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Identification of peanut and hazelnut allergens by native two-dimensional gel electrophoresis

A procedure for the native two-dimensional electrophoresis of peanut and hazelnut proteins is described. Proteins were solubilised after acetone treatment using a combination of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and tetramethylene sulphone. These extracts were analysed by a combination of isoelectric focusing in the presence of lactose in immobilized pH gradients followed by charge shift electrophoresis. Immunoblot analysis, using sera from nut allergic patients, allowed the identification of a peanut and hazelnut allergen with identical isoelectric point and apparent molecular mass. These proteins were recovered from duplicate gels using a mixture of formic acid, acetonitrile (ACN) and isopropanol. The molecular masses for both proteins, determined by matrix assisted laser desorption/ionisation-mass spectrometry (MALDI-MS), were 4826 Da.

Keywords: Native two-dimensional electrophoresis / Charge shift / Peanut / Hazelnut / Allergen / Matrix assisted laser desorption/ionization-time of flight-mass spectrometry EL 4063

1 Introduction

To date, the analysis of peanut and hazelnut allergens has exclusively used protein denaturing electrophoretic techniques [1–3]. These techniques have enabled the identification of a large number of polypeptide allergens, particularly in peanut. Many of these are low *pI* glycoproteins [4]. However, the technique has hindered the identification of common features of allergen native structure and has prevented the identification of discontinuous epitopes – epitopes formed through folding of the polypeptide chains of proteins in their native conformation. These discontinuous epitopes are mainly on the surface of native proteins and surface epitopes are likely to be responsible for the immediate clinical response to a food allergen.

We investigated the native 2-D electrophoresis of two nuts, known to contain allergens. Two significant problems with native electrophoresis had to be overcome, firstly, to obtain high resolution of the native proteins by IEF and secondly, to obtain a second-dimensional separation based on molecular mass of the proteins without incurring denaturation. We investigated the use of the nondenaturing detergent CHAPS [5] and the lipophilic protein solvent, tetramethylene sulphone [6], together with glycerol and sodium carbonate buffer to solubilise

proteins and the application of the lectin-specific sugar to separate glycoproteins from lectin. Pharmacia “clean-gel” technology was used to optimise extraction and resolution of proteins by native IEF before transferring the technology to Immobiline gels for the 2-D separation.

We investigated the use of blue native electrophoresis in gradient PAGE gels, as described as a first-dimensional separation by Schägger *et al.* [7], in which proteins are resolved on the basis of their native molecular mass without denaturation, by inducing a charge shift on the proteins imposed by the addition of CBB to the electrophoresis buffers. These methodologies were combined to create a 2-D separation technique which was applied to both peanut and hazelnut proteins to identify similar allergens in the two seeds by immunoblotting with sera from nut allergic patients. The native 2-D separation of seed proteins is an important advance since it can lead to an analysis of the native structure of the allergens.

2 Materials and methods

2.1 Materials

All apparatus for IEF (Multiphor II electrophoresis chamber, Immobiline DryStrip Kit, LKB Macrodrive 5 power supply) and 3–10 nonlinear 13 cm Immobilines were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The vertical PAGE equipment (Protean II xi electrophoresis cell), blotting tank (transblott) and PVDF membrane were purchased from Bio-Rad (Richmond, CA, USA). The Ultraturax was purchased from Scientific Laboratory Supplies (Nottingham, UK) and the MALDI-

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Abbreviation: PDA, diacrylylpiperazine

Table 1. Buffer for native charge shift electrophoresis

Gel buffer (3 ×)	1.8 M aminocaproic acid, 0.15 M Bis-Tris, pH 7
Equilibration buffer	Gel buffer (1 ×) containing 0.012% w/v CBB G-250 and 12% w/v glycerol
Sealing buffer	Gel buffer (1 ×) containing 0.002% w/v CBB G-250 and 0.5% w/v agarose
Colourless cathode buffer	0.05 M Tricine, 0.015 M Bis-Tris, pH 7
Blue cathode buffer	Colourless cathode buffer containing 0.002% w/v CBB
Anode buffer	0.05 M Bis-Tris

TOF-MS Voyager DE STR from PE Biosystems (Foster City, CA, USA). All chemicals unless otherwise stated were purchased from Sigma (St. Louis, MO, USA). Acrylamide, diacrylylpiperazine (PDA), Bis-Tris, CHAPS, ammonium persulphate and TEMED were purchased from Amresco (Solon, OH, USA). Low-fat milk protein (Marvel) was purchased from a local market. GelBond PAG film was purchased from Flowgen (Ashby de la Zouch, UK). Acetone, ACN, formic acid, glycerol, isopropanol, sodium bicarbonate, sodium carbonate, Tris base and TFA were purchased from Fisher Scientific (Springfield, NJ, USA). Raw fresh peanuts and hazelnuts were purchased from Holland and Barrett. Sera were collected from patients attending the Regional Immunology Service, Manchester, who reported allergic symptoms after exposure to peanuts. Total, peanut and hazelnut specific immunoglobulin E (IgE) antibody concentrations were evaluated using the IgE RAST-CAP assay (Pharmacia) according to the manufacturer's instructions. Goat anti-human IgE(ε) alkaline phosphatase-labeled affinity purified antibodies were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA).

2.2 Methods

2.2.1 Protein extraction procedure and sample preparation for electrophoresis

Shelled, skinned peanuts or hazelnuts were ground in a mortar and pestle and 1 g of the powder was added to 33 mL of 50 mM sodium bicarbonate buffer, pH 9.2, containing 30% w/v tetramethylene sulphone. The mixture was homogenised with an Ultraturrax for 1 min and then stirred at room temperature for 1 h. The homogenate was centrifuged at $10\,000 \times g$ for 10 min, the clarified extract mixed with acetone at a ratio of 9:1 v/v, acetone: extract and the protein precipitated at -20°C for 30 min. After centrifugation at $10\,000 \times g$ for 10 min at 4°C the protein solids were washed twice with butanol and allowed to dry. These solids were then reextracted at a ratio of 25 mg to 1 mL of extraction buffer containing 5 mM triethanolamine buffer, pH 8.5, 2% w/v CHAPS, 20% w/v tetramethylene sulphone and 2 mM EDTA. In addition, 5% w/v lactose

Table 2. Gel composition for charge shift native PAGE

	Gradient separation gel		
	Stacking gel 4%T, 3%C	6%T, 3%C	12%T, 3%C
Gel buffer (3 ×)	4.0 mL	4.16 mL	4.16 mL
40% w/v acrylamide	1.16 mL	1.75 mL	3.49 mL
10% w/v PDA	0.144 mL	0.22 mL	0.43 mL
20% w/v CHAPS	0.12 mL	0.12 mL	0.12 mL
Glycerol	1.2 g	1.2 g	3.0 g
TEMED	6 µL	6 µL	6 µL
10% Ammonium persulphate	60 µL	60 µL	60 µL
Total volume	12.0 mL	12.5 mL	12.5 mL

was added for peanut extracts only. In the case of denaturing 2-D electrophoresis, the proteins precipitated by acetone treatment were washed in butanol as described and then resolubilised in 8 M urea containing 2% w/v CHAPS at a ratio of 25 mg to 1 mL of extraction solution.

2.2.2 IEF

Clean-gels were prepared by bonding a 4.2%T, 3%C acrylamide and PDA gel onto GelBond PAG film, washing three times in distilled water and once in 2% w/v glycerol, before drying at room temperature overnight [8]. Clean-gels were rehydrated in 0.7% w/v CHAPS, 20% w/v glycerol, 3.65% v/v pH 3–10 Ampholines and 7.5% v/v pH 5–7 Ampholines, for 1 h at room temperature. Samples were applied at the cathode and electrophoresis was performed at 12°C , from 200 V, 6 mA, 6 W for 30 min, then 500 V, 8 mA, 8 W, then 2000 V, 14 mA, 14 W for 4 h followed by 2500 V, 14 mA and 16 W for 15 min. 13 cm non-linear pH 3–10 Immobiline DryStrips were rehydrated in buffer containing 200 µg protein extract in 0.2% w/v CHAPS, 20% w/v glycerol, and 1% v/v Ampholines, overnight, gel facing down, in a rehydration chamber under paraffin oil [9]. Strips were focused on a Multiphor II, under silicone oil for a total of 66 000 Vh, with stepwise increments from 300 V for 10 min, followed by 1000, 2000 and 3000 V for 30 min each and then 3500 V overnight.

2.2.3 Charge shift electrophoresis

The procedure described by Schagger and von Jagow [10] was adapted for use as a second-dimensional electrophoresis separation technique. Focused strips were recovered from the silicone oil, drained briefly, then equilibrated for 3 min in 1.5 mL of equilibration buffer (Table 1). Strips were immobilised on the top of charge shift gels (Table 2) with sealing gel (Table 1) at 50°C which was then allowed to set for 2 min. Electrophoresis was per-

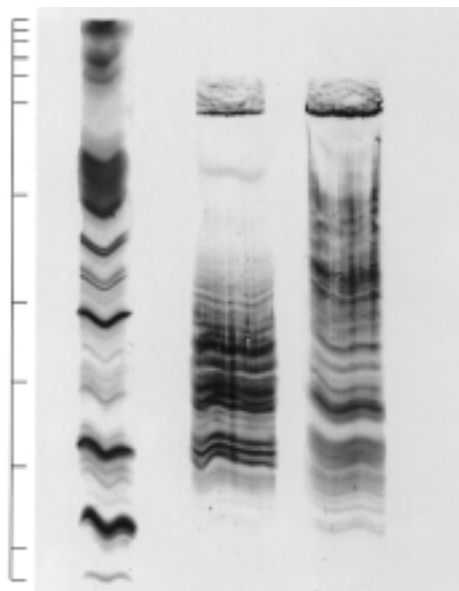


Figure 1. The IEF profile of peanut and hazelnut proteins after CBB staining. Left, pI marker; middle, 60 µg peanut proteins; right, 60 µg hazelnut proteins.

formed at 12°C, between blue cathode buffer and anode buffer (Table 1), at 100 V through the stacker, then 500 V through the gradient gel, until the dye front had reached one third of the total run distance, whereupon the cathode buffer was replaced with colourless cathode buffer (Table 1). At the end of the electrophoresis run, Immobiline strips were recovered and stained with CBB.

2.2.4 Immunoblotting

Proteins were transferred onto PVDF using Dunn's carbonate buffer (10 mM NaHCO₃, 3 mM NaCO₃, pH 9.9), at 12°C, overnight at 15 V. Staining of gels after transfer showed that these conditions transferred all of the detectable protein from the gel to the membrane. Membranes were blocked with PBS containing 22% w/v low-fat milk protein and 0.1% w/v Tween-20, for 4 h, then in PBS containing 10% w/v BSA and 0.1% w/v Tween-20 for 1 h. The PVDF membrane was found to be particularly difficult to block and high concentrations of low-fat milk protein and BSA were found to be necessary to minimise nonspecific antibody binding. The membranes were briefly washed three times in PBS before incubation overnight at room temperature with patient sera at a dilution of 1:10 in PBS containing 10% w/v BSA and 0.01% w/v sodium azide. After incubation the membranes were washed sequentially in PBS, then PBS containing 0.1% w/v Tween-20 and then PBS, before the addition of alkaline phosphatase-labelled anti-human antibody at 1:1000 dilution in PBS containing 5% w/v low-fat milk protein. After 2 h at

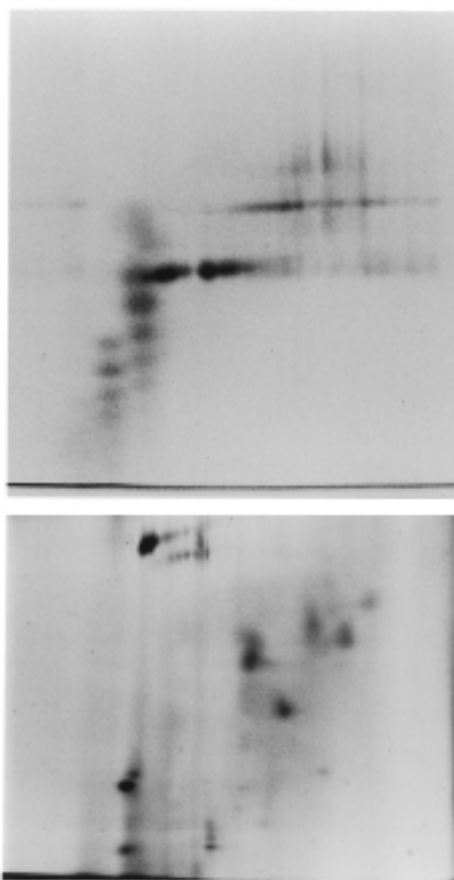


Figure 2. Native 2-D separation of peanut and hazelnut proteins (200 µg); CBB staining; Immobiline pH 3.5–10.0 nonlinear pH gradient; 6–12% PAG charge shift gel. Top, peanut; bottom, hazelnut.

room temperature the washing procedure was repeated and then the allergens visualised by incubation with Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium substrate.

2.2.5 Protein extraction from gels and MALDI-TOF-MS

Protein spots were excised from the gels after CBB staining and destaining. The gel pieces were dried under vacuum, then incubated in 50 µL of formic acid/ACN/isopropanol/water (50/25/15/10 v/v/v/v) in an ultrasonic bath at 35°C for 30 min [11]. The resulting solution was mixed 1:1 with 1% w/v cyano-4-hydroxycinnamic acid in 50% v/v ACN containing 0.1% w/v TFA and 1 µL spotted onto a stainless steel target and air-dried. MALDI-MS was performed with a TOF Voyager DE STR with a 337 nm nitrogen laser. Experiments were performed in linear mode with positive ion detection. Spectra were acquired in linear mode by summing 70–140 laser shots.

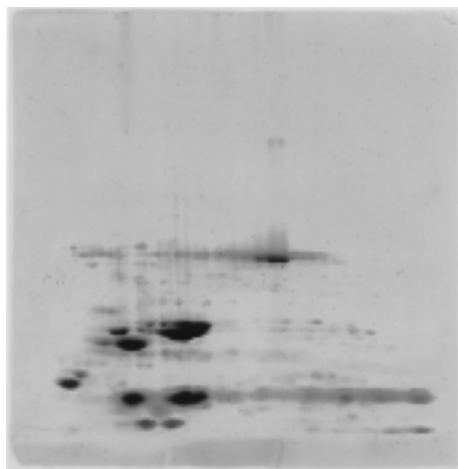


Figure 3. Denaturing 2-D separation of peanut proteins. Immobiline pH 3.5–10 nonlinear pH gradient urea IEF; 6–12% SDS-PAGE.

3 Results and discussion

3.1 Protein solubilisation

Obtaining sufficient protein solubilisation without the use of denaturing reagents was a recognised problem. Optimised soluble protein concentrations were obtained from acetone-washed ground peanut and hazelnut with increasing pH of extraction medium, 2 mM EDTA concentration and the addition of the nondenaturing detergent CHAPS and tetramethylene sulphone [5, 6]. Subsequent IEF of these extracts, however, gave poor resolution of protein bands. Optimum protein profiles could only be achieved with a combination of the removal of the testa from the nuts before protein extraction and the introduction of a butanol wash to remove residual fat. The total protein recovered from 1 g of peanut and hazelnut was 17 and 15.3 mg for peanut and hazelnut, respectively. This compares with only 8.2 mg obtained for peanut after extraction with Tris buffer at pH 7.0.

Immunoblotting of the peanut proteins with human sera after IEF showed severe underlying streaking not visible on the CBB-stained gels. It was suspected that this was caused by binding of glycoprotein to lectin in the extracts: both peanut lectin and low pI peanut glycoproteins are allergens. Peanut lectin binds to lactose and the addition of 5% lactose to the extraction media markedly improved the resolution of the peanut immunoblots. In a separate study, we have obtained similar improvements examining IEF immunoblots from soybean extract after the addition of 5% *N*-acetylgalactosamine (the sugar specific for the soybean lectin) to the extract, suggesting that it is the dissociation of the lectin glycoprotein complexes that improves the resolution of the immunoblots.

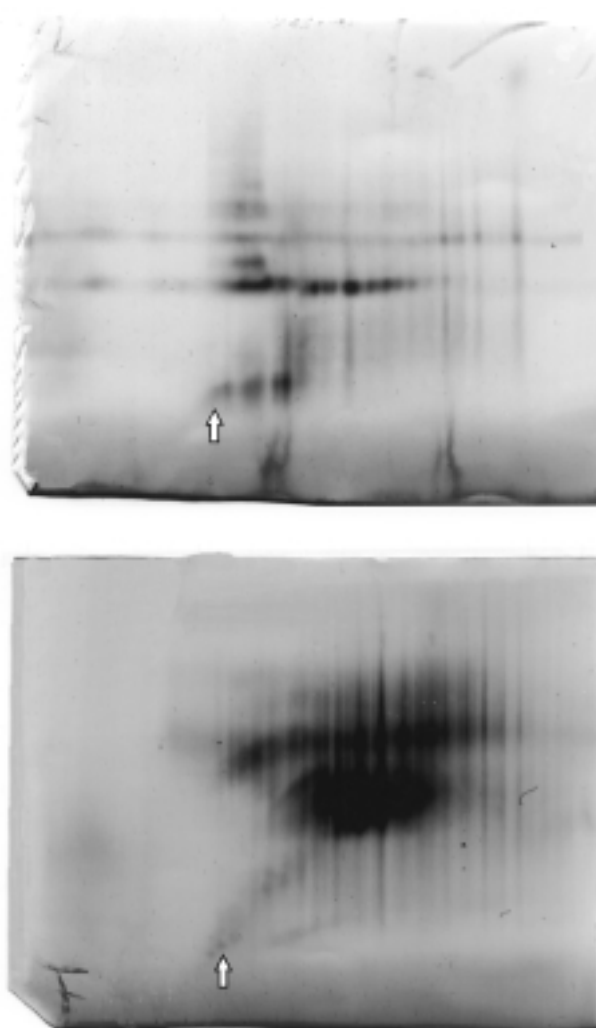


Figure 4. Immunoblots of native 2-D separated peanut and hazelnut proteins (200 µg) separated on Immobiline nonlinear gradient pH 3.5–10 and 6–12% PAGE charge shift gel. The arrow indicates the low molecular mass allergen identified in both peanut and hazelnut. Top, peanut; bottom, hazelnut.

3.2 IEF

We found that clear protein profiles (Fig. 1) could only be achieved by IEF after a number of measures had been taken. The use of rehydrated “clean-gels” (Amersham Pharmacia Biotech) was found to be essential for the optimum resolution of peanut and hazelnut protein by IEF. There was, however, significant variability in profiles of an extract run several times across the same gel when the wire electrodes were placed directly on the gel. This was only alleviated with the introduction of paper wicks, soaked in water, contrary to the recommended procedure [8]. Clear protein profiles were only achieved after the

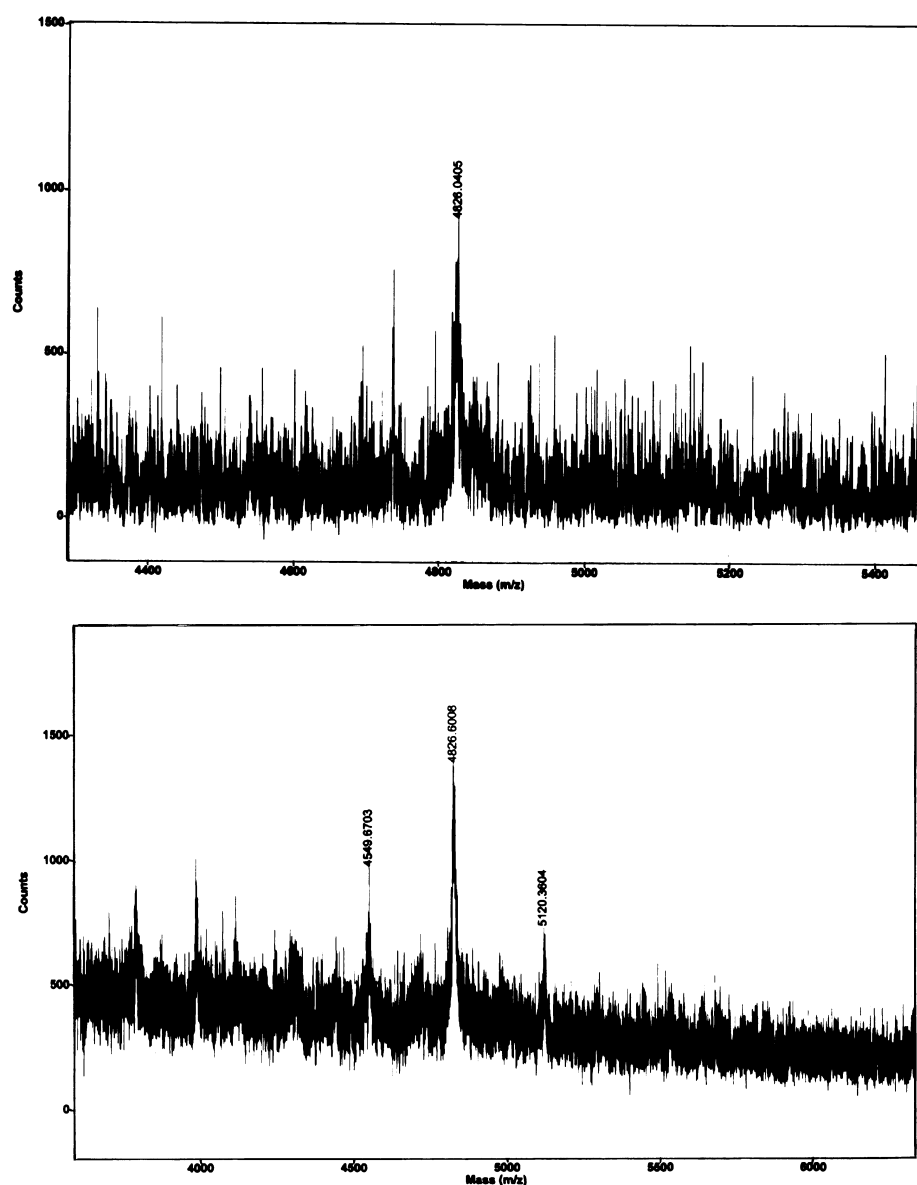


Figure 5. The MALDI-TOF profile of the two low molecular mass allergens identified by native 2-D electrophoresis of peanut and hazelnut proteins. The protein spots were excised from the gel, eluted in formic acid: ACN: isopropanol: water 50:25:15:10, and the resulting solution spotted onto the stainless steel MALDI target: Top, hazelnut; bottom, peanut.

addition of CHAPS to the rehydration buffer on the “clean gels” however, despite published reports of CHAPS enhancing blotting [12], 2% CHAPS completely inhibited the transfer of proteins from IEF gels onto PVDF membranes during electroblotting. CHAPS has zwitterionic properties and will not carry a charge during blotting, thereby probably suppressing the native protein charge during transfer. By reducing the CHAPS concentration in the IEF gel to below the critical micelle concentration it was possible to successfully electrotransfer proteins to PVDF membrane, although some protein streaking reappeared. Dunn's carbonate buffer was used for blotting since its high pH ensured that all proteins with p/s below 9.9 would be charged and would therefore transfer.

3.3 Charge shift PAGE

Charge shift electrophoresis, whilst akin to SDS denaturing PAGE, in distributing a uniform charge over the proteins, differs since it does so without denaturing the proteins. Charge shift utilises the binding power of CBB to proteins, giving all proteins a negative charge. Proteins then move through the gel driven by the CBB towards the anode. Using this technique, gels were obtained which showed a broad distribution of proteins across the gel for both peanut and hazelnut. This separation was further enhanced by adjusting the polyacrylamide gradient to retard the migration of larger proteins (Fig. 2). In comparison, the separation of peanut proteins by denaturing 2-D

electrophoresis under the same pH and gel gradient conditions showed a significantly different distribution of proteins across the gel (Fig. 3).

The conditions to effect optimum charge shift separation in the second dimension were changed slightly from the published method. A slightly higher concentration of aminocaproic acid was used than that reported (0.6 M instead of 0.5 M) since this higher concentration was found to improve the resolution of the protein profiles. PDA was used as the second-dimensional acrylamide cross-linker [13] to strengthen the gradient gel and decrease background staining when gels were subsequently silver stained. It was also necessary to incorporate CBB into the first-dimensional gel equilibration buffer and sealing buffer to facilitate the solubilisation and movement of the proteins from the first dimensional into the second. After CBB staining of the recovered Immobiline strips at the end of electrophoresis it was found that all the protein had moved from the first to the second dimensional since no banding was apparent.

3.4 Allergen identification and analysis

Analysis of immunoblots of the 2-D native profiles of hazelnut and peanut proteins using sera from four peanut-hazelnut allergic patients revealed two areas of allergen commonality on the gels with three of the sera, one a discreet low molecular mass allergen and a second allergen associated with a faint streak near the center of the gel (Fig. 4). Only the lower molecular mass allergen could be detected in both the hazelnut and peanut samples by immunoblotting after excision from the gel. Further analysis by MALDI-TOF-MS of this low molecular mass allergen revealed that both the hazelnut and peanut samples contained a protein of mass 4826 Da (Fig. 5). Additional work is required to confirm that the separated proteins are undenatured and unfragmented.

4 Concluding remarks

A method for native 2-D separation of proteins is described. Major problems associated with the development of a native 2-D electrophoretic separation are retaining solubility of the protein throughout the separation and

obtaining a separation based on different characteristics of the protein for each of the two dimensions. By manipulating concentrations of the nondenaturing detergents CHAPS, tetramethylene sulphone and aminocaproic acid, it was possible to solubilise high concentrations of proteins from both peanuts and hazelnuts and to couple native IEF to native charge shift electrophoresis. The resulting 2-D separation is based on native charge of the proteins in the first dimension and native molecular size in the second dimension, providing information for further protein purification.

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5 References

- [1] Burks, A. W., Cockrell, G., Connaughton, C., Karpas, A., Helm, R. M., *J. Allergy Clin. Immunol.* 1995, **95**, 607–611.
- [2] Burks, A. W., Cockrell, G., Stanley, J. S., Helm, R. M., Bannon, G. A., *J. Clin. Invest.* 1995, **96**, 1715–1721.
- [3] Hefle, S., Folger, J. P., Bush, R. K., Chu, F. S., *Food Agric. Immunol.* 1994, **6**, 197–208.
- [4] Taylor, S. L., Lehrer, S. B., *Crit. Rev. Food Sci. Nutr.* 1996, **36**, S91–S118.
- [5] Stark, R. E., Leff, P. D., Milheim, S. G., Kropf, A., *J. Phys. Chem.* 1984, **88**, 6063–6067.
- [6] Vecchio, G., Righetti, P. G., Zanoni, M., Artoni, G., Gianazza, E., *Anal. Biochem.* 1984, **137**, 410–419.
- [7] Schagger, H., Cramer, W. A., von Jagow, G., *Anal. Biochem.* 1994, **217**, 220–230.
- [8] Westermeier, R., *Electrophoresis in Practice*, VCH, Weinheim 1993, pp. 153–165.
- [9] Sanchez, J.-C., Rouge, V., Pisteur, M., Ravier, F., Tonella, L., Moosmayer, M., Wilkins, M. R., Hochstrasser, D. F., *Electrophoresis* 1997, **18**, 324–327.
- [10] Schagger, H., von Jagow, G., *Anal. Biochem.* 1991, **199**, 223–231.
- [11] Ehrling, H., Stromberg, S., Tjernberg, A., Noren, B., *Rapid Commun. Mass Spectrom.* 1997, **11**, 1867–1873.
- [12] Rabilloud, T., *Electrophoresis* 1996, **17**, 813–829.
- [13] Hochstrasser, D. F., Harrington, M. G., Hochstrasser, A.-C., Miller, M. J., Merrill, C. R., *Anal. Biochem.* 1988, **173**, 424–435.