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Two-dimensional electrophoresis replica blotting: A valuable technique for the immunological and biochemical characterization of single components of complex extracts

The combination of the high resolution electrophoresis (2-DE) with subsequent transfer onto a protein-binding membrane (blotting), immunological detection, and/or *N*-terminal sequencing is a powerful tool to identify and characterize single components of complex protein mixtures. Direct comparison of protein staining, immunological detection, and biochemical characterization of single protein spots was achieved by the replica blotting technique. The proteins were transferred from one two-dimensional gel onto several blotting membranes one after another. A canon of methods has been employed to identify and characterize allergens from different allergen sources. We have studied single major allergens as well as related major allergens from different grass species ("allergen groups") using patients' sera and allergen-specific monoclonal antibodies. The biochemical structure of the allergenic components has been analyzed by *N*-terminal and internal protein sequencing, precise mass determinations by matrix-assisted laser desorption/ionization mass spectrometry and investigations on post-translational modifications such as glycosylation. Here, we give a general survey of methods, and we describe an array of techniques suitable for characterization and identification of components of complex extracts, even if there is little or no previous information available.

Keywords: Allergens / Blotting / Carbohydrate structure / Two-dimensional gel electrophoresis
PRO 0441

1 Introduction

The aim of many bioscientific studies is to identify, characterize, and/or isolate single components from very complex protein mixtures such as blood, homogenates, or extracts. A lot of information is necessary in order to devise an efficient separation and purification strategy. By use of chromatographical procedures, separations can be performed according to size, hydrophobicity, isoelectric point (*pI*), affinity by gel filtration, reverse phase, chromatofocusing and affinity chromatography, respectively. Further purification steps may require several time-consuming steps and interpretation of chromatograms. Furthermore, these techniques require large amounts of source material. Each chromatographical run

is separated by only one parameter, and therefore several runs with different parameters must be performed to purify components. In contrast, 2-DE blotting is a technique that combines two physicochemical properties: *pI* and molecular mass. The experimental conditions can be optimized according to the proteins of interest. Narrow *pI* ranges and molecular mass ranges can be prepared. Frequently, separation according to just one physicochemical property is not sufficient, as is the case with grass pollen allergens. Only when two techniques (IEF and SDS-PAGE) are combined, can we separate the components from each other.

When we started 12 years ago to identify and characterize allergens of pollen (pollen may contain up to 1000 different components [1]), little was known about the number of relevant allergens and their biochemical structures. Approximately 20% of people in industrialized countries suffer from type I allergy, which is characterized by specific immunoglobulin E (IgE) antibodies against harmless components from the environment. For specific immunotherapy (hyposensitization), knowledge of their biochemical structure is necessary to create modified aller-

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Abbreviations: **BCIP**, 5-bromo-4-chloro-3-indolyl phosphate potassium salt; **Ig**, immunoglobulin; **NBT**, nitro-blue tetrazolium

gens that would allow a safer therapy [2]. Thus, 2-DE is a useful step (1) to get an overview of the components of the extract, (2) to determine the allergens, (3) to characterize them individually by immunological and biochemical techniques, and (4) to isolate single components. For steps (2) to (4) we performed blotting and employed specific detection and characterization techniques.

2 Materials and methods

2.1 Pollen extract

One gram of grass pollen (ARTU Biologicals, Lelystad, The Netherlands) was incubated in 50 mL of 0.1 M NH_4HCO_3 buffer (pH 8.0) for 30 min. Afterwards, the soluble fraction (the extract) was isolated by centrifugation at $17\,000 \times g$ for 30 min and dialyzed against double-distilled water overnight. The extract was lyophilized and stored at 4°C until use.

2.2 Patients' sera and monoclonal antibodies

For the identification of IgE-reactive proteins we used sera collected from several grass pollen-sensitive patients with high amounts of specific IgE antibodies (CAP class >IV) using the Pharmacia CAP System (radio allergosorbent test for the quantification of specific IgE, scored according to the manufacturer's protocols; Pharmacia Diagnostics, Uppsala, Sweden). Monoclonal antibodies had been raised against crude timothy pollen extract [3, 4].

2.3 SDS-PAGE

SDS-PAGE was performed as described by Laemmli *et al.* [5] on homogenous gels (15%T, 2.6%C) under reducing conditions. Samples were loaded at a concentration of 5 µg/cm onto the gel. The proteins were subsequently transferred to nitrocellulose membrane (0.45 µm pore size, Schleicher & Schüll, Dassel, Germany) by semidry blotting for 30 min at 0.8 mA/cm² [6].

2.4 2-DE blotting

2-DE was performed as described by Görg *et al.* [7]. First dimension IEF was carried out on polyacrylamide strips (4%T, 4C%; size $240 \times 80 \times 0.5 \text{ mm}^3$) with an immobilized pH gradient of 3.6–9.3 containing 8 M urea and 10 mM DTT. Either 200 µg or 500 µg of protein were applied to each lane and separated at 3000 V and 2 mA for 7 h. After incubation for 3 min in an equilibration solution containing 6 M urea, 65 mM DTT and 260 mM iodoacetamide,

SDS-PAGE was carried out in the second dimension using gradient gels (8–13T%, 4C%; size $240 \times 80 \times 0.5 \text{ mm}^3$) at 200 V and 30 mA. Molecular masses and p/s were determined by comparison with marker protein test mix 9 (pH range 3.5–10.5; Serva, Heidelberg, Germany) and low range SDS-PAGE standards (14.4–97.4 kDa; Bio-Rad, Richmond, CA, USA).

Immunological studies were performed by thermoblotting onto nitrocellulose membrane [8]. For structural determination (protein sequencing, amino acid composition, neutral sugar analysis) proteins were transferred onto Immobilon P membrane (Millipore, Eschborn, Germany) by electroblotting for 30 min at 200 mA using 10 mM CAPS (pH 11.0) supplemented with 10% v/v methanol as blotting buffer [9].

2.5 Protein staining and immunological detection

For protein staining on nitrocellulose and PVDF membranes we used 0.1% India ink (Pelikan, Hannover, Germany) in TBS [10] and 0.1% Coomassie G-250 in 50% methanol, respectively.

For the identification of antigens and allergens, blots were incubated with hybridoma supernatants and patients' sera, respectively. As secondary antibodies we used alkaline phosphatase-conjugated goat anti-mouse IgG/IgM (Dianova, Hamburg, Germany) and goat anti-human IgE (Allergopharma, Reinbek, Germany). The binding patterns were visualized by a substrate solution consisting of 0.033% nitro-blue tetrazolium (NBT) and 0.017% 5-bromo-4-chloro-3-indolyl phosphate potassium salt (BCIP) (Sigma, St. Louis, MO, USA) in 0.1 M TBS (pH 9.5) [11].

To study the carbohydrate moieties, blots were incubated with several digoxigenin-labeled, carbohydrate-specific lectins (Roche, Mannheim, Germany) [12]. Lectin binding was visualized by alkaline phosphatase-labeled digoxigenin and NBT/BCIP as chromogen/substrate mixture.

2.6 N-terminal microsequencing and amino acid analysis

After blotting for 30 min onto PVDF membranes, the blots were washed in double-distilled water, stained in 0.1% Coomassie R250 (Serva) in 50% methanol for 10 min, destained in 50% methanol, and air-dried. Protein spots containing the allergen were excised. Microsequencing was performed by Edman degradation using a 473 A protein sequencer with on-line PTH amino acid analyzer

(Applied Biosystems, Weiterstadt, Germany). For determination of the amino acid composition, the 420 H Applied Biosystems analyzer was used with hydrolysis and on-line PTC (phenylthiocarbamyl) amino acid analysis.

2.7 MALDI-MS

Pollen extracts were separated by 2-DE, electroblotted onto nitrocellulose membrane and stained by Ponceau S (Roche). An area about 3 mm² of the stained spots was excised and dissolved in 20 µL matrix solution (saturated α -cyano-4-hydroxycinnamic acid (Sigma, Deisenhofen, Germany) in acetone containing 1% TFA) [13]. Positive MALDI mass spectra were obtained on a REFLEXTM (Bruker-Franzen-Analytik, Bremen, Germany) in linear TOF mode at an acceleration voltage of 28.5 kV. Mass calibration was performed internally by adding a known amount of a dissolved dot blot of BSA to the first sample. This calibration constant was then used to analyze the remaining samples.

2.8 Carbohydrate detection and determination

Carbohydrate detection was performed using the glycan detection kit (Roche) according to the manufacturer's instructions [14]. Vicinal hydroxyl groups of carbohydrates were oxidized to aldehydes by 10 mM NaIO₄ diluted in 100 mM sodium acetate buffer (pH 5.5) for 20 min. Then the steroid hapten digoxigenin was covalently bound to the aldehyde groups via a hydrazide group. Glycoproteins were visualized by alkaline phosphatase labeled anti-digoxigenin followed by incubation in NBT/BCIP substrate.

2.9 Monosaccharide determination by neutral sugar analysis

Qualitative and quantitative determination of carbohydrate composition was performed according to Mankarios *et al.* [15]. The protein spots of fifteen 2-DE separated samples (each containing 400 µg timothy pollen extract) were excised from PVDF membranes and treated for 48 h by 0.1 M HCl, 500 ng of allose were added as internal standard. The samples were evaporized and afterwards diluted in 0.3 M NaBH₄ solution containing 0.25% w/v NaOH to reduce to the corresponding alditol derivatives. Peracetylation was performed overnight by a mixture of pyridine : acetic anhydride (3 : 1). For identification and determination of the monosaccharides, the samples were separated by gas phase chromatography (HP-5 cross-linked) on-line to MS (MS Engine HP 5989A mass spectrometer, Hewlett Packard, Avondale, PA, USA).

3 Results and discussion

3.1 2-DE and blotting for the detection of allergens

To obtain a general overview of the proteins in a particular extract/mixture by 2-DE, the gel compositions should allow for wide-range separation according to *pI* and molecular mass. In our case, we wanted to identify allergenic, relevant components in grass pollen extracts. The components were extracted from the pollen using an aqueous buffer (similar to the natural exposition by an allergic individual) and no problems concerning insolubility and precipitations occurred in the following experiments. We studied timothy grass pollen (*Phleum pratense*) as an important representative of the grasses; the extract was separated by 2-DE using a wide *pI* range (3.6–9.6) for the first dimension and an SDS-PAGE gradient gel that enabled protein separation in the molecular range from 10–100 kDa. The proteins were blotted by capillary force at 50°C (thermoblotting) from one 2-D gel onto several sheets of nitrocellulose membrane to obtain identical replicas of the protein pattern. This procedure saves times and allows the comparison of differently treated blots. For immunological studies the transferred protein concentration is sufficient, and the IgE-reactive, allergenic molecules were detected by immunostaining utilizing a mixture of sera of grass pollen-allergic patients. One replica was incubated with India ink for protein detection.

As shown in Fig. 1A, approximately 250 components were detected with *pI*s ranging from 4.6–9.6 and masses of 12–70 kDa [16]. However, only 16 intense spots showed IgE reactivity as identified by patients' IgE antibodies [17]. These components are indicated in Fig. 1B by numbers. Most allergens were detected as single spots within the range 38–32 kDa and a wide *pI* range of 5.0–9.5 as single spots. Thus, in contrast to the 1-D SDS-PAGE the components were clearly resolved from each other, since they focussed at different *pI*s. Additional IgE-reactive proteins were detected at about 13 kDa and a smear covering *pI* 6.0–8.0 and 55–60 kDa. Several components showed similar size but differed in their *pI*, e.g. for the most intense spots 1–4 and 5–8 we estimated a molecular mass of 38 and 32 kDa, respectively. Spots 9 and 10 revealed sizes of 35 kDa, spots 12 and 13 of 37 kDa and spot 11 (weak smear) of 33 kDa [19]. Surprisingly, as observed by direct comparison of Figs. 1A and B, India ink did not stain all proteins [20]. The allergenic components 1–8 and 14–15 were not detected. However, these spots were stained by AuroDye (Amersham Biosciences, Little Chalfont, Bucks, UK). These differences were also observed when Coomassie stain was used (data not shown). While the India ink-stainable components showed intense blue spots, red spots turned to a

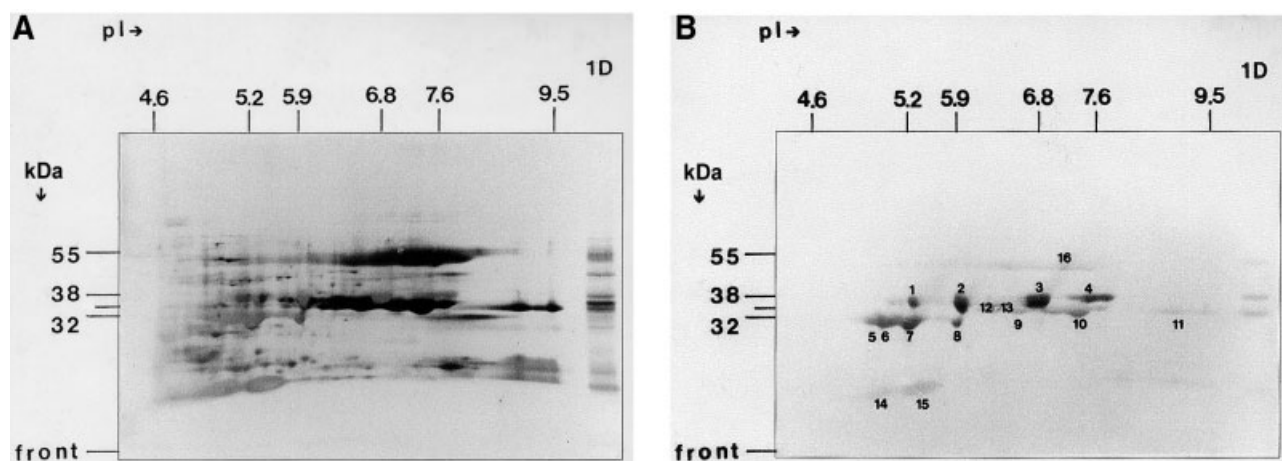


Figure 1. 2-DE blot of timothy grass pollen extract ([17] reproduced with permission). A, protein staining of nitrocellulose membrane by India ink (intense dark spots) and afterwards with AuroDye (gray spots). B, detection of allergenic components by use of patients' serum and identification of IgE-reactive proteins. Intense spots are indicated by numbers.

light blue after Coomassie staining. Even this “unspecific” protein staining might indicate a probable biochemical similarity.

3.2 Characterization of the protein structure

For immunological characterization and classification of the proteins of an extract, allergen-specific mAbs are valuable tools. We raised mAbs using crude timothy pollen extract to immunize BALB/c mice. Using the replica method we showed that IgE-reactive components were also recognized by mAbs. Using the 2-DE blotting technique with several identical imprints from one gel (replica technique) it was possible, even in the 30–40 kDa region with closely adjacent proteins, to correlate and classify the particular spots. Since each mAb is epitope-specific, the detection of several spots indicated structural similarity.

Figure 2 depicts the binding patterns of three monoclonal antibodies. The recognized spots correspond to the IgE-reactive proteins of number 1 to 8 [4]. While the mAbs Bo 1 recognized all of the eight spots, BG 6 detected spots 1–4 (the 38 kDa components) and Bo 9, spots 5–8 (the 32 kDa allergens). The results indicate that Bo 1 is directed against an epitope located on the 38 and 32 kDa allergens. BG 6 and Bo 9, on the other hand, just bound to protein spots of 38 or 32 kDa, respectively. Each time components of identical size were detected independently from differences in the pI, this indicates them as isoforms (molecules that differ slightly by microheterogeneity, for review [18]). Testing individual patient's sera we observed that approximately 90% of them

showed IgE reactivity to these components (major allergen). All of these sera detected spots 1–8, probably suffering several IgE-binding epitopes on these components.

To confirm the immunological data that indicate a relationship among distinct protein spots, biochemical structure analyses are mandatory. After 2-DE of the extract, electroblotting onto PVDF membrane and Coomassie staining, the particular protein spots were excised. Concentrations of 10 pmol are sufficient to determine the N-terminal sequence and the amino acid composition. Here, we performed Edman degradation of the eight protein spots.

As shown in Table 1, spots 1–4 and 5–8 have similar molecular masses, respectively, and identical N-termini, although they differed in pI. The 38 and 32 kDa proteins were similar in 60% of the 20 amino acid residues [17]. To evaluate the structural similarity of the 38 and 32 kDa proteins, we determined the amino acid composition and calculated the relationship among the different protein spots (according to the index of Marchalonis and Weltman [19]). The data support the notion that spots with identical molecular masses are indeed isoallergens. Slight differences may be due to minor microheterogeneity, since single amino acid exchanges may cause pI differences. According to these results, the 38 and 32 kDa components were distantly related with values lying only within the limits of the “weak” test.

In order to understand the structural relationship and cross-reactivity of grass pollens, we also performed 2-DE blotting on other grass pollen extracts frequently identi-

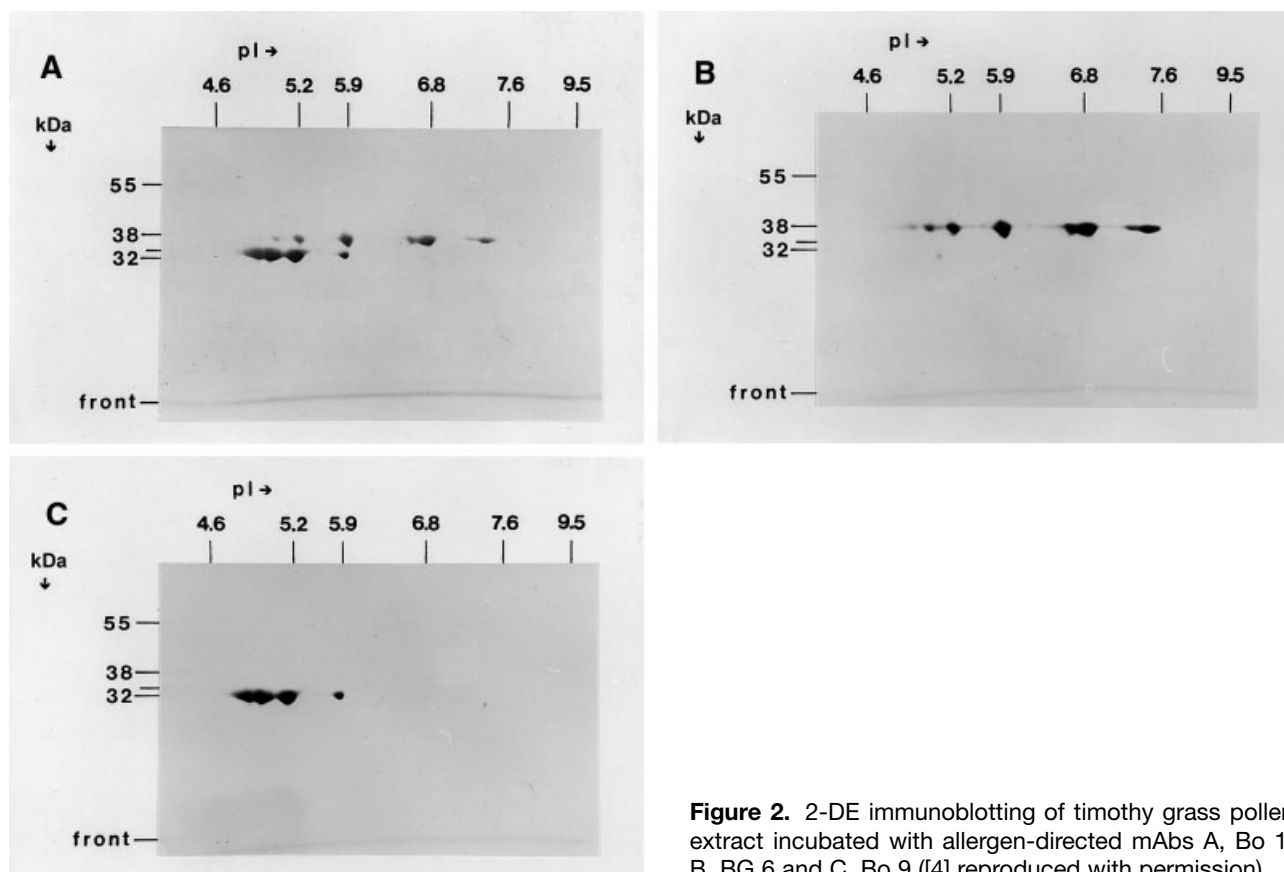


Figure 2. 2-DE immunoblotting of timothy grass pollen extract incubated with allergen-directed mAbs A, Bo 1; B, BG 6 and C, Bo 9 ([4] reproduced with permission).

fied as allergenic, such as ryegrass (*Lolium perenne*), velvet grass (*Holcus lanatus*) and rye (*Triticum aestivum*) and obtained quite similar binding patterns of patients' IgE and mAbs, respectively, as well as comparable protein sequencing results [20].

In order to determine structural differences between similar proteins, a comparison of the fragmentation patterns obtained after enzymatic or chemical digestion is useful.

Different fragments can easily be distinguished by a combination of electrophoretic separation, blotting and immunodetection. To study pollen allergens, specific endoproteinases (Glu-C and Lys-C) were also chosen, based on the amino acid composition of the allergens. We [4] excised the eight different protein spots from the Coomassie-stained 2-DE gel. The gel slices were transferred into the pockets of a SDS-PAGE gel and overlaid with endoproteinase Glu-C solution. After the protein and en-

Table 1. Microsequencing of the IgE-reactive fragments of the 38 and 32 kDa allergens after cleavage with endoproteinase Glu-C. N-terminal sequences of the original 38 and 32 kDa proteins are also stated.

Protein/Fragment	1	5	10	15	20															
38 kDa	A	D	L	G	Y	A	P'	A	T	P'	A	A	P'	A	A	G	Y	T	P'	A
20 kDa	A	D	L	G	Y	A	P'	A	T	P'	A	A	P'	A	A	G	Y	T	P'	A
14 kDa	V	K	V	I	P	A	G	E	L	Q	V	I	E	K	V	D	A	A	F	
32 kDa	A	D	A	G	Y	A	P'	A	T	P'	A	A	A	G	A	E	A	G	K	A

P', hydroxyproline

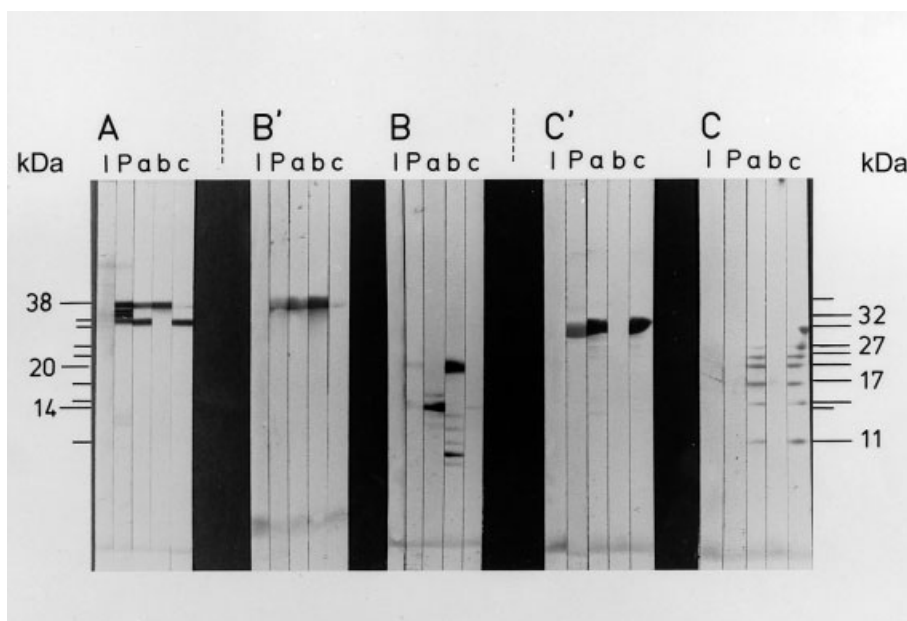


Figure 3. Fingerprint analysis of the 38 and 32 kDa allergens of timothy grass pollen digested by endoproteinase Glu-C and immunostained. Untreated pollen extract (A), 38 kDa allergens untreated (B') and digested by Glu-C (B), 32 kDa allergens untreated (C') and digested by Glu-C (C). I, protein staining by India ink; P, patients' IgE; a–c, mAbs Bo 1, BG 6 and Bo 9, respectively ([4] reproduced with permission).

zyme solution had reached the bottom of the sample gel, the run was interrupted to allow the enzymes to digest the allergens. After 30 min, electrophoresis was continued to separate the fragments. After blotting the fragmentation pattern was analyzed by patients' IgE and monoclonal antibodies.

Figure 3 shows that the cleavage products from the 38 kDa and 32 kDa spots differed in size and showed diverse binding patterns after incubation with patients' sera or the cross-reactive antibody Bo 1, respectively. No differences in the protein patterns were observed among the fragments of the 32 or 38 kDa isoforms [4]. No structural differences among the isoforms of different p/s were determined. The fragments were also used to determine internal protein sequences of the allergens (Table 1) [4]. This information was needed to design degenerated primers for screening a cDNA library of timothy grass pollen. Additionally, knowledge of the protein sequence was helpful to determine the DNA sequence from the ORF of the cloned/inserted gene. As shown for the 38 kDa allergen in Table 1, we obtained fragments containing new sequential parts as, for example, from the 14 kDa fragment, while the 20 kDa bears the *N*-terminal sequence of the 38 kDa protein.

Isolation of protein spots from the 2-DE blot can serve as a useful method to obtain purified material for antibody production. We [21] selected spot 15 (13 kDa) as a prominent, IgE-reactive protein. Since no mAb was available at that time, we excised the protein spot after 2-DE and transferred it onto nitrocellulose membrane, solubilized

it in DMSO and precipitated it afterwards as fine protein-bearing nitrocellulose particles by addition of phosphate buffer [22]. The particles were successfully used to immunize BALB/c mice. The hyperimmune serum obtained showed a similar reactivity to both 13 kDa proteins (spots 14 and 15), indicating that they are highly cross-reactive. Interestingly, a weak cross-reactivity was observed to spots 1–8, which suggested further structural similarity. This observation was later confirmed after cloning of the allergens: the 13 kDa allergen revealed a 50% sequence identity with the *N*-terminal sequence of the 38 and 32 kDa allergens.

3.3 Investigations on post-translational structures

After protein biosynthesis many proteins become post-translationally modified, e.g. by disulfide formation or by the attachment of carbohydrate structures [23]. Since these alterations can create new epitopes, investigation of post-translational modifications and their influence on the protein conformation and function are important. For general survey of the glycosylated components of an extract, the 2-DE blot can serve as a useful tool. After blotting, the nitrocellulose membrane was treated by periodate to oxidize adjacent hydroxyl groups in glycans to aldehydes. Digoxigenin-hydrazine derivative is afterwards bound to these aldehyde groups and is detected by an alkaline phosphatase labeled anti-digoxigenin conjugate.

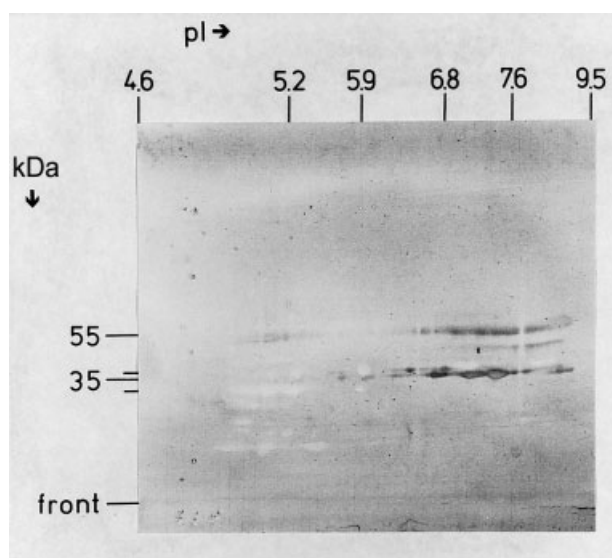


Figure 4. Detection of glycoproteins on nitrocellulose membrane after timothy grass pollen extract was separated by 2-DE blotting ([16] reproduced with permission).

Figure 4 shows the glycoproteins of timothy pollen extract components of 37 and 35 (referring to spots 9, 10, 12, 13) and the smear of about 55–60 kDa (spot 16) [16]. The white spots (negative staining) of several other proteins clearly confirm that these are not glycoproteins. Further information on specific glycan structures and linkages can be obtained from immunological studies using carbohydrate-specific lectins. Although the variety of different specificities of lectins is limited, investigation may give some helpful hints for probable carbohydrate structures. We [24] used ten lectins (glycan differentiation kit, Roche) and observed a strong reaction with *Aleuria aurantia* agglutinin (AAA) and a rather faint signal with Con A. While the latter recognizes several glycan structures that usually refer to *N*-glycans, AAA identifies the linkage of fucose residues. These structures have been identified as an IgE-binding epitope on other aller-

gens such as phospholipase A2 of bee venom [25, 26] and our own inhibition studies show the α 1,3-linked fucose on timothy grass pollen allergens as well [27].

To determine the amount of carbohydrate of a certain component, glycosidases may be good tools. However, many glycosidases do not cleave special configurations (e.g. *N*-glycosidase F), or cleavage sites are only accessible when the protein was cleaved before into short fragments (e.g. *N*-glycosidase A) [28]. After studies using mAbs and by *N*-terminal sequencing and DNA cloning we had already calculated the molecular mass of the grass pollen allergen (Phl p 1) which comprised protein spots 9–13. To estimate the glycan content, we [29] performed mass determination of each individual 2-D spot by sensitive and precise MALDI-MS analysis. The Ponceau S stained spots were cut from the nitrocellulose membrane, solubilized in acetone and cocrystallized with α -cyano-hydroxycinnamic acid.

Table 2 shows the molecular mass of four isoforms (the different isoforms vary by one amino acid position from each other; the concentration of spot 11 was too low). Interestingly, the sizes estimated by SDS-PAGE were higher and showed considerable deviation between the isoforms compared to the molecular masses determined by MALDI-MS. This can be explained by the fact that SDS-PAGE gives only a rough estimation of molecular mass. In contrast to chromatographically separated proteins, the mass peaks of the 2-D spots are broader, but allowed determination with a precision of ± 100 Da. The carbohydrate amount of each isoform was about 1.2 kDa corresponding to approximately ten monosaccharides. The glycans account for approximately 5% with each of the allergenic components (spots 9, 10, 12, 13).

To elucidate the carbohydrate structure of glycoproteins, we [24] analyzed the individual spots by performing neutral sugar analysis. Spots on PVDF membrane were hydrolyzed, derivatized and analyzed by gas chroma-

Table 2. Carbohydrate composition of the 35 and 37 kDa isoallergens by neutral sugar determination

Spot no.	Isoallergens	c protein (ng)	c carbohydrate (ng)	% carbohydrates	Fuc (ng)	Ara (ng)	Xyl (ng)	Man (ng)	GlcNAc (ng)
9	35 kDa, pI 6.5	990/1480	768/551	2.5–5.2	147/66	104/72	169/55	258/217	90/141
10	35 kDa, pI 7.1	1890/2400	1772/1822	4.9–6.4	330/472	242/278	216/236	712/490	322/294
12	37 kDa, pI 6.4	710/790	290/330	2.5–3.1	45/67	39/43	44/22	104/140	58/58
13	37 kDa, pI 6.6	390/740	184/232	1.7–3.2	30/36	6/22	21/0	98/105	29/69

Each determination was performed twice. Protein concentrations refer to amino acid analysis of one spot, carbohydrate concentrations refer to neutral sugar analysis of 15 spots.

Table 3. Comparison of pI and molecular masses of the spots 9–13

Spot no.	2-DE (kDa)	2-DE pI	MALDI (kDa)	PCGENE (kDa)	PCGENE pI
9	35	6.4	28.26	25.9	8.2
10	35	6.8	28.36		
12	37	6.4	28.06		
13	37	6.6	28.11		

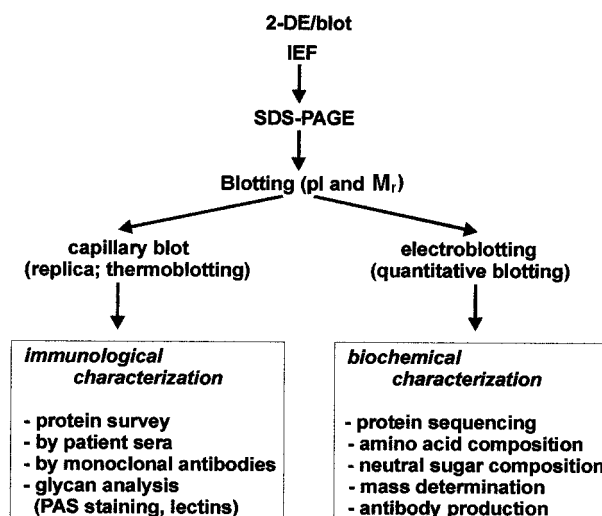
Determination of natural grass pollen components were performed by 2-DE and MALDI-MS, determination of the recombinant proteins were by computer prediction.

tography with on-line MS. Table 3 shows that each isoform is composed of the same monosaccharides: fucose, arabinose, xylose, mannose and *N*-acetylglucosamine. The ratios of the monosaccharides reveal some variation between the different isoforms. Carbohydrate concentrations of the samples were very low, possibly due to inaccuracy. However, because of the highly conserved pathway of *N*-glycan biosynthesis and the identification of fucose and xylose residues, our results clearly indicate the existence of a complex *N*-glycan of the pauci-mannose type. The detailed structure and configuration has already been described for horseradish peroxidase and bromelain and it is known for its IgE reactivity [28]. Thus, protein concentrations of the 2-D separated spots were sufficient to determine the specific structure of the carbohydrate moieties.

4 Concluding remarks

The versatility of 2-DE blotting was demonstrated using the analysis of timothy grass pollen as an example. Table 4 summarizes the techniques used for identification and determination of the specific allergenic components. These procedures can generally be used for the characterization of any protein from complex extracts. The 2-DE blot is a starting point to obtain a general overview of the components of an extract, to study the immunological and biochemical structure of single proteins, and finally isolate certain components for various further investigations in an efficient and economical way.

Recent problems concerning reproducibility of this technique have been overcome by the introduction of the Immobiline technique [30] and by the efficient way of obtaining up to 70 gel strips from one IEF gel. Variability can further be decreased by the use of replica blotting, which generates several identical imprints on blotting membranes from one 2-D gel. Still, one has to be aware that a lot of factors can cause alterations and/or addi-

Table 4. Procedure to characterize single components after 2-DE blotting

tional spots by artefacts, e.g. carbamylation of urea [31] and acrylamide monomers [32]. Other separation techniques might be more sophisticated and sensitive, but the simultaneous determination of structural data as pI, molecular mass and immunoreactivity of single extract components, the opportunity to get a comprehensive survey on the complete extract constituents and the possibility to zoom-up particular regions of the 2-DE separation by using narrow pI and/or molecular mass ranges [33], are unbeatable advantages of 2-DE blotting.

I would like to thank Dr. G. Reese and Dr. W. M. Becker for discussions and critical reading of the manuscript.

Received December 26, 2002

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