

## Immunoproteomics Analysis of Food Allergens

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### Abstract

Immunoblot-coupled proteomics based on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), namely, immunoproteomics, has been used for comprehensive identification of food allergens, because it is a simple and inexpensive tool for rapid identification of several IgE-binding proteins. In this section, we describe our protocols for identification of food allergens using immunoproteomics and discuss a few technical points in detail.

**Key words** Food allergy, Allergen, IgE, Immunoproteomics, Identification, 2D-PAGE, MS/MS

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### 1 Introduction

Some plants may trigger hypersensitivity reactions when patients with allergic diathesis eat parts of these plants or inhale their pollen. Most allergens in plants are proteinases, seed storage proteins, or enzyme (e. g., trypsin/amylase) inhibitor proteins. For identification of novel allergens, those allergens should have been extracted from food, purified and isolated by chromatography, such as ion-exchange and gel filtration, and their amino acid sequences decoded [1–3]. These methods involve complex steps, such as purifying the allergens from the crude extracts, and confirmation, at every step, by enzyme-linked immunosorbent assay (ELISA) or immunoblot analysis with the sera of the patients with food allergies, that the protein fraction has the capacity to bind IgE. Moreover, conventional methods to identify multiple allergens in foods are time-consuming, because only one allergen can be identified in a series of experiments. Proteomics has, therefore, been used for comprehensive identification of food allergens [4].

Immunoproteomics is a method coupling immunohistochemical analysis and proteomics based on 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE). For identification of allergens, immunoglobulin E (IgE)-binding protein spots are detected on a 2D map of the food extract, the spots are excised from the 2D gel,

**Table 1**  
**Summary of the reports of 2D-immunoblot analysis of plant allergens**

| Food plant | Identified allergen names                                                                                            | Authors        | Year | Reference no. |
|------------|----------------------------------------------------------------------------------------------------------------------|----------------|------|---------------|
| Celery     | Api g 1                                                                                                              | Vallier et al. | 1992 | [5]           |
| Hazelnuts  | Cor a 9 (11S globulin)                                                                                               | Beyer et al.   | 2002 | [6]           |
| Sesame     | Ses i 3 (7S vicilin-type globulin)<br>Ses i 2 (2S globulin)<br>Embryonic abundant protein<br>Seed maturation protein | Beyer et al.   | 2002 | [7]           |
| Lupin      | Conglutin $\gamma$<br>11S globulin basic subunits                                                                    | Magni et al.   | 2005 | [8]           |
| Peanuts    | Ara h 1<br>Ara h 3<br>Ara h 3/4<br>Ara h 4<br>Gly 1<br>iso-Ara h 3                                                   | Boldt et al.   | 2005 | [9]           |
| Peanuts    | Ara h 3 (basic subunit)                                                                                              | Restani et al. | 2005 | [10]          |
| Apple      | Mal d 1<br>Mal d 2<br>Mal d 3<br>Mal d 4<br>45 kDa basic protein                                                     | Herndl et al.  | 2007 | [11]          |
| Soybean    | Late embryogenesis abundant protein                                                                                  | Batista et al. | 2007 | [12]          |

followed by digestion with enzymes such as trypsin, and the allergens are identified by N-terminal amino acid sequencing or mass spectrometry/mass spectrometry (MS/MS) homology search. Immunoproteomics is simple and inexpensive method that allows rapid identification of several IgE-binding proteins at once. Since several IgE-binding proteins might be separated from crude extract using 2D-PAGE, minimum 5 short days would be needed to identify them in a food extract. In addition, protocols for 2D-PAGE have almost been optimized, whereas the protocols for allergen isolation need to be adjusted depending on the allergens. Therefore, the number of reports of identification of novel allergens and/or allergen location on 2D-map using immunoproteomics has been increasing recently [5–24] (Table 1).

In this section, we outline our protocols for the identification of food allergens using immunoproteomics and discuss a few technical points in detail. Of course, our protocols can be applied to 2D-western blotting to detect the target proteins using immunized animal antibodies [25, 26], and it is important to use optimal buffers for extracting allergens from food materials. The basic principle

is the same as that of 1D-immunoblotting, but double-staining of all protein spots and IgE-binding spots is adapted for precise localization of the IgE-binding spots. From our experience, 14 cm × 14 cm 2D-gels seems to be the optimal size for handling and obtaining good performance [11, 12, 18, 24].

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## 2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents.

Protect your eyes and hands when handling human serum, to prevent potential transmission of infections. Wearing gloves throughout the experiments is recommended for avoiding protein contamination.

### 2.1 Sample Preparation

1. Extraction buffer: 0.1 M phosphate-buffered saline (PBS, pH 7.2), 0.1 M Tris-HCl, pH 7.0, 1 M NaCl is frequently used (*see Note 1*).
2. 2-D Clean-Up Kit (GE Healthcare UK Ltd., Little Chalfont, UK): Store Wash buffer in the kit should be stored at  $-30^{\circ}\text{C}$ .
3. DeStreak Rehydration Solution (GE Healthcare): Before use, equilibrate at RT and dissolve urea crystals, and then add 0.5 % of IPG buffer and mix well.
4. Cellulose acetate 0.45  $\mu\text{m}$  filter (Dismic-45, Advantec Toyo Kaisha Ltd., Tokyo, Japan).
5. Pierce BCA Assay Kit (Thermo Fisher Scientific K.K., Yokohama, Japan) or 2D-Quant Kit (GE Healthcare).

### 2.2 Isoelectrophoresis and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Immobiline DryStrip pH3-10NL, 13 cm (GE Healthcare).
2. DryStrip holder, 13 cm (GE Healthcare).
3. IPG buffer pH3-10NL (GE Healthcare).
4. IPG phor III (GE Healthcare).
5. Cover oil fluid (GE Healthcare).
6. Equilibration buffer: 50 mM Tris-HCl, pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, and a small amount of bromophenol blue (BPB). Weigh 72.07 g of urea and 4 g of SDS and add 10 mL of 1 M Tris-HCl (pH 8.8), 69 mL of glycerol, and a small amount of BPB. Bring up the volume up to 200 mL and stir at room temperature (RT). Divide into aliquots and store at  $-20^{\circ}\text{C}$ .
7. Dithiothreitol: Just before use, add 100 mg (1 %) to 10 mL of equilibration buffer.
8. Iodoacetamide: Just before use, add 250 mg (2.5 %) to 10 mL of equilibration buffer.

9. Precast gels: Perfect NT gel S 10–20 %, 2-D well, 14 cm × 14 cm (D.R.C. Coop., Tokyo, Japan).
10. Electrophoresis system.
11. Agarose solution: 0.5 % low-melting agarose. Weigh 0.5 g of low-melting agarose and add 100 mL of SDS electrophoresis buffer. Warm to just before bubbling in a microwave. Divide 1 mL of agarose solution into microtubes and store at RT.
12. SDS electrophoresis buffer: 25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS. Dissolve 4.5 g of Tris base, 21.6 g of glycine, and 1.5 g of SDS in ultrapure water and top up the volume up to 1.5 L. In case of frequent use, make a tenfold concentrated stock solution and store at RT.
13. ECL Plex Fluorescent Rainbow Markers (GE Healthcare).

### **2.3 Double-Stained Immunoblotting**

1. Tank transfer system.
2. Blotting buffer: 25 mM Tris, 192 mM glycine. Dissolve 15 g of Tris base and 72 g of glycine in ultrapure water and top up the volume to 5 L. In case of frequent use, make a tenfold concentrated stock solution and store at RT.
3. Container: The area of the bottom and volume should be more than 14 cm × 14 cm and 250 mL, respectively.
4. Immun-Blot PVDF (Bio-Rad Laboratories, Hercules, CA, USA): Cut a sheet into 14 cm × 14 cm. Incubate in methanol for 5 min just before use.
5. Filter paper: 20 cm × 20 cm, for transfer grade.
6. Cy5 Mono reactive dye (GE Healthcare): Dissolve 1 vial with 100 µL of dimethylformamide, vortex, and add to 100 mL of PBS just before use.
7. Wash buffer: 0.05 % Tween-20/PBS. Add 500 µL of Tween-20 to 1 L of PBS, mix gently.
8. Blocking buffer: 0.5 % casein/PBS. Dissolve 1 g of casein to 200 mL of PBS. Heat the solution at 60 °C for 1 h, because casein dissolves with difficulty. After cooling to RT, store at 4 °C.
9. Horseradish peroxidase (HRP)-linked anti-human IgE (Nordic Immunology, Tilburg, Netherlands): Dilute 1:1,000 with 0.1 % casein/PBS just before use.
10. ECL Plus (Plex) Western Blotting Detection Reagents (GE Healthcare): available for detection by a fluorescence imager.
11. Image scanner: We use Typhoon 9400 from GE Healthcare.
12. Low-fluorescence glass plate (two plates): Large enough to cover a 14 cm × 14 cm membrane.

## 2.4 In Gel Digestion of Protein Spots

1. Quick-CBB plus (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or Silver Stain Kit for Mass Spectrometry (Thermo Fisher Scientific).
2. 1 M Ammonium bicarbonate: Weigh 67.9 mg and add 1 mL of ultrapure water. Prepare before every use.
3. Dehydration buffer: 50 % acetonitrile, 25 mM ammonium bicarbonate. Mix 1 mL of acetonitrile, 50  $\mu$ L of 1 M ammonium bicarbonate, and 950  $\mu$ L of ultrapure water.
4. Trypsin Gold (Promega): Dissolve in 100  $\mu$ L of ultrapure water. Divide aliquots and store at  $-80^{\circ}\text{C}$ .
5. Protease Max (Promega): Divide 10  $\mu$ L into each tube and store at  $-80^{\circ}\text{C}$ . Before use, add 990  $\mu$ L of 25 mM ammonium bicarbonate to make a 0.1 % solution.

## 2.5 Mass Spectrometry and Protein Identification

1. Zip-Tip C18 (Millipore Corp., MA, USA).
2.  $\alpha$ -Cyano-4-hydroxycinnamic acid ( $\alpha$ -CHCA, Sigma-Aldrich Corp., MO, USA): Add 50 % acetonitrile/0.1 % trifluoroacetic acid (TFA) to prepare a saturated solution (*see Note 2*). Dilute saturated  $\alpha$ -CHCA to 1:4 with 50 % acetonitrile/0.1 % TFA just before use.
3. Opti-TOF 384 well Insert (123 mm  $\times$  81 mm) (Life Technologies Corp., CA, USA).
4. 4800 MALDI TOF/TOF Analyzer (Life Technologies).
5. Mascot software (Matrix Science Inc., MA, USA).

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## 3 Methods

### 3.1 Protein Sample Preparation

1. Extract allergens from the plant specimens. Homogenize the plant specimens and add an adequate volume of extraction buffer. Rotate for more than 3 h to overnight at  $4^{\circ}\text{C}$  and centrifuge at  $8,000\times g$  for 10 min. Filter the supernatants using a 0.45  $\mu$ m filter.
2. Measure the protein concentration of the extracts with a Pierce BCA Assay Kit or 2-D Quant Kit, in accordance with the manufacturer's protocols. Divide into aliquots and store at  $-80^{\circ}\text{C}$ .
3. Purify and desalt an amount of extract containing 50  $\mu$ g proteins with a 2-D Clean-Up Kit (*see Note 3*).
4. Dissolve the protein pellet by vortexing in a 500  $\mu$ L of DeStreak Rehydration Solution to obtain a protein concentration of 0.1  $\mu$ g/ $\mu$ L. Divide the protein solutions into 25  $\mu$ g aliquots and store at  $-80^{\circ}\text{C}$ .

### **3.2 Isoelectric Focusing and SDS-PAGE (2D-PAGE)**

1. Pipette 250  $\mu$ L of the sample solution on a 13-cm strip holder and place an Immobiline DryStrip (pH3-10NL, 13 cm) on it, taking care to avoid formation of large air bubbles (*see Note 4*).
2. Overlay the strip with 800  $\mu$ L of Cover Oil Fluid (*see Note 5*).
3. Set and run an isoelectric focusing protocol as follows: Dehydration, 12 h; Step 1 (step-n-hold), 500 V for 4 h; Step 2 (gradient), 1,000 V for 1 h; Step 3 (gradient), 8,000 V for 2.5 h; Step 4 (step-n-hold), 8,000 V for 1.5 h; current limit 50  $\mu$ A/strip, run under 20 °C.
4. When the protocol is completed, pick the holders out. The total value of V-hrs will be 22,000 or more (*see Note 6*).
5. Pick the strip up from the holder and gently remove the oil, pushing it to a paper towel, then place the strip into a 60-mm dish so as to have the gel side face the inside of the dish. If not performing the next SDS-PAGE immediately, keep the strips stored at -80 °C until use.
6. Heat an Agarose solution tube at 100 °C on a heat-block.
7. If the strips had been kept in a freezer, place them at room temperature until they return to RT (*see Note 7*).
8. Add 10 mL of SDS-equilibration buffer containing 1 % DTT to the dish and gently shake for 15 min at RT.
9. Discard the buffer and replace with 10 mL of SDS-equilibration buffer containing 2.5 % iodoacetamide, and gently shake for 15 min at RT.
10. Pick the strip up and place it on the slot of an acrylamide gel plate with the acidic ends to the left (*see Note 8*).
11. Pipette slowly 1 mL of agarose solution on the strip without creating any air bubbles between the strip and the gel. Pipette 10  $\mu$ L of fluorescence rainbow marker into the marker slot of the gel before the agarose is set, and allow the gels to stand for 1–2 min until the agarose sets.
12. Fill an electrophoresis tank with 1,200 mL of SDS electrophoresis buffer. Avoid formation of bubbles around the inner tank.
13. Run the SDS-PAGE protocol at a constant 220 V for 3 h.
14. Stop the electrophoresis when the BPB line reaches ~5 mm from the bottom. After the electrophoresis, wash the gel in a container filled with ultrapure water, followed by immersion in blotting buffer (*see Note 9*).

### **3.3 Double-Stained Immunoblotting**

1. Incubate a 14 cm  $\times$  14 cm PVDF membrane in methanol at RT for 5 min. Sandwich the gel between a filter paper and PVDF membrane and place another filter paper on the membrane. Roll a cylinder glass tube on the surface of the membrane placed on the gel to remove any air bubbles. Fix them in a cassette,

and place the cassette in a transfer tank. Pour 5 L of transfer buffer into the tank, and transfer at 180 mA overnight under cooling (*see Note 10*).

2. After blotting, the membrane is picked up and incubated with Cy5/PBS solution to label the transferred proteins with Cy5 (*see Note 11*). Gently shake for 1 h at RT, avoiding exposure to light.
3. Wash the membrane with 100 mL of methanol. Gently shake for 10 min at RT and repeat two times with fresh methanol.
4. Wash the membrane with 100 mL of 0.05 % Tween-20/PBS. Gently shake for 10 min at RT and repeat two times with fresh solution.
5. Block the membrane with 100 mL of 0.5 % casein/PBS. Gently shake for 2 h at RT.
6. Pipette diluted serum (*see Note 12*) on the transferred side of the membrane. Incubate the membrane for 1 h at RT and then overnight at 4 °C under gentle shaking.
7. Wash the membrane with 100 mL of 0.05 % Tween-20/PBS (*see Note 13*). Gently shake for 10 min at RT and repeat two times with fresh solution.
8. Incubate the membrane in HRP-linked anti-human IgE (1:1,000 diluted with 0.1 % casein/PBS) solution for 1.5 h at RT under gentle shaking.
9. Wash the membrane with 100 mL of 0.05 % Tween-20/PBS. Gently shake for 10 min at RT and repeat two times with fresh solution.
10. Pipette ECL plus (plex) reagent on to the membrane, and after incubation for an appropriate period of time, move the membrane into ultrapure water (*see Note 14*).
11. Place the membrane on a clean, low-fluorescence glass plate with the stained side facing down. Gently pour a few milliliters of ultrapure water on the surface of the membrane and cover it with another clean glass plate (Fig. 1, *see Note 15*).
12. Place the gel plate on the scanning table of the image scanner. Scan gel images of Cy2 (458 nm, band-pass 40) for ECL plus (plex), and Cy5 (670 nm, band-pass 30) (Fig. 2).

### **3.4 In Gel Digestion of Protein Spots**

1. Prepare a gel separating 100 µg of proteins, stain with Coomassie brilliant blue or silver stain.
2. Excise gel pieces containing IgE-binding proteins from the 2D-gel and place them, one each in individual 1.5-mL centrifuge tubes.
3. The gel pieces are then decolorized with 50 µL of decolorizing buffer each. Replace fresh buffer and repeat washing the gel pieces until they are no longer stained.

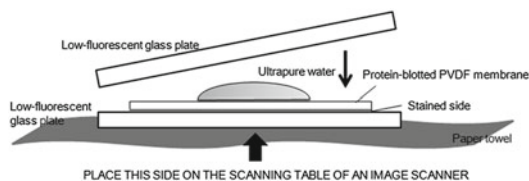


**Fig. 1** Detection of fluorescence of Cy5 and ECL-double stained membrane. After incubation with ECL plus (plex) substrate, place the membrane on a low-fluorescent glass plate with the stained side on which the proteins are stuck facing down. Drip a few milliliters of water on the back of the membrane and place another glass plate on the membrane to allow the water to spread over the entire surface of the membrane to remove air bubbles

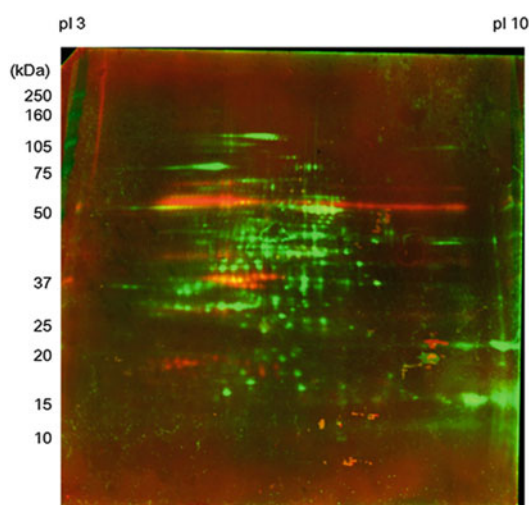


**Fig. 2** A merge image of all soluble rice seed protein- and IgE-binding protein-patterns. All transferred rice seed proteins were labeled with Cy5, and the IgE-binding proteins were labeled with ECL after incubation with the serum of a patient with rice allergy (diluted 1:100) followed by that with HRP-linked anti human IgE (diluted 1:1,000). The merged scan image of the Cy5 (*green*) and ECL (*red*) images is shown

4. Dehydrate the gel pieces with dehydration buffer followed by acetonitrile, and air-dry.
5. Add 10  $\mu\text{L}$  of trypsin digest solution (20 ng/ $\mu\text{L}$  of Trypsin Gold in 0.01 % Protease Max solution) to the completely dried gels. After keeping the tubes on ice for 15 min, incubate them for 2 h at 37 °C.

### 3.5 Mass Spectrometry and Protein Identification

1. Desalt the tryptic digests with Zip-Tip C18.
2. Mix 0.5  $\mu\text{L}$  of digest and 1  $\mu\text{L}$  of  $\alpha$ -CHCA and spot onto an Opti-TOF 384 well Insert plate.

3. Determine mass spectra and MS/MS fragment ion mass with a 4800 MALDI TOF/TOF analyzer.
4. Submit all product ions to a computer database search analysis with the Mascot MS/MS ion search.

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## 4 Notes

1. Appropriate extraction buffers for plant specimens or allergens should be selected. In general, soluble buffers such as PBS, Tris-HCl or NaCl solution are used as the extraction buffer. Commercial protein extraction kits for plant specimens may also yield good results within a short time. Prior to the 2D-immunoblotting, it should be confirmed by ELISA or immunoblotting using the patients' sera, that the extracts contain allergens.
2. After addition, vortex well and allow standing until the resins are completely settled. Do not use if the powder color changes to yellow, or the saturated solution is not clear.
3. Careful attention should be paid to desalt samples, since a high-salt buffer may be used for extracting the allergens. Most failures in spot focusing may be caused by not paying sufficient attention to this step.
4. Move the strip up and down so that entire surface of the gel comes in contact with the sample solutions.
5. Slowly drop the oil from the tip of the holder. Excessively rapid dropping of the oil would cause the samples to float above the gels. Be careful to avoid flooding with oil from the side of the holder.
6. We set long-time runs at low voltage so that any salt contaminating the samples moves away to the tip of the holder. It would not be a problem even if the voltage did not reach up to 8,000 V at the end of the run. If the voltage does not rise at all during the first step, it could be assumed that the sample preparation or rehydration has not been satisfactory.
7. Addition of buffers to a frozen strip might cause incomplete reduction and/or alkylation. Put the side of the strip on the bottom of the dish so that the gels get completely flooded by the buffer.
8. Cut 5 mm off both sides of the plastic sheet on the gels as to obtain a good fit into the gel slot. Place the plastic side of the strip on the longer plate and put it on the acrylamide gel with the flat side of a spatula. Be careful not to put it into the gels. If air bubbles are present between the strip and gel, remove them with a spatula.

9. When the gel is taken off from the glass plates, take care not to break the gel. Cut the acidic corner of the gel to see its direction.
10. Air bubbles between the gel and membrane should be removed. Change the time and/or the electric current, depending on the target range of molecular weight. Semi-dried transfer systems may also be used for blotting proteins to the membrane, while we have obtained good results using tank transfer systems. After the electrical transfer, cut the acidic corner of the membrane to see its direction.
11. Incubate the membrane face down so as to prevent drying of the surface of the membrane. After the incubation, check whether the entire surface of the membrane is stained blue.
12. The membrane should be incubated with more than 20 mL of serum, sufficient to cover the entire surface of the membrane. Take care to avoid drying of the surface of the membrane. The serum dilution could be changed depending on the total amount of IgE. Usually, we use 100-fold diluted serum (Fig. 2).
13. After incubation, the serum solution must be sterilized with 1 % sodium hypochlorite before it is disposed of.
14. ECL plus (plex) reagent should cover the entire surface of the membrane. Use of excessive reagent or a long reaction time might result in excessive chemical reaction, resulting in void spots. Dilution of the reagent with ultrapure water or a short reaction time might yield better results.
15. Insufficient water may cause drying of the surface of the membrane. Slowly put the glass plate down on the water hill to allow the water to spread over the entire surface of the membrane. Spotty air bubbles between the membrane and the glass plate would yield unclear images.

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