

Characterization of the isoforms of the group I allergen of *Cynodon dactylon*

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Background: The group I allergen of *Cynodon dactylon*, Cyn d I, was found to consist of four to 10 isoforms.

Methods: We studied the isoforms with the use of two-dimensional gel electrophoresis. The antigenic difference of the isoforms was evaluated by radioimmunoprecipitation with monoclonal antibodies (MAbs). The acidic isoforms and the basic and neutral isoforms were further isolated by MAb-affinity chromatography for RAST and competitive RAST. In addition, the N-terminal sequence was evaluated by microsequencing.

Results: A total of 11 isoforms were found in Cyn d I in extracts prepared from different sources of Bermuda grass pollen (BGP). They were either acidic (Cyn d I-A, I-B, I-C, I-D, I-E, I-F, I-G, I-H, and I-I), neutral (Cyn d I-X), or basic (Cyn d I-J). Cyn d I-G, with an isoelectric point of approximately 6.4, was constantly present in all the pollen preparations, whereas the content of the basic Cyn d I-J varied from less than 5% to greater than 20%. The molecular weight of the basic and neutral isoforms were slightly lower than those of the acidic isoforms. All isoforms shared a common antigenic determinant(s) recognizable by MAb 4-37, and the basic and neutral isoforms possessed a unique antigenic determinant(s) recognizable by MAb I-61. RAST showed that both the acidic Cyn d I and the basic and neutral Cyn d I were recognized by human IgE in the pooled sera of persons allergic to BGP. Competitive RAST showed a high crossreactivity between the acidic and the basic and neutral isoforms. A 95% sequence identity also existed between the N-terminal 20 amino acid residues of basic Cyn d I-J and the dominant acidic isoform Cyn d I-G.

Conclusions: The present study disclosed that basic Cyn d I-J is an important allergen and that the content of this isoform varies in different lots of BGP. (*J ALLERGY CLIN IMMUNOL* 1995;95:1206-14.)

Key words: *Cynodon dactylon*, grass pollen, group I allergen, isoform

Bermuda grass (*Cynodon dactylon*) pollen (BGP) is an important worldwide aeroallergen.¹⁻³ Its major allergenic component, Cyn d I, is a 32 to 34 kd protein that gives a 76% to 100% positive reaction rate with IgE in sera from patients with BGP allergy.⁴⁻⁸ Cyn d I has been purified either by a combina-

tion of concanavalin A-Sepharose and ion exchange chromatography or monoclonal antibody (MAb)-affinity chromatography.^{9,10} The 34 kd Cyn d I is easily degraded, mostly into a 29 kd polypeptide.¹⁰

The biochemical property of Cyn d I has been compared with that of Lol p I, the group I allergen of *Lolium perenne*.^{9,10} In addition to the similarity of amino acid composition, the N-terminal amino acid sequence of Cyn d I has a 60% homology with residues 1 to 25 of Lol p I, and that of the 29 kd degraded Cyn d I has a 68% homology with residues 31 to 68 of Lol p I. However, the immunologic property of Cyn d I seems to be different from that of Lol p I. The B-cell epitopes identified by our anti-Cyn d I MAbs are all absent in *L. perenne*.¹¹ The antigenicity and allergenicity of BGP was considered to be different from that of the other grass pollens.^{7,12}

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Abbreviations used

2D:	Two-dimensional
BGP:	Bermuda grass pollen
BSA:	Bovine serum albumin
cpm:	Counts per minute
<i>Cyn d</i> I:	Group I allergen of <i>Cynodon dactylon</i>
IEF:	Isoelectric focusing
<i>Lol p</i> I:	Group I allergen of <i>Lolium perenne</i>
MAB:	Monoclonal antibody
MW:	Molecular weight
PAGE:	polyacrylamide gel electrophoresis
PBS:	Phosphate-buffered saline solution
pI:	Isoelectric point
PVDF:	Polyvinylidene difluoride
RIP:	radioimmunoprecipitation
TRIS:	Tris(hydroxymethyl)aminomethane

We reported previously that *Cyn d* I consists of four to 10 isoforms with an isoelectric point (pI) ranging approximately from 5.6 to 7.3.^{8,10} They are designated as *Cyn d* I-A to *Cyn d* I-J according to their appearance (from acidic to basic) on isoelectric focusing (IEF) gel. All these isoforms are considered allergens because they react with human IgE from sera of patients with BGP allergy.

In the present study the isoforms of *Cyn d* I were further analyzed with two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE). More than 10 extracts from different lots of BGP were examined. The basic and neutral isoforms were found to have a relatively lower molecular weight (MW) than that of the acidic isoforms. According to the relative content of the basic *Cyn d* I-J, the *Cyn d* I from different batch of BGP extracts can be categorized into three patterns. With a radioimmunoprecipitation (RIP) assay it was found that all isoforms of *Cyn d* I share a common antigenic determinant and that the basic and neutral *Cyn d* I possess a unique epitope. The acidic and the basic and neutral isoforms were isolated by MAb-affinity chromatography, and their allergenic properties were studied by a competitive RAST. Finally, whether *Cyn d* I-J should be grouped into another (group V) allergen was evaluated by N-terminal amino acid sequencing.

METHODS

Pollen extracts

Defatted pollens were purchased from Allergon AB (Carthage, Mich.) and International Biologicals Inc. (Piedmont, Okla.). Each sample was extracted with gentle shaking at 4°C for 18 hours in 0.15 mol/L phosphate-buffered saline solution (PBS) (pH 8.0) at a

ratio of 1:10 (grams per milliliter) as previously described.⁸ After dialysis with distilled water at 4°C overnight, the protein content of the extract was determined by the dye-binding method of Bradford¹³ with bovine serum albumin (BSA) as a standard. The extract was then lyophilized and stored at -20°C.

Anti-*Cyn d* I MAbs

MAbs 4-37 and 1-61, which are specific for *Cyn d* I, were generated by fusion of spleen cells from BGP-immunized BALB/c mice and NS-1 myeloma cells as described by Chang et al.⁸

Allergic sera

Sera were collected at the Allergy Clinic, Veterans General Hospital, Taiwan, Republic of China, from patients with positive intradermal skin test reactions to BGP extracts (supplied by Taiwan Allergy Center, 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 according to reactivity in a RAST⁸ and pooled for subsequent experiments.

2D-PAGE and immunoblotting

2D-PAGE was performed according to the method of Shen et al.¹⁴ with minor modifications. Briefly, BGP was dissolved in the IEF sample buffer, which contained 9 mol/L urea, 4% (vol/vol) Nonidet P-40 nonionic detergent, 2% (vol/vol) ampholine (pH 3 to 10), and 2% (vol/vol) 2-mercaptoethanol. Separation was carried out on a 3% (wt/vol) polyacrylamide gel containing 0.18% (wt/vol) *N,N'*-methylenebisacrylamide, 8 mol/L urea, 1.5% (vol/vol) ampholyte (pH 5 to 8), 0.5% (vol/vol) ampholyte (pH 8 to 10.5), and 1.7% (vol/vol) of Nonidet P-40 detergent. The IEF gels were cast in glass tubes and preliminarily electrophoresed for 200 volt hours before sample loading. BGP extract (1 mg per 20 µl) was loaded onto each tube and focused for 14,000 volt hours. At the end of the focusing step, the gels were extruded from the glass tubes and equilibrated in a tris(hydroxymethyl)aminomethane (TRIS) buffer (125 mmol/L, pH 6.8) containing 10% (wt/vol) glycerol, 4% (wt/vol) sodium dodecyl sulfate, and 10 mmol/L dithiothreitol for 1 hour at room temperature. The pH gradient of each IEF gel was determined according to the method of Hjelmeland et al.¹⁵ After focusing, the IEF gel was cut into 0.5 cm pieces and immersed in degassed double-distilled water on a shaker. The pH of each extract was determined after an hour's extraction. 2D electrophoresis was carried out on 11% SDS-PAGE with low-MW standards (Bio-Rad, Richmond, Calif.) as markers.

Proteins were visualized with silver stain¹⁴ or were electrophoretically transferred. For immunoblotting the proteins were blotted onto a nitrocellulose membrane (0.45 µm; BA 85; Schleicher & Schüll, Dassel, Germany) in transfer buffer (25 mmol/L TRIS, 192 mmol/L glycine, and 20% [vol/vol] methanol, pH 8.3) as previously

described.⁶ For N-terminal sequence analysis the proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Mass.) with a semidry blotting unit.¹⁶ Blotting was performed at a constant current of 5 mA/cm² for 30 minutes with 10 mmol/L CAPS (pH 11.0) as blotting buffer. Protein spots on the membrane were visualized by staining with 0.1% (wt/vol) Coomassie Blue dye (Serva, Heidelberg, Germany) in 50% methanol for 1 minute and destained in the same solution without dye.

IgE-binding capacity

After electroblotting, the membrane was blocked by treatment with 3% (wt/vol) gelatin as previously described.⁶ The membrane was then incubated with the pooled patient sera (1:10 dilution) at 4° C overnight and washed with TRIS buffer (1 mol/L, pH 7.2) containing 0.5% (vol/vol) Tween-20. Iodine 125-labeled anti-human IgE MAb¹⁷ was added and incubated with the membrane at room temperature for 1 hour. After washing with TRIS buffer (0.1 mol/L, pH 7.2) containing 0.5% BSA and air drying, we performed autoradiography with Kodak XAR-5 films (Kodak, Rochester, N.Y.) and analyzed with a phosphorimager (model 425 E 120; Molecular Dynamics, Sunnyvale, Calif.) according to the manufacturer's instructions.

Radioimmunoprecipitation

RIP was performed as previously described.⁸ Briefly, BGP was iodinated by the chloramine T method.¹⁸ The labeling efficiency was about 10% and 20%. All isoforms of *Cyn d* I were labeled. Ascites (10 µl) containing anti-*Cyn d* I MAb 4-37 or 1-61 was added to 100 µl of Sepharose 4B beads precoupled with rabbit anti-mouse IgG and IgM antibodies. After the antibody-bead complex was shaken at room temperature for 45 minutes, it was then washed three times with PBS containing 0.5% (vol/vol) Nonidet P-40 detergent. ¹²⁵I-labeled BGP (5 × 10⁷ counts/min [cpm]) was added to the bead pellet. Incubation was carried out at room temperature for 45 minutes with shaking. Reaction was terminated by washing the bound immune complex eight times with PBS containing 0.5% (vol/vol) Nonidet P-40 detergent. After the final wash the beads were extracted with IEF sample buffer.

Isolation of acidic isoforms, and basic and neutral isoforms, of *Cyn d* I

The basic and neutral *Cyn d* I was isolated with an affinity column made of MAb 1-61, and the acidic isoform of *Cyn d* I was isolated first with an affinity column made of MAb 4-37 and followed by absorption with an immunosorbent made of MAb 1-61. The affinity chromatography was performed as previously described.¹⁰ Briefly, MAbs 4-37 and 1-61 were purified from ascites with a protein A column and coupled to cyanogen bromide-activated Sepharose 4B at a concentration of 4 mg/ml matrix, as suggested by the manufac-

turer (Pharmacia, Uppsala, Sweden). For affinity chromatography the immunosorbent column (1.5 × 15 cm) was preequilibrated with PBS and 40 mg of BGP extracts 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 off the column was immediately adjusted to 7.0 with 1 mol/L TRIS buffer (pH 10), then dialyzed against distilled water and lyophilized. For absorption the material eluted from the immunosorbent 4-37 was further incubated with the immunosorbent of MAb 1-61 at 4° C overnight and then dialyzed against distilled water. The protein content of the material obtained was determined. The product was then lyophilized and ready for studies.

RAST

For analysis of the IgE-binding capacity of the acidic and of the basic and neutral *Cyn d* I, the isolated material was coated onto wells of the polyvinyl plate at a concentration of 100 ng/well. The unsaturated sites were blocked with PBS and BSA. The pooled sera from persons with BGP allergy (1:3 dilution) and those from the normal persons were added to the wells (50 µl/well) and incubated at 4° C overnight. The plates were washed, and iodinated anti-human IgE MAb¹⁷ was added to the treated wells at a concentration of 5 × 10⁴ cpm/well. After thorough washing with PBS-BSA, the radioactivity in each well was determined by a gamma counter (LKB, 1271 RIAGamma, Turku, Finland).

Competitive RAST

To analyze the crossreactivity of the acidic isoforms and the basic and neutral isoforms, a competitive RAST was performed as described by Ayuso et al.,¹⁹ with some modifications. The pooled sera (1:3 dilution) from persons with BGP allergy was mixed (1:1, vol/vol) either with different concentrations (0.01 to 100 µg/ml) of the competitor (the acidic isoforms or the basic or neutral isoforms) or of control (PBS). The mixtures were incubated at 4° C for 2 hours, and aliquots of 50 µl were added to the isoform-coated radioimmunoassay plates and incubated at 4° C overnight. After being washed with PBS-BSA, the IgE bound to the wells was detected with ¹²⁵I-labeled anti-human IgE (5 × 10⁴ cpm/well). The radioactivity was determined as described previously, and the percentage of inhibition of RAST was calculated as follows: [Control (cpm) - Competitor (cpm)] ÷ Control (cpm).

N-terminal sequence analysis

Protein spots on PVDF membranes containing the isomeric subunits of *Cyn d* I were excised for protein sequence analysis. Automated cycles of Edman degradation were performed on an ABI gas/liquid-phase model 470A/900A sequencer equipped with an on-line

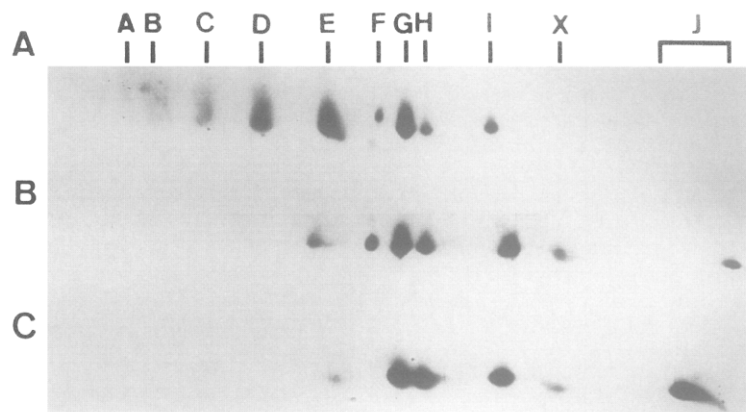


FIG. 1. Profiles of three patterns of *Cyn d I*. Isoforms of three representative *Cyn d I* (A, pattern I; B, pattern II; C, pattern III) were separated by 2D gel electrophoresis and silver stained. Approximate pI values of isoforms are 5.6 (*Cyn d I-A*), 5.8 (*Cyn d I-B*), 5.9 (*Cyn d I-C*), 6.0 (*Cyn d I-D*), 6.2 (*Cyn d I-E*), 6.35 (*Cyn d I-F*), 6.42 (*Cyn d I-G*), 6.48 (*Cyn d I-H*), 6.7 (*Cyn d I-I*), 7.0 (*Cyn d I-X*), and 7.3 (*Cyn d I-J*).

model 120A amino acid phenylthiohydantoin analyzer, according to the procedure of Hewick et al.²⁰ Sequencing of proteins from PVDF membrane was performed according to the method of Chang et al.¹⁶

RESULTS

Profiles of isoforms analyzed by 2D gel electrophoresis

The composition of the isoforms of *Cyn d I* extracted from different lots of BGP was analyzed by 2D gel electrophoresis, and the proteins were visualized by silver staining. We have described 10 isoforms (*Cyn d I-A*, *I-B*, *I-C*, *I-D*, *I-E*, *I-F*, *I-G*, *I-H*, *I-I*, and *I-J*) previously.¹⁰ In all preparations of BGP *Cyn d I-G* was constantly present in a large amount whereas the content of *Cyn d I-J* varied. We found that if the relative percentage of *Cyn d I-I* is used as a standard, all the *Cyn d I* identified can easily be grouped into three patterns. Pattern I contained a low level (<5%) of J isoform, whereas patterns II and III contained moderate (between 5% and 20%) and high (>20%) amounts of J isoform, respectively. The basic SDS-PAGE profile among samples of each pattern was similar, but variation of the relative percentage still existed.

Samples representing the three patterns are illustrated in Fig. 1. The 10 isoforms (*Cyn d I-A* to *Cyn d I-J*) previously identified were all observed in a pattern I sample (Fig. 1, lane A). In a pattern II sample (Fig. 1, lane B), *Cyn d I-A*, *Cyn d I-B*, and *Cyn d I-C* were not detected and *Cyn d I-D* was barely visible. In addition, a new isoform with a pI between those of isoforms I and J was observed. This new isoform is designated as *Cyn d I-X*. The

content of *Cyn d I-X* was very low in the pattern I sample. Pattern III (Fig. 1, lane C) contained mainly four dominant isoforms, *Cyn d I-G*, *Cyn d I-H*, *Cyn d I-I*, *Cyn d I-J*, and two minor isoforms, *Cyn d I-E* and *Cyn d I-X*.

The pI of these isoforms was estimated by slicing the gel and measuring the pH of each slice extract. The pI of *Cyn d I-A* to *Cyn d I-I* ranged from 5.6 to 6.7. The pI of *Cyn d I-X* was approximately 7.0. Because *Cyn d I-J* sometimes appeared as a smear at the most basic edge of our gel (pH 7.3), its pI is probably 7.3 or greater.

A minor MW difference can be found among these subunits. The acidic isoforms (*Cyn d I-A* to *Cyn d I-I*) have a slightly higher MW than that of the other subunits (*Cyn d I-X* and *Cyn d I-J*).

2D immunoblotting

We have shown previously with a pattern I sample that both the acidic and the basic isoforms are allergens.¹⁰ To clarify whether the newly identified *Cyn d I-X* is also an allergen, the immunoblotting experiment was performed with a pattern II *Cyn d I* and human sera from persons with BGP allergy. The results (Fig. 2) show that *Cyn d I-X* is recognized by ¹²⁵I-labeled anti-human IgE.

Antigenic difference among isoforms

The isoforms of *Cyn d I* can be distinguished by two anti-*Cyn d I* MAbs, 4-37 and 1-61. MAb 4-37 reacted with all isoforms whereas MAb 1-61 reacted with only the neutral and basic isoforms. All three patterns of *Cyn d I* showed similar results in a RIP assay, and the data from a pattern III *Cyn d*

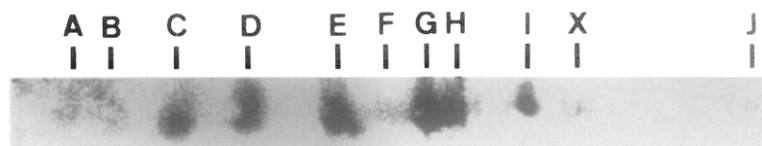


FIG. 2. IgE-binding capacity of pattern II *Cyn d I* sample. After 2D gel electrophoresis, gels containing isoforms of pattern II *Cyn d I* were electrotransferred onto slice of nitrocellulose membrane and reacted with pooled sera from patients with BGP allergy. Bound IgE was detected with ^{125}I -labeled anti-human IgE MAb (2×10^6 cpm/strip) and autoradiographed for 1 day.

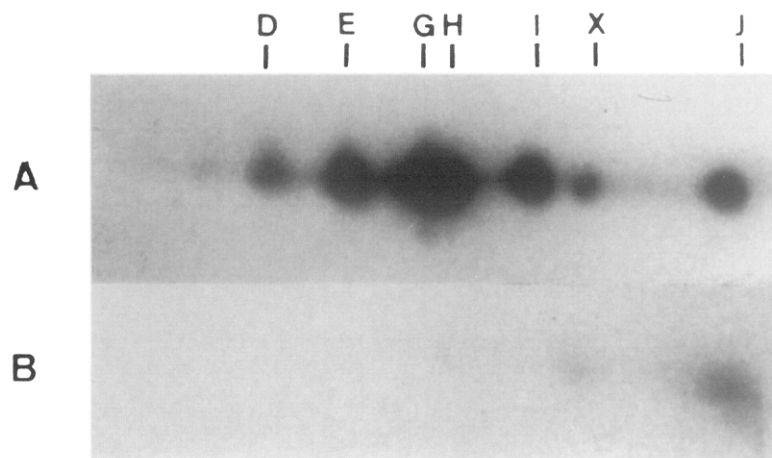


FIG. 3. RIP assay of *Cyn d I* (pattern III) with anti-*Cyn d I* MAbs 4-37 and 1-61. ^{125}I -labeled BGP (5×10^7 cpm) was precipitated by Sepharose 4B beads, which were coupled with either MAb 4-37 (A) or MAb 1-61 (B). Radioactivity of immunoprecipitates were 40,673 cpm (A) and 14,314 cpm (B). After 2D gel electrophoresis, gels were dried and autoradiographed for 8 (A) or 10 days (B).

TABLE I. RAST of acidic isoforms, and basic and neutral isoforms, of *Cyn d I*

Source of pooled sera	Acidic <i>Cyn d I</i> (cpm)	Basic/neutral <i>Cyn d I</i> (cpm)
Normal persons ($n = 10$)	96 ± 18	97 ± 13
Persons with BGP allergy ($n = 14$)	1649 ± 134	1228 ± 72

The acidic isoform and the basic and neutral isoform were coated onto wells of radioimmunoassay plates at a concentration of 100 ng/well. After blocking, pooled normal or allergic sera (1:3 dilution) were added to the wells and incubated at 4°C overnight. The IgE bound to the wells was then detected with ^{125}I -labeled anti-human IgE (5×10^4 cpm/well). Data are the average of triplicate experiments and are expressed as cpm.

I sample are presented in Fig. 3. As shown, MAb 4-37 immunoprecipitated all the detectable isoforms of *Cyn d I*: D (faintly), E, G, H, I, X, and J (Fig. 3, lane A). MAb 1-61 reacted only with *Cyn d I*-X and *Cyn d I*-J (Fig. 3, lane B).

RAST

Whether the isolated acidic isoform and the basic and neutral isoform retained their IgE-binding capacity was evaluated with a RAST. As shown in Table I, the IgE of the pooled sera from persons with BGP allergy ($n = 14$) responded strongly both to the acidic isoform and to the basic and neutral isoform, whereas that of the pooled normal serum had no response.

Competitive RAST

The binding of the IgE to the acidic *Cyn d I* was inhibited both by the acidic and by the basic and neutral *Cyn d I* (Fig. 4, A). A maximal inhibition ratio of 97% was observed, and the inhibition was dose dependent. A similar RAST inhibition was also observed when the basic and neutral *Cyn d I* was used as the antigen of the RAST (Fig. 4, B), and a maximal inhibition of 90% was observed.

The acidic isoforms and the basic and neutral isoforms showed similar competitive ability for the inhibition of the binding of IgE to the acidic *Cyn d I* (Fig. 4, A). On the contrary, the acidic isoform

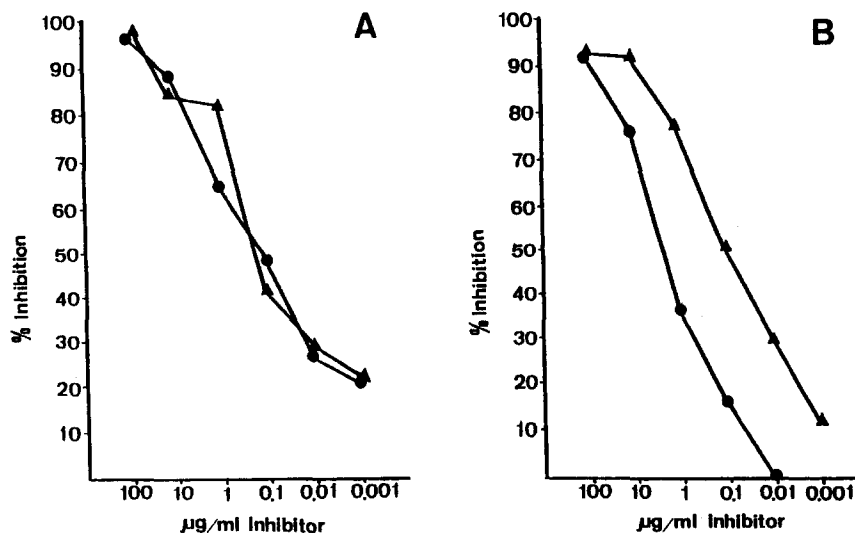


FIG. 4. Competitive RAST curves. Isolated isoforms of *Cyn d* I (A, acidic isoform; B, basic and neutral isoform) were coated onto wells of RIA plates and binding of specific IgE from pooled sera from persons with BGP allergy ($n = 14$) was inhibited by different concentrations (0.001 to 100 µg/ml) of competitors (acidic isoforms; ●; basic and neutral isoforms; ▲).

had a lower competitive ability than the basic and neutral *Cyn d* I for the RAST inhibition of the basic and neutral *Cyn d* I (Fig. 4, B).

N-terminal sequence analysis

A pattern III *Cyn d* I sample was chosen for N-terminal amino acid sequence analysis. The amount of *Cyn d* I-X in this sample was too low for sequencing. Therefore only subunits G, H, I, and J were excised from the PVDF membrane. The sequence of the first 20 N-terminal residues from these subunits are listed in Table II. A 90% (*Cyn d* I-G, *Cyn d* I-H, and *Cyn d* I-I) to 95% (*Cyn d* I-G and *Cyn d* I-J) sequence identity was found among these isoforms. Variation exists only at the thirteenth and the twentieth residues. At residue 13 of *Cyn d* I-G we detected two signals that represent threonine and lysine. Signals representing serine and threonine were found on *Cyn d* I-H and *Cyn d* I-I. The sequencer did not yield any signal from this cycle for *Cyn d* I-J. At the twentieth residue the sequencer yielded signals representing aspartic acid for *Cyn d* I-G, *Cyn d* I-I, and *Cyn d* I-J, and produced signals representing both leucine and aspartic acid for *Cyn d* I-H.

DISCUSSION

More than 10 lots of BGP supplied by two different companies were used in this study. The profile of the *Cyn d* I in different preparations as analyzed by 2D PAGE fell into three patterns (Fig.

TABLE II. N-terminal amino acid sequence of four dominant isoforms from a pattern III *Cyn d* I sample

<i>Cyn d</i> isoform	Sequence
I-G	Ala-Met-Gly-Asp-Lys-Pro-Gly-Pro-X-Ile-Thr-Ala-Thr-Tyr-Gly-Asp-Lys-Trp-Leu-Asp Lys
I-H	Ala-Met-Gly-Asp-Lys-Pro-Gly-Pro-X-Ile-Thr-Ala-Ser-Tyr-Gly-Asp-Lys-Trp-Leu-Asp Leu
I-I	Ala-Met-Gly-Asp-Lys-Pro-Gly-Pro-X-Ile-Thr-Ala-Thr-Tyr-Gly-Asp-Lys-Trp-Leu-Asp
I-J	Ala-Met-Gly-Asp-Lys-Pro-Gly-Pro-X-Ile-Thr-Ala-X-Tyr-Gly-Asp-Lys-Trp-Leu-Asp

X, Not determined.

1). Different lots of BGP from the same company sometimes have different profiles, whereas BGP from different companies can occasionally share the same profile. In all the pollen preparations (Fig. 1) *Cyn d* I-G with a pI of 6.4 was constantly present in large amounts, and it was the major isoform of *Cyn d* I. Matthiesen et al.⁷ reported that *Cyn d* I has a MW of 32 kd and a pI of 6.2. The *Cyn d* I that they studied is probably the G isoform. On the contrary, the content of the basic *Cyn d* I-J varies greatly. We do not know what caused this variation. However, the fact that the group I

allergens varied among different lots of BGP, especially *Cyn d* I-J, indicates the importance of the work to characterize these isoforms.

The basic and neutral isoforms of *Cyn d* I (*Cyn d* I-J and *Cyn d* I-X) had a slightly lower MW than the acidic isoforms (*Cyn d* I-A to *Cyn d* I-I) (Fig. 1). This agrees with the recent finding of Smith et al.²¹ They found that *Cyn d* I can be separated into an acidic *Cyn d* Ia and a basic *Cyn d* Ib with MWs of 32 and 31 kd, respectively. Whether the difference between the MWs of these isoforms is due to different degree of glycosylation or due to the sizes of themselves remains unclear.

The present study identified *Cyn d* I-X as an allergen with a pattern II material (Fig. 2). This isoform was not identified previously because the test material was a pattern I sample, which was low in neutral and basic isoforms. The IgE-binding capacity of the transferred isoforms (Fig. 2) was not in proportion to the relative protein percentages of the same tested sample (Fig. 1, lane B), and this is because the acidic isoforms were successfully transferred onto nitrocellulose membrane in the basic transfer buffer whereas the basic and neutral ones were not.

Because the immunoblot assay is not capable of showing the correct allergenicity among the isoforms, attempts were made to separate the acidic and the basic isoforms, and their allergenicity was studied. This was performed by taking advantage of the specificity of two anti-*Cyn d* I MAbs: MAb 4-37 reacts with all isoforms, whereas MAb 1-61 reacts only with the basic and neutral isoforms. The finding that MAb 4-37 reacts with all detectable isoforms of a pattern III *Cyn d* I in a RIP assay (Fig. 3, lane A) suggests that all these isoforms share a common antigenic determinant. In comparison, the finding that MAb 1-61 reacts only with *Cyn d* I-J and I-X suggests that the basic and the neutral isoforms of *Cyn d* I have their own unique antigenic determinant(s). The antigenic difference of the isoforms was studied with a RIP instead of an immunoblotting because the antigenic determinant recognized by MAb 1-61 is highly conformational¹¹ and is therefore inappropriate for studying by the 2D immunoblot assay.

The basic and neutral and the acidic *Cyn d* I isoforms were isolated either directly or indirectly, and their allergenic property was studied. The positive RAST (Table I) shows that the isolated material retained their IgE-binding capacity. In addition, it also suggests that the BGP allergics had IgE antibodies against both the acidic isoforms and the basic and neutral isoforms (Table I). The

findings that the acidic *Cyn d* I was capable of competitively inhibiting (>90%) the binding of IgE to the basic and neutral *Cyn d* I and that the basic and neutral *Cyn d* I was also capable of inhibiting the binding of the IgE to the acidic *Cyn d* I (>95%) (Fig. 4) suggest that most of the IgE-binding allergic epitopes are common to both the acidic *Cyn d* I and the basic and neutral *Cyn d* I. These shared allergenic epitopes are also responsible for most of the allergenic activity of *Cyn d* I. Furthermore, the finding that basic and neutral *Cyn d* I had a higher RAST inhibition ratio than that of the acidic *Cyn d* I on the RAST of the basic and neutral *Cyn d* I (Fig. 4, B) but not on the RAST of the acidic *Cyn d* I (Fig. 4, A) suggests that the basic and neutral *Cyn d* I bears some unique allergenic determinants. The allergenic property correlates with the antigenic property and suggests *Cyn d* I-J to be an important allergen and antigen of *Cyn d* I, because it processes not only the allergenic and antigenic epitope(s) of the acidic isoforms but also has its unique allergenic and antigenic epitope(s).

Five patients allergic to BGP were tested; all of them showed stronger skin reactions to the basic and neutral *Cyn d* I than to the acidic *Cyn d* I (unpublished results). Therefore a proper diagnostic *Cyn d* I should contain not only the acidic isoforms but also the basic and neutral isoforms. Furthermore, a patient who is not sensitized to the unique epitopes on the basic isoform may elicit an immune response against such epitopes; thus it may be preferable to separate the preparation used for hyposensitization into acidic *Cyn d* I and neutral and basic *Cyn d* I.

Singh et al.²² identified in ryegrass pollen, besides *Lol p* I (a 35 kd major allergen), another 31 kd major allergenic protein, *Lol p* Ib. *Lol p* Ib, which differs from *Lol p* I in having a lower MW, a basic pI, and a totally different N-terminal amino acid sequence, is a different allergen. *Lol p* Ib was later designated as a group IX allergen.²³ It has recently been identified to be a group V allergen²⁴ on the basis of the fact that these two groups of allergens have a high N-terminal sequence homology^{25, 26} and antigenic crossreactivity.²⁷

Our findings that the basic *Cyn d* I differs from the acidic one in having a lower MW (Fig. 1) and a more basic pI, and in possessing unique antigenicity (Fig. 3), raise the possibility that, as in *Lol p* Ib, *Cyn d* I-J may represent another structurally different allergen, the group V allergen. However, this is not supported by our observation because the N-terminal sequence of *Cyn d* I-J (Table II)

does not contain the group V (IX)-restricted characteristic N-terminal sequence (Ala-Asp-Ala-Gly-Tyr).²⁶ In addition, data summarized in Table II show that *Cyn d* I-J and *Cyn d* I-G share a 95% sequence homology for the N-terminal 20 amino acid residues, whereas a 10% variation (2/20) for the N-terminal 20 amino acid residues has been observed among the acidic isoforms G, H, and I. Putting these results together, we thus conclude that *Cyn d* I-X and I-J are isoforms of *Cyn d* I rather than different groups of allergens.

During peptide sequencing analysis the sequencer did not yield any signal for residue 13 of *Cyn d* I-J, suggesting that this is probably a glycosylation site or a cysteine residue. So far this is the only difference that we could find at the protein level between the acidic and the basic isoforms.

The isomeric peptides of other grass pollens have also been studied. Ekramoddoullah²⁸ documented that the N-terminal sequence for two of the four isomeric subunits of *Poa p* I are alike. In reporting the complete amino acid sequence for two isoforms of *Lol p* I, Perez et al.²⁹ found very few amino acid changes (4/270). On the contrary, there is a 10% (2/20) variation among the N-terminal of the dominant acidic isoforms of *Cyn d* I (Table II), suggesting a relatively higher number of amino acid changes. A 20% variation (4/20) between the N-terminal of the *Cyn d* Ia and *Cyn d* Ib was found by Smith et al.²¹ The high variation among isoforms is probably one of the reasons that *Cyn d* I has heterogeneous biochemical and immunologic properties, and its antigenicity is considered to be distinct from that of the others.⁷

In conclusion, the present study provides important information to clinical allergists by showing that the basic *Cyn d* I-J is an important allergen of *Cyn d* I and that the content of this isoform varies in different lots of BGP. Therefore a proper diagnostic BGP extract should contain not only the acidic isoforms of *Cyn d* I but also the basic isoform of *Cyn d* I.

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