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Purification and Characterization of a Novel Isoallergen of a Major Bermuda Grass Pollen Allergen, Cyn d 1

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Key Words
Bermuda grass pollen · Allergen · Purification · Carbohydrate

Abstract
A novel immunoreactive isoallergen of a major Bermuda grass pollen allergen, Cyn d 1, was purified by the use of a combination of various chromatographic techniques, including high-performance liquid chromatography. This new isoallergen has a pI value of 9.1 and shows significant N-terminal sequence homology with other isoforms. Carbohydrate composition analysis revealed a 10.4% carbohydrate content consisting of 7 different sugar moieties, including arabinose, fucose, galactose, glucose, mannose, xylose and N-acetylglucosamine, as well as a trace amount of rhamnose. Upon periodate oxidation, the binding activities of the Cyn d 1 isoform to murine monoclonal antibodies and human serum IgE and IgG were reduced, suggesting the importance of the carbohydrate moiety in the immune response. The availability of the purified Cyn d 1 basic isoform will allow for further structural and immunological characterization, and ultimately for the design of an appropriate therapy.

Introduction

The importance of Bermuda grass *(Cynodon dactylon)* pollen as a respiratory allergen which causes allergic reactions is well documented [2, 20, 30]. It contains multiple IgE-binding proteins [7, 14, 24, 27]; some of these proteins have been isolated and proven to be allergens [8, 10, 15, 16, 21, 23, 25, 27–29]. One of the IgE-binding proteins, Cyn d 1, a major allergen of Bermuda grass pollen, contains 11 isoforms whose pI values range from 5.6 to 7.4, with N-terminal amino acid sequences which show significant sequence homology [3]. Of the 11 isoforms, 3 have been isolated and partially characterized [8, 15, 25]; however, pI values have been reported for only 2 isoforms, namely 6.5 and 7.4, respectively. Cyn d 1 is the most abundant protein in Bermuda grass pollen, representing 15% (wt/wt) of the whole-pollen extract [15]. Although Cyn d 1 has been shown to bind to the lectin concanavalin A [10], little information is available about its carbohydrate moiety.

During the course of a study characterizing the 60-kD Bermuda grass pollen allergen BG60, a basic isoform of Cyn d 1 was found with a much higher pI value than those previously reported [3, 25]. In this paper, we report on the purification and characterization of this new isoallergen;

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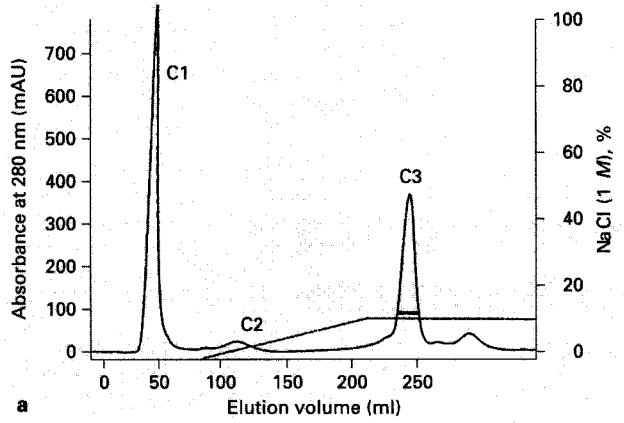

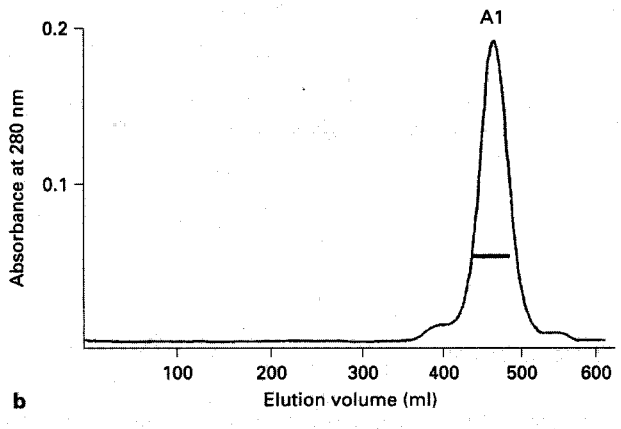

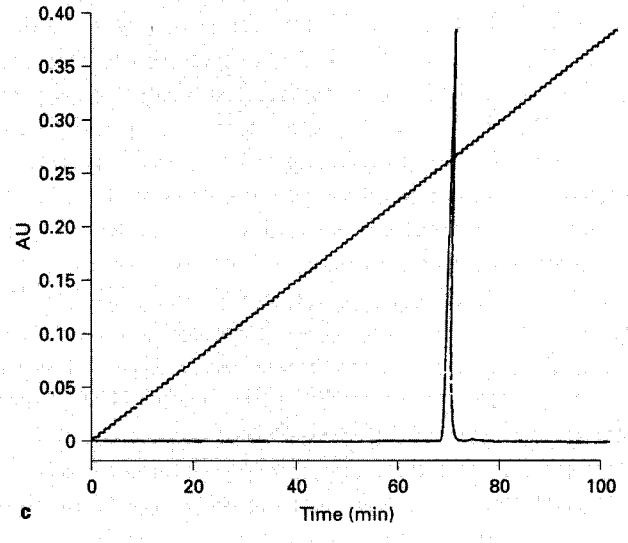

in addition, we analyzed its immunologic and physicochemical properties, especially its carbohydrate composition.

Materials and Methods

Preparation of a 70–90% Ammonium Sulfate Fraction

All experiments were carried out at 4°C unless otherwise indicated. Bermuda grass *(C. dactylon)* pollen (defatted) was purchased from International Biologicals (Piedmont, Okla., USA). The G1 fraction was obtained as described previously [28]; solid ammonium sulfate was added to achieve a 70% saturated ammonium sulfate solution which was stirred at 4°C for 40 min. The precipitate (designated AS1) was collected after centrifugation, and the supernatant was supplemented with additional solid ammonium sulfate to achieve a 90% saturated ammonium sulfate solution which was stirred as before. Again, the precipitate formed was collected after centrifugation, dissolved in a small volume of phosphate buffer (20 m*M*, pH 6.0, 0.02% sodium azide) and dialyzed thoroughly against the same buffer. After extensive dialysis, a small amount of the insoluble material was removed by centrifugation, and the supernatant (designated AS2) was used for further experiments as described below.

Carboxymethyl-TSK Column Chromatography

An aliquot of AS2 (8 mg/200 µl) obtained from the previous step was applied to a carboxymethyl (CM)-TSK column (1.6 × 33 cm, Tosoh Co., Tokyo, Japan) equilibrated with phosphate buffer (20 m*M*, pH 6.0, 0.02% sodium azide). Elution was carried out at a flow rate of 60 ml/h by increasing the salt gradient (NaCl). The experiment was carried out at 4°C using an AKTA purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden). Fraction C3 (indicated by a black bar in fig. 1a) was concentrated by adding solid ammonium sulfate to 90% saturation with stirring at 4°C for 40 min. The precipitate was collected after centrifugation (designated C3), dissolved in PBS and used for the following experiment.

Gel Filtration Chromatography and Reversed-Phase High-Performance Liquid Chromatography

Sample C3 (12 mg/ml) was applied to an Ultrogel AcA 44 (IBF, Garenne, France) column (2.5 × 130 cm) equilibrated with PBS and was eluted with PBS at 4°C with a flow rate of 48 ml/h. Cyn d 1 was found to be a dominant protein in the major peak (A1). Fraction A1 was pooled, concentrated and dialyzed against distilled water; the resultant protein (500 µg/100 µl) was applied to a semipreparative reversed-phase high-performance liquid chromatography (RP-HPLC) column (C_4, 10 × 200 mm, Vydac, Hesperia, Calif., USA) equilibrated with 0.1% trifluoroacetic acid. Elution was carried out across a continuous gradient of increasing acetonitrile from 0 to 100% for 100 min at a flow rate of 1 ml/min.

Fig. 1. a Chromatogram of fraction AS2 (8 mg) on a CM-TSK column (35 × 1.6 cm). Fractions containing the Cyn d 1 basic isoform were collected as indicated by the black bar and were designated C3. **b** Chromatogram of fraction C3 (12 mg) on an Ultrogel AcA 44 column (133 × 2.5 cm). Fractions containing the Cyn d 1 basic isoform were collected as indicated by the black bar and were designated A1. **c** Chromatogram of fraction A1 (500 µg) on a semipreparative RP-HPLC column (C_4, 10 × 200 mm). Elution was carried out with a continuous gradient of increasing acetonitrile from 0 to 100% over 100 min at a flow rate of 1 ml/min.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Protein content was determined according to the method of Bradford [1] using bovine serum albumin as a standard. The highly purified Cyn d 1 basic isoform was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [12] with a 5% stacking gel and a 12.5% separating gel. After electrophoresis, protein was either stained with Coomassie brilliant blue or transferred to PVDF strips (Millipore, Bedford, Mass., USA) for reaction with human IgE, IgG, monoclonal antibodies (mAbs) and lectins.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was carried out essentially as described by Voller et al. [32]. In general, 5 washings with distilled water were performed between each step, unless otherwise indicated. ELISA plates were coated with 50 µl of the antigen (2 µg/ml) diluted in carbonate buffer (15 m*M*, pH 9.6) and allowed to sit overnight at 4°C. Wells were blocked with blocking solution (1% normal goat serum in PBS containing 0.3% Tween 20) for 30 min at 37°C. For IgE binding, allergic serum was diluted 100-fold with blocking solution and incubated overnight at 4°C. However, for IgG binding, the serum was diluted 5,000-fold with blocking solution and incubated for 3 h at 37°C, after which the alkaline phosphatase-conjugated mouse anti-human (IgE, IgG) antibody (diluted 1,000-fold; Pharmingen, San Diego, Calif., USA) was added and incubated for 3 h at 37°C. For the mAb-binding study, horseradish peroxidase-conjugated goat anti-mouse IgG antibody (diluted 5,000-fold) was added and incubated for 3 h at 37°C. Alkaline phosphatase activity was determined using disodium *p*-nitrophenyl phosphate as a substrate. Horseradish peroxidase activity was measured using 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) and by measuring the absorbance at 405 nm after incubation for between 10 and 30 min.

Lectin-Binding Activity

Lectin-binding activities of the Cyn d 1 basic isoform were investigated by ELISA as described above. Briefly, 50 µl (2 µg/ml) of the Cyn d 1 basic isoform diluted in carbonate buffer (15 m*M*, pH 9.6) was coated onto a 96-well microtiter plate (Costar, Cambridge, Mass., USA) overnight at 4°C. After washing and blocking, a panel of 6 biotinylated lectins (100 ng/well), including castor bean, pokeweed, Osage orange, *Wisteria floribunda*, jack bean and jacalin (Sigma, St. Louis, Mo., USA) was added and incubated for 3 h with constant shaking at room temperature. Then, avidinylated horseradish peroxidase was added and incubated for 3 h at room temperature. Lectin-binding activities were determined by measuring the horseradish peroxidase activity as described above.

Human IgG, IgE and mAb-Binding Study

Patients who were selected for this study had been referred to the allergy clinic at the University of Florida because of a history of seasonal allergic rhinitis for at least 1 year; furthermore, they had never received Bermuda grass pollen immunotherapy. Their ages ranged from 15 to 35 years. Serum samples, obtained when patients visited the clinic for the first time, were stored in 1-ml aliquots at −20°C until use. Skin tests were applied to each patient's back and included the standardized allergic extracts of bahia, Bermuda, Johnson, red top and timothy grass pollens (Miles Laboratories, Spokane, Wash., USA). Wheal reactions of greater than 5 mm over albumin-saline controls were considered positive. Classification of the skin reaction ranged from 0 to 4+. Twenty patients included in this study had registered at least 3+ by the prick test with the Bermuda grass pollen extract. Three normal (i.e. nonatopic) serum samples were used as negative controls and showed very low absorbance values (0.05–0.08), which were considered nonreactive. Two patients with asthma who were allergic to Bermuda grass pollen were subjected to skin testing by a subcutaneous injection of 25 µl of the purified protein (2 µg/ml) in PBS. Wheal reading was taken within 15 min after the injection, and a wheal greater than 8 mm over the PBS control was considered a positive response.

mAbs of the Cyn d 1 basic isoform were prepared similarly to that for BG60 as described previously [28]. Briefly, female BALB/c mice were immunized intraperitoneally with 0.2 ml of an emulsion containing 50 µg of highly purified antigen and Freund's complete adjuvant. The mice were boosted at least twice at 3-week intervals with the same material. A final booster injection (antigen in saline) was administered to the mice 3–4 days before they were sacrificed to obtain their spleens for hybridization. Mice exhibiting serum antibodies against the Cyn d 1 basic isoform as determined by ELISA were chosen for fusion. Hybridization of spleen cells and a myeloma cell line (FO) was carried out essentially as described by Koehler and Milstein [11].

Enzymatic Deglycosylation and Periodate Oxidation

To examine the role of the carbohydrate moiety of the Cyn d 1 isoforms in the immune response, enzymatic cleavage and chemical modification of the carbohydrate moiety of the Cyn d 1 isoforms were performed by treatment with an enzymatic deglycosylation kit (Bio-Rad Laboratories, Richmond, Calif., USA) and sodium periodate, respectively. Enzymatic deglycosylation was performed according to the manufacturer's instructions. Bovine fetuin was used as a positive control. Periodate oxidation of the Cyn d 1 isoforms was performed as described elsewhere [22]. Briefly, the antigen was transferred to a PVDF membrane after SDS-PAGE, then the strips were oxidized in a sodium periodate solution (20 m*M* in 50 m*M* acetate buffer, pH 4.5) for 18 h at 4°C in the dark and washed with TBST (50 m*M* Tris-buffered saline, 0.15 *M* NaCl and 0.05% Tween 20, pH 7.5). Then, sodium borohydride (50 m*M*) was added to the strips, and they were incubated for 30 min at room temperature. After washing, the strips were blocked with TBST containing 1% gelatin for 30 min at room temperature and washed again. Mock-treated Cyn d 1 isoforms were prepared the same way except without sodium periodate oxidation. Allergic serum (diluted 1/4 in TBST) was then added to the strips and incubated overnight at 4°C. For IgE binding, allergic serum was diluted 4-fold (for IgG binding, serum was diluted 100-fold) with blocking solution and incubated overnight at 4°C. After this, the alkaline phosphatase-conjugated mouse anti-human (IgE, IgG) antibody (Pharmingen) was added (diluted 100-fold for IgE assay and 1,000-fold for IgG assay) and incubated for 3 h at 37°C. Alkaline phosphatase activity was determined using 5-bromo-4-chloro-3-indolyl phosphate as a substrate. For mAb-binding studies, periodate-treated and mock-treated Cyn d 1 basic isoforms were prepared in a manner similar to that described for BG60 [29], and binding activities were determined by ELISA as described previously [29].

Carbohydrate Composition Analysis

For monosaccharide composition analysis, samples were methanolyzed with 0.5 *M* methanolic-HCl (Supelco, Bellefonte, Pa., USA) at 80°C for 16 h, re-*N*-acetylated with 500 µl of methanol, 10 µl of pyridine and 50 µl of acetic anhydride, and then treated with the Sylon HTP® trimethylsilylating reagent (Supelco) for 20 min at room

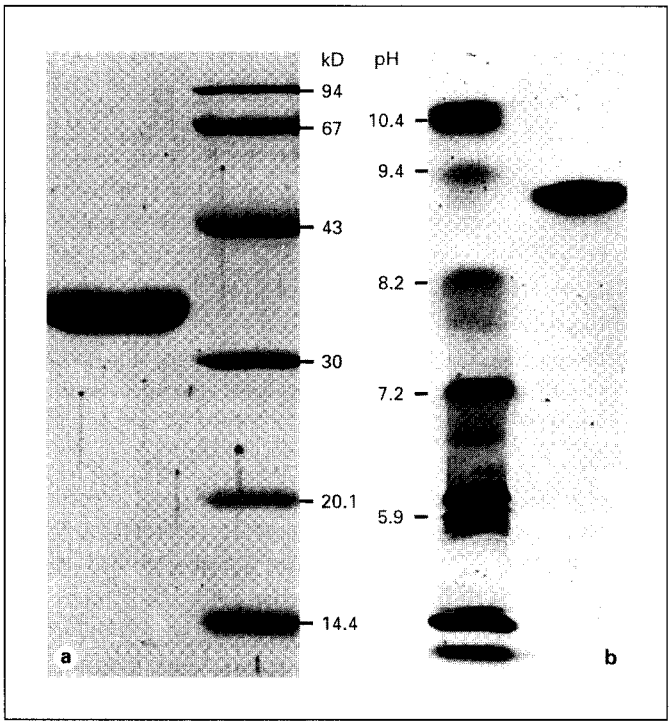

Fig. 2. a The major peak obtained from RP-HPLC as electrophoresed on SDS-PAGE (12.5%) for Coomassie blue staining and molecular mass estimation. **b** The pI value of the Cyn d I basic isoform as measured using isoelectrofocusing-PAGE (the pH ranged from 3.5 to 10.5).

temperature, after which they were dried and redissolved in hexane. GC-MS analysis of the trimethylsilylated derivatives was performed on a Hewlett-Packard (HP) gas chromatograph 6890 connected to an HP 5973 mass selective detector fitted with an HP-5MS fused silica capillary column (30 m × 0.25 mm internal diameter, HP). The column head pressure was maintained at around 56.55 Pa to give a constant flow rate of 1 ml/min using helium as the carrier gas. The initial oven temperature was held at 60 °C for 1 min, increased to 140 °C at 25 °C/min, to 250 °C at 5 °C/min and then to 300 °C at 10 °C/min. The reference sugars used to identify unknown compounds were arabinose, fucose, galactose, glucose, mannose, rhamnose, N-acetylgalactosamine, N-acetylglucosamine and N-acetylneurominic acid. Total carbohydrate content was estimated from the amount of detected monosaccharides.

Amino Acid Analyses and N-Terminal Sequencing of the Cyn d 1 Isoforms

About 1.0 nmol of each of the Cyn d 1 isoforms (acidic, neutral and basic) was hydrolyzed with 300 µl of 6 *N* HCl in a vacuole at 150 °C for 90 min. Amino acids were analyzed with a Beckman 6300 amino acid analyzer. N-terminal Edman degradation of the Cyn d 1 basic isoform was performed on a PROCISE 494 protein sequencer (Perkin Elmer/ABI, Foster City, Calif., USA) equipped for on-line detection of phenylthiohydantoin derivatives.

Results

Purification of the Cyn d 1 Basic Isoform

To enrich the Cyn d 1 allergen, defatted Bermuda grass pollen was first subjected to a stepwise ammonium sulfate precipitation and dialysis, resulting in 90% purity of the AS2 fraction. When the AS2 fraction was chromatographed on a CM-TSK column, 2 major peaks and a few minor peaks were obtained (fig. 1a). The major component of each of the 3 peaks (C1, C2 and C3) was found to be Cyn d 1 isoforms. Peaks C1 and C2 were both directly eluted from the CM-TSK column, indicating that the proteins of these 2 peaks are either acidic or neutral. However, peak C3 was eluted from the column with buffer containing salt, indicating that peak C3 contains basic proteins. Peak C3 was further fractionated by gel filtration chromatography on an Ultrogel AcA 44 column (fig. 1b), where 1 major peak (A1) and 2 minor peaks were found. The Cyn d 1 basic isoform was found to be a dominant protein of the A1 fraction. The A1 fraction was further purified by RP-HPLC, with 1 major peak found (fig. 1c). The major peak was shown to contain only a single band by both SDS-PAGE (fig. 2a) and isoelectrofocusing-PAGE (fig. 2b). The pI of the basic isoform of Cyn d 1 was estimated to be 9.1. The content of the highly basic isoform was measured to be approximately one-third of the total Cyn d 1, and the yield of this basic protein was approximately 0.3 mg protein/g pollen.

Antigenic and Allergenic Properties of the Cyn d 1 Basic Isoform

The antigenicity and allergenicity of the Cyn d 1 basic isoform were assayed in vitro. Sera from twenty patients with clinical allergy to Bermuda grass pollen extract by the skin prick test were analyzed. As shown in table 1, whereas all 20 samples contained high titers of specific IgG antibodies (3+), they had different IgE-binding activities: 2 had low titers (1+), 3 had medium titers (2+) and 15 had high titers (3+). These results indicate that the Cyn d 1 basic isoform is a strong immunogen in humans. In addition, the allergenicity of the Cyn d 1 basic isoform was also confirmed by a positive skin test reaction in 2 asthmatic patients who were allergic to Bermuda grass pollen (data not shown).

Carbohydrate and Amino Acid Composition Analyses and N-Terminal Analysis of the Cyn d 1 Basic Isoform

The carbohydrate content of the Cyn d 1 basic isoform was about 10.4% as measured from the detected monosaccharides after GC-MS (gas chromatography-mass

Table 1. IgE-binding reactivity of the Cyn d 1 basic isoform in Bermuda grass pollen-allergic patients (rhinitis and bronchial asthma)

Serum No.	ELISA positivity, +
3	1
12	3
14	3
19	2
21	2
24	3
25	3
27	3
28	3
29	1
31	2
32	3
102	3
114	3
146	3
159	3
173	3
203	3
226	3
267	3

The positivity scale for ELISA was arbitrary, taken from the net value of the serum as follows: $0.02 < 1+ < 0.05$, $0.05 < 2+ < 0.1$, and $0.1 \leq 3+$. The reactivity of the 3 nonallergic sera was <0.01. All sera showed IgG reactivity of 3+.

Table 2. Monosaccharide composition of the Cyn d 1 basic isoform

Monosaccharide	Molar ratio
Arabinose	7.6
Galactose	4.6
Glucose	5.0
Fucose	2.4
Mannose	11.9
Rhamnose	trace
Xylose	1.0
N-acetylglucosamine	3.1

The monosaccharide composition is based on GC-MS analysis of trimethylchlorosilane derivatives. The calculated molar ratios are based on an average from two separate runs. Xylose was arbitrarily taken as 1.0.

Table 3. Comparison of amino acid composition of the Cyn d 1 isoforms

Amino acid	Molar ratio		
	C1	C2	C3
Asp/Asn	12.89	11.90	12.15
Thr	3.23	2.82	3.18
Ser	5.28	5.17	4.79
Glu/Gln	10.68	9.93	8.40
Pro	6.94	7.25	10.23
Gly	12.97	12.92	12.97
Ala	9.47	8.49	10.24
Cys	0.88	0.84	0.95
Val	3.36	3.16	3.48
Met	3.90	4.30	1.67
Ile	3.26	3.09	3.62
Leu	5.69	5.58	5.43
Tyr	3.40	3.47	3.77
Phe	3.81	3.85	4.47
Lys	9.86	11.45	10.25
His	1.49	2.03	2.02
Arg	2.89	3.73	2.36
Try	ND	ND	ND
Total	100	100	100

The pI values of the C1, C2 and C3 isoforms are as follows: $C1 < 6.0$, $6.0 < C2 < 9.1$, and $C3 = 9.1$. ND = Not determined.

spectrometry) analysis. The chromatograms of the sugar analyses showed 7 kinds of sugars, including arabinose, fucose, galactose, glucose, mannose, xylose and N-acetylglucosamine, in addition to a trace amount of rhamnose. The molar ratio of the 7 sugars was further analyzed and is summarized in table 2.

The amino acid composition of the Cyn d 1 basic isoform is summarized in table 3. The amino acid compositions of the other Cyn d 1 isoforms are also included in table 3. The Cyn d 1 isoforms contain most of the naturally occurring amino acids. Values for cysteine and tryptophan were not corrected due to destruction during hydrolysis. The N-terminal amino acid sequence of the Cyn d 1 basic isoform is shown in table 4 and is compared with those of the other isoforms. The sequencer yielded no signal for the ninth residue of the Cyn d 1 basic isoform. We considered that it might be a glycosylation site (Asn) or Lys by comparison with the ninth residues of other known sequences in table 4.

Table 4. Comparison of the N-terminal amino acid sequence of the Cyn d 1 isoforms

Isoform	N-terminal amino acid sequence	Reference
Cyn d 1 (9.1)	A**I**GDKPGP**X**ITATYG**S**KWL**E**AK	
Cyn d 1 (peptide N)	A**I**GDKPGP**X**ITATYG**S**KWL**E**AKATFYGXN	8
Cyn d 1.CD1	A**I**GDKPGP**N**ITATYG**S**KWL**E**ARATFYGSNPRGAA	26
Cyn d 1b (7.4)	A**I**GDKPGP**K**ITATYX**X**KWL**E**AKATFYGSNPRGAA	25
Cyn d 1.3	A**X**GDKPGP**N**ITATYG**D**KWL**E**AKATFYGSDPRGA	26
Cyn d 1	A**M**GDKPGP**K**ITATYG**S**KWL**D**AKATF	8
Cyn d 1	A**M**GDKPGP**X**ITATYG**D**KWL**D**AKATFYG	15
Cyn d 1a (6.5)	A**M**GDKPGP**X**ITATYG**D**KXL**D**AKXAFD	25
Cyn d 1-G (6.42)	A**M**GDKPGP**X**ITATYG**D**KWL**D**	3
Cyn d 1-H (6.48)	A**M**GDKPGP**X**ITASYG**D**KWL**D**	3
Cyn d 1-I (6.72)	A**M**GDKPGP**X**ITATYG**D**KWL**D**	3
Cyn d 1-J (7.30)	A**M**GDKPGP**X**ITAXYG**D**KWL**D**	3

Figures in parentheses are the pI value for each isoform indicated. The letters in bold indicate the amino acid differences between two groups of the isoforms.

Lectin-Binding Reactivity of the Cyn d 1 Basic Isoform

Cyn d 1 has been shown to bind to a concanavalin A affinity column [10]. Thus, the binding activity of the Cyn d 1 basic isoform to different lectins was examined. A panel of 6 lectins, including castor bean, pokeweed, Osage orange, *W. floribunda*, jack bean and jacalin, was found to bind to the Cyn d 1 basic isoform with differential binding activities (fig. 3). The binding activities of the lectins could be divided into two groups: group 1 had low binding activities (pokeweed, Osage orange and *W. floribunda*) and group 2 had high binding activities (castor bean, jack bean and jacalin). These results suggest that this basic isoform of the Cyn d 1 isoallergen is a glycoprotein with a complex composition of carbohydrate moieties.

Enzymatic Deglycosylation and Periodate Oxidation

The role of the carbohydrate moiety of the Cyn d 1 isoforms in antigenicity and allergenicity was investigated by enzymatic deglycosylation and periodate oxidation. Enzymatic deglycosylation of the Cyn d 1 isoforms was not successful in the presence of denaturing reagents (such as NP-40 and SDS). After enzymatic treatment with the endoglycosidases NANase II, O-glycosidase DS and PGNase F, the molecular mass of the three groups of Cyn d 1 isoforms was found to be similar to that of untreated native Cyn d 1 isoforms (data not shown). Although the carbohydrate moiety of fetuin (the positive control) was enzymatically removed, the carbohydrate moiety of the Cyn d 1 isoforms could not be removed by the three endoglycosidases. Thus, there were no deglycosylated Cyn d 1 isoforms for further antibody-binding studies. Therefore, we used the periodate-treated Cyn d 1 isoforms to examine the role of carbohydrate in antibody-binding activities. As is evident from figure 4a, the binding activities of the periodate-treated Cyn d 1 basic isoform to IgE antibodies were significantly reduced (lane 4) when compared to those of the mock-treated Cyn d 1 basic isoform (lane 3). Moreover, the binding activities of the periodate-treated Cyn d 1 isoforms from peaks C1 and C2 (fig. 4a, lanes 2 and 6, respectively) were also significantly reduced when compared to those of the mock-treated Cyn d 1 isoforms (fig. 4a, lanes 1 and 5, respectively). The binding activities of the periodate-treated Cyn d 1 isoforms were decreased in pooled allergic sera by about 60–70%. Moreover, binding activities of human IgG antibodies were also reduced in a similar fashion to those of the periodate-treated Cyn d 1 isoforms (data not shown). Furthermore, binding activities of the periodate-treated Cyn d 1 basic isoform to the mAb (549-56) decreased by about 15–65% as the concentration of sodium periodate increased from 0.1 to 20 m*M* (fig. 4b). These results provide evidence for the involvement of the carbohydrate moiety of Cyn d 1 in the immune response.

Discussion

In this report, a novel, basic isoallergen of Cyn d 1 with a pI value of 9.1 was purified by a combination of ammonium sulfate fractionation, CM-TSK, gel filtration and

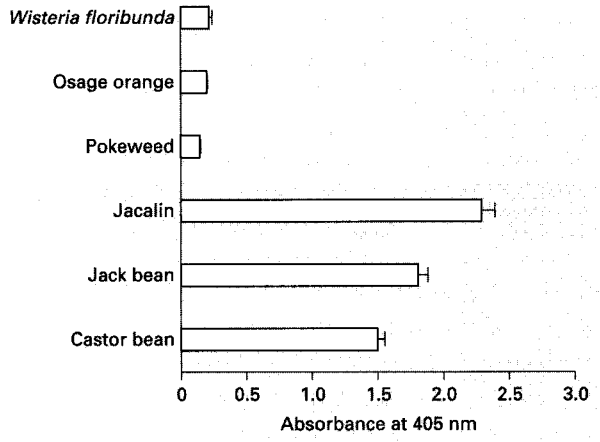

Fig. 3. Lectin-binding reactivity of the Cyn d 1 basic isoform. Binding of lectins to the Cyn d 1 basic isoform was investigated using ELISA with a panel of 6 lectins as shown in the figure. The results are the means of triplicate samples. Error bars indicate the standard deviation for triplicate samples.

RP-HPLC. The use of 70–90% ammonium sulfate fractionation was found to be critical for the purification of this basic isoform of Cyn d 1. More than 90% of the protein in this fraction is Cyn d 1 (including its acidic, neutral and basic isoforms), and no other degraded form, such as the 29-kD isoform, was observed [15, 25]. The Cyn d 1 isoforms were separated into 3 peaks (C1, C2 and C3) with high purity (>90%) which showed similar apparent molecular masses of about 34 kD. Smith et al. [25] showed that the molecular mass of the acidic isoform (32 kD, pI 6.5) is slightly larger than that of the basic isoform (31 kD, pI 7.4). Chang et al. [3] also showed similar results. The apparent molecular masses of the Cyn d 1 isoforms in this study are at variance with those in the literature. The reason for this discrepancy is not clear at the present time.

The N-terminal amino acid sequences of all the Cyn d 1 isoforms display high homology. In fact, 12 mAbs against the basic isoform showed cross-reactivity to both the acidic and neutral isoforms. The cross-reactivity between Bermuda grass pollen and other tree/grass pollens has been demonstrated with both rabbit anti-Cyn d 1 antibody and murine anti-Cyn d 1 mAbs [15, 25]. We also found that the mAbs against the Cyn d 1 basic isoform cross-reacted with other pollens (data not shown). Based on their N-terminal amino acid sequences, the Cyn d 1 isoforms can be divided into two groups: group 1 isoforms

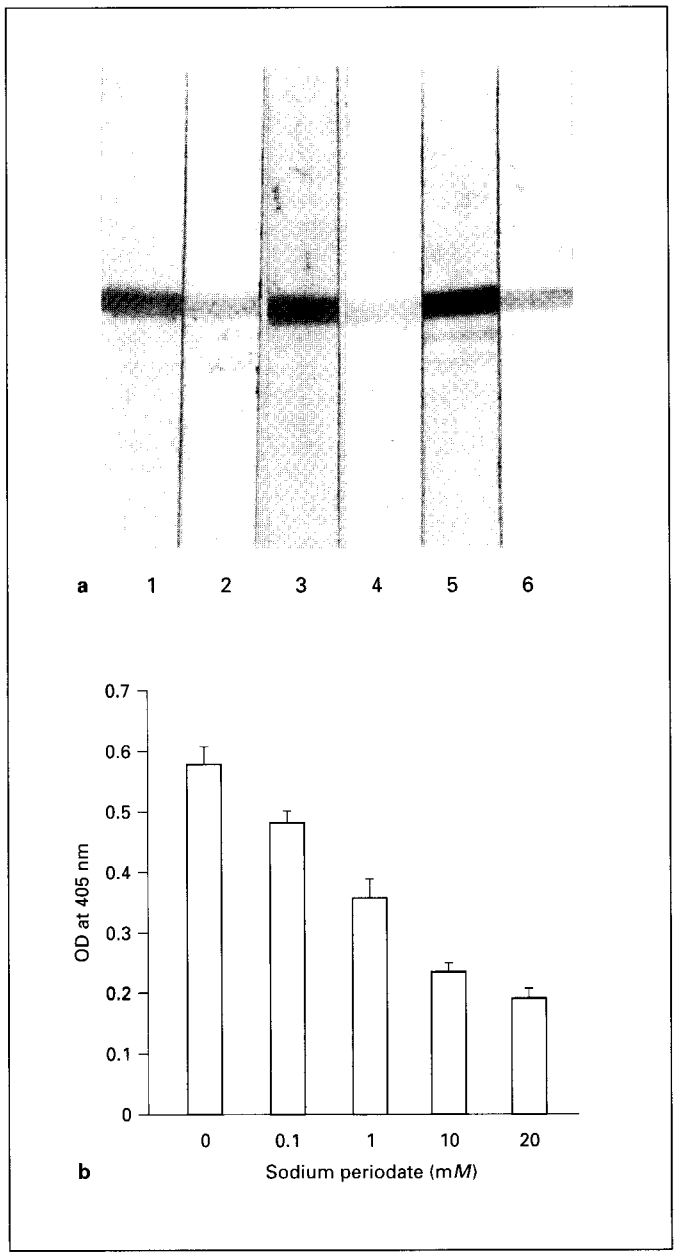

Fig. 4. a Effect of periodate oxidation on the binding of Cyn d 1 isoforms to antibodies. Binding of human IgE to the three groups (C1, C2 and C3 of fig. 1a) of Cyn d 1 isoforms treated with 20 m*M* sodium periodate. Lanes 1, 3 and 5 are mock-treated Cyn d 1 isoforms of C1, C3 and C2, respectively. Lanes 2, 4 and 6 are periodate-treated Cyn d 1 isoforms of C1, C3 and C2, respectively. **b** Binding of mAb (549-56) to the Cyn d 1 basic isoform treated with different concentrations (0.1–20 m*M*) of sodium periodate. The results are the means of triplicate samples. Error bars indicate the standard deviation for triplicate samples. OD = Optical density.

contain isoleucine (designated I) at the second residue and glutamic acid (designated E) at the twentieth residue, and group 2 isoforms contain methionine (designated M) at the second residue and aspartic acid (designated D) at the twentieth residue. According to these two major differences, isoleucine (I) may be assigned to the unknown amino acid (X) of the second residue of Cyn d 1.3 [26]. The first 22 amino acid residues of the N-terminal amino acid sequence of our Cyn d 1 basic isoform show complete identity with the N-terminal amino acid sequence of peptide N [8]. This finding indicates that these two isoforms may be the same. However, no data are currently available regarding peptide N, except for its N-terminal amino acid sequence.

Analysis of the carbohydrate composition of the Cyn d 1 basic isoform revealed a relatively large amount of arabinose, representing the second largest amount of sugar besides mannose in the carbohydrate moiety of the Cyn d 1 basic isoform. Havvik et al. [9] also reported that an allergen from timothy grass contains arabinose. This finding suggests that the Cyn d 1 basic isoform and the other isoforms may be arabinogalactan proteins. Arabinogalactan proteins have been isolated from plant tissues, including stigma surfaces, and seem to be involved in cell adhesion [4, 6]. The physiological role of the Cyn d 1 isoforms has not yet been characterized; the possibility that this major allergen is involved in cell adhesion needs to be further investigated.

To examine the role of the carbohydrate moiety of the Cyn d 1 isoforms in antibody binding, sodium periodate oxidation was performed. Sodium periodate has been shown to selectively alter the glycan structure without affecting the protein epitopes [33]. The antibody-binding activities of Cyn d 1 (including the acidic, neutral and basic isoforms) were reduced after periodate oxidation. Similar results have been obtained for other Bermuda grass pollen allergens such as BG60 [29] and Cyn d Bd46k [34]. Furthermore, other studies have also shown that the carbohydrate moiety is important in the immune response [5, 13, 17–19, 31]. The results of oxidation experiments reveal that the carbohydrate moiety of Cyn d 1 may also play an important role in the immune response. Moreover, the recombinant proteins of Cyn d 1 have been expressed in *Escherichia coli* and yeast, but only the protein expressed in yeast possesses IgE-binding reactivity [26], suggesting that glycosylation of the recombinant protein is essential for binding to IgE. However, it has been suggested that the carbohydrate moiety is not required for IgE binding to natural Cyn d 1, because enzymatic deglycosylation of natural Cyn d 1 did not abolish IgE recognition [26]. In this study, the carbohydrate moiety of Cyn d 1 could not be removed by endoglycosidases in the presence or absence of denaturing reagents (data not shown), suggesting that Cyn d 1 might not be accessible to the enzymes. The controversial role of the carbohydrate moiety of recombinant versus natural Cyn d 1 still remains to be clarified. However, our results of periodate oxidation support the notion that the carbohydrate moiety of natural Cyn d 1 plays a role in the immune response.

In conclusion, the newly identified basic isoform of Cyn d 1 has the highest pI value among all Cyn d 1 isoforms known. The highly basic isoform of Cyn d 1 contains about 10.4% carbohydrates, which consist of 7 sugars, and it may be an arabinogalactan protein. Interestingly, the binding ability of Cyn d 1 to mAbs and human IgE and IgG antibodies was inhibited by periodate treatment, suggesting that the carbohydrate moiety of Cyn d 1 may play an important role in the immune response.

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