonate
2 ml water

**10mM DTT**
1.5 mg DTT
1 ml 100mM ammonium bicarbonate

**100 mM Iodoacetamide**
18 mg iodoacetamide
1 ml 100 mM ammonium bicarbonate

**Trypsin stock solution**
20 µg trypsin
100 µl HCl 1mM
Aliquot in 10 µl and store at -20°C

**Trypsin solution**
10 µl trypsin stock solution
150 µl of 5 mM ammonium bicarbonate to obtain a concentration of 12.5 ng/µl
Prepare just before using and keep on ice until utilized

**Extraction buffer**
10 ml of acetonitrile
5 ml water
1 ml formic acid
Water to total volume 20 ml

**Day one**

1. The spot was cut from the gel and placed in an eppendorff tube. First step; Distaining in 50% acetonitrile in 2.5 mM ammonium bicarbonate, 2 h two times.
2. Dehydrate the gel pieces 10 min at room temperature in 100 µl of pure acetonitrile, two times. When dehydrated, the gel pieces will have an opaque white color and will be significantly smaller in size.
3. Carefully remove the acetonitrile from the sample and discard.
4. Store at -70°C.

**Day two**

5. Completely dry the gel pieces at room temperature in a vacuum centrifuge for 2-3 min.
6. Add 30 µl of 10 mM DTT and reduce the protein for 30 min at room temperature.
7. Carefully remove the DTT solution from the sample and discard.
8. Add 30 µl of 100 mM iodoacetamide and alkylate the protein at room temperature for 30 min.
9. Carefully remove the iodoacetamide solution from the sample and discard.
10. Add 100 µl of acetonitrile and dehydrate the gel pieces for ~5 min at room temperature.
11. Carefully remove the acetonitrile from the sample and discard.
12. Rehydrate the gel pieces in 100 µl of 100mM ammonium bicarbonate, incubating the samples for 10 min at room temperature.
13. Carefully remove the ammonium bicarbonate from the sample and discard.
14. Add 100 µl of acetonitrile and dehydrate the gel pieces for ~5 min at room temperature.
15. Carefully remove the acetonitrile from the sample and discard.
16. Completely dry the gel pieces at room temperature in a vacuum centrifuge for 2 to 3 min.
17. Digest the sample by adding 10 µl of trypsin solution, incubate 30 min in ice for rehydration of gel, and then remove the supernatant and add 10-15 µl of 5 mM ammonium bicarbonate and incubate overnight at 37°C to carry out the digestion.

**Day three**

18. Extract the peptides produced by the digestion in three steps.

i) Add 30 µl of 5 mM ammonium bicarbonate to the digest and incubate the sample for 10 min with occasional gentle vortex mixing. Drive the digest to the bottom of the tube by centrifuging the sample for 30 sec. Carefully collect the supernatant and transfer the sample to a 0.5 ml plastic micro-centrifuge tube.

ii) Add 30 µl of extraction buffer to the tube containing the gel pieces, and incubate the sample for 10 min with occasional gentle vortex mixing. Drive the extract to the bottom of the tube by centrifuging the sample for 30 sec. Carefully collect the supernatant, and combine the extract in the 0.5-ml plastic micro-centrifuge tube.

iii) Add a second 30 µl aliquot of the extraction buffer to the tube containing the gel pieces, and incubate the sample for 10 min with occasional gentle vortex mixing. Drive the extract to the bottom of the tube by centrifuging the sample for 30 sec. Carefully collect the supernatant, and combine the extract in the 0.5 ml plastic micro-centrifuge tube.

19. Reduce the volume extract to ~10 µl by evaporation in a vacuum centrifuge at ambient temperature. Do not allow the extract to dry completely.