

REGULAR ARTICLE

Sub-proteome analysis of novel IgE-binding proteins from Bermuda grass pollen

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Bermuda grass (*Cynodon dactylon*) pollen (BGP) is one of the most common causes of airway allergic disease, and has been shown to contain over 12 allergenic proteins on 1-D immunoglobulin E (IgE) immunoblots. However, only a few allergens have been identified and characterized. Cyn d 1 is a major allergen and the most abundant protein in BGP, representing 15% of the whole-pollen extract. To investigate variability in the IgE-reactive patterns of BGP-sensitized patients and to identify other prevalent allergens, a BGP extract was passed through an affinity column to remove Cyn d 1, and the non-bound material was collected and analyzed by 2-DE. IgE-reactive proteins were subsequently characterized by immunoblotting using serum samples from ten BGP-allergic patients. The prevalent IgE-reactive proteins were identified by MALDI-TOF MS, N-terminal sequence similarity, and LC-MS/MS. Here, we present a sub-proteome approach for allergen investigation and its use for determining BGP 2-DE profiles and identifying six novel allergens.

Received: September 10, 2004

Revised: November 18, 2004

Accepted: December 27, 2004

Keywords:

Allergen / Bermuda grass pollen / Immunoglobulin E-reactive protein / Proteome analysis

1 Introduction

Grass pollen allergens trigger immunoglobulin E (IgE)-mediated type I allergic diseases, such as allergic rhinitis, conjunctivitis, and bronchial asthma [1–3]. Bermuda grass (*Cynodon dactylon*) pollen (BGP) is one of the causative agents of asthma [4–6]. It is endemic in warm temperate and

subtropical climates, such as the southwestern United States, northern and central Australia, South Africa, and Taiwan. In Taiwan, approximate 27% of asthmatic patients have positive immediate skin hypersensitive responses to the crude pollen extract [7]. A PBS crude extract of BGP contains a very complex mixture of IgG- and IgE-reactive components [8], but only a few allergens (Cyn d 1, Cyn d 7, Cyn d 12, BG60, and Cyn d Bd46K) have been identified [9–13]. The major allergen, Cyn d 1, is the most abundant protein (15% of the total crude extract) in BGP and gives a 76–100% positive reaction rate with IgE in the sera of patients allergic to BGP [14–16]. Cyn d 7 is a calcium-binding protein, and its allergenicity and cross-reactivity have been investigated in other pollens, such as rape [17] and birch [18]. Cyn d 12 is a member of the profilins, which have been described as pan-allergens responsible for about 20% of the cross-reactivity seen in patients with pollen and food allergies [19]. Two other

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Abbreviations: BGP, Bermuda grass pollen; IgE, immunoglobulin E; dC1BCE, Cyn d 1-depleted BGP crude extract; EF-2, elongation factor 2; MDH, malate dehydrogenase; PR, pathogenesis-related protein

allergens, BG60 and Cyn d Bd46K, have been identified as a flavoprotein and a cytochrome *c* oxidase homologue, respectively [12, 13].

The identification of allergens is essential for investigations on hypersensitivity and for clinical treatment. However, traditional methods for the identification and characterization of common allergens require extensive effort and a large amount of starting material. With the advent of proteomics, approaches involving the combined application of separation techniques, MALDI-MS, LC-MS/MS, and bioinformatics have been proposed for the identification and characterization of proteins in a complex biological mixture in various experimental contexts [20, 21]. The aim of our study was to investigate the 2-DE protein profile and IgE-reactive patterns of important allergenic components present at low abundance in BGP and to identify them. To do so, we used affinity chromatography to remove the major component, Cyn d 1, from the crude BGP extract, and then applied 2-DE and 2-D IgE immunoblotting to perform sub-proteome analysis. IgE-reactive proteins were identified by PMF, N-terminal sequence similarity, and LC-MS/MS.

2 Materials and methods

2.1 Patient's sera and measurement of specific IgE

The patients selected for this study had been referred to the allergy clinic because of a history of seasonal allergic rhinitis or asthma for at least 1 year and had never received BGP immunotherapy. Serum samples, obtained when patients visited the clinic for the first time, were stored in 1 mL aliquots at -70°C until use. For this study, 12 allergic patients and 12 sex- and age-matched normal subjects were selected from the allergic population ($n > 300$). All the selected patients had registered a score of at least 3+ on the prick test using the BGP extract, and all the normal sera used as negative controls gave a very low absorbance value of 0.05 in the CAP assay and were considered nonreactive. All patients and normal subjects gave their written informed consent to the study. Specific IgE antibodies to *Dermatophagoides pteronyssinus* and BGP were measured using a Pharmacia CAP system IgE Fluorescence Enzyme-linked ImmunoAssay kit (Amersham Biosciences, Uppsala, Sweden), according to the manufacturer.

2.2 Protein extraction and sample preparation

BGP was obtained from International Biologicals (Piedmont, OK, USA). The BGP crude extract was prepared by extracting defatted pollen with PBS (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 140 mM NaCl, 2.7 mM KCl, pH 7.4) for 16 h at 4°C with constant stirring, filtration of the extract through a Whatman No. 1 filter and centrifugation at $800 \times g$ for 30 min at 4°C , and dialysis of the supernatant at 4°C against phosphate buffer (20 mM Na_2HPO_4 , pH 7.2). The dialyzed sample was loa-

ded on to an affinity column (anti-Cyn d 1 mAb-conjugated Sepharose 4B, 1.6×5 cm; mAb provided by Dr. Song-Nan Su, Department of Medical Research and Education, Veterans General Hospital, Taipei, Taiwan) to deplete Cyn d 1, and the column washed with the same phosphate buffer. The column effluent (Cyn d 1-depleted BGP crude extract; dC1BCE) was collected and stored at -20°C . After elution of dC1BCE, the affinity column was washed with 0.004 N HCl for regeneration, and only Cyn d 1 was shown on the SDS-PAGE analysis of the wash-out fraction. Proteins in dC1BCE were precipitated by adding TCA (final concentration 20%), washed with cold acetone, and vacuum dried in a SpeedVac concentrator. The dried powder was used for 1-DE and 2-DE. When tested on 12.5% SDS-PAGE, the material showed no CBB-stained band corresponding to Cyn d 1; a band at 32 kDa was shown not to be Cyn d 1 by PMF.

2.3 1-DE and 2-DE

dC1BCE proteins were separated by 1-DE (12.5% SDS-PAGE) or 2-DE as described previously [22]. For 1-DE, 20 μg dC1BCE was incubated for 10 min at 95°C with 20 μL 1-DE sample buffer (50 mM Tris-HCl, pH 6.8, 0.4% SDS, 20% 2-mercaptoethanol), then loaded into the well of a 12.5% polyacrylamide gel. M_r standards ranging from 97 to 14.4 kDa (Amersham Biosciences) were also included. Electrophoresis was performed in running buffer (25 mM Tris, 192 mM glycine, pH 8.8, 1.0% SDS) using an EIDO NA-1010 apparatus (Nihon EIDO, Tokyo, Japan) at 16 mA. For 2-DE, 1 mg dC1BCE was incubated with 120 μL 2-DE sample buffer (8 M urea, 2% pharmalyte pH 3–10, 60 mM DTT, 0.5% Triton X-100, bromophenol blue) and loaded through sample cups. The first dimension of the 2-DE was run on IPG strips (Immobiline DryStrip pH 3–10, 11 cm; Amersham Biosciences) on a horizontal electrophoresis system (Multiphor II system equipped with an EPS 3500 XL power supply; Amersham Biosciences). The IPG strips were focused in a four-step procedure, the voltage being ramped to 300 V for 0.1 min, maintained at 300 V for 5 h, linearly increased to 2000 V over 8 h, and maintained at 2000 V for 9 h at 20°C . Prior to the second dimension, the focused strips were equilibrated in 50 mM Tris-HCl, pH 6.8, 6 M urea, 1% SDS, 0.25% DTT for 15 min at room temperature, then placed on the 12.5% SDS-polyacrylamide gel, and separation performed at 20 mA.

2.4 Protein staining, glycoprotein staining, and IgE-binding protein detection

For protein profiling, the separated proteins on the gel were stained with CBB (Amersham Biosciences), as described previously [22]. Glycoprotein staining was performed using a GelCode glycoprotein staining kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer. Briefly, 1.5 mg dC1BCE was separated by 2-DE, and the electro-

phoresed gel incubated with 50% methanol for 30 min, washed with 3% acetic acid for 10 min, then transferred to oxidizing solution for 15 min. After three washes, the gel was transferred to GelCode glycoprotein staining reagent for 15 min, then to reducing solution for 5 min, and washed extensively with 3% acetic acid and ultrapure water. Glycoproteins appeared as magenta spots.

For immunostaining, proteins on the 1-D or 2-D gels were transferred to PVDF membranes (Millipore, Bedford, MA, USA) by semidry blotting (Nihon EIDO; blotting time 3.5 h, current/area 1 mA/cm²). The membranes were then blocked with skimmed milk, and incubated for 16 h at 4°C with a 1:5 dilution of serum from a BGP-hypersensitive patient or control. Bound IgE was detected using a 1:1500 dilution of alkaline phosphatase-labeled goat anti-human IgE antibody (BioSource International, Camarillo, CA, USA) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as the substrate system [22].

2.5 N-terminal sequencing

After 2-DE, the blotted proteins were stained with CBB, and the protein spots corresponding to those recognized by the serum samples from BGP-hypersensitive patients were excised and subjected to N-terminal sequence analysis in a Procise 494 protein sequencer (Applied Biosystems).

2.6 In-gel digestion and protein identification

The CBB-stained protein spots corresponding to IgE-binding were excised, destained with 1 mL 50% ACN in 50 mM NH₄HCO₃, and dried in a SpeedVac concentrator as described previously [23]. The protein was then digested for 16 h at 37°C with sequencing-grade trypsin (0.1 mg/mL; Promega Madison, WI, USA) in 50 mM NH₄HCO₃, and the resulting peptides extracted sequentially with 50 µL 1% TFA and 50 µL 0.1% TFA/50% ACN. The combined extracts were lyophilized, resuspended in 10 µL 0.1% TFA, desalted on a ZipTip C18-microcolumn (Millipore), vacuum dried, and resuspended in 0.5% TFA/30% ACN. Protein identification was carried out by MALDI-TOF MS or nanoLC-ESI-MS/MS analysis. The database search was performed using MS-Fit software for MALDI-TOF MS (<http://prospector.ucsf.edu>) and MASCOT software (<http://www.matrixscience.com>) for nanoLC-ESI-MS/MS.

2.7 MS

2.7.1 MALDI-TOF MS analysis

Peptide extract (0.5 µL) was mixed with CHCA (0.5 µL; 5 mg/mL) on the MALDI sample plate and introduced into the mass spectrometer after drying. PMFs were obtained using a MALDI-TOF mass spectrometer (Voyager DE-STR, Applied Biosystems, Framingham, MA, USA). Ions were accelerated at 20 kV and reflected at 21.5 kV, and all spectra

were acquired in positive ion reflector mode and in delayed extraction. Each single mass spectrum was the result of the summation of 100 laser shots. Spectra were externally calibrated using a standard solution containing angiotensin I, adrenocorticotrophin, and bovine insulin (Applied Biosystems).

2.7.2 NanoLC-ESI-MS/MS analysis

Samples that did not return any confident matches from the MALDI-TOF MS database searches were subjected to MS/MS analyses. NanoLC-ESI-MS/MS analysis was performed on an integrated nanoLC-MS/MS system (Micromass, Manchester, UK) comprising a three-pump Micromass/Waters CapLC system with an autosampler, a stream select module configured for the precolumn and analytical capillary column, and a Micromass Q-ToF Ultima API mass spectrometer fitted with a nano-LC sprayer, operated under MassLynx 4.0 control. Injected samples were first trapped and desalted isocratically on an LC-Packings PepMap C18 µ-precursor cartridge (5 µm bead size, 300 µm id × 5 mm; Dionex, Sunnyvale, CA, USA) for 2 min with 0.1% formic acid delivered by an auxiliary pump at 30 µL/min, then the peptides were eluted from the precolumn and separated on an analytical C18 capillary column (15 cm × 75 µm id, packed with 5 µm Zorbax 300 SB C18 particles; Micro-Tech Scientific, Vista, CA, USA), connected inline to the mass spectrometer, at a flow rate of 300 nL/min using a 40-min fast gradient of 5–80% ACN in 0.1% formic acid.

3 Results and discussion

3.1 Detection of IgE-binding proteins in dC1BCE by 1-D immunoblotting

BGP gives complicated 1-D IgG- and IgE-binding patterns [8]; however, the most abundant allergen, Cyn d 1, greatly interferes with the detection of low abundant IgE-reactive proteins. Using the depletion process, our 1-D IgE-binding profile of dC1BCE showed more allergenic components [8]. Figure 1 shows the results for IgE immunoblotting of the proteins using sera from 12 patients allergic to BGP (lanes a–l) and one of the normal controls (lane n) with no specific IgE to BGP or house dust mite; all 12 patients had seasonal allergic rhinitis and high levels of IgE to BGP, and some were also sensitized to house dust mite and had high levels of specific IgE (Table 1). Complex patterns of IgE-binding proteins were detected, the molecular masses of the IgE-binding components ranging from 14 to 97 kDa. Bands with approximate molecular masses of 89, 66, 60, 45, 32, 28, and 18 kDa showed the strongest responses and were seen at a high frequency.

Previously, studies on allergenic components in BGP have focused on the major or a single IgE-reactive protein, and only a few allergens have been characterized, e.g.,

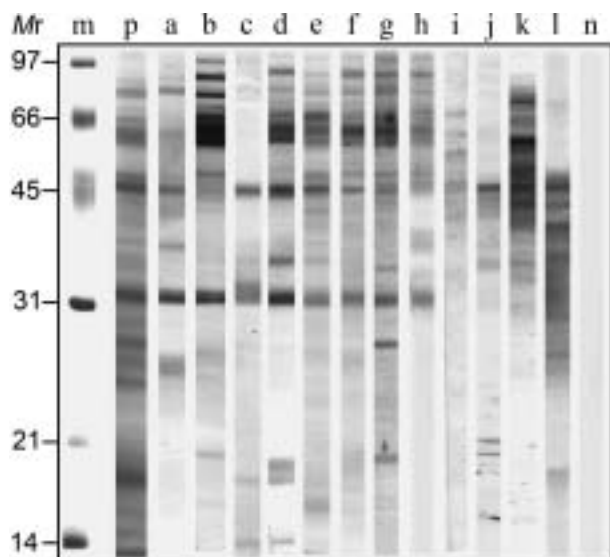


Figure 1. 1-D immunoblotting of dC1BCE proteins. Lane m, molecular weight markers; lane p, CBB-stained protein profile; lanes a–l, detection of IgE-binding proteins using sera from different allergic patients; lane n, a negative control using one of the non-atopic sera.

Table 1. Clinical characteristics of the 12 patients and the control subjects

Patient	Symptoms	Skin prick test to BGP	Specific IgE (kU/L)	
			Mite	BGP
a	SAR	>4+	5.00	2.35
b	SAR	3+	0.97	5.47
c	SAR	3+	1.82	0.64
d	SAR	3+	<0.35	1.27
e	SAR	3+	0.66	1.57
f	SAR	>4+	66.40	6.65
g	SAR	4+	0.54	12.30
h	SAR	>4+	46.80	6.11
i	SAR	3+	<0.35	2.35
j	SAR	3+	<0.35	3.60
k	SAR	4+	4.73	5.36
l	SAR	>4+	0.39	23.80
Control (<i>n</i> = 12)	None	NT	<0.35	<0.35

SAR, seasonal allergic rhinitis; NT, not tested. Controls are sex- and age-matched to allergic subjects.

Cyn d 1, calcium-binding protein (Cyn d 7), and profilin (Cyn d 12) [9–11]. Figure 1 shows that our approach allows the characterization of many allergens.

3.2 2-DE protein and IgE immunoblotting patterns

For a more detailed analysis of the dC1BCE allergens, 2-DE and IgE immunoblotting were performed to characterize the proteins and IgE-binding patterns. Figure 2A shows the

CBB-stained 2-D gel, which demonstrates that most of the proteins had molecular masses between 66 and 14 kDa and neutral or acidic pIs. However, some proteins with molecular masses of 60, 32, 28, 21, and 16 kDa with a pI > 8 were also detected.

Carbohydrates play an important role in IgE reactivity [24, 25] and IgE cross-reactivity [26, 27]. Most of the allergens in BGP are glycoproteins [9, 12, 13] and the carbohydrate moieties contribute to IgE reactivity [12, 13, 16]. As shown in Fig. 2B, glycoprotein staining gave more than 40 magenta glycoprotein spots.

When 2-D IgE immunoblotting was performed using sera from ten allergic patients (a, c, e–l), each gave a different pattern; one of the immunoblots probed with serum from patient g is shown (Fig. 2C). Taking together the results for the ten sera, over 60 different IgE-reactive spots were detected, with molecular masses and pIs ranging, respectively, from 14 to 89 kDa and 3.2 to 8.8. Spots recognized by serum samples from two or more patients were numbered as 1a to 8; their labeling patterns with each serum sample are shown in Fig. 3.

In the present study, we first used a depletion method to remove the major component Cyn d 1 before using the extract for the detection of IgE-reactive proteins. This improved the detection of low-abundant, but important, IgE-reactive proteins by reducing interference by Cyn d 1 (data not shown). Cyn d 1 is recognized by sera from up to 76% of BGP allergic patients [14]; our 2-D immunoblots showed dozens of IgE-reactive components in dC1BCE (Fig. 2C), some of which were also recognized at a high frequency (100% (no. 1), 90% (no. 2), and 50% (no. 3)) (Fig. 3).

3.3 Protein identification by MS, MS/MS, and N-terminal sequencing

Sixteen spots (1a–1g, 2, 3, 4, 5a, 5b, 6, 7a, 7b, and 8) were excised from the gel and identified by MALDI-TOF MS PMF and N-terminal sequence similarity analysis. The PMF results showed that the 16 spots belonged to eight different proteins. Two were well-known allergens, BG60 (no. 3) and Cyn d 12 (no. 8), but six had not previously been described as IgE-reactive proteins in BGP. As shown in Table 2, these were enolase 2 (nos. 1a–g), aldolase (no. 2), elongation factor 2 (EF-2) (no. 4), pathogenesis-related protein 1 (PR-1) (nos. 5a, 5b), malate dehydrogenase (MDH) (no. 6), and Phl p 11-like proteins (nos. 7a and 7b). N-terminal sequences of these IgE-reactive proteins were also obtained for similarity analysis, and showed high similarity of up to 100% except for spot 5a and 5b (Table 2). However, the peptide mass matches and sequence coverage of the identified proteins were relatively low, ranging from 4% to 10%, and 9% to 20%, respectively.

To further investigate the novel allergenic proteins, spots 1a, 2, 4, 5a, 6 and 7a were analyzed by LC-MS/MS; the sequences matched using high-quality MS/MS are summarized in Table 3. The number of identified peptides for each

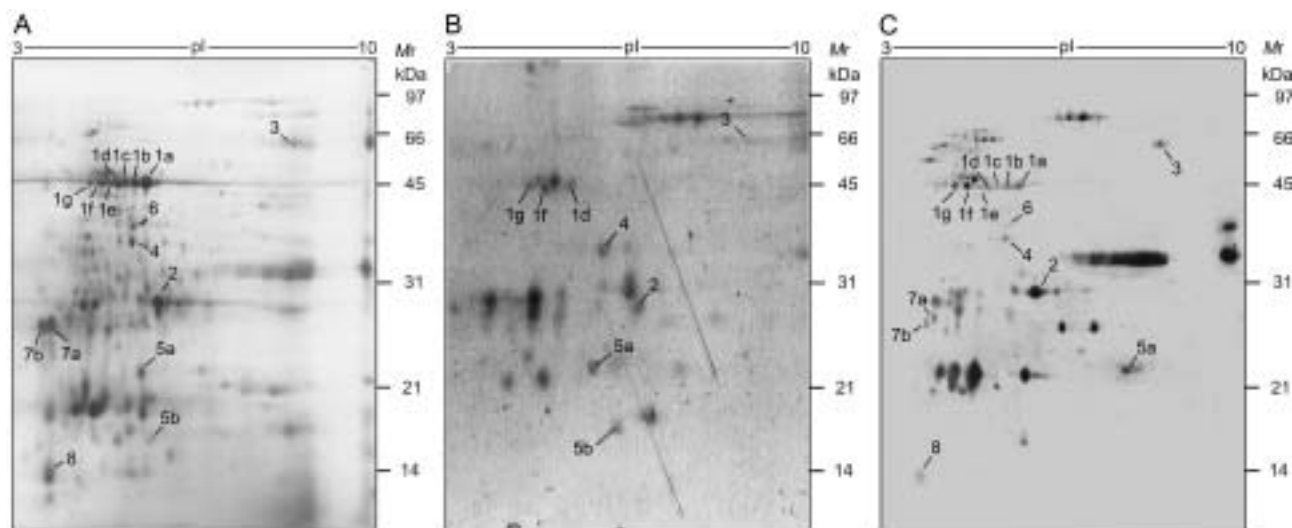


Figure 2. 2-DE analysis of dC1BCE and corresponding immunoblot using serum from one allergic patient. dC1BCE proteins (1 mg) were separated by 2-DE using a linear pH 3–10 gradient in the first dimension, followed by 12.5% SDS-PAGE in the second dimension. The separated proteins were stained with CBB (A) or for glycoproteins (B) or transferred to a PVDF membrane, then probed with serum from a patient allergic to BGP (patient g) (C). Selected protein spots from (A) were numbered and collected for identification.

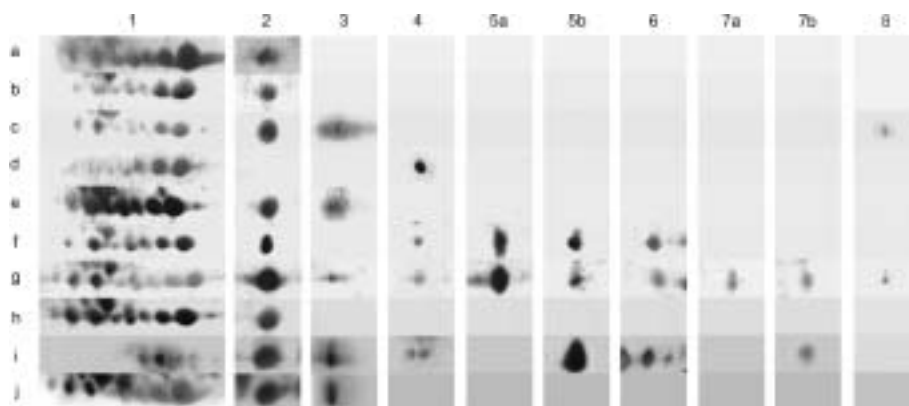


Figure 3. 2-D IgE-binding patterns on immunoblots. dC1BCE proteins (1 mg) were separated by 2-DE and transferred to a PVDF membrane. The binding of IgE from the sera of ten individual patients allergic to BGP (patients a, c, e to j) to selected spots (nos. 1a–8) is shown.

of the four proteins was 3–10, covering 8–37% of the protein, and each identified peptide sequence was specific to the corresponding protein. These results show that the sequence of a component allergen can be rapidly identified by searching databases with MS data in conjunction with MS/MS sequencing. In instances when the identity of the protein is unknown, such searches can still prove useful when homologous proteins appear in the database.

These six novel allergenic proteins in BGP have been reported as allergens in other species. For example, enolase 2 is an important inhalant allergen from several allergenic species, such as baker's yeast (*Saccharomyces cerevisiae*) [28], *Candida albicans* [29], natural rubber latex (*Hevea brasiliensis*) [30], *Penicillium citrinum* and *Aspergillus fumigatus* [31]. The

N-terminal sequence (Table 2) and the peptide sequences identified by LC-MS/MS (Table 3) showed 100% identity with rice enolase 2, and a homology search showed 72–95% similarity with enolase 2 from various flowering plants, fungi, and yeast (data not shown), suggesting that this enzyme might be a common allergen in such species. Spots 1a–g were also confirmed as enolase using specific anti-enolase antibody (Wako, Chuo-Ku, Osaka, Japan).

Aldolase, EF-2, and MDH are also highly conserved proteins and have been reported, respectively, as an allergen of wheat grain (*Triticum aestivum*) [32], natural rubber latex (*Hevea brasiliensis*) [33], and *Malassezia furfur*, a member of the human skin microflora [34]. The N-terminal sequence (Table 2) of spot 2 and spot 6 showed 80% and 87% similarity

Table 2. Identification of IgE-reactive proteins from BGP

Spot	Experimental		Identified ID/ accession no.	Identified N-terminal sequence	Theoretical		N-terminal similarity (%)	Glyco- protein staining
	pI	Mass (kDa)			pI	Mass (kDa)		
1	a	6.1	Enolase 2/AAP94211	KGVSK AVNNV NSIIG	5.7	41.7	100	–
	b	5.9		KGVSK AVNNV NSIIG	5.7	41.7	100	–
	c	5.7		KGVSK AVNNV NSIIG	5.7	41.7	100	–
	d	5.5		KGVSK AVNNV NSIIG	5.7	41.7	100	+
	e	5.3		KGVSK A	5.7	41.7	100	–
	f	5.1		KGVSK A	5.7	41.7	100	+
	g	4.8		KGVSK A	5.7	41.7	100	+
2		6.2	Aldolase/BAA02729	ESTAE LGKKI NKVVL	6.6	38.6	80	+
3		8.8	BG60/AY451241	N.D.	9.3	54.9	–	+
4		5.8	Elongation factor 2/ BAB91927	GAEII VSPPV VSFRE	5.9	27.5	93	+
5	a	5.9	Pathogenesis-related protein 1/P08299	STQLP SDEPL NGKND	5.9	21	33	+
	b	5.8	Pathogenesis-related protein 1/P08299	STQLP S	5.9	21	50	+
6		5.8	Malate dehydrogenase/ AAM64855	TPASG QPERK VAILG	8.5	35.8	87	–
7	a	4.5	Phl p 11-like protein/ AAN32987	FTVTG EVYXD PXRA	5.0	15.8	80	–
	b	4.3		FTVVG EVYXD	5.0	15.8	90	–
8		4.1	Profilin (Cyn d 12)/ CAA69670	SXQAY VDDHL	4.9	14.1	90	–

ND, not determined (blocked)

with rice aldolase and thale cress MDH, respectively, and the LC-MS/MS identified peptide sequences of the two spots (Table 3) also presented 100% identity with rice aldolase and thale cress MDH, respectively. EF-2 (BAB91927) has a theoretical molecular mass of 88.4 kDa, contains 795 amino acids, and shows 63–93% similarity in *Oryza sativa*, *Beta vulgaris*, *Chlorella kessleri*, and *Botryocladia uvarioides*. Spot 4 had a molecular mass of 34.3 kDa. Since N-terminal sequencing of spot 4 gave a very similar sequence (93%) corresponding to region 548–562 of EF-2 and since all the peptide fragments predicted by PMF from MALDI-MS or identified by LC-MS/MS had sequences corresponding to region 574–768, this suggests that spot 4 corresponded to a C-terminal fragment of EF-2.

Although their N-terminal sequences showed low similarity with the corresponding PR-1 sequences in the database, spots 5a and 5b gave four peptide sequences identified by LC-MS/MS (Table 3) showing 39–54% similarity with the corresponding region of PR-1 (P35793, *Hordeum vulgare*) (data not shown). Although members of the PR family have been reported as allergens in many plants, *e.g.*, Cup a 3 (PR-5, *Cupressus arizonica*) [35], thaumatin-like kiwi allergen (PR-5, *Actinidia deliciosa*) [36], SAM22 (PR-10, *Glycine max*) [37], Bet v 1 (PR-10, *Betula pendula*) [38], and Mal d 1 (PR-10, *Malus*

domestica) [39], this is the first report showing that a member of the PR-1 subfamily is an allergen. The identification of spot 5a and 5b as PR-1 was also confirmed by specific anti-PR-1 antibody (supplied by Dr. Song-Nan, Su; data not shown).

Spots 7a and 7b were identified as Phl p 11 homologues [40, 41] and their N-terminal sequences were, respectively, 80 and 90% identical to that of Phl p 11, an allergen from timothy grass (*Phleum pratense*) (Table 2). However, the four digested peptide sequences identified by LC-MS/MS (Table 3) only showed 31–58% similarity to the corresponding region of Phl p 11 (data not shown). This might be because the two Phl p 11 homologues are not highly conserved in different species, as shown by similarity searches performed using the NCBI protein-protein BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) (data not shown).

Two well-known allergens, BG60 (spot 3) and Cyn d 12 (spot 8), were identified by MALDI-MS PMF in our study. BG60 is a flavoprotein containing nickel and copper ions [42] and shows some structural homology with berberine bridging enzyme of California poppy (*Eschscholtzia californica*) [43]. It exists in two isoforms, a long form with a blocked N terminus and a short form lacking the N-terminal region of the long form [42]. In our study, since the N terminus was blocked, BG60 was also identified on 2-D immunoblots

Table 3. LC-MS/MS identification of IgE-binding proteins from the 2-D gel

Spot	Protein ID Species/ accession no.	LC-MS/MS analysis				
		Cover (%)	MH+ submitted (Da)	MH+ matched (Da)	MH+ delta (Da)	Peptide sequence
1a	Enolase 2 <i>O. sativa</i> / AAP94211	35	1578.88	1579.89	−1.01	AVDNVNSVIAPALIGK
			1678.91	1678.95	−0.04	KIPLYQHIANLAGNK
			445.11	445.29	−0.18	SVIK
			2322.98	2323.04	−0.06	YGQDATNVGDEGGFAPNIQENK
			1999.94	1999.96	−0.02	VVIGMDVAASEFYNDKDK
			899.42	899.44	−0.02	TYDLNFK
			1572.83	1572.84	−0.01	VNQIGSVTESIEAVK
			2235.12	2235.13	−0.01	SGETEDTFIADLAVGLATGQIK
			1490.71	1490.76	−0.05	IEEELGAAAVYAGAK
2	Aldolase <i>O. sativa</i> / BAA02729	8	976.50	977.50	−1.00	FRAPVEPY
			989.50	989.50	0.00	GTIEVAGTDK
			828.38	828.38	0.00	YYEAGAR
			1345.73	1345.72	0.01	VAPEVIAEYTVR
			1222.59	1222.59	0.00	ILSEEFGWDK
			1062.57	1062.57	0.00	GVQYLNIEIK
			1118.52	1118.50	0.02	EGALAEENMR
			954.52	954.52	0.00	GGGQVIPTAR
			2939.57	2939.60	−0.03	LLEPVYLVEIQAPENALGGIYGVLNQK
5a	Pathogenesis-related protein 1 <i>N. tabacum</i> / P08299	37	1800.55	1799.84	0.71	ATGFYNNAPHFCFVGR
			2412.02	2411.30	0.71	QNPLLWTCAGVVGLGVSLLSWK
			1398.61	1398.74	−0.13	EEGLVTEPLVWK
			1308.57	1308.75	−0.18	VTKHPSGITTLR
6	Malate dehydrogenase <i>A. thaliana</i> /AAM64855	16	1920.15	1920.16	−0.01	KVAILGAAGGIGQPLALLMK
			1792.06	1792.06	−0.00	VAILGAAGGIGQPLALLMK
			1318.71	1317.69	1.01	DDLFINAGIVK
			1218.69	1218.70	−0.01	LFGVTTLDVVR
			1388.69	1388.69	0.00	RTQDGGTEVVEAK
			1232.59	1232.59	0.01	TQDGGTEVVEAK
7a	Phl p 11-like protein <i>P. pratense</i> /AAN32987	37	1785.91	1785.93	−0.02	HSLETCEYEPLFFFR
			1866.04	1867.98	−1.93	FPLEIVDAVAKEIGPDR
			1265.69	1265.76	−0.07	IQIRVGSIPAGR
			891.51	891.41	0.10	MTSGAAAGR

using an anti-BG60 mAb (supplied by Dr. Song-Nan, Su; data not shown). Cyn d 12, a profilin, was cloned and expressed in the previous study [44], and profilins have been reported as allergens in other pollens [45–48]. No 2-D analysis information was previously available for BG60 and Cyn d 12; in the present study, we identified these two allergens and determined their positions on 2-D maps.

More investigation is needed to determine the actual allergenicity of IgE-reactive proteins. Based on the information from our proteome analysis and identification, we have purified enolase 2 and PR-1 from dC1BCE for further immunological studies. Our preliminary data showed that enolase 2 could stimulate histamine release and PR-1 could trigger a positive wheal-and-flare response in allergic patients, and these results confirmed their allergenicity to

elicit the immediate hypersensitive reactions (data not shown). Moreover, specific antibodies generation, cDNA cloning and recombinant protein expression for these newly identified IgE-reactive proteins are also in progress.

Only a few BGP proteins have been identified and characterized, the BGP genome has not been completely sequenced, and no proteome analysis of BGP has been reported. Because of this, although the MALDI-MS PMF provided efficient and useful information for protein identification of the more than 30 IgE-reactive protein spots on the 2-D gel digested and analyzed by MALDI-MS, only 16 were unambiguously identified by LC-MS/MS, N-terminal similarity analysis, and specific antibody detection. The remaining spots are now being processed to identify and characterize other important allergenic proteins.

4 Concluding remarks

Here, we provide the 2-DE protein and glycoprotein patterns and IgE-reactive profiles of a BGP sub-proteome, dC1BCE, and identify six novel important allergenic proteins by a combination of a depletion approach and MALDI-MS PMF, N-terminal sequence similarity, and LC-MS/MS analysis. This proteomic approach provides a more efficient and accurate method for the identification of allergenic components than traditional methods. Our IgE-reactive proteome analysis of BGP not only provides an overall map of the allergenic proteins, but also useful information (kinds of allergens, their normal function, and their prevalence) for further systematic research on pollen allergens and for individual desensitizing treatments and therapeutic development.

This work was supported in part by the Promotion of Research-Oriented University Program from the Ministry of Education and Grant NSC 92-2320-B-002-174 from the National Science Council of the Republic of China. LC-MS/MS analyses were performed by the Core Facilities for Proteomics Research located at the Institute of Biological Chemistry, Academia Sinica, supported by a National Science Council grant (NSC 91-3112-B-001-010-Y) and the Academia Sinica.

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