

2D-Electrophoresis and Immunoblotting in Food Allergy

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Abstract

Two-dimensional polyacrylamide gel electrophoresis (2DE) and Western immunoblotting have proven to be invaluable and fundamental techniques for comprehensive characterization of food allergens. Here, we describe and discuss detailed protocols used in our studies on identification of allergenic proteins in shrimp, sesame, hazelnut, and pistachio.

Key words Food allergen, Proteomics, 2D electrophoresis, Immunoblotting, Hazelnut, Pistachio

1 Introduction

In the last two decades, food allergies have been increasingly important health concern worldwide [1, 2]. Identification and characterization of food allergens are essential for understanding mechanisms, improving diagnostic and prognosis of the disease and for assessing food safety. A remarkable effort made by many research teams including ours has yielded in identification at molecular level of more than 600 food allergens with over 200 officially registered by the allergen nomenclature subcommittee at International Union of Immunological Societies (IUIS) (<http://www.allergen.org>).

Owing to complexity of food products, investigation of food allergens has always required implementation of a multistep procedure involving a variety of separation and analytical methods including immuno-assays (enzyme-linked immunosorbent assay (ELISA) and immunoblotting), gel electrophoresis and isoelectric focusing, liquid chromatography, molecular cloning, protein and nucleic acid sequencing, and introduced later on mass spectroscopy. While most of these techniques have been extensively used since pioneering studies, overall approach and methodology for identification and characterization of food allergens have remarkably changed over the last decades [3–12]. Introduction of advanced high-resolution mass spectrometry, improved bioinformatics tools and highly efficient separation techniques has widened experimental horizons and

provided a platform for large-scale proteomics-based research of food allergens. For more discussion on application of proteomics methodology in food allergen studies and on basics of individual techniques, we would like to refer you to a wealth of recent excellent reviews, tutorials, and books [8–12]. In this chapter, we are focusing on detailed procedure of two-dimensional polyacrylamide gel electrophoresis under denaturing conditions and Western blot analysis which have been extensively used for identification and characterization of food allergens in many laboratories including ours ([7], and references in [13–21]).

High-resolution modification of 2D-PAGE using denaturing conditions in both dimensions has been a powerful tool for analysis of complex protein mixture from biological samples since it was introduced by Patric O’Farrell in 1975 [22]. This technique allows separation of hundreds to thousands of proteins in gel and has been recognized over the years as robust and fundamental method in protein studies. However, there are well-understood built-in limitations of 2-DE including low reproducibility, difficulties in separation of hydrophobic proteins and resolving basic proteins with pI above 7, low dynamic range and protein losses in different steps of the workflow [11, 12, 23]. Early days low reproducibility caused by instabilities of the pH gradients was overcome later on with introduction of immobilized pH gradients (IPG) which also allow using narrow range pH gradients for more accurate analysis of closely arrayed protein spots [11, 12, 23]. Furthermore, non-equilibrium pH gel electrophoresis (NEPHGE) techniques have been developed to resolve proteins with high pI (≥ 7) which cannot be separated by conventional isoelectrofocusing [11, 12, 23]. At the same time, while there are approaches to address difficulties in separation of hydrophobic proteins by IEF and low dynamic range, these two remain unsolved built-in limitations of the method [11, 12, 23]. Despite of these technical drawbacks, 2-DE continues to be one of the best methods suitable for discovery phase of research on proteome. Furthermore, 2-DE coupled with downstream Western immunoblotting and mass spectroscopy remains powerful and essential approach in proteomics.

In the next sections, detailed protocols for 2-DE and immunoblotting are described. Combination of these methods was instrumental in our work on identification and characterization of novel allergenic proteins from shrimp, sesame, hazelnut, and pistachio nuts [16, 17, 19, 20]. We made use of commercially available ready-to-use immobilized pH gradient (IPG) strips and pre-cast polyacrylamide mini-gels followed by immunoblotting with sera from subjects with confirmed sensitization to hazelnut and pistachio. Final identification of each protein spot was done by mass spectroscopy and by internal Edman sequencing.

Recently, detailed outline of 2-DE and Western blotting procedures have been described ([7, 23], for example). While the

already published protocols and the one discussed below are similar in many aspects, there are differences as well. We hope that all this information should be helpful to researchers in designing procedure most suitable for their goals.

2 Materials

Prepare all solutions using ultrapure water (preferably MilliQ quality or similar) and analytical grade reagents. All reagents should be prepared and stored at room temperature unless indicated otherwise.

2.1 IEF

1. Reagent 2, lyophilized (Bio-Rad). Add 6 mL of water to lyophilized Reagent 2, dissolve by gentle mixing. Make 1 mL aliquots in 1.5 mL polypropylene tubes. Store aliquots at -80°C .
2. 200 mM of Tributylphosphine (TBP) stock solution, stored at -20°C (*see Note 1*).
3. 7 cm IPG gel strips with immobilized linear pH gradient: broad range, 3–10 and narrow range, 4–7 and 5–8 (Bio-Rad). Strips are stored at -20°C . Take appropriate number of strips from the freezer and bring them to room temperature.
4. Rehydration Buffer: Reagent 2 containing 2 mM TBP (*see Notes 1 and 2*).
5. Protein extract prepared in 1× phosphate buffered saline (PBS) with protease inhibitor cocktail.
6. 0.5 M of Dithiothreitol (DTT) stock solution (*see Note 2*).
7. Electrode wicks (Bio-Rad).
8. Tweezers to handle IPG strips.
9. Mineral oil (any PCR quality oil).
10. The focusing tray (Bio-Rad).
11. Protean IEF cell (Bio-Rad).

2.2 SDS PAGE and Transfer

1. SDS PAGE running buffer: Make 1 L of 1× running buffer from 20× stock MES NuPAGE buffer. Add 50 mL of 20× running buffer to 1 L cylinder and bring up to 1 L with water.
2. Precast NuPAGE gradient (4–12%) Bis-Tris ZOOM gels (Invitrogen).
3. 1 M Tris-HCl (pH 8.8): Add about 400 mL water to a glass beaker. Weigh 157.6 g of Tris-HCl and transfer to a beaker with water. Mix and adjust pH with NaOH. Top up to 500 mL with water; filter the solution by using any 500 mL Filter System; use in the next step.

4. 2D Equilibration buffer stock: 6 M Urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% Glycerol. Weigh 80 g of urea and dissolve it in about 200 mL of water in a glass beaker by mixing. Add to Urea solution 10 g of SDS, 182.5 mL of 1 M Tris-HCl stock solution, 100 mL of glycerol and mix. Transfer the buffer into 500 mL cylinder and adjust the volume to 500 mL. Filter Equilibration buffer by using any 500 mL sterile unit.
5. 2D Equilibration buffer I: 6 M Urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% Glycerol, 5 mM TBP; usually prepared immediately prior to use (*see* **Notes 2 and 4**).
6. Iodoacetamide (optional, *see* **Note 3**).
7. NuPAGE LDC Sample buffer (4×) (Invitrogen).
8. 2D Equilibration buffer II: 1× NuPAGE Sample buffer.
9. SeeBlue Plus2 Prestained Standard (Invitrogen or equivalent).
10. Disposable rehydration/equilibration tray (Bio-Rad).
11. SimplyBlue Protein Stain.
12. Heat Block (water bath) at 70 °C.
13. XCell *SureLock*TM Mini-Cell system with Blot Module and Blotting Pads.
14. Methanol.
15. NuPAGE[®] MES 20× Transfer buffer. Make 1 L of transfer buffer by adding 50 mL of 20× stock solution and 200 mL methanol (20% methanol for 2 gels) to 750 mL deionized water.
16. Immobilon-P transfer membrane, PVDF, 0.45 µm (Millipore) or similar quality.
17. Whatman No. 3 Filter paper.
18. Plastic container.
19. Power Supply.

2.3 Immunoblotting

1. Phosphate buffered saline (PBS; 10× and 1×).
2. 1× PBS containing 0.05% Tween-20 (PBST).
3. Bovine serum albumin (BSA) grade V.
4. Antibody dilution buffer: 10% normal goat serum (NGS), 1% BSA in PBST. Prepare 500 mL of antibody dilution buffer: Dissolve 5 g BSA in 400 mL of PBST, add 50 mL NGS and adjust the volume to 500 mL with PBST. Filter the buffer by using sterile filter unit. Make 45 mL aliquots in 50 mL conical tubes and store at -20 °C (*see* **Note 4**).
5. 0.05 M Tris (pH 9.6): Dissolve 6.05 g of Tris-base in 400 mL of water in a glass beaker. Mix and adjust pH with ~10 M HCl. Transfer the solution into the cylinder and adjust the volume to 500 mL.

6. Phosphatase substrate BCIP/NBT.
7. Kodak X-OMAT Imagine Film.
8. Cassette with mounted intensifying screen on both sides (*see Note 5*).

2.4 Antigens and Conjugates

1. Subject' serum is diluted five to twenty times depending on the level of antigen-specific IgE in diluent solution. Choice of secondary antibody (*see Note 6*).
2. Iodine-125-labeled goat anti-human IgE antibody.
3. Phosphatase-conjugated goat anti-human IgE antibody.

3 Methods

All procedures are performed at room temperature unless otherwise specified. The following protocol was optimized for our specific research projects.

To identify all potential allergenic proteins, protein extracts should be first separated on IPG gel strips with broad range of pH. Then resolve proteins by molecular weights on SDS PAGE and transfer to ImmobilonP membranes (Figs. 1 and 2). Identify the allergens by immunolabeling of the separated proteins with

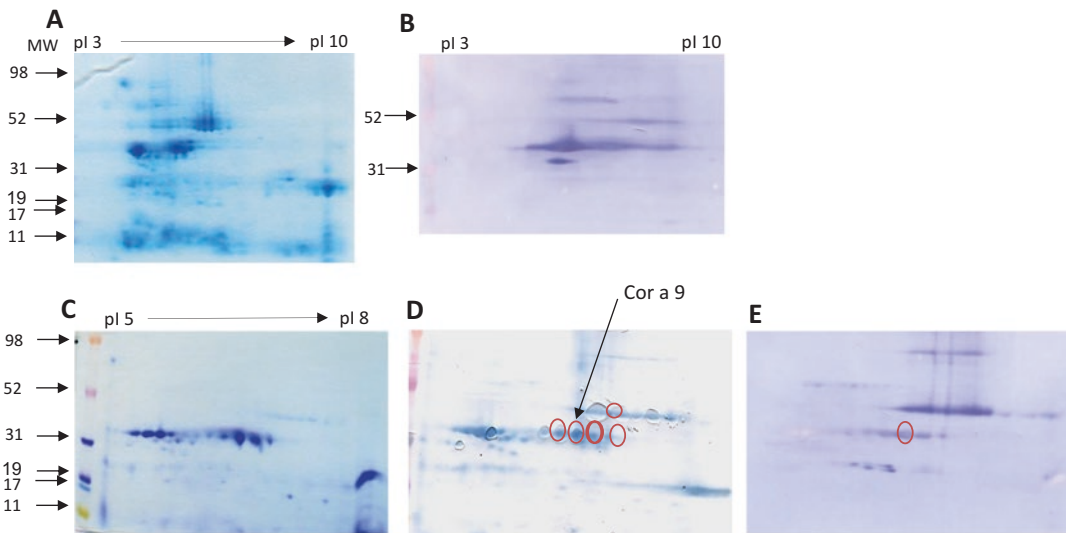


Fig. 1 2D Proteomics map of hazelnut proteins. 50 μ g of the extract was first separated at broad pH range (3–10) and stained with Colloidal Blue stain for total protein analysis (a). The transferred proteins were immunolabeled with serum pool of five subjects to identify the clusters of allergenic proteins (b). For identification of the individual allergens, the extract was separated at narrow pH (5–8) range. The gel was stained with Coomassie Blue stain (c). The membrane with transferred protein was stained with Amido Black stain to confirm the efficiency of the transfer (d). Immunolabeling with individual patient serum is shown (e). Alkaline Phosphatase-conjugated secondary antibody diluted five thousand times was used for immunolabeling

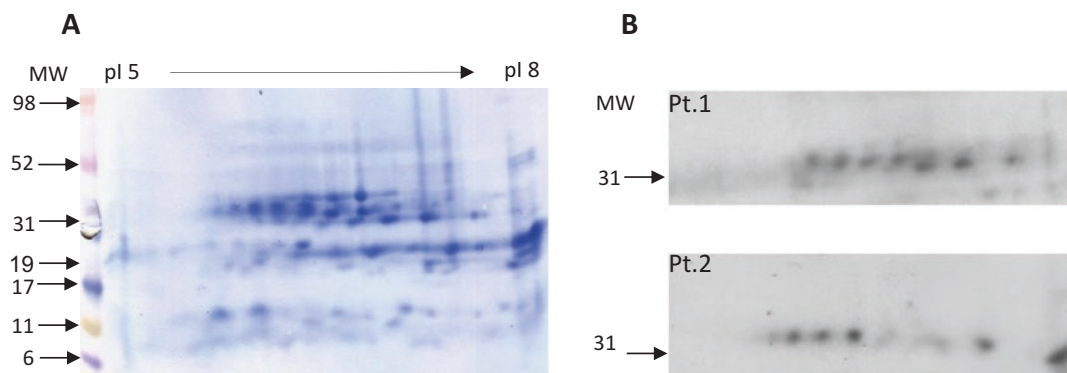


Fig. 2 Two-dimensional proteomic map of pistachio proteins separated at narrow pH range. The membrane was stained with Coomassie Blue stain (**a**) and immunolabeled with two representative patients (**b**). For identification of pistachio allergens, iodinated secondary antibody was used at dilution factor of 5

the pool of diluted sera from five to ten allergic subjects. For identification of the individual proteins use IPG gel strips with immobilized narrow pH range. Two strips need to be tested for validation of replicability.

3.1 Isoelectric Focusing

In our isoelectrofocusing procedure, we always use the rehydration method with sample.

1. Prepare the sample in Rehydration buffer enough for two to four IPG strips. Sample loading volume per strip should be at 150 μ L. Add no more than 10–15 μ L (50 μ g of protein) of extract to Rehydration buffer (*see* **Notes 7** and **10**).
2. Add 150 μ L of the sample to each well of the focusing tray. Remove the protective cover from the IPG strip and place the strip into a tray well with the gel side facing down. Make sure that the strips are positioned in the focusing tray in correct polarity. Wet the strip by sliding it through the rehydration solution. Slide the damp electrode wicks between electrodes and the strip. Overlay each strip with mineral oil and cover the tray with the lid.
3. Place the tray on the Peltier platform. Use a preset method with linear voltage gradient and active rehydration. It takes about 18 h for the isoelectric focusing procedure with rehydration step to be completed (*see* **Note 8**).
4. Upon completion of the isoelectrofocusing step transfer the IPG strips into equilibration tray or plastic container with the gel side facing up and keep at 4 $^{\circ}$ C for a short time (1–2 days). For longer storage, transfer the strips to -20° C freezer.

3.2 SDS PAGE and Transfer

Always wear gloves and use tweezers when working with ImmobilonP transfer membrane.

1. Take needed number of IPG strips (add two more strips for replicability test, which will not be used for immunoblotting) from the freezer, place into equilibration tray and warm up to room temperature.
2. Prepare 2D equilibration buffer I for both strips (enough to use twice). Mix 25 μL of TBP stock with 975 μL of stock of 2D equilibration buffer and add about 450 μL to each strip. The buffer should cover the strip. Place the tray on the rocking table for 10 min, then replace equilibration buffer I in the tray with a fresh aliquot of the same solution and equilibrate for another 10 min.
3. Transfer the strips into the same volume of equilibration buffer II and continue the incubation for 10 min. While the strips are being incubated in equilibration buffers I and II, prepare NuPAGE Bis-Tris ZOOM gels, 1 $\times$ NuPAGE running buffer and 1 $\times$ transfer buffer (*see Note 9*).
4. Take the strip from the sample buffer, cut plastic ends of the strip and load the strip on the gel. Load 5 μL of Plus2 standard in the single well and electrophorese at 200 V until the dye front has reached the bottom of the gel (Fig. 1a, c, *see Note 10*).
5. Start preparing membrane, filter paper, and sponges for transfer when the dye front is about 1 inch from the bottom of the gel. Place sponges in the container, add transfer buffer, enough to cover, and use roller to remove any trapped air bubbles. Wet ImmobilonP membrane in methanol for 15 s, then rinse in water for 2 min and leave membrane in transfer buffer until the gel is ready (5–10 min).
6. Following SDS PAGE, immediately remove the gels from the running buffer. Open the plates with the help of a spatula. Rinse the gel in transfer buffer and assemble the gel membrane sandwich. Transfer two gels in one Blot Module at 30 V for 1 h.
7. After transfer is completed, rinse ImmobilonP blots in 100% methanol for 5 min.
8. Transfer all blots onto filter paper to remove the excess of the liquid.
9. Leave the blots between filter paper to dry overnight or over the weekend (*see Note 11*).

3.3 Immunoblotting

For identification of allergenic proteins, modified rapid immunodetection procedure suggested by Millipore were followed. To avoid any unspecific binding, the membranes have to be completely dry before starting immunolabeling with subjects' sera.

1. Bring the antibody dilution buffer to room temperature. Keep subject's and control sera on wet ice (*see Note 10*).

2. Dilute serum pool in antibody dilution buffer according to the specific IgE (sIgE) levels as follows: sera with sIgE levels at <5 kU/L were diluted four times, with sIgE at 5–10 kU/L-five times, with sIgE at 10–20 kU/L-ten times, and with above 20 kU/L of sIgE-twenty times. As a control for unspecific binding, negative control serum diluted four times should be used (*see* **Note 12**).
3. Place each blot in a dish and add diluted pool or individual serum enough to cover the blots.
4. Incubate the blots with agitation for 1 h. The solution should move freely across the surface on the membrane. At the end of the incubation with sera, prepare a dilution of the secondary antibody.
5. Remove antibody and add PBS to the blots.
6. Wash blots with PBS four times for 10–15 s each.
7. Add phosphatase-conjugated secondary antibody at 5000 times dilution and incubate with agitation for 45 min. [¹²⁵I]-labeled secondary antibody is to be used at five times dilution.
8. Repeat the washes as in **steps 5** and **6**. For phosphatase-conjugated secondary antibody, follow **steps 9a–12a**. For [¹²⁵I]-labeled secondary antibody, skip **steps 9a–12a** and follow **steps 9b–13b**.
- 9a. Aspirate PBS and rinse the blots in 50 mM (pH 9.6) Tris buffer for 1 min.
- 10a. Add Phosphatase substrate (BCIP/NBT) enough to cover the membrane and leave blots in the substrate for 5–10 min.
- 11a. As soon as the protein bands are visible on the blot with negative control serum, rinse the membranes in water and dry the blots with filter paper.
- 12a. Analyze images of the blots on any available Imaging System (Fig. 1b, e).
- 9b. If using [¹²⁵I]-labeled secondary antibody, after **step 8** from above: Remove the blots from PBS; dry the excess of the liquid with filter paper; mount the membranes on the new sheet of filter paper; wrap in clear plastic film.
- 10b. Place the wrapped blots in the cassette. In the dark room place Kodak X-OMAT Imagine Film on the blots.
- 11b. Insert the cassette in the light protective bag and leave the cassette at –80 °C for 2–3 h or overnight (*see* **Note 13**).

- 12b. Before developing the film, bring the cassette to room temperature. Usually it takes about 2 h.
- 13b. Develop the film (Fig. 2b, *see* **Notes 6** and **13**).

4 Notes

1. TBP is a toxic reagent and should be always opened under the fume hood. The reagent is not stable at lower concentrations. Any buffer containing TBP has to be prepared immediately before use.
2. TBP could be replaced with DTT in Rehydration and Equilibration buffers. Prepare 0.5 M stock solution: Dissolve 0.78 g of DTT in 10 mL of water, filter through 0.22 μ m syringe filter, make 0.5 mL aliquots and store the tubes at -20°C .
3. We have tested Rehydration buffer prepared with DTT at concentrations 0.05, 0.075, and 0.1 M. With 0.05 M DTT in the solution, the proteins were well separated. However, protein extracts separation showed better resolution in TBP containing buffer.
4. Leave one or two aliquots of the diluent in the refrigerator if using it next day.
5. The [^{125}I]-labeled antibody has very low specific radioactivity. Therefore, intensifying screens have to be placed on both sides of the cassette.
6. Advantages of using [^{125}I]-secondary antibody are clean Negative Controls and generation of multiple X-Ray images with various intensity of exposure. Disadvantages are the cost, requirement of Lab License for Isotope use and limited shelf life (about 2 months).
7. Salt concentration in the sample is most critical during electrofocusing procedure. The volume of the extract mixed with Rehydration buffer should be less than 10% of the total sample volume. The protein concentration in the extract loaded on IPG strips has to be at 5 mg/mL or higher. Use any desalting/concentrating columns with cut off ≥ 3 kDa to concentrate the extract. It is convenient to precipitate the proteins and then dissolve the pellet in Rehydration buffer. In our experiment we used "Perfect Focus" kit from Geno Technology Inc. for protein precipitation.
8. We found that it is practical to start the separation of the proteins in first dimension early afternoon. Then on next day morning, IPG strips are ready for equilibration and proteins separated in 1D would be loaded on SDS PAGE gels. First of all it is a must to validate IEF for replicability. Use two strips

for the second dimension and stain the gels with protein stain. Then the proteins from the rest of the strips could be transferred onto the membranes.

9. If DTT is used instead of TBP in Equilibration buffer, two buffers have to be prepared and used -I-1 and I-2. Equilibrate the IPG strips for 10 min in each. To make up I-1 buffer add 25 μ L of 0.5 M DTT to 975 μ L of 2D stock equilibration buffer (enough for two strips). Buffer I-2 contains 0.135 M of Iodoacetamide (25 μ g per 1 mL of buffer). It is important to equilibrate IPG strips in the buffer with DTT first followed by incubation in the Iodoacetamide containing buffer.
10. It is important at the beginning to separate the extract at broad pH range to see the map of all proteins and stain 2 gels for replicability of isoelectrofocusing. Coomassie Blue protein stain is less sensitive than Colloidal Blue stain but it is easy to use. In our experiments, we used Colloidal Blue stain for staining the extract separated at 3–10 pH range. Then the proteins were transferred onto the membrane and tested with serum pool from five patients to identify the clusters of allergens (Fig. 1a, b). For identification of individual allergenic proteins, extract was isoelectrofocused at narrow pH range. Membrane with transferred proteins could be stained with Amido Black or Coomassie Blue stain (Figs. 1d and 2a). Sensitivity of both stains are similar.
11. This is an important step before starting the immunolabeling, which helps to avoid unspecific binding to separated proteins. In addition, put a heavy weight (book or catalog usually works well) on the filter paper/membrane sandwich to keep the blots flat. The membranes with transferred proteins could be stored between filter paper up to 1 month at room temperature.
12. In our immunolabeling experiments, we used sera from non-food allergic subjects as a control for unspecific binding.
13. In case specific IgE of tested sera are very high, keep the cassette at room temperature for 2–3 h, develop the film, then put a new film and leave it overnight in the freezer. Always include negative control serum in each immunolabeling experiment.

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