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Proteomics of cypress pollen allergens using double and triple one-dimensional electrophoresis

Italian cypress (*Cupressus sempervirens*, *Cups*) pollen causes allergic diseases in inhabitants of many of the cities surrounding the Mediterranean basin. However, allergens of *Cups* pollen are still poorly known. We introduce here a novel proteomic approach based on double one-dimensional gel electrophoresis (D1-DE) as an alternative to the 2-DE immunoblot, for the specific IgE screening of allergenic proteins from pollen extracts. The sequential one-dimensional combination of IEF and SDS-PAGE associated with IgE immunoblotting allows a versatile multiplexed immunochemical analysis of selected groups of allergens by converting a single protein spot into an extended protein band. Moreover, the method appears to be valuable for MS/MS identification, without protein purification, of a new *Cups* pollen allergen at 43 kDa. D1-DE immunoblotting revealed that the prevalence of IgE sensitization to this allergen belonging to the polygalacturonase (PG) family was 70% in tested French allergic patients. In subsequent triple one-dimensional gel electrophoresis, the *Cups* pollen PG was shown to promote lectin-based protein-protein interactions. Therefore, D1-DE could be used in routine work as a convenient alternative to 2-DE immunoblotting for the simultaneous screening of allergenic components under identical experimental conditions, thereby saving considerable amounts of sera and allergen extracts.

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homologous with Cup s 3, a pathogenesis-related (PR-5) protein peculiarly expressed in polluted environments [11]. However, it has been stated that besides Cup s 1 and Cup s 3, *Cups* pollen contains other proteins distributed over a wide range of molecular weights (14–95 kDa) some of which bind to specific IgE of sensitized patients [9, 12]. In the present work, we focused on the allergen repertoire of *Cups* pollen grains using a proteomic approach in order to expand the restricted panel of well-characterized allergenic molecules implicated in this pollinosis.

1.2 Allergomic studies

The term allergomics has recently been used to define the application of proteomics to the study of allergen repertoires [13, 14]. 2-DE, associated with immunoblotting using sera from allergic patients, is most commonly used in allergomic studies to isolate, characterize and identify IgE reactive proteins in complex extracts [14–19]. However, one of the drawbacks of this technique is that only a single supplementary experiment can be performed on each single 2-DE protein spot (e.g. mass spectrometry or immunoreactivity after Western blotting) [20]. Therefore, additional functional or immunological characterizations will require either protein purification (e.g. for multiplexing through ELISA or micro-array technologies [21–23]) or replicates of 2-DE [24]. Another disadvantage of this technique is often the lack of visualization of low-abundance protein spots that constitute an important part of any proteome [25].

In 2-DE 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, which is then individually incubated with a sizeable volume of serum (at least 300 μ L for a membrane of 100 cm²) [9, 19, 26]. To overcome these drawbacks, we propose to perform a 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 (M_r) under identical experimental conditions.

1.3 D1-DE

Initially reported in 1975 by Altland et al. [27], the concept of D1-DE was mainly used to study protein genetic variants [28–30] and 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 high resolution and simultaneous separation of proteins of interest in dual sorting parameters. In the present study, 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

that obtained with a standard 2-DE IEF/SDS-PAGE, but its abundance is many times higher than what can be found in a 2-DE single spot. We applied this technique to the study of the allergen repertoire from *Cups* pollen, which represents a widespread and highly invasive allergenic source in Southern Europe [31].

By using a combination of standard 2-DE and D1-DE followed by immunoblotting, we unraveled a basic allergen with a molecular mass close to the already described major allergen (Cup s 1) and established a prevalence of sensitization for these two allergens. Furthermore, a triple one-dimensional (T1-DE) is also reported for the study of molecular complexes.

2 Materials and methods

2.1 Patient data

Sera were drawn after obtaining informed consent from individuals suffering from cypress pollen allergy. Sera from cypress allergic patients were selected according to their symptoms and to a positive ImmununoCAP[®] (t23, Phadia, Uppsala, Sweden) to Italian cypress pollen extracts (with serum-specific IgE higher than 0.71 kU_A/L: CAP class 2). They represented residues of IgE titer evaluations (performed in the Biomnis medical laboratory at Lyon, France). For each analysis, the serum from a healthy individual with a normal total IgE concentration (less than 0.10 kU_A/L) was chosen as control.

2.2 Cypress pollen extracts

C. sempervirens pollen was supplied by Allergon AB (Angelholm, Sweden). For the preparation of pollen extracts, 100 mg (1/10 w/v) was incubated overnight and under rotation in 1 mL phosphate-buffered saline (PBS) pH 7.4. The suspensions were centrifuged at 14 000 $\times$ g for 20 min at 4°C and the supernatants were collected and stored as aliquots at –20°C until use. Protein concentration of samples was measured using the Bradford protein assay with bovine serum albumin (BSA) as standard.

2.3 Two-dimensional gel electrophoresis

Cups pollen extract was first separated by IEF performed in a polyacrylamide 4%T, 3%C (Clean gel, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) hydrated in a solution containing 5% v/v Servalyt[®] pH 2–11 (Serva, Heidelberg, Germany) on a flat bed electrophoretic chamber (Multiphor II, GE Healthcare) cooled at 15°C. Samples (55–60 μ g of proteins from the total pollen protein extract) were loaded on 10-cm-long and 0.7-cm-wide strips of a dry Whatman[®] 3 MM paper (GE Healthcare) placed directly on top of the gel (anode side).

After IEF separation, 5-mm-wide strips of the gel were cut, incubated in the equilibration buffer (114 mmol/L Tris pH 6.8 containing 12% w/v SDS) for 30 min and submitted to the second dimension: an SDS-PAGE separation on an 8–18% gradient gel, allowing a wide-range separation according to M_r on a flat bed electrophoretic chamber. Two-DE gels were either silver-stained according to Blum et al. [32], Coomassie blue-stained or blotted onto a cyanogen bromide-activated nitrocellulose (NCa) (Optitran[®] BA-S 83, Schleicher and Schuell, Dassel, Germany) sheet [33].

2.4 Principles of D1-DE

Like standard 2-DE, the D1-DE described here consists of IEF as a first dimension followed by SDS-PAGE. The difference between the two techniques resides in the migration axis of the second separation, which is orthogonal for standard 2-DE and parallel for D1-DE. This means that an acidic, neutral or basic horizontal band from the first dimension (IEF) is submitted to SDS-PAGE (Fig. 1).

For the IEF first step, the pollen extract was loaded in the same conditions as for 2-DE. The pI ranges 7–7.5 and 8.5–9.5 corresponding to Cup s 1 and the novel basic allergens, respectively, were then horizontally cut into 10-cm-

long strips. After incubation in the equilibration buffer, the strips were placed gel to gel and run on SDS-PAGE. Therefore, proteins are finally isolated as a long continuous band with purity comparable to what is obtained for a single spot in a conventional 2-DE (IEF/SDS-PAGE). In the present survey, the sequential one-dimensional combination of IEF and SDS-PAGE followed by immunoblotting was used for the screening, under identical experimental conditions, of allergen-specific IgE in sera of 30 cypress pollen allergic patients. After the IEF separation, the basic and neutral allergenic fractions at pI 7–7.5 and 8.5–9.5, respectively, were horizontally cut out and submitted to SDS-PAGE in an 8–18% gradient gel. For a better correlation between patterns obtained by 2-DE and D1-DE analysis, we employed the same IEF gel (12 × 25 cm, hydrated in an ampholyte solution) used as the first dimension of our 2-DE immunoblotting. Therefore, this kind of IEF gel allows the cutting of at least 10-cm-long IEF strips in a selected narrow pI range.

2.5 T1-DE

For T1-DE, the allergenic fraction of 60 kDa obtained by D1-DE was cut out and submitted to a second SDS-PAGE

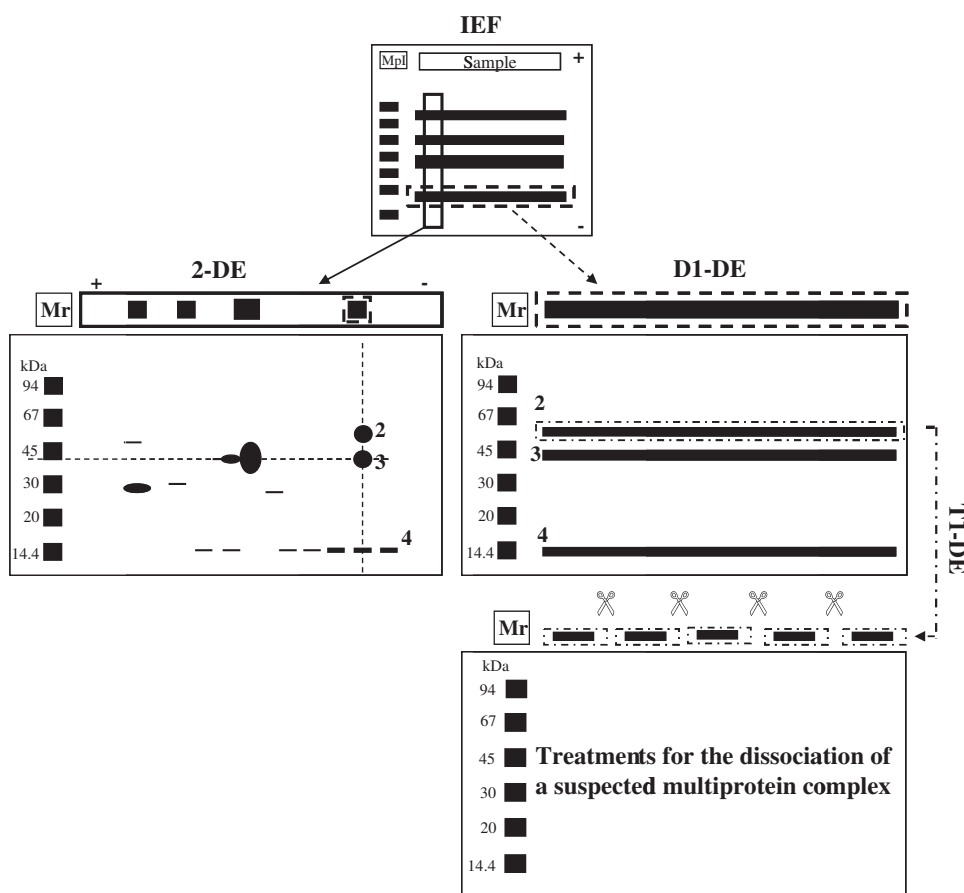


Figure 1. Schematic illustration of the IEF basic strip separated by D1-DE. Both 2-DE and D1-DE described in this study consist of the sequential combination of IEF and SDS-PAGE. The difference between the two techniques resides in the axis of migration of the second separation which is orthogonal for standard 2-DE and parallel for D1-DE (unchanged migration axis). Protein bands separated by D1-DE can be further characterized using T1-DE.

separation in order to establish whether this protein represents a multiprotein complex. For this purpose, the 60 kDa strip was cut out from the D1-DE separation and divided into five equal parts. Before SDS-PAGE, four parts were subjected to different biochemical treatments by adding to the equilibration buffer: (i) carbohydrate, i.e. 10 mM α -D-methyl mannopyranoside and 100 mM α -D-methyl glucopyranoside (Sigma Biochemicals, St. Louis, USA), (ii) 20 mM EDTA, (iii) 3% β -mercaptoethanol (2-Me, Sigma), (iv) high salt concentration (300 mM NaCl). The fifth part corresponds to the equilibration buffer itself as control.

2.6 Immunoblotting

Electroblotting of separated proteins was performed onto 0.2 μ m NCA sheets with a semidry Novablot apparatus (LKB, Uppsala, Sweden) following manufacturer's instructions (1 h, 1 mA/cm²). The membranes were then dried and blocked with PBS containing 0.3% v/v Tween 20 (PBS-Tw, Sigma) for 1 h at ambient temperature. For D1-DE screening, each NCA was then cut into 2.5-mm-wide strips that were individually incubated with 1:10 diluted patient sera in PBS-Tw 0.1% (overnight at 20°C). For 2-DE analysis, the whole NCA membranes were incubated with individual sera. Each membrane was washed three times for 10 min in PBS-Tw v/v and incubated for 2 h at 20°C with a 1:700 dilution of alkaline phosphatase (AP)-conjugated goat anti-human IgE (Sigma). The AP activity was detected using 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) and nitro blue tetrazolium (NBT, Sigma) in 0.1 mol/L Tris acetate buffer pH 9.5.

3 Results

3.1 Two-DE map of *Cups* pollen salt-extractable proteins: Localization of novel basic allergens

Upon 2-DE analysis of PBS-soluble proteins in the wide pH range 2–11, we could detect several as yet undescribed basic spots (nos. 2–4) with heterogeneous M_r (60, 43 and 14 kDa) and similar pI 9–9.5 (Fig. 2A). On the other hand, as also depicted in Fig. 2A, two proteins (nos. 1 and 3) exhibited similar M_r (~43 kDa) and different pI (7.5 and 9.0, respectively).

Two-DE-immunoblot analysis (Fig. 2B) using the serum from a cypress pollen allergic patient revealed that the IgE reactive 43–45 kDa fraction of *Cups* pollen extracts included not only Cup s 1 (pI 7–7.5), as previously reported, but also another basic allergen (pI 9–9.5, no. 3).

To evaluate the rates of sensitization to these allergenic proteins, without going through a purification process, we opted for a new approach based on a D1-DE gel separation followed by IgE immunoblotting.

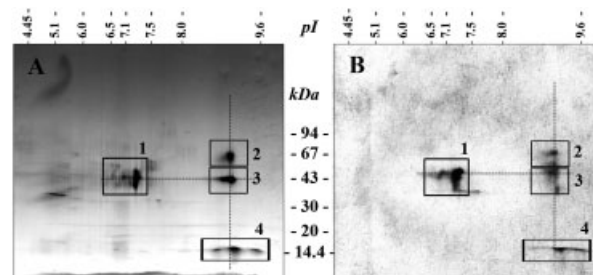


Figure 2. Two-DE immunoblotting of PBS-extractable proteins. Silver-stained 2-DE of PBS pollen extract (A) showed 3 as yet undescribed basic spots (nos. 2–4). M_r and pI are indicated on the right and at the top of the silver-stained 2-DE, respectively. Allergenic components are revealed by 2-D IgE immunoblot (B) probed with serum from a cypress pollen allergic patient.

3.2 D1-DE protein characterization and immunoblotting: A multiplexed and rapid immunoassay for detection of specific IgE directed against allergens of interest

In the silver-stained D1-DE of the basic fraction (pI 8.5–9.5), we detected all three basic proteins of 60, 43 and 14 kDa (Fig. 3B, lane Pb, nos. 2–4) previously visualized in our 2-DE separation (Fig. 2A, nos. 2–4). For the neutral fraction (7–7.5), the profile was also in accordance with our 2-DE map (Fig. 2A, no. 1) and only a single band of 43 kDa (Cup s 1) was clearly visualized (Fig. 3C, lane Pn, no. 1).

Figure 3B demonstrates that among 30 tested patients, 21 (70%) showed a positive IgE response to the novel 43 kDa basic allergens, while 22 (~73%) were shown to be sensitized to Cup s 1 (Fig. 3C). The prevalence of IgE sensitization to the basic proteins of 14 and 60 kDa was 17 and 23%, respectively. Interestingly, in the neutral fraction we also observed weak specific IgE reactivities in M_r (60, 43 and 14 kDa) corresponding to basic allergens in the D1-DE immunoblot. This fact could be due to the presence of low-abundance neutral isoforms of these three allergenic polypeptides, which were not visible with standard silver staining.

3.3 One gel, several applications

To demonstrate the versatility of this technique, we performed a D1-DE analysis for protein sequencing and MS characterization (Fig. 4A), protein immunoblotting (Fig. 4C) and as a preparative separation for triple 1-DE SDS-PAGE (Fig. 4B). Forty 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 allergic patients. Preliminary MS analysis showed that both basic proteins of 60 and 43 kDa are homologous to already sequenced cypress-cedar glycoprotein polygalacturonases (see Supporting Information). This result suggested that the 60 kDa

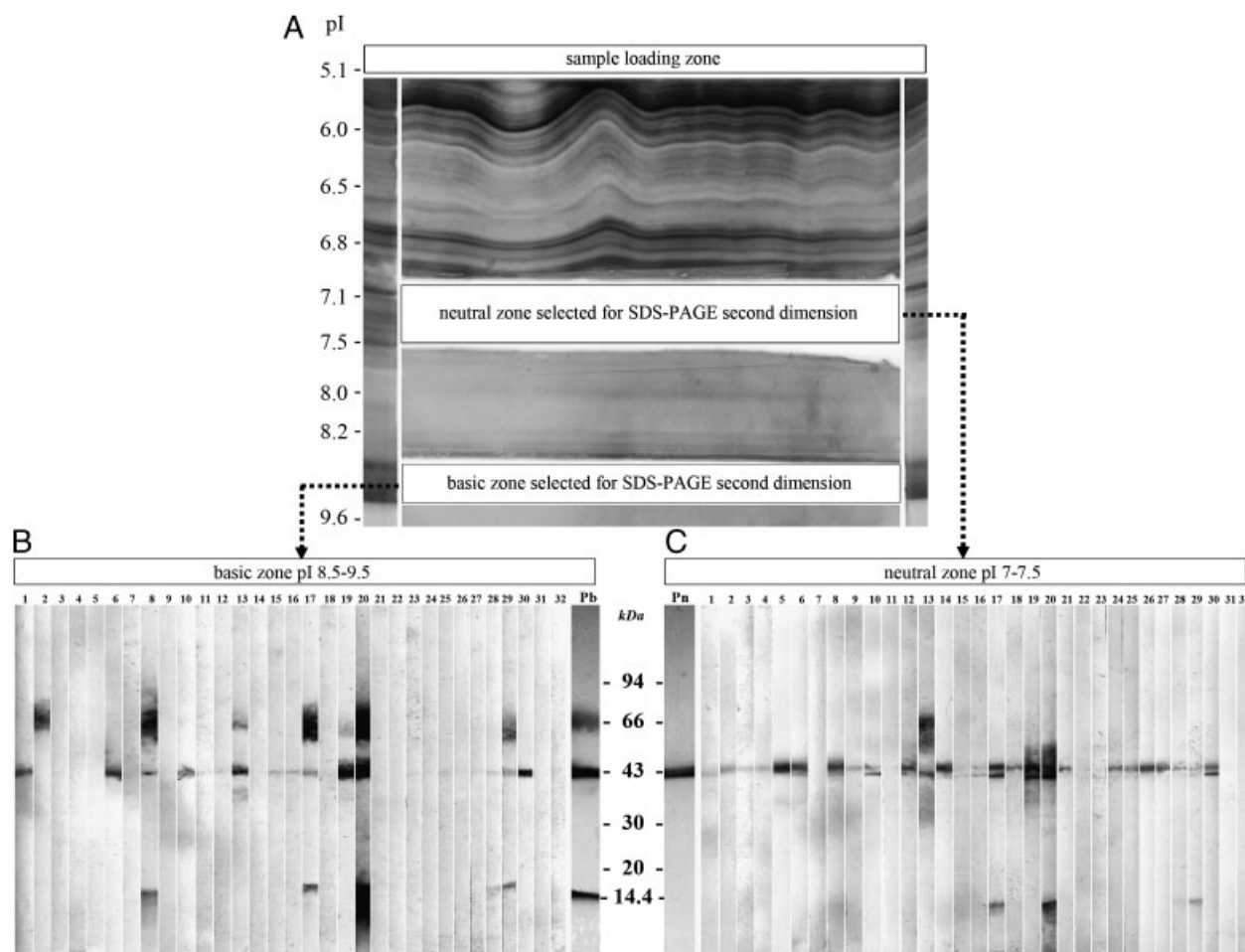


Figure 3. D1-DE followed by IgE immunoblotting. (A) represents the initial IEF separation. The silver staining was performed after excision of the basic and neutral bands. In D1-DE separations, the lanes Pb and Pn correspond to silver-stained basic and neutral allergenic proteins, respectively; IgE immunoblots of the basic (B) and neutral (C) proteins used sera of 30 cypress pollen allergic patients: lanes 1–30; lane 31, healthy donor serum; lane 32, no serum (negative control).

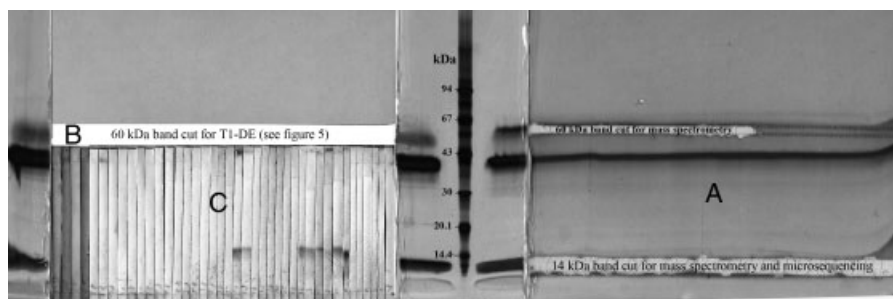


Figure 4. Multiple applications of D1-DE in allergomic studies. Part A was used for gel excision and MS/MS identification of IgE-binding proteins; part B was used to isolate the 60 kDa PG for subsequent T1-DE using different biochemical treatments (see Fig. 5); part C was used for Western blotting on the 43 and 14 kDa basic proteins.

band might be a protein complex including the 43 kDa polygalacturonase. To approach this question the 60 kDa protein band obtained by D1-DE (Fig. 4B) was cut out, divided into five equal parts and subjected to different biochemical treatments, including a mixture of carbohydrates to disrupt a possible glycoprotein–lectin interaction, a chelating agent to detect the presence of a possible divalent ion-dependent complex, a reducing agent to break disulfide

bonds, and finally a high salt concentration to destabilize ionic strength-sensitive protein complexes.

These D1-DE treated fractions were subsequently submitted to a third separation using SDS-PAGE. From the D1-DE treated fractions submitted to T1-DE, we found that the 60 kDa constituted a protein complex that is dissociated either totally by adding a carbohydrate mixture (Fig. 5, lane 2) or partially by the three other treatments, and can be visual-

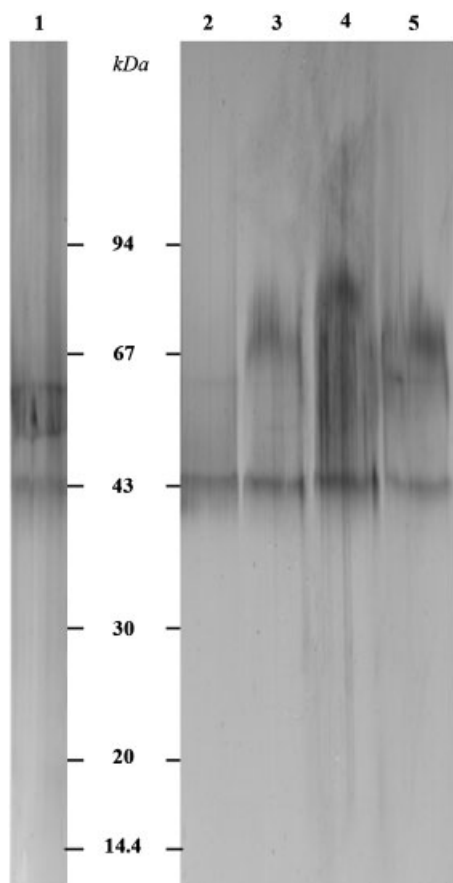


Figure 5. T1-DE separation of the 60 kDa protein band cut from a preparative D1-DE. Different biochemical treatments during the equilibration step before SDS-PAGE separation were performed: lane 1, the equilibration buffer (EB); lane 2, EB containing a mixture of two carbohydrates; lane 3, EB with 20 mM EDTA; lane 4, EB incorporating 3% 2-Me; lane 5, EB with 300 mM NaCl.

lized by the presence of smears (Fig. 5, lanes 3–5). A band at M_r 43 kDa is clearly highlighted. The total extinction of the 60 kDa protein in the carbohydrate part together with the presence of the 43 kDa protein indicates binding of the 43 kDa glycoprotein to an unknown lectin-like protein which is not visible in the silver-stained T1-DE.

4 Discussion

The present study describes a new procedure to identify and characterize allergenic components present in a complex pollen extract by using D1-DE (Fig. 6). A common strategy in allergy research, aimed at inducing tolerance toward immunotherapeutic antigens, requires the identification of allergens contained in a given allergenic source [34]. Two-DE immunoblotting is an outstanding proteomics tool used for allergen identification and for evaluation of IgE sensitization profiles of allergic patients [14, 17–19, 24, 35, 36]. The protein spots, previously shown to be recognized as allergens by specific patient IgE, are then excised from a

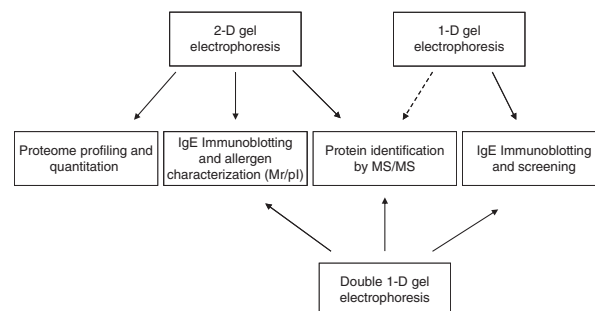


Figure 6. Schematic description of the main applications of 2-DE, 1-DE and D1-DE in allergomic studies. The dotted arrow points out that MS/MS analysis performed from 1-DE gel separations is often not precise enough to identify the IgE-binding protein, as the excised protein bands generally include a mixture of several proteins.

stained 2-DE (IEF/SDS-PAGE) gel and identified using MS/MS analysis. In fact, in order to obtain these two kinds of data, IgE reactivity and MS identification, two replicates of the same 2-DE are required. A third element relating to the protein spot of interest, e.g. immunoblot ligand inhibition, will require a third replicate of the 2-DE and so on for each new investigation. The D1-DE that we propose allows this multi-characterization in a single step since it provides a higher quantity of proteins by converting a protein spot into a protein band. D1-DE is thus appropriately adapted to allergomic studies. It is an extension of what was originally reported by Atland et al. in 1975 using slab gels [27]. In the present study, the use of commercially available GelBond-backed gels considerably facilitates the handling, cutting and combination of electrophoretic separations and significantly improves the repeatability of D1-DE analysis. The technique was used here to establish the prevalence of a newly described basic allergen (M_r 43 kDa) in the pollen of *C. sempervirens* by using one-step IgE reactivity screening of 30 allergic patients. Such prevalence analysis is usually performed using Western blotting after one-dimensional electrophoretic techniques (1-DE) and/or ELISA [37, 38]. In 1-DE, the resolution of proteins is not optimal as isolated bands often include a mixture of several proteins [35]. In ELISA, a pure protein needs to be coated on microtitration plates [7, 37, 39]. Purification steps such as chromatographic methods can be time- and material-consuming and for low-abundance components often lead to protein loss and poor yields [40, 41]. Besides multiplexing for immunoblotting, this method circumvents the paucity of proteins often encountered in one single protein spot, which generally makes MS and microsequencing experiments difficult. The whole protein band can be excised, digested and processed for analysis of more complex protein extracts such as latex [42] and *Arabidopsis thaliana* pollen extracts [43].

In the present work, D1-DE allowed the characterization of a *Cups* pollen polygalacturonase (PG) of 43 kDa. This protein overlapped in 1-D SDS-gels with *Cup s* 1, the major cypress pollen allergen abundantly present in aqueous/salt *Cups* pollen extracts, constituting a single allergenic protein

band of 43–45 kDa [9]. Although PGs represent the second group of pollen allergens in several Cupressaceae species [44–48] (i.e. *Juniperus ashei*, *Chamaecyparis obtusa*, *Cryptomeria japonica*), no protein from this family has yet been reported and identified in cypress (*Cupressus*) pollen. Therefore, it seems that the overlapping of this protein with the group 1 of cypress pollen allergens in 1-D SDS-PAGE as well as its instability during extraction and dialysis procedures may partly explain why it has not yet been identified.

By providing high resolution of *Cups* pollen proteins present in a narrow pI-range, D1-DE allowed the simultaneous one-step IgE screening of low-abundance basic proteins with molecular masses of 14, 43 and 60 kDa. Using this approach we showed that the prevalence of IgE sensitization to the cypress pollen PG is high and reaches 70% in tested cypress pollen allergic patients. The frequency of IgE recognition is higher for this PG than for its 60 kDa isoenzyme (~23%). This fact reflects potential differences between specific IgE-binding epitopes involved in the recognition of these two cypress pollen PGs.

Finally, a D1-DE followed by a T1-DE has been developed in order to detect and characterize potential protein-protein interactions among *Cups* pollen allergens. After submitting the suspected 60 kDa protein complex to T1-DE, we demonstrated that carbohydrates successfully dissociated 100% of the 60 kDa protein band into the 43 kDa polygalacturonase. This result implies the interaction of the cypress pollen PG with one or more proteins exhibiting lectin-like properties. In some fruits such as tomato, it has already been shown that PGs may be associated with other proteins to form a multiprotein complex [49–51]. In tomato fruits, an 8-kDa protein belonging to the class of non-specific lipid transfer proteins has recently been isolated as part of the PG1 multiprotein complex [52]. Therefore, the detection of a PG multiprotein complex in cypress pollen could be of special value in opening up new perspectives for the isolation, purification and functional characterization of PGs in many other pollen grains.

To conclude, D1-DE is an analytical as well as a preparative method that allows, in one step, a multiplexed analysis of proteins of interest. Moreover, because samples were assayed in the same run and in strictly similar conditions, comparative screening results are reliable, which is not always the case in replicates of standard 2-DE where inter-assay variations may occur from one gel to another. This outstanding proteomic tool is virtually applicable to the study of proteins present in many types of complex biological extracts or fluids. With its advantages in terms of low consumption of serum and material, versatility, speed and technical simplicity, D1-DE may be applied to other clinical fields such as cancer biomarkers, infectious and autoimmune diseases.

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The authors have declared no conflict of interest.

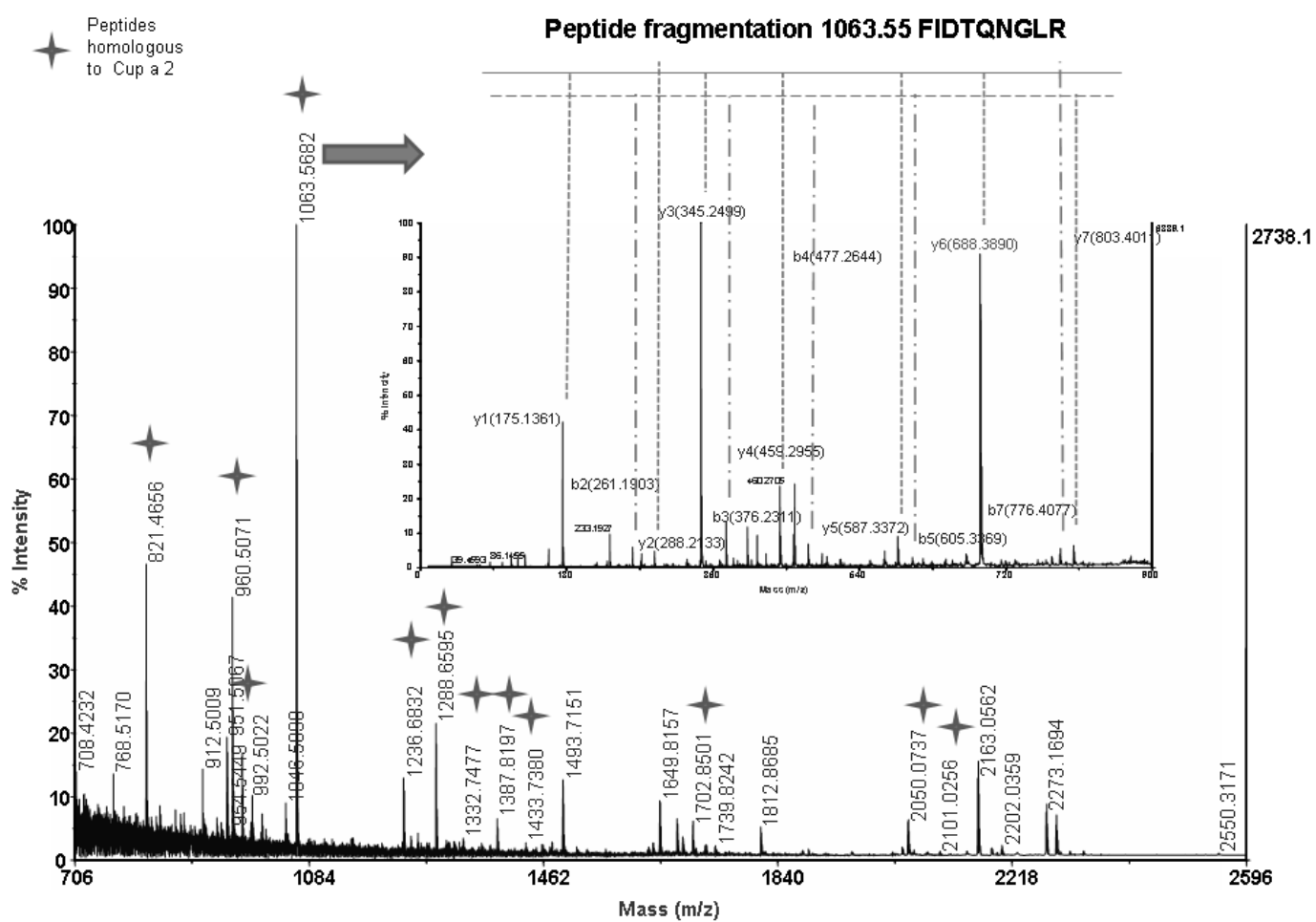
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MS/MS analysis on the novel 43 kDa basic allergen of Italian cypress pollen

Peptides homologous to Cup a 2



Cup a 2 -----HDVAIVF 8
Jun a 2 MSMKFMAALAFALQLIVMAAGEDQSAQIMLDSDTKQYHRSSRLRKRHHARHDVAIVF 61
Cha o 2 MGMKFMAAVAFALQLIVMAAAEDQSAQIMLDSIEQYLRNRSRLKKLVHS-RHDAATVF 60
Cry j 2 MAMKFIAPMAFVAMQLIIMAAAEDQSAQIMLDSIEQYLRNRSRLRKVEHS-RHDAINIF 60
*****:

Cup a 2 NVEHYGAVGDGNHDSSTDAFEKTWNEACKT**LSAVFLVPANKK**FVNNLVFYGPCQPHFSFK 68
Jun a 2 NVEHYGAVGDGKHDSSTDAFEKTWNAACN**KLSAVFLVPANKK**FVNNLVFYGPCQPHFSFK 121
Cha o 2 NVEQYGAVGDGKHDSSTDAFEATTWNAACK**KASAVLLVPANKK**FVNNLVFRGPCQPHLSFK 120
Cry j 2 NVEKYGAVGDGKHDSSTDAFEATWQAACK**KPSAMLLVPGNKK**FVNNLVFFNGPCQPHFTFK 120
*****:

Cup a 2 **VDGIIAAYPDPVK**WKN**SKIWMHFAR**LTDNFLMGTGVIDGQGSKWWS**DQCKTVNGR****TVCND** 128
Jun a 2 **VDGTIAAYPDP**AKWKN**SKIWMHFAR**LTDNFLMGTGVIDGQGNRWWS**DQCKTINGR****TVCND** 181
Cha o 2 **VDGTIVAQDP**ARWKN**SKIWLQFAQ**LTDNFLMGTGVIDGQGQWWS**WAGQCKVNGR****TVCND** 180
Cry j 2 **VDGIIAAYQNP**ASWKN**NRILWLQFAK**LGTFTLMGKGVIDGQGWWS**WAGQCKWNGR****REICND** 180
*****:

Cup a 2 **KGRPTAIKIDFSK**SVTV**KELTLMNSPEFHLVFG**EC**DGVKIQGIKIK**APKESPN**TDGIDIF** 188
Jun a 2 **KGRPTAIKIDFSK**SVTV**KELTLTNSPEFHLVFG**EC**DGVKIQGIKIK**APRDSPN**TDGIDIF** 241
Cha o 2 **RNRPTAIKIDYSK**SVTV**KELTLMNSPEFHLVFG**EC**EGVKIQGLKIK**APRDSPN**TDGIDIF** 240
Cry j 2 **RDRPTAIKFDFST**GLI**IQGLKLMNSPEFHLVFG**NC**EGVKIIGISIT**APRDSPN**TDGIDIF** 240
*****:

Cup a 2 GSK**RFEIEK**CTIGTGDDCVAIGTGSSNITIK**DLTCGPGHGM**SIGSLGKGN**SRSEVSFVHL** 248
Jun a 2 ASK**RFEIEK**CTIGTGDDCVAVG TGSSNITIK**DLTCGPGHGM**SIGSLGKGN**SRSEVSFVHL** 301
Cha o 2 ASK**RFHIEK**CVIGTGDDCIAIGTGSSNITIK**DLICGPGHG**ISIGSLGRDN**SRAEVS**HVHV 300
Cry j 2 ASK**NFHLQK**NTIGTGDDCVAIGTGSSNIVIE**DLICGPGHG**ISIGSLGR**ENSRAEVS**YVHV 300
*****:

Cup a 2 **DGAKFIDTQNGLR**IKTWQGGSGSLASHITYENVEMVNAENPILINQFYCTSAAC**ENQRSA** 308
Jun a 2 **DGAKFIDTQNGLR**IKTWQGGSGSLASHITYENVEMINAENPILINQFYCTSAAC**KNQRSA** 361
Cha o 2 **NRAKFIDTQNGLR**IKTWQGGSGSLASYITYENVEMINSENPIINQFYCTSAAC**QNQRSA** 360
Cry j 2 **NGAKFIDTQNGLR**IKTWQGGSGSMASHIYENVEMINSENPIINQFYCTSAAC**QNQRSA** 360
*****:

Cup a 2 **VKIEDVTFK**NIHGTSATAAAIQLMCSDSVPCSNIKLSNVVLKLSSGKVATCVNK**ANANGYY** 368
Jun a 2 **VKIQDVTFK**NIHGTSATTAIQLMCSDSVPCSNIKLSNVFLKLTS**SGKVATCVNK****ANANGYY** 421
Cha o 2 **VQIQGVTYK**NIHGTSATAAAIQLMCSDSVPTGIQLSNVSLKLTS**SGKPASCVDK****NARGFY** 420
Cry j 2 **VQIQDVTYK**NIHGTSATAAAIQLKCSDSMPCCKINLSDISLKLTS**SGKIASCLND****ANANGYF** 420
*****:

Cup a 2 **TNPLNPPCK**SLHPG----- 382
Jun a 2 **TNPLNPSCK**SLHPGRTPKELELHQPTTLLMDEK-----MGASLNSSPPNCKNKCKG 473
Cha o 2 **SGRLIPTCK**NLRPGPSPKEFELQQPTTVMDEKNGACAKGDSTCISLSSPPNCKNKCKG 480
Cry j 2 **SGHVI**PACKNLSPSAKRKESKSHKHPKTMVENMGAYDKGNRTRILLGSRPPNCTNKCHG 480
*****:

Cup a 2 -----
Jun a 2 CQPCPKPLIIVHPNPEDYYPQRWVC**SCHNKIYNP** 508
Cha o 2 CQPCPKPLIIVHPNKPQDYYPQKWVC**SCHNKIYNP** 515
Cry i 2 CSPCKAKLVIVHRIMPOEYYPORWMCS**CHGKIYHP** 515

Figure S. MS/MS analysis of the *Cups* pollen polygalacturonase (PG) and coverage map obtained by the resulting sequenced peptides. The basic IgE binding protein of 43 kDa previously visualized in the figure 3 were in-gel excised, digested and analyzed by MS/MS. Figure SA represents the raw mass spectrum of the *Cups* pollen PG. Peptides matching to Cup a 2 are shown by black stars (n=11). The black arrow indicates the peptide MS/MS fragmentation of one of the amino acid sequences (FIDTQNGLR) showing 100% homology with all already cloned Cupressaceae PGs. Coverage map obtained by the resulting sequenced peptides is illustrated in the figure SB and aligns Cup a 2, Jun a 2, Cha o 2 and Cry j 2 representing major cypress-cedar PGs. Matching amino acids are shown by bold characters and the shaded area corresponds to the fragmented peptide 1063.55 illustrated in the figure SA.