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The classical proteomics approach for the identification of allergen candidates consists on the separation of proteins by high-resolution two-dimensional electrophoresis (2-DE) with subsequent IgE immunoblotting and further analysis of IgE-reactive protein spots with mass spectrometry. In this approach at least two gels must be run. One gel is used for staining and the other is for immunoblotting by antibodies labeled with specific immunostains. Additional functional characterizations require either protein purification or 2-DE replicates and appear to be time- and reagent-consuming. Here we described a modified double one-dimensional electrophoresis (D1-DE) allowing the conversion of a protein spot previously visualized by 2-DE into an extended protein band. In D1-DE, the purity of the protein of interest is similar to 2-DE spots, but its abundance is many times higher than what can be found in a 2-DE single spot allowing many other functional analyses from a single D1-DE separation.

1 Introduction

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immunoblotting, although disease-associated proteins often cover a very small percentage of the whole 2-DE separation, the entire gel must be transferred onto a membrane and individually incubated with a sizeable volume of serum (at least 250–300 μL for a membrane of 100 cm^2) [5–8]. In addition, with regard to allergomic studies, since the 2-DE has not yet been miniaturized, this method appeared to be tedious, time- and reagent-consuming when several allergenic sources have to be studied [9]. To overcome these drawbacks, we propose the use of a modified double one-dimensional electrophoresis (D1-DE) allowing the simultaneous screening of at least 30 allergic patient sera on the same blotted protein separated according to the isoelectric point (pI) and molecular mass (Mr), just as a 2-DE spot [10]. It is an extension of the first D1-DE concept originally reported by Atland et al. (1981) to study protein genetic variants [11]. This method consists of the sequential one-dimensional combination of two electrophoretic separations while the migration axis remains unchanged. The main advantage of this technique was obviously in the simultaneous separation and high resolution of proteins of interest in dual sorting parameters. For allergomics application, we designed and developed a D1-DE for the conversion of a protein spot previously visualized by 2-DE into an extended protein band. Therefore, the purity of the protein of interest is similar to 2-DE spots, but its abundance is many times higher than what can be found in a 2-DE single spot allowing many other functional analyses from a unique D1-DE separation.

2 Materials

2.1 Equipment

1. Horizontal flatbed electrophoresis apparatus (e.g., Multiphor II, GE Healthcare, Uppsala, Sweden).
2. Electrophoresis power supply apparatus of minimum 1000 V (e.g., EPL 3501 XL, GE Healthcare).
3. Thermostatic circulator (e.g., Multitemp II, GE Healthcare).
4. Semidry blotting apparatus (e.g., Multiphor II electro-semidry transfer apparatus, GE Healthcare).
5. Gel rehydration pool (e.g., GelPool, GE Healthcare).
6. Rehydration tray for IEF strips (e.g., rehydration trays, Serva, Heidelberg, Germany).
7. Flat Forceps.
8. A pair of scissors.
9. Magnetic stirrer.
10. Water bath.

2.2 First Dimension IEF

1. Polyacrylamide gel 4%T, 3%C (CleanGel, GE Healthcare BioSciences AB, Uppsala, Sweden).
2. Carrier ampholytes for IEF (Servalyt pH 2–11) were from Serva, Heidelberg, Germany.
3. Cathode and anode buffers (Serva IEF buffers, Serva, Heidelberg, Germany).
4. Electrode GF/B glass fiber strips (Whatman).

2.3 Second Dimension Separation Using SDS-PAGE

1. Chemicals composing the equilibration buffer: 114 mmol/L Tris pH 6.8 and 12% w/v sodium dodecyl sulfate (SDS) were all supplied by Sigma-Aldrich (St Louis, MO, USA).
2. Polyacrylamide 8–18% gradient gel (ExcelGel gradient 8–18%) were from GE Healthcare.
3. ExcelGel SDS buffer strips (anode and cathode; GE Healthcare).
4. Molecular masses (Mr) standard protein mixture was from Bio-Rad (Hercules, CA, USA).
5. Whatman N° 1 and 3 paper (GE Healthcare).

3 Methods

3.1 Principles of D1-DE

Like standard 2-DE, the D1-DE reported here consists of IEF as a first dimension followed by SDS-PAGE. The difference between the two techniques mainly resides in the migration axis of the second separation. This means that an acidic, neutral, or basic horizontal band from the first dimension (IEF) is transferred to SDS-PAGE (Fig. 1). Therefore, proteins are isolated as a long continuous band with purity comparable to 2-DE spots. In the present protocol, the sequential one-dimensional combination of IEF and SDS-PAGE followed by immunoblotting is described for the IgE screening of patients sera under equal conditions. For a better correlation between 2-DE and D1-DE patterns, the same IEF gel could be used as the first dimension of both 2-DE and D1-DE immunoblotting.

3.2 Preparation of IEF

1. A polyacrylamide gel 4%T, 3%C (CleanGel, GE Healthcare BioSciences AB, Uppsala, Sweden) is first hydrated in a solution containing carrier ampholytes (CleanGel Dry IEF-12.5 × 26.0 cm).
2. For this purpose, first prepare the rehydration buffer by adding 5% v/v Servalytes pH 2–11, (40% carrier w/v solution in water) (Serva, Heidelberg, Germany) in distilled water.
3. To rehydrate a complete CleanGel, pipet 25 mL rehydration solution into a Gelpool (tray for rehydration).

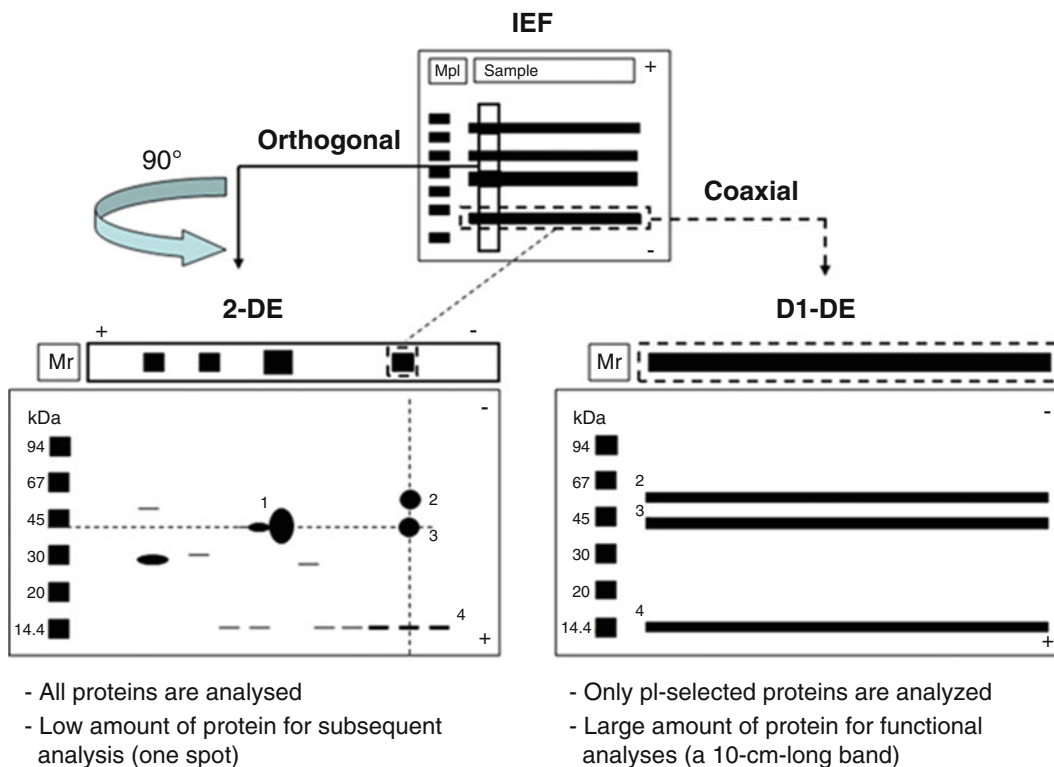


Fig. 1 Schematic illustration of a D1-DE adapted from Shahali et al. (2012) [10]. Both 2-DE and D1-DE consist of the sequential combination of IEF and SDS-PAGE. The difference between the two techniques resides in the axis of migration of the second separation

4. Place the CleanGel IEF precast gel into the pool for rehydration by laying the edge of the CleanGel, with the gel surface facing down into the rehydration buffer, slowly lower it in order to avoid air bubbles.
5. Using forceps, lift the film at the edges up to the middle and lower it again without catching air bubbles to distribute evenly the liquid. Very even rehydration is also obtained by shaking the GelPool at a slow rotation rate.
6. Meanwhile, switch on the cooling system (thermostatic circulator or Peltier cooling plate) of the horizontal flatbed apparatus.
7. Remove the gel from the Gelpool after 1 h making sure the rehydration has been evenly achieved.
8. Remove the excess buffer from the gel surface using the edges of a Whatman filter paper N° 1, until the gel surface is completely dry (*see Note 1*).
9. The rehydrated gel is now ready for IEF run.
10. Pipet a low amount of kerosene (about 2.5 mL) onto the horizontal cooling plate (e.g., Multithpor II apparatus) to improve the cooling contact.

11. Place the gel (gel surface up/gel film or GelBond down) onto the center of the cooling plate.
12. Cut the electrode strips at the gel length (26 cm).
13. Align strips on the cathodal and anodal edges of the gels, the electrode strips overlapping the gel-edge with about 3 mm.
14. Apply evenly the cathode and anode buffers (Serva IEF buffers, Heidelberg, Germany) on the cathode and anode strips, respectively.
15. Cut two 10-cm-long and up to 0.7-cm-wide strips of a dry Whatmans N°1 paper (GE Healthcare) for sample application on anode side.
16. Cut a small piece of Whatman N° 1 paper (0.5 × 0.5 cm) for IEF protein standards (pI marker) and place it directly onto the upper center of the gel (anode side).
17. Use forceps to align the strips on both sides of the pI marker at the same distance from the paper piece (minimum 1 cm).
18. Pipet 2 µL of pI marker (Bio-Rad) for wide or short range IEF as comparative references.
19. Load samples (55–60 µg of proteins from the allergen extract) using a micropipette on each 10-cm-long sample paper piece.
20. Clean platinum electrode wires with a wet tissue paper.
21. Then, move electrodes on anode/cathode strips to ensure a complete overlapping between buffer strips, gel, and electrode wires.
22. For Multiphor, connect the cables of the electrodes to the electrophoresis power supply apparatus (EPS 3501 XL, GE Healthcare). Finally lower and close the safety lid.
23. Select the running program and start the IEF migration (*see Note 2*).
24. Stop the migration and wash the cathode and anode borders of the gel with PBS pH 7.5.
25. Lift the film at the edges up to the middle to form a U shape and pour promptly PBS on the middle of the gel.

3.3 Preparation of D1-DE

1. After IEF running, cut out the gel at the pI marker along with a small part of the sample separation for Coomassie blue or silver staining.
2. Lay the gel face down on a clean plastic film or a glass plate. Mark the area that should be cut out for transferring. The size of this area should be 10 cm long and up to 7 mm wide.
3. Cut out horizontally the IEF strip in the selected narrow pI range containing the allergenic fraction of interest by reference to pI markers and sample stained.

4. At this step, the IEF strips could be transferred to the SDS-PAGE separation or placed in a sealed bag until use, conserve the gel at -20°C until use (up to 1 years).
5. Otherwise, incubate the IEF strips in the equilibration buffer containing 114 mmol/L Tris pH 6.8 and 12% w/v SDS for 3×10 min.
6. Meanwhile, switch on the thermostatic circulator adjusted to 12°C .
7. Pipet a low amount of kerosene (about 2.5 mL) onto the horizontal cooling plate (e.g., flatbed electrophoretic chamber, Multiphor II, GE Healthcare) to improve the cooling contact.
8. During the equilibration procedure, place the thin 8–18% gradient polyacrylamide gel (ExcelGel; GE Healthcare) surface up/gel film down onto the center of the cooling plate.
9. Align the ExcelGel SDS buffer strips (anode and cathode; GE Healthcare) on the cathodal and anodal edges of the gels, the electrode strips overlapping the gel-edge with at least 5 mm.
10. Cut a small piece of Whatman N°1 paper (0.5×0.5 cm) for molecular masses protein standards (Mr) and place it directly onto the upper center of the gel (anode side).
11. Remove the 10-cm IEF strips from the equilibration trays.
12. Remove the excess buffer using the edges of a Whatman filter.
13. Using two forceps, place each IEF strip gel-side down into the top of the SDS ExcelGel and push them carefully toward the anode.
14. Pipet 2–5 μL of Mr (Bio-Rad) onto the small piece of Whatman paper.
15. Clean platinum electrode wires with a wet tissue paper.
16. Then, move electrodes on anode/cathode strips to ensure a complete overlapping between buffer strips, gel, and electrode wires.
17. For Multiphor, connect the cables of the electrodes to the basic electrophoresis power supply apparatus. Finally lower and close the safety lid.
18. Select the running program and start the SDS-PAGE migration (*see Note 3*).
19. Stop the migration and wash carefully the entire SDS gel with PBS pH 7.5. At this step, DI-DE separations could either be silver-stained according to Blum et al. [12], Coomassie blue-stained or blotted onto a cyanogen bromide-activated nitrocellulose (Fig. 2) (Optitrans BA-S 83, Schleicher and Schuell, Dassel, Germany) sheet [13] for further functional analyses (*see Note 4*) [10, 14].

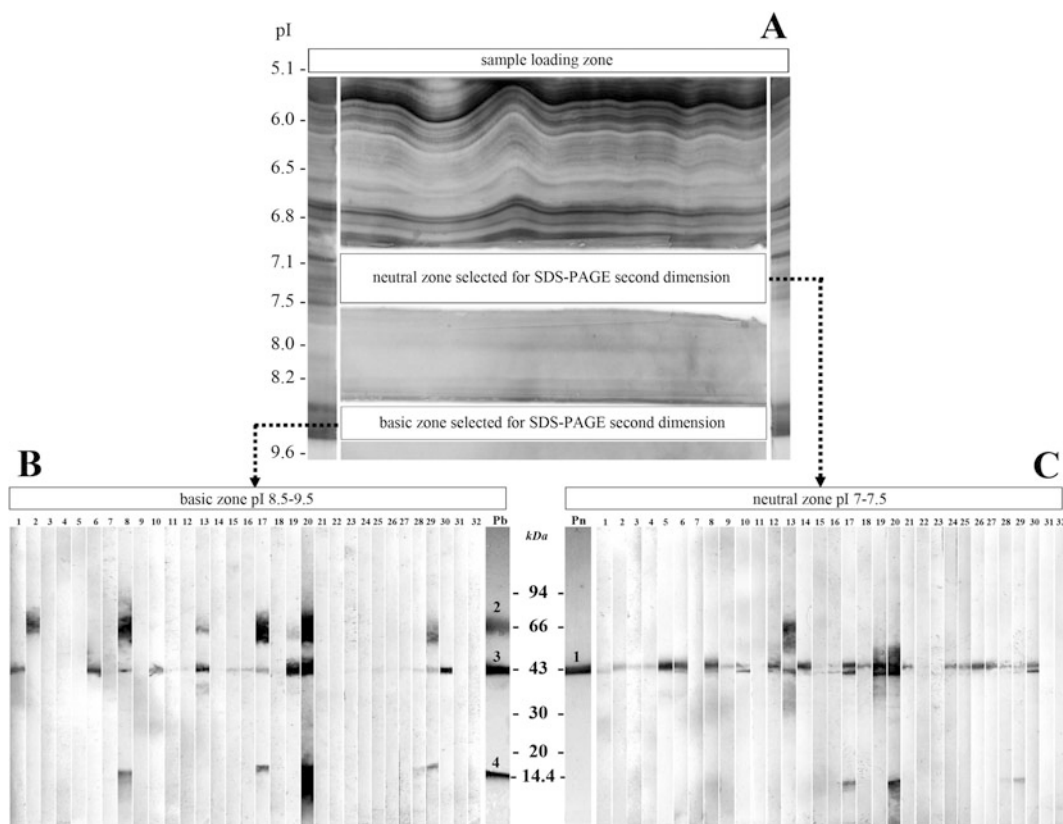


Fig. 2 D1-DE combined with IgE immunoblots (adapted from Shahali et al. 2012) [10]. (a) Represents the initial IEF separation. The silver staining was performed after excision of the basic and neutral bands. IgE immunoblots of the basic (b) and neutral (c) proteins of cypress pollen (CP) extracts probed with sera of 30 CP allergic patients: lanes 1–30; lane 31: healthy donor serum; lane 32: no serum (negative control). D1-DE allowed the MS/MS characterization of an allergenic polygalacturonase (PG) of 43 kDa which overlapped (in previous 1-DE experiments) with another CP major allergen belonging to the pectate lyase (PL) family referenced as Cup s 1 (*see Note 6*)

20. After staining, the protein band of interest could be cut out, digested, and submitted to mass spectrometry analyses (*see Note 5*) [10, 14].

4 Notes

1. Remove excess buffer from the gel surface with a piece of Whatman N° 1 paper until you can hear a “squeaking.”
2. Running conditions and parameters for IEF experiment:
Running temperature: 12 °C; total running time: 2 h 30 min.
Phase 1: Set the voltage as constant at 50 V during 60 min.
Remove the strips at the end of this phase.

- Phase 2: Set the voltage as constant at 200 V during 60 min.
Phase 3: Set the voltage as constant at 150 V during 40 min.
Phase 4: Set the watt as constant at 1 W during 90 min.
Phase 5: Set the watt as constant at 2 W during 50 min.
Phase 6: Set the watt as constant at 3 W during 120 min.
3. Stop the IEF when cytochrome C (red color) marker is near cathode side
- Running conditions and parameters for SDS-PAGE. Underlined values are constant.

Time (min)	Voltage (V)	Current (mA)	Power (W)	Temperature (°C)
75	100	40	40	12
110	750	20	15	12
120	900	25	15	12

4. Up to 40 blotted strips could be tested with various antibodies, with or without inhibitors, which gave rise to fundamental results concerning the specificity of the allergen recognition among cypress pollen allergic patients [14].
5. Besides multiplexing for immunoblotting, the D1-DE circumvents the problem of low protein amounts often encountered in a single 2-DE spot, which generally makes MS and microsequencing experiments difficult. The whole protein band can be excised, digested, and processed for analysis. A novel low-abundant allergen has been recently identified and characterized in cypress pollen using this approach [15].
6. D1-DE IgE screenings on the neutral and basic IEF fractions of CP extracts demonstrate that among 30 tested patients, 21 (70%) showed a positive IgE response to the novel 43 kDa basic allergen (PG), while 22 (~73%) were sensitized to Cup s 1 (PL). This novel CP major allergen has been recently indexed as Cup s 2 by the WHO/IUIS allergen nomenclature [14].

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