

Use of monoclonal antibodies to isolate and characterize *Cyn d I*, the major allergen of Bermuda grass pollen

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Background: *Cyn d I* has been found to be the major allergen of Bermuda grass (*Cynodon dactylon*) pollen, but its exact nature remains to be clarified.

Methods: *Cyn d I*, the major allergen of Bermuda grass (*Cynodon dactylon*) pollen, was purified by monoclonal antibody (MoAb) affinity chromatography, and its biochemical and immunologic properties were characterized. Anti-*Cyn d I* MoAb 4-37, which recognizes all of the isoallergens of *Cyn d I*, was chosen as the immunosorbent.

Results: The purified protein has an amino acid composition similar to that of the group I allergens of other grass pollens. It appears as a single 34 kd band or as a mixture of 34 and 29 kd polypeptides in sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis. The hydrophobicity of these two polypeptides is similar because they have the same retention time on a C_{18} reverse-phase column when a trifluoroacetic acid/ H_2O/CH_3CN buffer system is used. The N-terminal amino acid sequence of the 34 kd component has a 60% homology with residues 1-25 of Lol p I, whereas that of the 29 kd component has a 68% homology with residues 31-68 of Lol p I. In addition, this 29 kd polypeptide can be recognized by another anti-*Cyn d I* MoAb 1-61.

Conclusions: These results suggest that the 29 kd component is derived from *Cyn d I*. In spite of the similarity in the amino acid composition between *Cyn d I* and group I allergens of other grass pollens, none of our four anti-*Cyn d I* MoAbs cross-reacted with 10 other grass pollens tested, including ryegrass pollen. Despite biochemical similarity with other group I allergens, the B-cell epitopes on *Cyn d I* are different from those on other grass pollens. (*J ALLERGY CLIN IMMUNOL* 1993;92:549-58.)

Key words: *Cyn d I*, purification, characterization, monoclonal antibody affinity chromatography

Bermuda grass (*Cynodon dactylon*) pollen (BGP) is an important worldwide aeroallergen.¹⁻³ Two allergens (BGP-1 and BGP-2) from the extract of BGP were initially identified by Orren

and Dowdle.⁴ BGP-1 (molecular weight [MW] = 30 kd) was found to be a major allergen that elicits a 100% positive immediate cutaneous reaction in all BGP-sensitive subjects tested. BGP-2 (MW = 14 kd) was found to be a minor allergen with a 75% positive rate in skin tests. With the immunoblotting technique, the major allergen from BGP was further characterized by Ford and Baldo⁵ and by Shen et al.⁶ A 32 to 34 kd component was found to be reactive with IgEs in sera from BGP-allergic patients with a positive rate of 76% to 100%.

The major allergenic component of BGP was also identified as antigen 34/35 by Matthiesen et al.⁷ It exists in isomeric forms with a MW of 32 kd and reacts with 97% of the allergic sera tested. This component was later purified by concanavalin A Sepharose and ion exchange chromatogra-

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Abbreviations used:

<i>Amb e:</i>	<i>Ambrosia elatior</i> (ragweed) pollen extract
<i>Ant o:</i>	<i>Anthoxanthum odoratum</i> (sweet vernal grass) pollen extract
BGP:	Bermuda grass pollen
<i>Cyn d I:</i>	Group I allergen of <i>Cynodon dactylon</i>
<i>Cyn d Bd35K:</i>	Unpurified <i>Cyn d I</i> , 35 kd band component of <i>Cynodon dactylon</i>
<i>Dac g:</i>	<i>Dactylis glomerata</i> (orchard) pollen extract
<i>Fes e:</i>	<i>Festuca elatior</i> (meadow fescue grass) pollen extract
<i>Hol l:</i>	<i>Holcus lanatus</i> (velvet grass) pollen extract
<i>Lol p:</i>	<i>Lolium perenne</i> (perennial ryegrass) pollen extract
MoAb:	Monoclonal antibody
MW:	Molecular weight
<i>Pas n:</i>	<i>Paspalum notatum</i> (Bahia grass) pollen extract
PBS:	Phosphate-buffered saline
<i>Pha c:</i>	<i>Phalaris canariensis</i> (reed canary) pollen extract
<i>Phl p:</i>	<i>Phleum pratense</i> (timothy grass) pollen extract
<i>Poa p:</i>	<i>Poa pratense</i> (Kentucky bluegrass) pollen extract
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
<i>Sor h:</i>	<i>Sorghum halepense</i> (Johnson grass) pollen extract

phy.⁸ The purified component (*Cyn d I*) consists of a dominating 32 kd band and a minor 29 kd band in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Recently, we have generated a panel of 16 anti-BGP monoclonal antibodies (MoAbs) for the characterization and purification of the allergenic components from BGP.⁹ When radioimmunoprecipitation was combined with two-dimensional gel electrophoresis for characterization, the proteins recognized by these MoAbs were designated as *Cyn d Bd200K*, *Cyn d Bd67K*, *Cyn d Bd58K*, *Cyn d Bd46K*, *Cyn d Bd35K*, *Cyn d Bd25K*, *Cyn d Bd12K*, and *Cyn d Bd68K*, 48K, 38K⁹ according to the nomenclature system recommended by the International Union of Immunological Societies.¹⁰ Among these 16 anti-BGP MoAbs, four recognized *Cyn d Bd35K*, an isomeric protein that is most strongly recognized by allergic human IgEs in a modified RAST with a recognition frequency

of 91%.⁹ *Cyn d Bd35K* is considered to be the major allergen of BGP, *Cyn d I*.

In the present study, we report the single-step purification of *Cyn d I* with an MoAb affinity column. The purified product appears as a single 34 kd protein or as a mixture of 34 and 29 kd polypeptides. The 29 kd component appears to be a degraded product of the 34 kd protein. The hydrophobicity, N-terminal sequence, and amino acid composition of these components were characterized. In addition, the immunologic property was also studied with the use of four anti-*Cyn d I* MoAbs.

METHODS**Pollen extract**

Defatted dry BGP pollen was purchased from Allergon AB (Carthage, Mich.) and International Biologicals, Inc. (Piedmont, Okla.). It was extracted with 0.15 mol/L phosphate-buffered saline (PBS) (pH 8.0) at a ratio of 1:10 (grams per milliliter) with gentle shaking at 4° C for 18 hours. The extract was dialyzed (MW cutoff = 3500 d) against distilled water for 24 hours, and the protein content was determined by the dye-binding method of Bradford¹¹ with bovine serum albumin as a standard. The lyophilized material was stored at -20° C.

Other pollens used in the cross-reaction are: sweet vernal grass (*Anthoxanthum odoratum*, *Ant o*), orchard grass (*Dactylis glomerata*, *Dac g*), meadow fescue grass (*Festuca elatior*, *Fes e*), velvet grass (*Holcus lanatus*, *Hol l*), perennial ryegrass (*Lolium perenne*, *Lol p*), Bahia grass (*Paspalum notatum*, *Pas n*), reed canary grass (*Phalaris canariensis*, *Pha c*), timothy grass (*Phleum pratense*, *Phl p*), Kentucky bluegrass (*Poa pratense*, *Poa p*), Johnson grass (*Sorghum halepense*, *Sor h*), and ragweed (*Ambrosia elatior*, *Amb e*). They were extracted as described above.

Anti-*Cyn d I* MoAbs

MoAbs 4-37, 10-7, 11-7, and 1-61 (all IgG1), which are specific for *Cyn d Bd35K*, were generated by the fusion of spleen cells from BGP-immunized BALB/c mice and NS-1 myeloma cells as described by Chang et al.⁹

Allergic sera

Sera were collected at the Allergy Clinic, Veterans General Hospital-Taipei (Taiwan, Republic of China) from allergic patients with positive intradermal skin test results to BGP extracts (supplied by Taiwan Allergy Centre, Taipei, Taiwan, Republic of China). Histamine (0.1 mg/ml) and PBS were used as positive and negative controls, respectively. The collected sera were further selected on the basis of their strong reactivity in a RAST as previously described.⁹ They were pooled for the studies in this report.

Gel electrophoresis and immunoblotting

SDS-PAGE analysis of pollen extracts was performed on gels (T = 11%, A = 0.29%) according to the method of Laemmli.¹² Two-dimensional gel electrophoresis was performed as described by Shen et al.¹³ For SDS-PAGE, rabbit muscle phosphorylase b (97 kd), bovine serum albumin (66 kd), ovalbumin (45 kd), carbonic anhydrase (31 kd), soybean trypsin inhibitor (21 kd), and hen egg white lysozyme (14 kd) were used as standards and included in each gel. In isoelectric focusing the isoelectric point was determined according to the method of Hjelmeland et al.¹⁴ After focusing, a slice of the control isoelectric focusing gel was cut into 0.5 cm pieces, immersed in degassed distilled water, and extracted at 4° C on a shaker. The pH of each extract was determined after 1-hour extraction.

After electrophoresis, proteins were visualized with silver staining¹⁵ or electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) and treated with 3% wt/vol gelatin as previously described.⁶ The membrane was incubated either with culture supernatant containing MoAb (1:4 diluted) at room temperature for 1 hour or with patient sera at 4° C overnight and was then washed with Tris buffer (1 mol/L, pH 7.2) containing 0.05% Tween 20.

The MoAb-treated membrane was then incubated with 1:1500 diluted peroxidase labeled goat anti-mouse IgG (Bio-Rad Laboratories, Chemical Div., Richmond, Calif.) for 1 hour. After thorough washing, the bound peroxidase was detected by adding a freshly prepared solution of 0.05% (wt/vol) 4-chloro-1-naphthol (Bio-Rad Laboratories, Chemical Div.) in methanol and 0.015% H₂O₂ in Tris buffer (20 mmol/L, pH 7.2). The membrane was then washed with distilled water and air dried. Alternatively, the human sera-treated membrane was incubated with iodine-125-labeled anti-human IgE MoAbs, which were prepared according to the method of Chang et al.¹⁵ After incubation at room temperature for 1 hour, the membrane was washed with Tris buffer (0.1 mol/L, pH 7.2) containing 0.5% bovine serum albumin and air-dried; autoradiography was then performed with Kodak XAR-5 films (Eastman Kodak Co., Rochester, N.Y.).⁹

In another experiment proteins were separated by SDS-PAGE, transferred to PVDF membrane, and incubated with [¹²⁵I]-labeled MoAb 4-37 (1 × 10⁶ cpm/strip) at room temperature for 1 hour. Autoradiography was performed as described above.

Preparation of MoAb affinity column

MoAb 4-37 was purified from ascites with a protein A column¹⁶ and coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at a concentration of 4 mg/ml. The gel matrix was then reacted with ethanolamine and washed with sodium acetate and sodium bicarbonate buffers before it was packed into a glass column as suggested by the manufacturer.

Affinity chromatography

The immunosorbent column¹⁷ (1.5 cm × 15 cm) was preequilibrated with PBS, and 40 mg of BGP extract was loaded onto the column at a flow rate of 5 ml/hr with a peristaltic pump. Unabsorbed proteins were removed by washing with PBS, and the column was developed with two bed volumes of elution buffer (0.1 mol/L citric acid, pH 3.0) at a flow rate of 2 ml/min. The pH of the protein eluted from the column was immediately adjusted to 7.0 with 1 mol/L Tris buffer (pH 10) and then dialyzed against distilled water and lyophilized.

High-performance liquid chromatography analysis

High-performance liquid chromatography (HPLC) analysis was performed on an ABI model 140B solvent delivery system (Applied Biosystems Inc., Foster City, Calif.) equipped with a model 1000S diode array detector. Protein separation was carried out on a 5 μmol/L Vydac C₁₈ reverse-phase column (0.21 cm i.d. × 25.0 cm) (HP Analytical, Hefparia, Calif.). Solvent A was 0.08% (vol/vol) trifluoroacetic acid in water and solvent B was 80% (vol/vol) acetonitrile in water containing 0.05% (vol/vol) trifluoroacetic acid. The column was equilibrated with 20% buffer B and eluted at a rate of 0.3 ml/min. A linear gradient of 1% per minute of buffer B was started 10 minutes after sample injection.

Protein sequencing and amino acid analysis

Automated cycles of Edman degradation were performed on an ABI gas/liquid-phase model 470A/900A sequencer (Applied Biosystems, Inc.) equipped with an on-line model 120A amino acid phenylthiohydantoin analyzer according to the procedure of Hewick et al.¹⁸ The sequencing of proteins from PVDF membrane was performed as described by Chang et al.¹⁹

Proteins were hydrolyzed in the gas phase (24 hours at 110° C) with 6 mol/L HCl containing 1% phenol. After hydrolysis, samples were analyzed in a Waters PicoTag amino acid analysis system (Waters Chromatography Div., Millipore, Milford, Mass.) according to the manufacturer's instructions.

Radioimmunoassay

Pollen proteins were coated onto wells of 96-well polyvinyl plates (Costar, Cambridge, Mass.) (20 μg/ml, 50 μl/well). The cross-reaction between four anti-Cyn d I MoAbs and 10 grass pollens was tested by radioimmunoassay.⁹ BGP and ragweed pollen were used as positive and negative controls. After coating, the wells were saturated with 5% bovine serum albumin, and then 50 μl of MoAb supernatants was added. Anti-BGP MoAb 9-13 was used as the antibody control. After incubation at 37° C for 1 hour and four washings with PBS (1 mol/L) containing 0.5% bovine serum albumin, the bound antibody was quantitated by incubation with

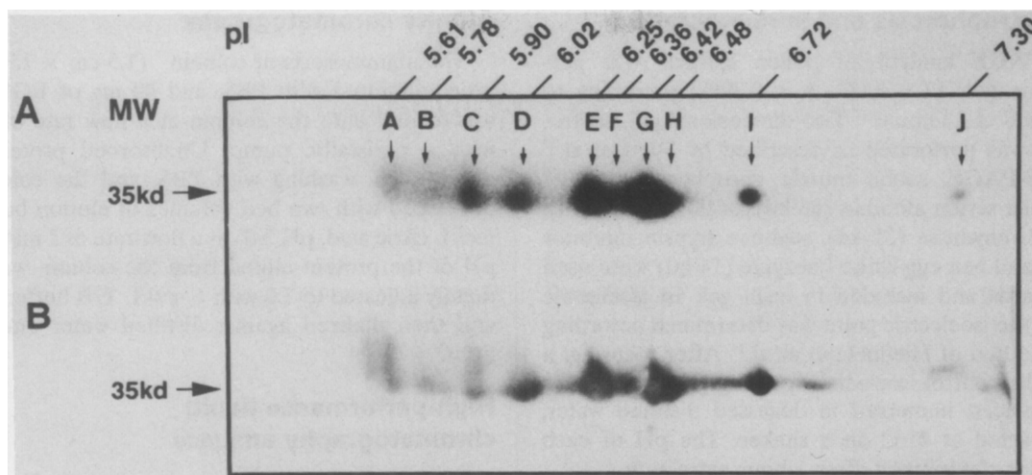


FIG. 1. The two-dimensional gel immunoblotting profiles of the 35 kd major allergen of BGP and *Cyn d* Bd35K. BGP extracts were separated by isoelectric focusing and SDS-PAGE and transferred onto PVDF membranes to react with (A) IgEs in pooled sera from BGP-allergic patients and (B) MoAb 4-37.

[^{125} I] rabbit-anti-mouse Ig(G + M) (5×10^4 cpm/well) (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). Radioactivity was measured by a gamma counter and expressed as counts per minute. Data are the average of triplicates.

RESULTS

Two-dimensional polyacrylamide gel electrophoresis and immunoblotting

Whether isomeric subunits of *Cyn d* Bd35K are all allergenic was evaluated and is illustrated in Fig. 1. After two-dimensional gel electrophoresis, gel sections containing 30 to 36 kd components were cut and transferred onto PVDF membranes to react with IgEs in BGP-allergic sera (Fig. 1, A) or MoAb 4-37 (Fig. 1, B). Normal sera and nonspecific MoAbs were used as controls, and the results were all negative (data not shown). As shown in Fig. 1, A, there were 10 isoallergens in *Cyn d* Bd35K, and they are designated as *Cyn d* I-A to *Cyn d* I-J. Among these isoallergens, seven of them (C, D, E, F, G, H, I) showed strong IgE-binding capacity. The immunoblotting profiles in Fig. 1, A and B are similar, which suggests that all of the isoallergens recognized by human IgEs are reactive to MoAb 4-37.

Purification of *Cyn d* I by affinity chromatography

MoAb 4-37 was used as immunosorbent for *Cyn d* Bd35K purification. Elution buffers such as citric acid (0.1 mol/L, pH 3.0), glycine-HCl (1 mol/L, pH 2.8), and sodium thiocyanate (3 mol/L, pH 7.0) had been tested in preliminary studies.

Citric acid (0.1 mol/L) was chosen because it is relatively mild and because the affinity column can be reused two to three times.

We routinely recovered approximately 2.5 mg of purified protein from 40 mg of crude BGP extract, which represents a 6.25% yield. *Cyn d* I isolated with the MoAb affinity column was analyzed on an SDS-polyacrylamide gel, and the crude extract of BGP was included for comparison (Fig. 2, A). The proteins were visualized by silver staining, and the purified *Cyn d* I appeared as a single polypeptide of 34 kd (lane 2) or as a mixture of 34 and 29 kd proteins of different proportions, depending on the preparation (lanes 3 and 4). A single 34 kd component could be observed only when elution was completed within 5 minutes. A mixture of 34 kd and 29 kd components would be obtained with an increase in the elution time. These two components were also observed in the eluent when other *Cyn d* I MoAbs, instead of MoAb 4-37, were used as the immunosorbent.

To study the nature of the 29 kd component, protein fractions from the MoAb 4-37 affinity column were subjected to SDS-PAGE separation and then transferred onto a PVDF membrane before reacting with either [^{125} I]-labeled goat-anti-mouse Ig or [^{125} I]-labeled anti-*Cyn d* I MoAb 1-61. The goat-anti-mouse Ig did not react with the protein fractions eluted from the column, indicating that these fractions are not derived from mouse immunoglobulins (data not shown). The [^{125} I]-labeled anti-*Cyn d* I MoAb 1-61, which only recognizes the 34 to 35 kd (*Cyn d* Bd35K)

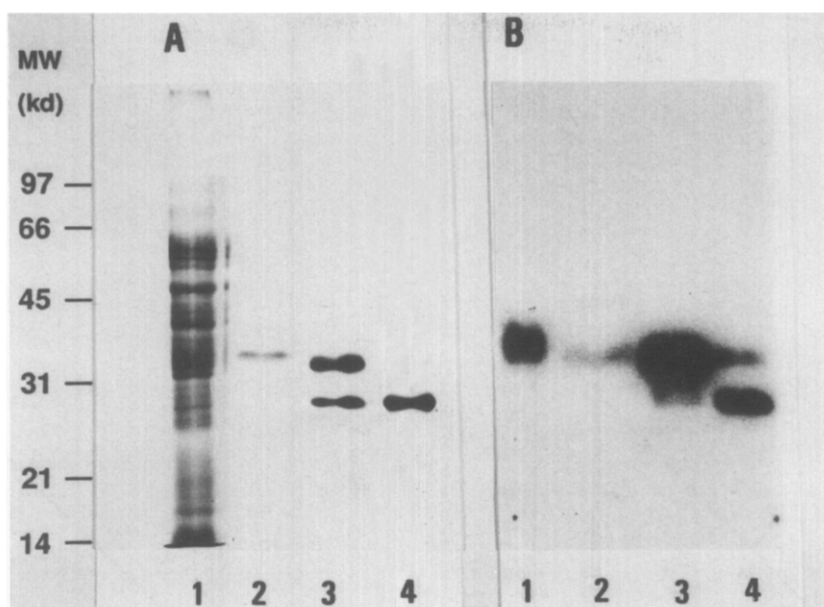


FIG. 2. SDS-PAGE patterns and immunoblotting profiles of *Cyn d* I. Crude extracts of BGP (lane 1) and different preparations of *Cyn d* I purified with an MoAb affinity column (lanes 2-4) were separated on an 11% gel. Proteins were visualized by silver staining (panel A) or transferred onto PVDF membrane and reacted with [^{125} I]-labeled MoAb 1-61 and visualized by radiography (panel B).

polypeptide in the crude BGP extract (Fig. 2, B, lane 1), reacted with all of the protein fractions (the 34 kD and the 29 kD proteins) eluted from the affinity column (Fig. 2, B, lanes 2-4). In addition, a concoction of BGP crude extract and affinity-column-purified *Cyn d* I (34 + 29 kD mixture) produced only two bands on an immunoblot (data not shown). This suggests that *Cyn d* Bd35K and the 34 kD and 29 kD proteins are sharing the same epitope.

HPLC analysis of *Cyn d* I

Different preparations of *Cyn d* I were analyzed on a C_{18} reverse-phase column. The HPLC profiles of the 34 kD *Cyn d* I protein (Fig. 2, lane 2) and a *Cyn d* I preparation that contains both 34 kD and 29 kD peptides (Fig. 2, lane 3) are presented in Fig. 3, A and B, respectively. The 34 kD *Cyn d* I protein was eluted from the column with 53% buffer B. The 34 and 29 kD peptide mixture was also eluted from the column in a single peak with 53% buffer B. This suggests that the hydrophobicity of these proteins is similar. In addition, a small peptide (peptide N), which comigrated with the dye front during SDS-PAGE analysis, was eluted from the column with 34% buffer B (Fig. 3, B). The MW of the proteins eluted from the column was confirmed by SDS-PAGE analysis (data not shown).

N-terminal sequence analysis

A *Cyn d* I sample that contains mainly the 34 kD peptide was further purified on a reverse-phase column (Fig. 3, A), and the major peak (peak I) was subjected to protein sequence analysis. The sequence of the first 25 residues from the N-terminus is summarized in Table I. We detected two signals for residues 2, 5, 8, 13, and 16. Sequence of these peptides was found to have a 60% (15 of 25 residues) homology to that of *Lol p* I.

The sequence of the N-terminal 30 amino acids from peptide N in Fig. 3, B was ascertained and is listed in Table I. The sequencer did not yield any signal for the ninth residue, which may be a cysteine residue or a glycosylation site. The result indicates that peptide N has a sequence nearly identical to that of the 34 kD *Cyn d* I (24 of 25 residues). Therefore this peptide is probably the N-terminal fragment of one of the *Cyn d* I isomers.

The *Cyn d* I 29 kD peptide, either isolated on reverse-phase column (peak I, Fig. 3, B) or separated on SDS-polyacrylamide gel and then transferred onto PVDF membrane, was also subjected to sequence analysis (Table I). The first 38 residues of the 29 kD peptide of *Cyn d* I obtained by the formal method were found to have a 68% homology with the thirty-first to sixty-eighth resi-

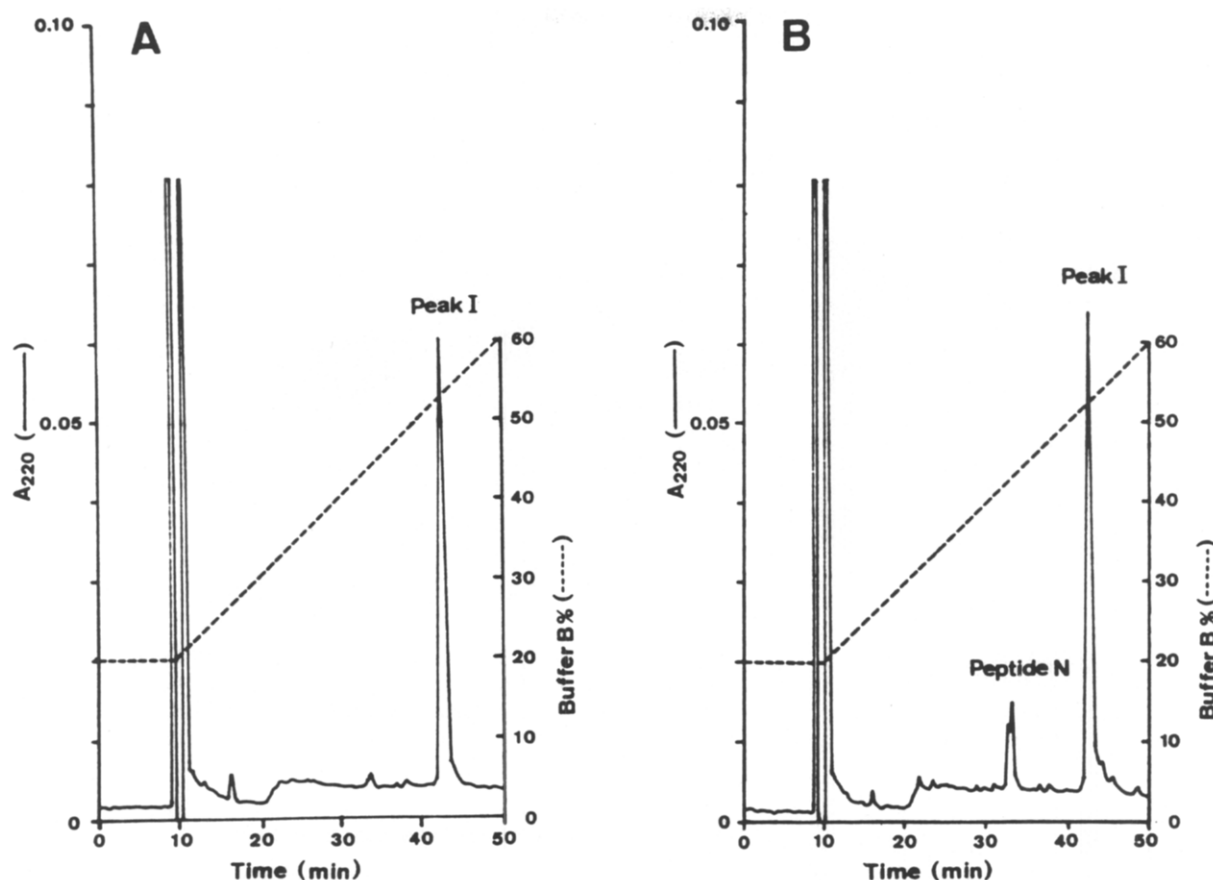


FIG. 3. HPLC profile of the 34 kD *Cyn d I* protein (**panel A**) and a *Cyn d I* preparation that contains both 34 kD and 29 kD peptides (**panel B**).

TABLE I. Amino acid sequence of *Cyn d I*

<i>Cyn d I</i> *	A	M	G	D	D	P	G	P	K	I	T	A	T	Y	G	S	K	W	L	D	A	K	A	T	F													
		I						K						K			K																					
<i>Cyn d I</i>	A	I	G	D	K	P	G	P	/	I	T	A	T	Y	G	S	K	W	L	E	A	K	A	T	F	Y	G	/	N	P								
Peptide N																																						
<i>Lol p I</i> †	I	A	K	V	P	P	G	P	N	I	T	A	E	Y	G	D	K	W	L	D	A	K	S	T	Y	Y	G	K	P	T								
<i>Poa p I</i> ‡	I	A	K	V	/	P	G	/	/	I	T	A	E	Y	G	D	K	W	L	D	A	K	S	T	W	Y	G											
<i>Cyn d I</i> *	G	A	A	P	D	D	H	G	G	A	/	G	Y	K	D	V	D	K	P	P	F	D	G	M	T	A	/	G	N	E	P	I	F	K	D	D	L	S
29 kd																																						
<i>Cyn d I</i> §	G	A	A	P	D	D	H	G	G	A	/	G	Y	K	D	V	D	K	P	P	F	D	G	M	T	A	/	G	N	E								
29 kd																																						
<i>Lol p I</i>	G	A	G	P	K	D	N	G	G	A	C	G	Y	K	D	V	D	K	A	P	F	N	G	M	T	G	C	G	N	T	P	I	F	K	D	G	R	G

Virgules (/) represent amino acids that were not detected.

*Sample after affinity and reverse-phase chromatography.

†Data from Perez et al.²⁰

‡Data from Cottam et al.²²

§Sample isolated on SDS-polyacrylamide gel and electroblotted onto PVDF membrane.

||Data from Perez et al.,²⁰ residues 31-68.

dues of *Lol p I*, and the first 21 residues of the 29 kD peptide of *Cyn d I* obtained by the latter method were found to have a 57% homology with the thirty-first to fifty-second residues of *Lol p I*.²⁰

Amino acid analysis

Cyn d I sample from the MoAb affinity column was further purified with a Superose 12 gel filtration column (Pharmacia) and eluted with 10

TABLE II. Amino acid composition (residues per molecule) of *Cyn d* I

Amino acid	No. of amino acids				
	<i>Cyn d</i> I*	<i>Cyn d</i> I†	<i>Lol p</i> I‡	<i>Poa p</i> IC§	<i>Poa p</i> IB§
Asp/Asn	28	27	28	37	37
Glu/Gln	13	20	20	34	28
Thr	12	11	18	18	18
Ser	14	15	12	23	18
Gly	32	26	28	34	28
Ala	27	19	16	30	30
Ile	17	11	11	14	13
Leu	17	14	8	20	16
Met	3	5	2	0	8
Val	12	10	16	22	22
Pro	19	16	16	17	20
Phe	12	9	8	12	12
Tyr	9	9	9	13	13
His	7	5	3	0	4
Arg	6	6	6	13	11
Lys	31	24	26	30	36
Cys	ND	7	7	0	0
Trp	ND	ND	6	ND	ND

ND, Not determined.

*Data from this study. The number of amino acid residues was calculated by using 34 kd as the maximum MW of *Cyn d* I.

†Data from Matthiesen et al.⁸

‡Amino acid composition deduced from *Lol p* I clone 1A.²⁰

§Data from Ekramoddoullah.²¹

TABLE III. Cross-reaction between anti-BGP MoAbs and ten other grass pollens

Pollen extracts	MoAb				
	1-61	10-7	4-37	11-7	9-13
<i>Ant o</i>	51	99	73	55	2950
<i>Cyn d</i>	2854	2990	4291	1480	850
<i>Dac g</i>	82	114	91	101	4591
<i>Fes e</i>	67	124	73	137	4772
<i>Hol l</i>	90	119	203	133	4678
<i>Lol p</i>	31	91	50	131	4814
<i>Pas n</i>	94	185	204	61	3315
<i>Pha c</i>	86	141	80	32	3154
<i>Phl p</i>	77	99	72	105	4499
<i>Poa p</i>	72	99	91	64	4183
<i>Sor h</i>	38	103	56	71	3786
<i>Amb e</i>	98	115	53	38	133

mmol/L potassium phosphate, pH 7.0, and 100 mmol/L KCl. The resulting protein fraction, which consisted of 34 kd and 29 kd peptides in a ratio of approximately 9:1, was subjected to amino acid composition analysis. The results are summarized in Table II together with data from Matthiesen et al.⁸ on *Cyn d* I, Perez et al.²⁰ on *Lol p* 1, and Ekramoddoullah²¹ on *Poa p* I for comparison.

Cross-reactivity

The epitope specificity of four anti-*Cyn d* I MoAbs was examined against 10 grass pollen extracts. MoAb 9-13, which cross-reacts with canary grass, corn grass, cane and annual bluegrass, as previously reported,⁹ was used as the positive control. As shown in Table III, all four anti-*Cyn d* I MoAbs reacted strongly with BGP but not with 10 other grass pollens. On the other hand, the

control MoAb 9-13 cross-reacted with all grass pollens tested but not with ragweed pollen.

DISCUSSION

Previously, we had demonstrated the distribution of different protein components of BGP on two-dimensional gels.⁹ *Cyn d* Bd35K, the major allergenic component of BGP,⁶ which is recognized by MoAbs 4-37, 11-7, 10-7, and 1-61, had also been examined with a radioimmunoprecipitation test; it was found to consist of at least four isomeric proteins.⁹ Whether all or part of these isomeric components can be recognized by human IgE antibodies is not clear. By means of immunoblotting, the present study further demonstrated that there are 10 isomeric components and that they all possess epitopes recognized by human IgEs (Fig 1, A) and MoAb 4-37 (Fig 1, B).

Cyn d I, purified with the MoAb affinity column, appeared as a single protein of 34 kd or as a mixture of 34 and 29 kd polypeptides on SDS-PAGE analysis. The small difference in MW between the purified *Cyn d* I and *Cyn d* Bd35K reported previously is probably due to the concentration of polyacrylamide used in electrophoresis. Both the 34 kd and the 29 kd components could be recognized by IgEs in pooled sera of BGP-allergic patients and could inhibit IgE binding as revealed by a BGP RAST (data not shown). These findings are similar to those reported by Mathiesen et al.⁸

In most preparations, the affinity-column-purified *Cyn d* I appeared as a mixture of 34 and 29 kd polypeptides (Fig. 2, lanes 3 and 4). The finding that anti-*Cyn d* I MoAb 4-37 (Fig. 1) or 1-61 (Fig. 2, B, lane 1) reacted only with a 34 to 35 kd component in BGP extracts suggests that *Cyn d* Bd35K is the sole constituent in BGP that can be recognized by these two anti-*Cyn d* I MoAbs. On purification with an MoAb 4-37 affinity column, an additional 29 kd peptide was generated. That the 29 kd peptide could also be recognized by anti-*Cyn d* I MoAb 1-61 (Fig. 2, B, lanes 3 and 4) suggests that it has the antigenic determinants recognized by MoAbs 4-37 and 1-61. Furthermore, this peptide did not appear if the column elution process was completed within 5 minutes. We tentatively conclude that the 29 kd peptide is a degraded product of *Cyn d* Bd35K.

The N-terminal amino acid sequence of *Cyn d* I was determined, and the result is shown in Table I. The 34 kd peptide, purified by antibody-affinity and reverse-phase chromatography, yielded two phenylthiohydantoin amino acids for residues 2, 5,

8, 13, and 16. Peptide N, a small peptide isolated on reverse-phase column (peptide N, Fig. 3, B) was found to have an amino acid sequence identical to that of the 34 kd preparation except that Glu was substituted for Asp for the twentieth residue. The sequence for residues 2, 5, 8, 13, and 16 of peptide N was identical to either one of the two phenylthiohydantoin amino acids found in the 34 kd peptide. These protein sequencing data suggest that *Cyn d* I may consist of peptides having similar but not identical amino acid sequences instead of peptides of identical amino acid sequences with different degrees in glycosylation or amidation as suggested by Cottam et al.²² Studies are now in progress to determine the N-terminal sequence of individual isomeric subunits of *Cyn d* I.

Matthiesen et al.⁸ purified *Cyn d* I by a combination of concanavalin A Sepharose and ion-exchange chromatography. The purified sample was also a mixture of 32 and 29 kd components, which is in close agreement with our observation. With the exception of residue 16, the N-terminal amino acid sequence of our *Cyn d* I 34 kd protein was identical to that of the 32 kd component reported by Matthiesen et al. This indicates that the *Cyn d* I purified by us with an affinity column is the same as that purified by Matthiesen et al. by biochemical methods. The dissimilarity in MW between the two samples may be attributed to the difference in acrylamide concentration used in the gel system. Nonetheless, we cannot exclude the possibility that these proteins contain different types or degrees of posttranslational modification or C-terminal processing.

Ekramoddoullah²¹ has recently reported that *Poa p* I, the group I allergen of Kentucky bluegrass, is composed of four isomers (with MWs from 33 to 35.8 kd, isoelectric points from 6.4 to 9.1). *Poa p* I B, *Poa p* I C, and *Lol p* I¹⁹ have almost identical N-terminal sequences (Table I), whereas the first 25 amino acids of *Cyn d* I show more than 50% sequence similarity with these proteins.

The amino acid compositions of these proteins (*Cyn d* I, *Poa p* I B, *Poa p* I C, and *Lol p* I) are listed in Table II for comparison. The data for *Lol p* I (deduced from DNA sequence) and *Cyn d* I are similar, whereas a larger deviation is observed when the compositions of *Poa p* I (samples on PVDF membrane) and *Cyn d* I are compared. It has been demonstrated that amino acid analysis at moderate to high sensitivities lacks precision and consistency. Few laboratories

can routinely provide compositions that are at least 90% correct.²³ In addition, analysis of samples on PVDF membrane has been known to be problematic, with only 83% accuracy.²⁴ Therefore the deviations observed among *Cyn d* I, *Poa p* I B, and *Poa p* I C can be ascribed to either technical difficulties or differences in composition.

The assumption that the 29 kd component is a degraded product of *Cyn d* Bd35K is further supported by the results from our biochemical analysis (Fig. 3 and Table I). As shown, the hydrophobicity of these two proteins is similar, because they are inseparable on a C₁₈ reverse-phase column (Fig. 3, A and B). In addition, the sequence of the 29 kd protein has a 68% homology with the thirty-first to sixty-eighth residues of *Lol p* I. We speculate that *Cyn d* I (34 kd protein), which is composed of most acidic proteins⁹ with an above average number of proline residues (7.3%), is unstable under acidic conditions²⁵ and yields a 39 kd degradation product during the purification process.

To simulate the condition that leads to the degradation of *Cyn d* Bd35K, several methods were used to extract *Cyn d* Bd35K from GBP. Although vigorous extraction did not produce the 29 kd component, an additional 20 kd band was obtained (data not shown). Generation of the 20 kd band could be avoided by adding phenylmethylsulfonyl fluoride, a protease inhibitor, to the extraction buffer. Our finding that *Cyn d* I can be easily degraded is in close agreement with that of Orren and Dowdle,⁴ who reported that BGP-1 may degrade into BGP-2.

Assuming that the 29 kd peptide is a degraded product of the 34 kd protein, the amino acid sequence determined in this report (Table I) represents approximately one fifth of the complete sequence of *Cyn d* I and obviously has a high degree of homology to that of *Lol p* I. However, because most of the B-cell epitopes are conformational rather than sequential,²⁶ this biochemical similarity does not mean that there should be antigenic cross-reactivity among *Cyn d* I, *Poa p* I, and *Lol p* I.

In addition to the biochemical characterization, immunologic methods were also used to analyze *Cyn d* I. The MoAb reacts with a single epitope; consequently, it is a better tool for studying the cross-reaction of antigenic components. In this study the cross-reactivity of *Cyn d* I with other grass pollens was evaluated by anti-*Cyn d* I MoAbs 4-37, 10-7, 11-7, and 1-61 (Table III).

These anti-*Cyn d* I MoAbs have recently been identified as reacting with three distinct allergenic epitopes on *Cyn d* I.²⁷ The finding that none of these anti-*Cyn d* I MoAbs cross-reacts with extracts from 10 different grass pollens, including *Poa p* and *Lol p* (Table III), suggests that the epitopes recognized by these MoAbs are not present in these pollens.

Matthiesen et al.⁸ raised rabbit anti-sera against *Cyn d* I, which only weakly precipitated allergens from other grass species in an immunoelectrophoresis assay. Consequently, they suggested that *Cyn d* I has some unique immunochemical properties. They also generated four anti-*Cyn d* I MoAbs, which, however, cross-reacted with pollen components from other grass species in different patterns.⁸ Whether their MoAbs are active against allergenic epitopes remains unknown.

In contrast, MoAbs 4-37 and 1-61 recognize major allergenic epitopes.²⁷ In a RAST inhibition assay, these two MoAbs inhibited more than 30% of the IgE binding of sera from patients allergic to *Cyn d* I. The absence of these allergenic determinants in other grass pollens correlates with the findings of Schmachter et al.,²⁸ who demonstrated that patients sensitized to BGP do not possess IgEs that significantly cross-react with other grass pollens.

The possibility that there are common epitopes shared by *Cyn d* I and other group I allergens still exists. The difference between our anti-*Cyn d* I MoAbs and those generated by Matthiesen et al.⁸ remains unclear. One of the possible factors is that our hybridomas had been chosen with a radioimmunoprecipitation assay before being kept for further growth.⁹ This screening system may favor MoAbs against dominant epitopes. Most of all, it guarantees that our MoAbs are directed against antigenic determinants on *Cyn d* I but not against the possible contaminating materials.

In the present study we purified *Cyn d* I with a MoAb affinity column and characterized its biochemical and immunologic properties. The purified allergen should be an ideal material for basic study and clinical use. In addition, we demonstrated that in spite of the high sequence homology between *Cyn d* I and *Lol p* I, the allergenic B-cell epitopes identified by our anti-*Cyn d* I MoAbs were not present in 10 other grass pollens. This provides clinical allergists with a reasonable explanation as to why BGP has been considered to have allergenicity different from that of other grass pollens.²⁹⁻³¹

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