

Monoclonal Antibody-Based ELISA to Quantify the Major Allergen of *Cynodon dactylon* (Bermuda Grass) Pollen, Cyn d 1

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Key Words

Allergen · Bermuda grass · Cyn d 1 · *Cynodon dactylon* · Immunoassay · Monoclonal antibody · Pollinosis

Abstract

Background: Pollen of Bermuda grass (*Cynodon dactylon*) is an important cause of pollinosis in many areas of the world. Most patients show sensitivity to the major allergen Cyn d 1, a glycoprotein composed of a number of isoforms with a molecular mass of 31–32 kDa. The aim of this work was to develop a monoclonal antibody (mAb)-based ELISA to quantify Cyn d 1, and to assess the correlation of the allergen content with the biological activity of *C. dactylon* pollen extracts. **Methods:** After fusion of myeloma cells with spleen cells from a BALB/c mouse immunized with *C. dactylon* pollen extract, Cyn d 1-specific mAbs secreting hybridomas were selected, and the antibodies characterized. One of them (4.4.1) was used as the capture antibody in an ELISA method for Cyn d 1 quantitation. An anti-Cyn d 1 rabbit serum was used as the second antibody. Cyn d 1 was purified by immunoaffinity chromatography with mAb 4.4.1, characterized, and used as the standard in the assay. **Results:** The identity, purity and isoallergen composition of affini-

ty-purified Cyn d 1 was confirmed by N-terminal amino acid sequencing, SDS-PAGE, Western blot and 2D electrophoresis. The Cyn d 1 ELISA is highly specific and sensitive, with a detection limit of 0.24 ng/ml and a linear range of 1.1–9.2 ng/ml. An excellent correlation was found when the content of Cyn d 1, measured in 16 different extracts, was compared with the allergenic activity of the same extracts determined by RAST inhibition. **Conclusions:** The results prove the usefulness of the Cyn d 1 ELISA for the standardization of *C. dactylon*-allergen products on the basis of major allergen content.

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Introduction

Grass pollens are among the leading sources of seasonal aeroallergens worldwide. In warm temperate, subtropical and tropical environments, pollen of Bermuda grass (*Cynodon dactylon*) is a prevalent cause of allergies [1–5]. Bermuda grass, belonging to the subfamily Chloridoideae, has been reported to have a limited antigenic and allergenic cross-reactivity with grasses of the Pooideae subfamily [5–9], thus specifically requiring *C. dactylon*-allergenic extracts for diagnosis and treatment of allergic

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patients [8, 10]. The allergenic composition of this pollen has been investigated in several studies. All of them reported that, in the different populations of Bermuda grass-allergic individuals studied, most patients showed sensitivity to one allergenic component of the extract in the molecular weight range of 31–34 kDa on SDS-PAGE [4, 11–13]. This major allergen, Cyn d 1, belongs to the grass group 1 allergen family, to which a plant cell wall loosening (β -expansin) function and a probable serine protease activity have been assigned recently [14, 15]. Nevertheless, the identity of the amino acid sequence of Cyn d 1 with group 1 allergens from the Pooideae subfamily is only about 70%, and a low degree of antigenic and IgE-binding similarities has been observed [16, 17]. Cyn d 1 is a glycoprotein composed of a number of isoforms with pI values ranging from 5.6 to 7.4 [18, 19]. Several full-length cDNAs encoding Cyn d 1 isoforms have been identified and sequenced, and two main groups of isoforms, acidic and basic, have been established, with amino acid variations between both groups of 14% [20–22]. A minor 29 kDa component of Cyn d 1 was described in the first reports on the characterization of the allergen, which turned out to be a degradation product of the allergen lacking the first 31 N-terminal amino acid residues [23].

Here we present the production of a monoclonal antibody (mAb) that recognizes acidic and basic isoforms of Cyn d 1. The availability of this mAb enabled us to purify Cyn d 1 by immunoaffinity chromatography and to develop a two-site ELISA to quantify it in complex mixtures, such as pollen extracts. The analytical features of this assay and the correlation of the Cyn d 1 content with the allergenic potency of Bermuda grass pollen extracts are also reported. This method will be a valuable tool to improve the standardization of allergenic products intended for clinical use.

Material and Methods

Source Material and Preparation of Extracts

C. dactylon pollen was extracted at a 1:10 (w/v) ratio in PBS, pH 7.5, in the presence of 1 mM phenyl-methylsulfonyl fluoride (Sigma, St. Louis, Mo., USA), with magnetic stirring for 90 min at 4°C. The soluble fraction was separated by centrifugation at 22,000 *g* for 20 min at 4°C and filtered through 0.22 μ m filters (Sartorius, Göttingen, Germany). For allergen purification, the extract was used immediately whereas for other uses, it was stored frozen at –40°C until use.

C. dactylon pollen used for mice immunization and allergen purification was purchased from Allergen (Aengelholm, Sweden). Other allergenic extracts used in this work were obtained from ALK-Abelló

(Madrid, Spain) and had been prepared from different batches of pollens of *C. dactylon* and other grasses purchased from several suppliers.

Protein concentration was determined by the method of Lowry et al. [24].

Human Sera

A human serum pool, prepared by mixing equal-volume aliquots of sera from 30 allergic patients with hypersensitivity to *C. dactylon* pollen, as demonstrated by clinical history, positive skin reaction to a commercial in vivo diagnostic product (ALK-Abelló) and RAST classes 3 and 4, was used throughout this work.

Production of Antibodies

Female BALB/c mice (CRIFFA, Barcelona, Spain) were injected intraperitoneally with 100 μ g of *C. dactylon* pollen extract in Freund's complete adjuvant. On days 15 and 30 after the injection, mice were boosted with an identical amount of antigen in Freund's incomplete adjuvant. On day 52, mice were boosted intravenously with the same dose of antigen in PBS and, 3 days later, spleen cells from immunized mice were fused with P3.X63.Ag8.653 myeloma cells according to the method of Galfré and Milstein [25]. Ten days after the fusion, cell culture supernatants were screened for anti-Cyn d 1-specific antibodies by an ELISA with a preparation of Cyn d 1, purified by a combination of concanavalin A-Sepharose affinity chromatography and carboxymethyl-Sepharose chromatography according to the method described by Matthiesen et al. [17], on the solid phase. The P3.X63.Ag8 mouse myeloma culture supernatant was used as a negative control. The positive hybridomas were cloned and subcloned by limiting dilution.

Monospecific polyclonal antibodies against Cyn d 1 were produced in New Zealand rabbits as previously reported [17]. Both monoclonal and polyclonal antibody production was carried out in accordance with the principles of good laboratory practice issued by the Organization for Economic Cooperation and Development (as revised in 1997) and European Council Directive No. 609 (1986).

Purification and Characterization of Antibodies

Selected monoclonal antibodies were purified from hybridoma culture supernatants using a protein G Sepharose affinity column (Amersham Biosciences, Uppsala, Sweden), following the manufacturer's instructions. The isotype of the antibodies was determined by ELISA with anti-mouse subclass antisera (Nordic, Tilburg, The Netherlands).

Purification of Cyn d 1 by Immunoaffinity Chromatography

An immunosorbent column was prepared by coupling the anti-Cyn d 1 mAb 4.4.1 (20 mg) to 0.75 g of CNBr-activated Sepharose 4B gel (Amersham Biosciences), following the manufacturer's recommendations. *C. dactylon* pollen extract in PBS was passed through the column and, after extensive washing with PBS, Cyn d 1 was eluted using 100 mM glycine-HCl, pH 2.5. 1 ml fractions were collected in tubes containing 50 μ l of neutralizing buffer (1 M Tris buffer, pH 9.0). Fractions were pooled on the basis of protein content, dialyzed against distilled water, lyophilized and stored at 4°C until use. Purity and identity of Cyn d 1 was assessed by electrophoretic methods, N-terminal amino acid sequencing and amino acid composition analysis.

SDS-PAGE, 2D Electrophoresis and Immunoblotting

Electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed using Novex tricine gradient gels (10–20% acrylamide, Invitrogen, Carlsbad, Calif., USA), essentially following the method of Schägger and von Jagow [26]. 2D electrophoresis was carried out using the ZOOM IPGRunner System (Invitrogen), according to the manufacturer's instructions. Briefly, samples were dissolved in IPG gel rehydration buffer containing 8 M urea, 2% w/v 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% v/v carrier ampholytes, pH range 3–10, and 0.002% bromophenol blue, and the IPG strips were rehydrated in this solution for 16 h at room temperature. Separation of the proteins in the first dimension was followed by an equilibration step of the IPG strip in NuPage LDS sample buffer (Invitrogen), in the absence of reducing agent, for 15 min, after which proteins were separated by SDS-PAGE using NuPage Novex Bis-Tris gels (4–12% acrylamide gradient, Invitrogen). Bio-Rad standards were used as molecular weight markers (Bio-Rad, Hercules, Calif., USA), and the protein bands were detected with Coomassie brilliant blue. For Western blot, separated proteins were electrotransferred to nitrocellulose membranes according to the method of Towbin et al. [27]. Immunodetection of IgE-binding proteins using a serum pool from *C. dactylon*-allergic patients was accomplished with ¹²⁵I-anti-human IgE mAb HE2 [28].

Amino Acid Composition and N-Terminal Sequence Analysis

Amino acid composition analysis was carried out using reagents (AccQ.Tag) and equipment from Waters (Milford, Mass., USA).

N-terminal amino acid sequencing was carried out using an Applied Biosystems Procise 494 liquid-pulse sequenator (Applied Biosystems, Foster City, Calif., USA).

Similarity searches were performed in protein and DNA sequence databases (Gen Bank, EMBL, PIR, NBR, NBR3D and Swiss-Prot) using the BLAST program [29].

RAST Inhibition

The allergenic activity of *C. dactylon* extracts was evaluated by RAST inhibition, essentially following the method described by Ceska et al. [30], and expressed in BU/ml by comparison with an in-house reference, which had previously been tested for the presence of *C. dactylon* allergens by Western blot and calibrated by skin prick test [31].

Cyn d 1 ELISA

96-well microtiter plates (Costar, Cambridge, Mass., USA) were coated overnight at 4°C with 100 µl of anti-Cyn d 1 mAb 4.4.1 at 10 µg/ml in PBS. After blocking with 1% (w/v) BSA, 0.05% (v/v) Tween 20 in PBS (PBS-BSA-Tween), wells were sequentially incubated with samples and references, anti-Cyn d 1 rabbit serum (1:10,000 dilution), and goat anti-rabbit immunoglobulin antibodies conjugated with horseradish peroxidase (Calbiochem, San Diego, Calif., USA). Samples, controls and reagents were diluted in PBS-BSA-Tween, and all incubations were carried out for 1 h at room temperature with intermediate washes with 0.05% (v/v) Tween 20 in PBS between successive steps. Detection was accomplished by incubation in the dark for 30 min with a solution of *o*-phenylenediamine (DAKO, Glostrup, Denmark). The color reaction was then stopped by adding 2 N HCl, and the optical density was read at 492 nm with a 650-nm reference filter. A standard curve was constructed with eight twofold dilutions of affinity-purified Cyn d 1, starting from 50 ng/ml, and the variables optical density (OD₄₉₂) and Cyn d 1 concentration

were adjusted to a four-parameter logistic curve by the least-square method [32]. The limit of detection of the assay was calculated as the concentration of Cyn d 1 that corresponds to a response equal to the ordinate at the origin plus the standard deviation, using the logistic curve equation with the values for this parameter obtained in ten independent assays. The Cyn d 1 content in the test samples was determined by interpolation in the linear part of the standard curve. The precision of the assay was determined by analyzing six replicates of samples in the low, intermediate and high regions of the linear range in eight independent assays.

Results

Affinity Chromatography Purification of Cyn d 1

Eleven anti-Cyn d 1 monoclonal antibodies of IgG class were obtained from the fusion of P3.X63.Ag8.653 myeloma cells and spleen cells from a mouse immunized with *C. dactylon* pollen extract. Cross-competition experiments for the binding of ¹²⁵I-labelled Cyn d 1 among these mAbs enabled us to define two groups of mAbs recognizing partially overlapping epitopes (data not shown). The mAbs with the highest affinities for ¹²⁵I-labelled Cyn d 1 from each group were selected for preliminary experiments to develop an ELISA method for Cyn d 1 quantitation. All possible combinations of the antibodies, including the use of an anti-Cyn d 1 rabbit serum as the second antibody, were tested. The best results in terms of sensitivity and linearity were found with the mAb 4.4.1 (isotype IgG1, κ) as the capture antibody and an anti-Cyn d 1 rabbit serum as the second antibody. This mAb was also found to be very useful for the efficient purification of Cyn d 1 from a *C. dactylon* pollen extract by immunoaffinity chromatography. Approximately 0.5% of the total protein of the pollen extract applied on top of the column, measured by the Lowry method, was recovered as pure Cyn d 1. The high degree of purity achieved with this single-step purification procedure was assessed by SDS-PAGE. The electrophoretic patterns of Bermuda grass pollen extract, employed as the starting material for the purification, and affinity-purified Cyn d 1 are shown in figure 1. Cyn d 1 displayed a single band with an apparent molecular weight of 31.5 kDa (fig. 1B). This band was immunostained with the sera of *C. dactylon*-allergic patients in Western blot (fig. 1C), demonstrating that the purification process had not abolished the IgE-binding activity of Cyn d 1.

The identity of the purified protein was established by N-terminal amino acid sequencing and amino acid composition analysis. The first fifteen residues of the N-terminal amino acid sequence (NH₂-AI/MGDKPGPXITA-

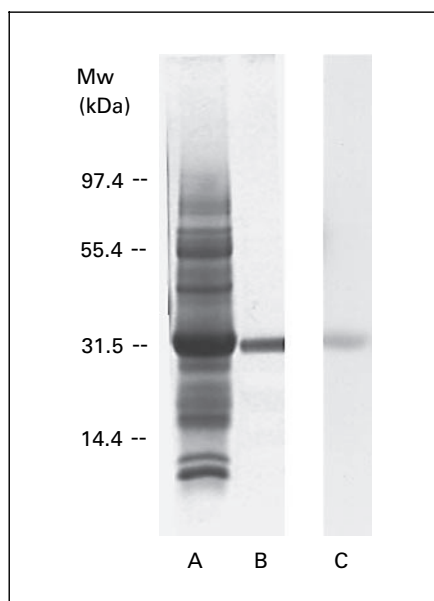


Fig. 1. SDS-PAGE analysis of *C. dactylon* pollen extract (100 µg, lane A) and Cyn d 1 allergen purified by affinity chromatography with mAb 4.4.1 (5 µg, lane B). Proteins were stained with Coomassie brilliant blue. In lane C, immunoblot of a sample of purified Cyn d 1, as in B, probed with a serum pool from Bermuda grass-allergic patients.

TYG) matched the sequence translated from the cDNA sequence of mature Cyn d 1 [20–22]. The unidentified residue at position nine corresponds to the N-glycosylated asparagine residue [19, 20]. The polymorphism observed at position two indicates that the isolated natural Cyn d 1 consists of a mixture of acidic and basic isoforms, as the presence of methionine or isoleucine at this position differentiates both groups of Cyn d 1 isoforms [22]. Moreover, the amino acid composition of immunoaffinity-purified natural Cyn d 1 agreed with that expected from a mixture of the amino acid sequences deduced from cDNA sequences [22] (data not shown).

The microheterogeneous composition of affinity-purified natural Cyn d 1 was confirmed by 2D electrophoresis. Figure 2A shows the Coomassie blue-stained pattern of this preparation. At least 8 spots within a pI range between 5.6 and 7.1 were visualized in the 31 kDa molecular weight region, in agreement with previously reported 2D patterns for Cyn d 1 [19]. Moreover, this pattern accurately corresponded with that obtained in a Western blot of the *C. dactylon* extract with a pool of sera from Bermuda grass-allergic patients (fig. 2B), demonstrating that the

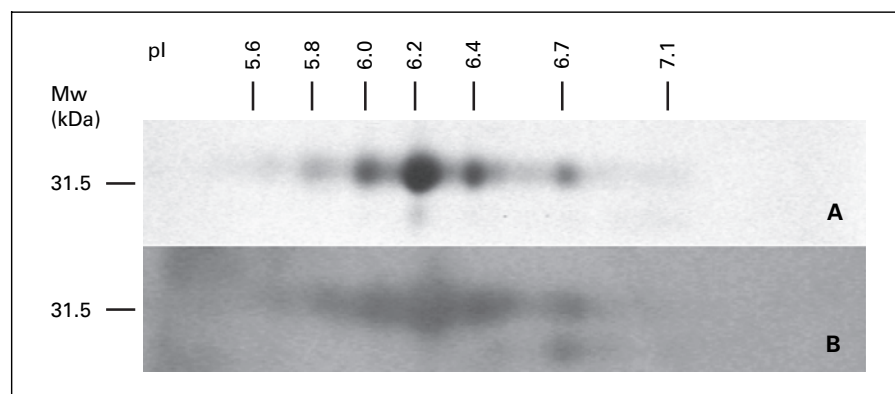


Fig. 2. **A** 2D electrophoretic analysis of affinity-purified Cyn d 1; 10 µg of Cyn d 1 was loaded and proteins were stained with Coomassie brilliant blue. **B** Western blot of a 2D electrophoresis of *C. dactylon* extract probed with a serum pool from human allergic patients; 200 µg of extract was loaded on the gel and 2D electrophoresis run on the same conditions as in **A**. Bound IgE was detected with ^{125} I-labelled anti-human IgE as described in Material and Methods.

mAb 4.4.1 recognizes all the major allergenic isoforms of Cyn d 1. A faint spot in the 29 kDa molecular weight region, at pH 6.2, was observed in the Cyn d 1 2D electrophoresis pattern, whereas no protein bands with this molecular weight were detected in SDS-PAGE. N-terminal amino acid sequencing of this protein suggested that it is a degradation product of the Cyn d 1 allergen, as it had also been observed for a 29 kDa contaminating band in Cyn d 1 preparations by other authors [23]. Thus, a possible explanation for the origin of this spot might be the degradation of the 31.5 kDa Cyn d 1 allergen during the hydration of the first-dimension isoelectric focusing gel, which was carried out with a solution of the allergen for 16 h at room temperature according to the manufacturer's instructions.

ELISA for Cyn d 1 Quantitation

As mentioned above, the method we have developed to quantify Cyn d 1 is a sandwich ELISA with the mAb 4.4.1 on the solid phase and an anti-Cyn d 1 rabbit serum as the second antibody. Detection of the rabbit antibodies bound is accomplished with goat anti-rabbit immunoglobulin antibodies conjugated with horseradish peroxidase.

The dose-response curves obtained in the ELISA with affinity-purified Cyn d 1 and *C. dactylon* pollen extract were parallel (fig. 3), which suggests that the allergen had not been antigenically altered during the purification process and that the assay was measuring the same molecule in the different preparations. Therefore, the concentra-

Table 1. Specificity of Cyn d 1 ELISA

Extract	Activity or concentration	OD ₄₉₀
Grasses		
<i>Cynodon dactylon</i>	100 BU/ml	3.848
<i>Avena sativa</i>	100 BU/ml	0.028
<i>Dactylis glomerata</i>	100 BU/ml	0.014
<i>Festuca pratensis</i>	100 BU/ml	0.016
<i>Hordeum vulgare</i>	100 BU/ml	0.081
<i>Lolium perenne</i>	100 BU/ml	0.019
<i>Phleum pratense</i>	100 BU/ml	0.011
<i>Poa pratensis</i>	100 BU/ml	0.011
<i>Secale cereale</i>	100 BU/ml	0.016
<i>Triticum sativum</i>	100 BU/ml	0.083
<i>Alopecurus pratensis</i>	100 BU/ml	0.025
<i>Anthoxanthum odoratum</i>	100 BU/ml	0.031
<i>Holcus lanatus</i>	100 BU/ml	0.017
<i>Phragmites communis</i>	100 BU/ml	3.728
<i>Zea mays</i>	5% (w/v)	0.123
<i>Agropyron repens</i>	5% (w/v)	0.216
<i>Agrostis alba</i>	5% (w/v)	0.067
<i>Arrhenatherum elatius</i>	5% (w/v)	0.106
<i>Bromus mollis</i>	5% (w/v)	0.082
<i>Cynosurus cristatus</i>	5% (w/v)	0.065
Weeds		
<i>Parietaria judaica</i>	100 BU/ml	0.029
<i>Plantago lanceolata</i>	100 BU/ml	0.026
<i>Artemisia vulgaris</i>	100 BU/ml	0.016
Trees		
<i>Olea europaea</i>	100 BU/ml	0.027
<i>Betula verrucosa</i>	100 BU/ml	0.019

Values of absorbance at 490 nm in Cyn d 1 ELISA. Extracts were assayed at a 1:50 dilution.

tion of affinity-purified Cyn d 1 was accurately measured by quantitative amino acid analysis, and this preparation was used as the primary standard in the ELISA method.

The assay developed in this work is very sensitive, with a detection limit of 0.24 ng/ml and a linear range of 1.1–9.2 ng/ml. The lower limit of this range (1.1 ng/ml) can be considered the limit of quantitation of the method. Moreover, the assay is highly reproducible. The mean intra-assay coefficient of variation in this ELISA was 6.8, 5.3 and 6.2% in the low, intermediate and high regions of the linear range, and the interassay coefficient of variation was 15.4, 13.6 and 14.5%, respectively.

The Cyn d 1 ELISA method showed a high specificity when it was evaluated against a large battery of extracts from pollens of grasses, trees, and weeds (table 1). Extracts from pollens other than grasses were negative and,

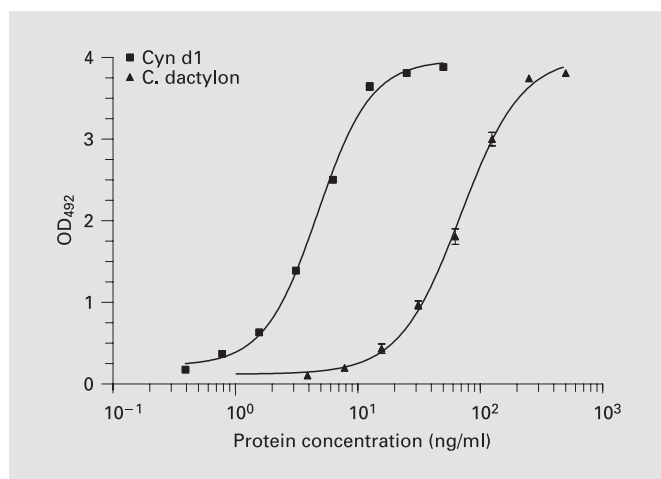


Fig. 3. Dose-response curves obtained in the mAb-based Cyn d 1 ELISA for a Bermuda grass pollen extract and affinity-purified Cyn d 1 allergen. Determinations were carried out in triplicate. Bars indicate standard errors of the mean.

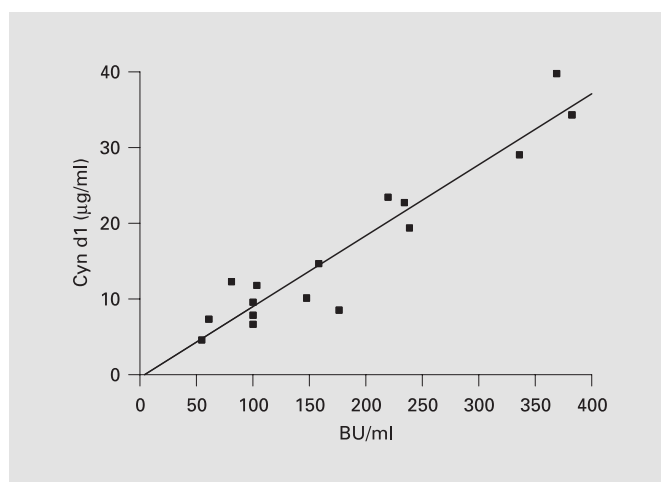


Fig. 4. Correlation between Cyn d 1 content (in µg/ml; measured by mAb-based ELISA, ordinate) and allergenic activity values (in BU/ml; determined by RAST inhibition, abscissa) in sixteen *C. dactylon* pollen extracts.

among grasses, only *Phragmites communis* gave a high response, *Agropyron repens* gave a very low response, and the rest were negative.

Quantitation of Cyn d 1 in *C. dactylon* Extracts and Correlation with Allergenic Activity

In order to assess the relationship between Cyn d 1 content and allergenic activity, we analyzed, by means of

Cyn d 1 ELISA and RAST inhibition, sixteen extracts from different batches of *C. dactylon* pollen. A comparison of the results obtained by both techniques is shown in figure 4. A very good correlation was observed, suggesting that Cyn d 1 content is highly representative of the total allergenic potency of the whole pollen extract. Moreover, an average Cyn d 1 content of 9.1 µg/ml in a *C. dactylon* extract with an activity of 100 BU/ml was estimated from the equation of the line of best fit.

Discussion

Monoclonal antibodies are very useful tools in many fields of biology and medical research as well as for diagnostic and therapeutic applications. Particularly in allergy, the quantitation of main allergens in mass units using monoclonal antibody-based ELISAs has notably improved the quality of allergen products intended for clinical use in the last years. In connection with this, we report here the production of monoclonal antibodies against the major allergen from the pollen of a widely distributed, clinically relevant grass, *C. dactylon*, and the use of one of these mAbs to purify this allergen and to develop an immunoassay to measure its content in allergenic extracts. The allergen was purified using an efficient affinity chromatography procedure based on the specificity of the mAb 4.4.1. The results obtained using different techniques to characterize the purified allergen allowed the unambiguous identification of the protein as Cyn d 1.

Cyn d 1 appeared as a single protein band at 31.5 kDa on SDS-PAGE. Nevertheless, it is worth mentioning that great precautions had to be taken to avoid the degradation of Cyn d 1 during the pollen extraction and purification processes. In agreement with observations by other authors [4, 23] regarding the lability of Cyn d 1 to the action of proteases and acidic conditions, we detected the presence of additional bands at 23 and 29 kDa on SDS-PAGE when the pollen extraction was carried out in the absence of protease inhibitors, and the neutralization of the acidic buffer used to elute Cyn d 1 from the affinity chromatography column was not carried out immediately after elution (data not shown). This instability of Cyn d 1 might also be the origin of the faint spot with a pI of 6.2 and a molecular weight of 29 kDa detected in the Coomassie blue-stained 2D electrophoresis pattern.

The existence of different allergen isoforms is a factor to be taken into account when designing an mAb-based immunoassay, as the epitope defined by the mAb might be absent in certain isoforms and, consequently, these

would not be detected in the immunoassay, rendering underestimated concentrations of the allergen. For this reason, we performed some experiments to characterize the protein recognized by the mAb 4.4.1. Thus, the polymorphism at position two of the amino acid sequence of the immunoaffinity-purified Cyn d 1 revealed that the mAb 4.4.1 had bound both acidic and basic Cyn d 1 isoallergens [22] from the pollen extract. Moreover, the 2D electrophoresis pattern of purified Cyn d 1 showed that the mAb recognized all the major allergenic isoforms that are detected in a Western blot with the polyclonal IgE from a pool of sera from Bermuda grass-allergic patients. Therefore, the mAb 4.4.1 was considered suitable to be used in a quantitation method for Cyn d 1.

Cyn d 1 was purified in an immunoreactive state, as shown not only from the results of immunoblotting after SDS-PAGE, but also by the fact that the dose-response curves obtained in the ELISA with the pure allergen and the pollen extract were parallel, indicating that the epitopes defined by the mAb and the polyclonal rabbit serum were equally recognized in both preparations, and hence that the conformation of the allergen had been preserved during the purification process. Furthermore, this parallelism enabled the use of a preparation of pure Cyn d 1, in which the protein concentration was accurately determined, as the primary standard to measure the concentration of the allergen in pollen extracts. This quantitation method has proved to be highly reproducible, with very low coefficients of variations, and sensitive.

A number of comparative studies have shown that Bermuda grass pollen possesses antigenic and allergenic properties different from those of other grass pollens [5–9], particularly of the Pooideae subfamily, which is mainly due to the absence of the group 5 allergen and the distinctive immunochemical characteristics of Cyn d 1 as compared to the group 1 from other grasses [16, 17, 23]. Nevertheless, some mAbs recognizing Cyn d 1 and group 1 from other grasses have been described, indicating that they might also share some antigenic determinants [17, 18]. We studied the specificity of the ELISA quantitation method we have developed by testing a large number of extracts from grass pollens. The only extract that gave a significant response was *P. communis*, belonging to the Arundinoideae subfamily, which has been reported to be allergenically different from grasses of the Pooideae subfamily [9]. This result reveals that there is a group 1 allergen in *P. communis* pollen that shares epitopes with Cyn d 1, particularly that defined by the mAb 4.4.1. This opens the possibility of purifying this homologous *P. communis* allergen by affinity chromatography in order to ascertain

its antigenic and allergenic properties, which have not been studied so far.

Finally, the good correlation obtained between the allergen content, determined by the Cyn d 1 ELISA, and the allergenic activity, determined by RAST inhibition, for sixteen *C. dactylon* pollen extracts clearly supports the previous findings that Cyn d 1 is the major allergen of *C. dactylon* pollen, contributing a high percentage of the total allergenic activity [17]. Therefore, the Cyn d 1 ELISA represents a very useful tool for the standardiza-

tion of *C. dactylon* allergen products in mass units of its major allergen, providing in addition a reliable indication of the allergenic activity of the whole extract.

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