

Two-Dimensional Electrophoresis and Identification by Mass Spectrometry

Fernando de la Cuesta, Gloria Alvarez-Llamas, and Maria G. Barderas

Abstract

Two-dimensional electrophoresis (2-DE) is a classical separation technique for complex protein mixtures based on a first-separation step by isoelectric point (pI) followed by SDS-PAGE. Due to its high resolving power (including protein isoforms) and the capability to display post-translational modifications, 2-DE has been the technique of choice in many studies focused in characterizing allergens. In this chapter, we describe a standard protocol which enables efficient protein separation and subsequent protein identification by tandem mass spectrometry (MALDI-MS/MS).

Key words 2-DE, Two-dimensional electrophoresis, MALDI-TOF, Mass spectrometry, Allergens

1 Introduction

Two-dimensional electrophoresis (2-DE) is a classical technique able to provide an extended picture of the proteins present in the sample of interest. It is based on a first-separation step by protein isoelectric point, followed by a second separation by molecular weight (SDS-PAGE) [1]. The result is a protein map highly resolved where one spot usually contains a unique protein. Despite of the most recent appearance of mass spectrometry-based approaches for protein separations (LC-MS/MS) [2], 2-DE is still the technique of choice in many studies aimed at the characterization of allergens, particularly when SDS-PAGE protein separation does not provide sufficient resolution to guarantee a unique protein per gel band [3–5]. 2-DE has high resolution power to separate complex mixtures and proved capacity to identify up- or down-regulation in a biological context when combined with quantitative assays based on fluorescence labeling as DIGE [6]. Additionally, 2-DE allows displaying post-translational modifications (PTMs) as glycosylation, phosphorylation, oxidation, and nitrosylation. Main limitation of

2-DE is the difficulty to study certain subsets of proteins which show impaired separation affecting subsequent MS identification, such as membrane proteins, proteins with isoelectric point higher than 11 or lower than 3, and proteins with molecular weight higher than 120 kDa or lower than 7 kDa.

The successful implementation of 2-DE in the laboratory demands a previous careful optimization of the experimental protocol. Sample preparation should guarantee an efficient protein extraction and subsequent removal of potential matrix interferents to ensure compatibility with the isoelectric focusing (IEF) step, otherwise resulting in a defective protein separation. Instrumental parameters (IEF program) should be set-up and optimum conditions should be adjusted for every sample type. Once proteins have been separated, bidimensional immunoblot may follow or direct protein identification from spots of interest by MALDI-MS/MS. In this case, the method of choice for protein spots visualization should be compatible with mass spectrometric analysis, and proteins have to be extracted from the gel spot and efficiently enzymatically digested. Resulting peptides are extracted from the gel and analyzed by mass spectrometry.

In this chapter, we describe a general procedure to carry out 2-DE analysis and protein identification by MALDI-MS/MS which can be applied to allergens characterization.

2 Materials

2.1 Equipment

2.1.1 Two-Dimensional Gel Electrophoresis

1. Electrophoresis apparatus (Protean isoelectric focusing (IEF) cell, PROTEAN II, Bio-Rad, Hercules, CA).
2. ReadyStrip immobilized pH gradient (IPG) 17 cm 4.0–7.0, 3.0–10.0, 6.0–9.0 (Bio-Rad).
3. Protean IEF cell (Bio-Rad).
4. Mili Q® System (Millipore, Bedford, MA).
5. Shaker.
6. Thermomixer compact.
7. Ultrasonic bath.
8. Glass plates 16 × 20 cm (Bio-Rad).
9. Reswelling cassette (Bio-Rad).
10. Clamps (Bio-Rad).
11. Scanner (DUO Scan Hid, Agfa-Geavaert, Marstel, Belgium).

2.1.2 Identification of Allergens by MS/MS

1. ExQuest Spot Cutter.
2. 5424 Microcentrifuge.
3. Ettan Digester (GE Healthcare).
4. Speedvac vacuum concentrator.

5. MALDI plate (AB Sciex).
6. 4800 MALDI TOF/TOF Analyzer (AB Sciex).
7. 4000 Series Explorer Software (AB Sciex).
8. GPS Explorer Software (AB Sciex).

2.2 Reagents

2.2.1 Two-Dimensional Gel Electrophoresis

1. Bradford protein assay (Bio-Rad).
2. Sample solubilization buffer: 7 M Urea, 2 M Thiourea, 4% CHAPS, 1% dithiothreitol (DTT). To prepare 50 mL of solubilization buffer, dissolve 7.02 g of thiourea, 21.02 g of urea, and 2 g of CHAPS in deionized water and make up to 50 mL, stir, and filter. Sample solubilization buffer can be stored at -80°C in small aliquots of 0.5 mL. Never refreeze.
3. Rehydration solution: 8 M urea, 0.5–4% CHAPS, 20–100 mM DTT, 0.5–2% Pharmalyte. It is important not to heat urea solutions above 37°C in order to reduce the risk of protein carbamylation.
4. Equilibration buffer: 8 M urea, 1.5 M Tris-HCl, 87% glycerol (99% purity), 2% sodium dodecyl sulfate (SDS) (97% purity), bromophenol blue. During the first equilibration step, 100 mg of DTT is added to 10 mL equilibration buffer. In the second step, 250 mg of iodoacetamide is added to 10 mL equilibration buffer.
5. Acrylamide/*bis*-acrylamide solution: Acrylamide/*bis*-acrylamide 30%. Stir for 10 min, filter, and fill up to 100 mL with deionized water.
6. Ammonium persulfate solution: 10% Ammonium persulfate (98% purity).
7. Phosphated-buffered saline (PBS) buffer pH 7.2: NaCl (99.5% purity), KCl (99% purity), Na_2HPO_4 (99.5% purity), KH_2PO_4 (99% purity).
8. Fixation solution: 30% Ethanol (99.8% purity), 5% acetic acid.

2.2.2 Identification of Allergens by MS/MS

1. 100 mM ammonium bicarbonate (99% purity; Scharlau) in water. Make daily and discard after use.
2. DTT solution: 10 mM dithiothreitol (DTT) (Sigma-Aldrich) in 100 mM ammonium bicarbonate. Prepare shortly before use.
3. Iodoacetamide solution: 55 mM iodoacetamide (Sigma-Aldrich) in 100 mM ammonium bicarbonate. Prepare shortly before use.
4. Methanol (gradient, HPLC grade, Scharlau).
5. Acetonitrile (gradient, HPLC grade, Scharlau).
6. Trypsin buffer: Modified porcine trypsin (sequencing grade; Promega) 20 ng/ μL in 20 mM ammonium bicarbonate. Prepare shortly before use.
7. Trifluoroacetic acid (99.5% purity; Sigma Aldrich).

3 Methods

3.1 Two-Dimensional Gel Electrophoresis

3.1.1 Sample Preparation

1. Solubilize 300 µg of protein in 200 µL of solubilization buffer and freeze at -80°C until use (*see Note 1*).
2. Sonicate the protein mixture for 1 min at room temperature.
3. Heat the protein sample at 80°C for 5 min.
4. Cool the sample at room temperature.
5. Incubate the protein mixture with 200 µL of Benzonase to eliminate nucleic acids.
6. Incubate again for 50 min at room temperature.
7. Quantify the protein content using the Bradford method [7].
8. Dilute 200–250 µL in rehydration solution (350 µL final volume).

3.1.2 Sample Application

1. 250 µg of proteins are mixed with 350 µL of rehydration solution.
2. Pipet the entire sample containing rehydration solution into the groove of a swelling tray.
3. Peel off the protective cover sheets from the IPG Gel Strips (17 cm, pH 3.0–10.0, 4.0–7.0, 6.0–9.0, 6.0–11.0) and position them such that the gel of the strip is in contact with the sample (*see Note 2*).
4. Cover the IPG Gel Strips and the sample with mineral oil.

3.1.3 First Dimension: IEF Separation

IEF is performed in a Protean IEF Cell (Bio-Rad). The strips are actively rehydrated for 12 h at 30 V to enhance protein uptake and then the voltage is increased according to the following program:

1. 1 min at 50 V (ramping) for a total of 300 V.
2. 30 min at 300 V (ramping) for a total of 1000 V.
3. 1 h and 30 min at 1000 V (ramping) for a total of 5000 V.
4. 1 h. and 30 min at 5000 V (ramping) for a total of 29,000 V.

For strips in the 6.0–9.0, 6.0–11.0 pH range a different protocol is used:

1. 16 h of rehydration at 30 V.
2. 30 min at 500 V.
3. 1 h at 1000 V.
4. 30 min at 5000 V.
5. 8000 V for a total of 60,000 V.

3.1.4 Second Dimension: SDS-Polyacrylamide Gel Electrophoresis

A number of gel concentrations and gradients as well as different buffering systems can be used in the second dimension [8]. We perform SDS-polyacrylamide gel electrophoresis according to the

Laemmli system [9], as previously described [10], using a Protean II system (Bio-Rad). Prepare a 12.5% acrylamide gel. Perform the equilibration as follows:

1. Place the IPG Gel Strips in a clean cassette for equilibration. Add 5 mL of equilibration buffer with DTT and rock for 15–20 min on a shaker. After that, discard the equilibration buffer and rinse with deionized water.
2. Add 5 mL of equilibration buffer with iodoacetamide and equilibrate for another 15–20 min on a shaker. Finally rinse again with deionized water and put on a piece of filter paper to remove excess of equilibration buffer (*see Note 3*).

Transfer the equilibrated IPG gel strips onto the top of the SDS gel and run it at 25 mA/gel at 4°C.

3.1.5 Fixing Gels

Remove the gels from the apparatus and separate the glass plates and place them fixation solution overnight.

3.1.6 Staining

When gels are fixed, stain with Silver staining kit (GE-Healthcare) or with different reagents (Coomassie Blue (Bio-Rad), Sypro Ruby Protein Gel Staining (Thermo Fisher Scientific, etc.)).

3.2 Identification of Allergens by MS/MS

From Shevchenko et al. with modifications [11] (*see Note 4*).

1. Excise the positive band/s from the 1-DE gel or spot/s from the 2-DE gel (*see Note 5*).
2. Cut bands into 1 × 1 mm cubes to facilitate in-gel digestion.
3. Transfer gel pieces into a microcentrifuge tube or 96-well plate, spin them down in a bench-top microcentrifuge (*see Note 6*).

3.2.1 Destaining of Coomassie Blue Stained Bands and Spots

1. Cover gel pieces with 100 µL (*see Note 7*) of 50 mM ammonium bicarbonate, 50% acetonitrile (mix 1:1 100 mM ammonium bicarbonate and acetonitrile). Incubate under constant agitation for 30 min at room temperature. Remove supernatant. Repeat until gel pieces have been mostly destained of Coomassie blue (*see Note 8*).
2. Shrink gel pieces by adding 100 µL acetonitrile for 5 min. Gel pieces should look whitish.

3.2.2 Reduction and Alkylation

1. Incubate with 50 µL of DTT solution for 30 min at 37 °C in a thermomixer under constant agitation.
2. Remove DTT solution (*see Note 9*).
3. Add 100 µL of 100 mM ammonium bicarbonate to let DTT come out of the gel pieces, incubate for 5 min at room temperature under constant agitation.

4. Shrink gel pieces by adding 100 μ L of acetonitrile for 5 min.
5. Incubate with 50 μ L of iodoacetamide solution for 20 min at room temperature in the dark.
6. Remove iodoacetamide solution.
7. Add 100 μ L of 100 mM ammonium bicarbonate to let iodoacetamide come out of the gel pieces, incubate for 5 min at room temperature under constant agitation.
8. Wash with 50 mM ammonium bicarbonate in 50% methanol for 5 min at room temperature under constant agitation.
9. Repeat gel shrinking by adding acetonitrile and dry in a Speedvac.

3.2.3 Trypsin Digestion

1. Add 50 μ L of trypsin buffer, or as much as needed to cover all gel pieces.
2. Incubate at 37 °C overnight (*see Note 10*).

3.2.4 Elution of Peptides and MALDI-TOF/TOF

1. Extract peptides from gel pieces with 60% acetonitrile and 0.1% formic acid for 15 min at 37 °C under constant agitation.
2. Mix an aliquot of the trypsin digestion solution with an aliquot of α -cyano-4-hydroxycinnamic acid in 30% aqueous acetonitrile, 15% 2-propanol and 0.1% trifluoroacetic acid.
3. Deposit onto the MALDI plate and allow drying at room temperature.
4. Obtain MALDI-MS and MS/MS data in a MALDI TOF/TOF Analyzer (*see Note 11*).

4 Notes

1. Sample preparation and solubilization are crucial factors for the performance of the 2-DE technique. Protein complexes and aggregates should be completely disrupted in order to allow the appearance of all possible spots.
2. For the correct development of the first dimension, it is very important that when the strip is placed over the sample no bubbles are present.
3. DTT is used to facilitate the conversion of disulfide bonds to free sulfhydryl groups. Sulfhydryl groups are then alkylated with iodoacetamide (carboxyamidomethylation), since the reducing reaction is reversible and the re-formation of the disulfide bridges must be prevented.
4. Special care has to be taken in order to avoid contamination with dust and particularly hair and skin keratins. Wear gloves at all times, rinse or dispose gloves if in contact with uncleaned surfaces. Perform all manual procedures in a laminar flow hood.

5. For excising positive band/s from a 1-DE gel or spot/s from a 2-DE gel, an automated picker can be used or can be done manually if not available. For the latter, excise positive band/s from the 1-DE gel using a sterile scalpel. For excising spot/s from the 2-DE gel, a 100–1000 μ L pipette tip can be used. Keep gels humid with double-distilled water during whole process. Carefully excise gel areas avoiding contamination with other bands or spots, which may result in misleading identification of potential allergen(s).
6. If in-gel digestion protocol is going to be performed in an automatic digester, gel pieces should not be smaller than 1×1 mm as they might be absorbed by the digester's suction tubes and get trapped, or clog pipette tips if doing manually.
7. The volumes specified during the whole protocol may be increased in order to cover all gel pieces.
8. Normally two washes of 30 min are enough to destain gel bands and spots. The majority of Coomassie blue has to be destained, but there is no problem if a bit is remaining.
9. Notice that most of the volume would have been absorbed by the dehydrated gel pieces.
10. After 30 min, check volume and add more trypsin buffer to cover entire gel pieces, if needed.
11. Analysis of mass data may be performed using the 4000 Series Explorer Software. MALDI-MS and MS/MS data are combined through the GPS Explorer Software to search a non-redundant protein database, such as Swiss-Prot or nrNCBI. Identification of allergens by MS/MS coming from positive bands excised after overlapping immunoblot and Coomassie blue-stained gels can result in identification of one or more top abundant proteins in the band. Bands from 1-DE gels (and sometimes 2-DE gel spots too if the extract is very complex) would normally contain more than one protein and special care has to be taken before affirming the identification of a novel allergen. Confirmation by additional experiments, such as inhibitory assays, is needed to prove these findings.

Acknowledgments

This work was supported by grants from the Instituto de Salud Carlos III (FIS PI070537, IF08/3667-1, PI11-02239, PI14/01917, PI11/01401, PI11/02432, PI13/01873, PI13/01746, PI13/01581, PI14/01650, PI14/01841) by PT13/0001/0013, IDC Salud (3371/002) and Redes Temáticas de Investigación Cooperativa (FONDOS FEDER, RD06/0014/1015, RD12/0042/0071).

References

1. Bruno B (2012) Two-dimensional gel electrophoresis (2-DE), gel electrophoresis—principles and basics. In: Sameh M (ed) InTech. doi: [10.5772/38312](https://doi.org/10.5772/38312)
2. Monaci L, Visconti A (2009) Mass spectrometry-based proteomics methods for analysis of food allergens. Trends Anal Chem 28(5):581–591
3. Castro L, Crespo JF, Rodríguez J et al (2015) Immunoproteomic tools are used to identify masked allergens: Ole e 12, an allergenic iso-flavone reductase from olive (*Olea europaea*) pollen. Biochim Biophys Acta 1854(12): 1871–1880
4. Abou Chakra OR, Sutra JP, Demey Thomas E et al (2012) Proteomic analysis of major and minor allergens from isolated pollen cytoplasmic granules. J Proteome Res 11(2):1208–1216
5. Perez-Gordo M, Cuesta-Herranz J, Maroto AS et al (2011) Identification of sole parvalbumin as a major allergen: study of cross-reactivity between parvalbumins in a Spanish fish-allergic population. Clin Exp Allergy 41(5):750–758
6. Unlü M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18(11):2071–2077
7. Ausubel FM, Brent R, Kingston RE et al (2005) Current Protocols in Molecular Biology, vol 10. John Wiley & Sons, New York, NY, pp 97–106
8. Gorg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4(12):3665–3685
9. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259): 680–685
10. Barrio E, Antón L, Marques G, Sánchez A, Vivanco F (1991) Formation of covalently linked C3-C3 dimers on IgG immune aggregates. Eur J Immunol 21(2):343–349
11. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1(6):2856–2860