

Cloning and high level expression of *Cynodon dactylon* (Bermuda grass) pollen profilin (Cyn d 12) in *Escherichia coli*: purification and characterization of the allergen

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Summary

Background Profilin, an actin-binding protein, was previously described as a panallergen which is involved in about 20% of the crossreactivity found among pollen and food allergic patients. This allergen is usually under-represented in natural extracts used for allergy diagnosis.

Objectives To obtain an immunologically active and soluble recombinant profilin from *Cynodon dactylon* pollen which could be used for diagnostic and therapy.

Methods Isolation of cDNA clones was performed by polymerase chain reaction amplification using degenerate primers. Expression in *Escherichia coli* BL21 (DE3) was carried out using vector pKN172, and the expressed product was isolated by affinity chromatography on poly L-proline-Sepharose.

Results Four cDNA inserts coding for *Cynodon dactylon* (Bermuda grass) pollen profilin (Cyn d 12) were cloned and sequenced. Full-length *C. dactylon* profilin gene was expressed in *Escherichia coli* as non fusion protein. Induced cells could produce high amounts of recombinant Cyn d 12, and after a single purification step on poly (L-proline)-Sepharose, up to 45 mg of pure allergen per litre culture could be obtained. The reactivity of recombinant Cyn d 12 with IgE antibodies present in sera from Bermuda grass-allergic patients is comparable to that of the natural Bermuda grass allergen. Recombinant Bermuda grass pollen profilin was shown to share B-epitopes with sunflower profilin.

Conclusions Our results showed that this heterologous expression system and purification procedure are suitable for the production of large amounts of pure allergen which can be used for the characterization of allergenic epitopes recognized by T and B cells and finally for diagnostic and therapeutic purposes.

Keywords: cDNA cloning, Bermuda grass, sequencing, PCR, recombinant allergen, expression, Cyn d 12

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Introduction

Type I allergy is a clinical disorder that affects up to 20% of the human population in developed countries [1]. Allergens for diagnostic and therapeutic purposes are usually extracted from natural sources, such as pollens. These extracts represent complex mixtures of proteins, and only a few of those

are allergens. Such preparations exhibit batch to batch variations in both quality and allergen content, due mainly to environmental conditions and proteases attack. The advent of molecular biology can overcome these problems by cloning and expression of the genes for the more common allergens, because the recombinant products are expected to represent pure, well defined, and reproducible compounds [2].

Bermuda grass (*Cynodon dactylon*) pollen (BGP) is an important worldwide aeroallergen in warm temperate and

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subtropical climates, such as Mediterranean area, south-western United States, northern and central Australia, South Africa, and Taiwan. Its major allergic component, Cyn d 1, has been found to consist of up to 11 isoforms with different isoelectric points [3–5]. Profilins, a ubiquitous family of actin-binding proteins [6], are prominent allergens in pollen of trees, grasses, and weeds, and in many fruits and vegetables. Although profilin is considered a minor allergen, except in the euphorbiacea *Mercurialis annua* where it is a major allergen [7], this allergen is recognized by 20% of all pollen-allergic patients [8–11]. Because of this broad allergic incidence, profilins have been described as pan-allergens [8,12,13].

The characterization of the individual IgE-reacting pattern can be achieved with a defined panel of major and minor recombinant allergens [14,15], and may consequently lead to the development of a patient-tailored immunotherapy. In the present study the cDNA encoding the BGP profilin has been analysed, and immunologically active and soluble recombinant allergen from *C. dactylon* pollen has been produced in *E. coli*.

Materials and methods

Isolation of Poly(A⁺)-mRNA and First-Strand cDNA Synthesis

Poly(A⁺) enriched RNA was isolated from 100 mg of BGP (Allergon AB, Valinge, Sweden) using the Quick Prep MicroRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). First strand cDNA was synthesized from 1 µg Poly(A⁺) enriched RNA using random hexadeoxynucleotide primers and Moloney murine leukaemia virus reverse transcriptase (First-Strand Synthesis kit, Pharmacia Biotech) as recommended by the manufacturer.

Amplification of BGP profilin cDNA by PCR

Degenerate oligodeoxynucleotide primers (Genosys Biotechnologies Inc., Cambridge, UK) for cDNA amplification were designed on basis of the amino acid sequence comparisons of different plant profilins. The primers used were 5'-AGA GAATTC CATATG TCGTGGCA(A/G)(A/G)CGT-ACGT and 5'-AGA AAGCTT (C/T)TACA(G/T)GCC(C/T)TGTTT(G/A/T)A(G/T/C)(G/A/C)AGGTA (where the slash between bases means that they were added at that particular position in equimolecular amounts during the synthesis of the primer). The regions corresponding to the 5' and 3' sequences, respectively, of the full-length cDNA encoding profilin are marked in bold, and the *EcoRI*, *NdeI*, and *HindIII* restriction sites are in italics. Products of the first-strand cDNA synthesis (5 µL) and primers were mixed in PCR standard mixture. After denaturation at 94 °C for

4 min, the sample was subjected to five cycles of denaturation at 94 °C (1 min), annealing at 45 °C (2 min), and extension at 72 °C (2 min), followed by 30 cycles at 94 °C (1 min), 56 °C (2 min), and 72 °C (2 min), and a final incubation at 72 °C (6 min) in a Robocycler 40 (Stratagene, La Jolla, CA, USA) with Taq DNA polymerase (Bioline, London, UK). PCR products were fractionated on 2% agarose. A DNA fragment from the predominant band (400 bp) was isolated by GeneClean (Bio101, La Jolla, CA, USA) and after cutting with *NdeI* and *HindIII*, cloned into the expression vector pKN172 [16]. The ligation mixture was used to transform competent cells of *E. coli* DH5α by the rubidium chloride method [17]. Restriction analysis and recombinant DNA techniques were performed by standard methods [18] or as recommended by the manufacturer.

Sequencing of BGP profilin cDNA

The nucleotide sequence of cDNA inserts was determined by the dideoxy chain termination method [19]. Standard protocols of the manufacturer for Taq DNA-polymerase-initiated cycle sequencing reactions with fluorescence-labelled dideoxynucleotide termination (Applied Biosystems Inc., Foster City, CA, USA) were used. The sequencing reactions were analysed on an ABI 373 A DNA sequencer. Commercial or vector-specific primers were used for sequencing both strands. Sequences analysis were done using the GCG Computer Package (Computer Group Inc., Madison, WI, USA).

Expression of BGP recombinant profilin

The T7 system, in which the gene of interest is expressed under the control of ϕ 10 promoter, was used. Competent *E. coli* BL21(DE3) [20] cells were transformed with the resulting recombinant plasmids. The cells were grown in LB supplemented with 200 µg/mL ampicillin to an absorbance of 0.6 at 600 nm. IPTG was then added to the culture to a final concentration of 0.6 mM, and incubation continued for another 3 h. After expression of the recombinant proteins, the cells were harvested by centrifugation. The pellet was resuspended in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and disrupted by sonication. Insoluble material was removed by centrifugation at 20 000 × *g* for 30 min.

Purification of BGP profilin

Proteins in the supernatant were applied to a 1 × 24 cm column of PLP-Sepharose column [21]. Briefly, the gel was prepared by coupling 30 mg poly (L-proline) (INC Biochemicals, Aurora, OH, USA) to 2 g of CNBr-activated Sepharose 4B (Pharmacia Biotech). The column was washed with 10 volumes of PBS, then washed with four volumes of 2 M urea in PBS, and finally the profilin was eluted with 6 M urea in

PBS. Protein containing fractions were pooled, dialysed overnight against water, lyophilized, and dissolved in PBS. Natural BGP profilin was purified by PLP-Sepharose chromatography from 500 mg of lyophilized BGP extract prepared as described [22]. Protein concentration was calculated, and the sample was dialysed, aliquoted and lyophilized. Determination of the protein concentration was performed according to Bradford [23].

Electrophoresis and immunoblotting

Natural and recombinant proteins were analysed by SDS-polyacrylamide gel electrophoresis [24] in 12.5% polyacrylamide gels under reducing conditions. Proteins were visualized by Coomassie Brilliant Blue R-250 staining or electrophoretically transferred to PVDF membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA) [25]. For IgE-binding analysis, PVDF membranes (containing dot-blotted or electroblotted proteins) were incubated overnight at 4 °C with BGP-allergic patients sera. Alternatively, the membranes were incubated for 60 min at 37 °C with rabbit polyclonal diluted serum (1:3000) against sunflower purified profilin. Immunochemical staining was performed as previously described [22]. Image analysis was performed using BioImage software (Millipore).

Antisera

Sera of BGP-allergic patients were selected according to case histories, the display of symptoms after inhalation of

BGP, positive skin-prick test, and radioallergosorbent test class greater than 2. Rabbit polyclonal antibodies against purified sunflower profilin (Arilla MC, unpublished results) were also employed to characterize recombinant protein.

Inhibition assays

For ELISA inhibition assays, microtitre plates were coated with 100 μ L/well of natural BGP profilin (1 μ g/mL) in 0.1 M bicarbonate buffer pH 9.6 and blocked with blocking buffer (PBS supplemented with 0.05% Tween 20 and 1% BSA). 100 μ L aliquots of profilin antisera (1:6000 dilution in blocking buffer), previously incubated with different concentrations of natural and recombinant BGP profilin, were subsequently added and incubated for 1 h at 37 °C.

For immunoblot-inhibition studies, 1 mL of 1:2 diluted pool of five sera from BGP-allergic patients was incubated with 10 μ g purified recombinant profilin or 10 μ g BSA (negative control) overnight at 4°C. The pre-adsorbed serum was then used for immunoblot experiments.

Results

Amplification of BGP profilin cDNA and sequence analysis

PCR amplification of cDNAs using degenerate primers produced two main fragments of 310 and 400 bp. The largest one, corresponding in size with the full-length profilin gene, was subcloned in the expression vector pKN172. Four

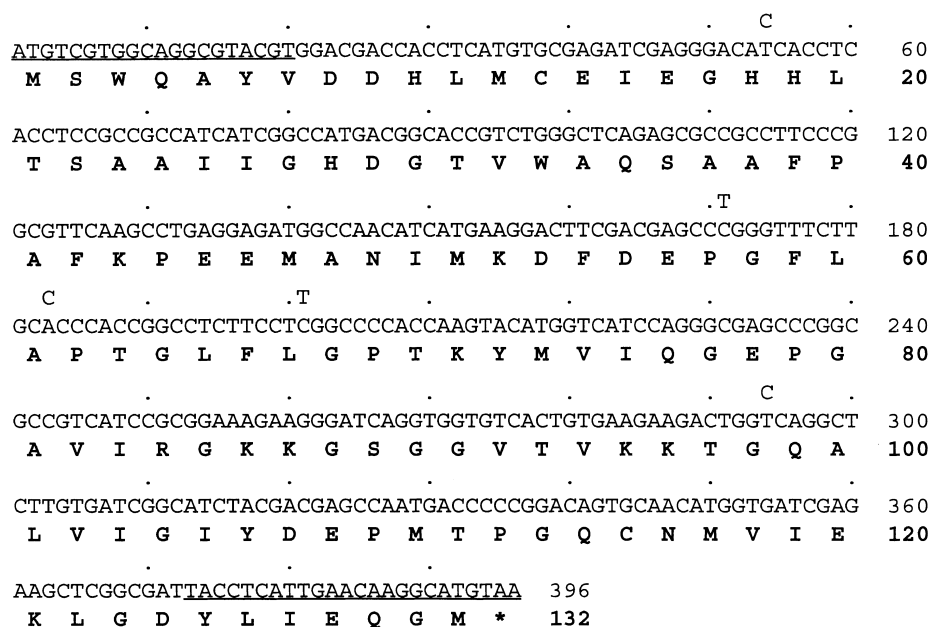


Fig. 1. Completed nucleotide and amino acid deduced sequences of BGP profilin: CdPRO1 and CdPRO2. Only different nucleotides from CdPRO1 are shown in CdPRO2 sequence. Primers used for cDNA amplification are underlined. The nucleotide sequences reported have been submitted to the GeneBank/EBI Databases under accession numbers Y08390 (CdPRO1) and Y08389 (CdPRO2).

	1				50
Cynodon	MSWQAYVDDH LMCEIEGH	HLTSAAIIG	HDGTVWAQSA	AFPAFKPEEM	
Zea1	...T...E.	V. ...AT...T ...E.....			
Phleum	...T...E.	..A...L.	D..Q.....I		
Phaseolus	...T.....L.....N	...H...L. Q..S...K.. S..Q.....I			
Triticum1	...T.....C...D.Q	L. ...S...TE.P N..K.....I			
Hordeum	...T.....C...D.Q	L. ...R..V..P N..Q.....I			
Betula	...T...E. ...D.D.QAS	NS. AS..V. ...S.....S S..Q...Q.I			
Nicotiana	...T.....AD...QQG	H..AA...L. ...S.....S T..K.....I			
	51				100
Cynodon	ANIMKDFDEP GFLAPTGLFL	GPTKYMVIQG	EPGAVIRGKK	GSGGVTVKKT	
Zea1	.A.....H.....I. .G.....		I.....		
Phleum	TG.....H.....M.V	AGA.....	...R.....A..I.I...		
Phaseolus	TG..N..N..T.....YI	.G.....	...S.....P.....		
Triticum1	.G.V...E..H.....	.G.....	...V.....T..I.I...		
Hordeum	.G.I.....H.....	.G.....	...V.....T..I.I...		
Betula	TG....E..H.....H.	.GI.....	.A.....I..I...		
Nicotiana	T.....H.....	.GA.....	I..I...		
	101				140
Cynodon	GQALVIGIYD EPMTPGQCNM	VIEKLG DYLI	EQGM		
Zea1	..S.I.....L	.V.R.....L			
Phleum	V.....	.V.R.....V			
Phaseolus	NL.....	IV.R.....L			
Triticum1	.M..IL....L	.V.R.....D..YWVPSSN			
Hordeum	.MP..IL....L	.V.R.....V	...F		
Betula	F...E..V.....	.V.R.....D..L			
Nicotiana	N...IF...E..V.....	.V..IR...V	D..Y		

Fig. 2. Comparison of different plant profilins: *Zea mays* profilin 1 (X73279), *Phleum pratense* (X77583), *Phaseolus vulgaris* (X81982), *Triticum aestivum* profilin 1 (X89825), *Hordeum vulgare* (U49505), *Betula verrucosa* (M65179), and *Nicotiana tabacum* (X82120). Points indicate identity of residues with respect to the upper sequence.

cDNA inserts turned out to be two very similar sequences, CdPRO1 and CdPRO2, differing by five changes in the third position of each codon and hence representing silent mutations (Fig. 1). Both sequences encoded a polypeptide of 131 amino acids with a predicted molecular mass of 14.1 kDa. Comparison of the deduced amino acid sequence of the BGP profilin revealed high homology with other described plant profilins [9,26–30]. Similarities among the different proteins were apparent throughout the entire sequence, although several regions of high sequence conservation were obvious in the alignment (Fig. 2). Pairwise alignments indicated the highest homology with *Zea mays* profilins (88% identity average) and the lowest with Bet v 2 (74% identity) from birch (*Betula verrucosa*). BGP profilin was consequently named Cyn d 12 in accordance with IUIS nomenclature [31]. Much lower identities were found with nonplant profilins: *Acanthamoeba castellanii*, 46%; *Physarum polycephalum*, 42%, and human, 32%.

Expression and purification of Cyn d 12 protein

A non-fusion form of Cyn d 12 was expressed in *E. coli* BL21 (DE3) after induction with IPTG. The yield of rCyn d

12 produced using the described conditions (cell density of 0.6, 0.6 mM IPTG, and 3 h expression) was 30–40% of total protein. No appreciable increase in yield was found at higher cell densities, increased IPTG concentrations, or longer expression times. More than 95% of expressed protein was soluble. The soluble material was chromatographed over a PLP-Sepharose affinity column, to which profilin specifically was bound. After elution with 6 M urea in PBS, rCyn d 12 showed a single band with an apparent molecular mass of 14.5 kDa, as determined by Coomassie Brilliant Blue-stained SDS-PAGE (Fig. 3a). Image analysis of the silver stained-gels showed more than 95% purity of the 6 M eluted rCyn d 12. The yield of purified recombinant Cyn d 12 was about 45 mg/L of bacterial culture. Natural Cyn d 12 was isolated from BGP extracts by PLP-Sepharose affinity chromatography giving a yield of 9 mg purified protein per 600 mg BGP extract protein (100 g BGP). Both recombinant and natural Cyn d 12 showed similar apparent molecular mass (Fig. 3).

Immunological characterization of recombinant Cyn d 12

Soluble proteins of cells expressing rCyn d 12 as well as

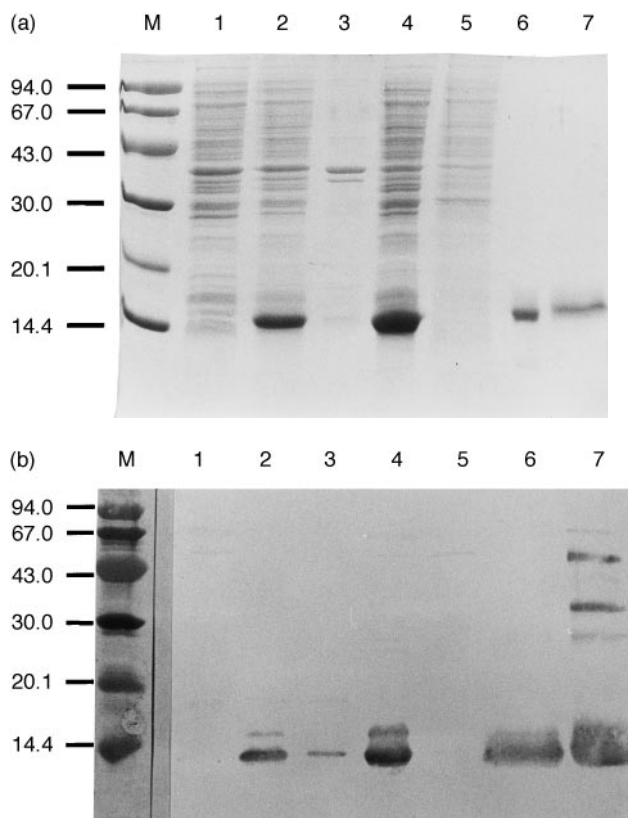


Fig. 3. Purification of recombinant BGP profilin expressed in *E. coli*. (a) 12% SDS-PAGE analysis of the purification procedure. (b) Western blotting of (a) using rabbit antiprolin serum. Lane M, molecular mass markers; lanes 1 and 2, total cell lysate from *E. coli* BL21 (DE3) containing pKN172 alone and pKN172 with CdPRO1 insert, respectively; lanes 3 and 4, insoluble and soluble fractions of sample on lane 2, respectively; lane 5, PLP-Sepharose column non-retained fraction; lane 6, 8 M urea elution of profilin from PLP-Sepharose column; lane 7, natural BGP profilin.

purified rCyn d 12 were used for immunological assays. After Western blotting and immunostaining, recombinant and natural BGP profilin were recognized by a rabbit anti-serum obtained against sunflower profilin (Fig. 3b). No antibody recognition was found using preimmune rabbit sera (data not shown). These data show the existence of some IgE-binding sites shared by natural and recombinant BG profilin and sunflower pollen profilin.

Cyn d 12 from BGP extracts gave rise to 20% positive reaction rate with IgE in sera from 30 patients with BGP allergy. Sera from eight selected BGP allergic individuals and serum pool from grass-allergic patients were tested for IgE-reactivity to native (dot-blotted) and denatured/reduced (SDS-PAGE electroblotted) purified rCyn d 12. In both assays rCyn d 12 showed comparable IgE-binding properties. IgE from all patients bound mainly to a 32 kDa band, probably the major allergen of BGP (Cyn d 1) [3]. All patients, except

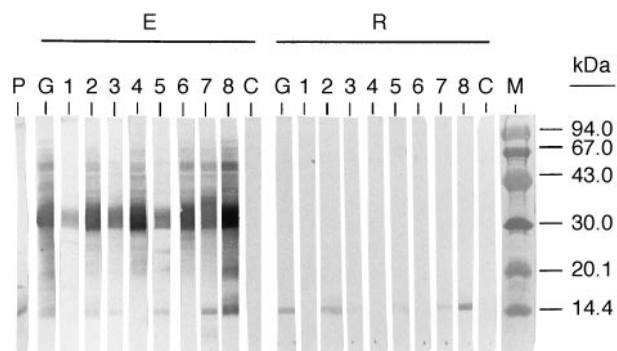


Fig. 4. Immunoblot-comparison of the IgE-reactivity of BGP allergic patients with natural BGP proteins (E) and purified recombinant BGP profilin (R). Proteins transferred onto PVDF-membranes were probed with sera from BGP allergic patients (lanes 1–8), serum pool from grass allergic patients (lane G) and serum pool from healthy individuals (lane C). Molecular mass markers (lane M) and purified recombinant BGP profilin (lane P) were stained with Amido Black B.

#1, #4, and #6, showed IgE reaction with the 14.5 kDa band of Cyn d 11 from both BGP extract and recombinant allergen (Fig. 4).

Inhibition experiments

To perform ELISA inhibition experiments, microtitre plates were coated with nCyn d 12 and incubated with profilin-specific antibodies previously preincubated with different

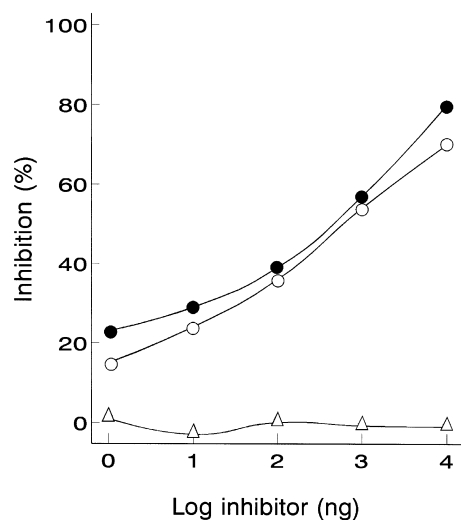


Fig. 5. Competitive inhibition of the binding of BGP profilin rabbit IgG antibodies to solid-phase natural BGP profilin by natural (●) or recombinant (○) BGP profilin. Control experiments were performed with BSA (△).

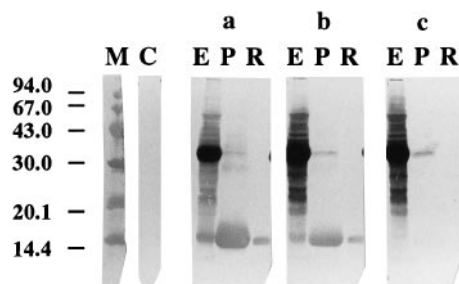


Fig. 6. Immunoblot-inhibition. Protein extract (E), purified natural (P) and recombinant (R) profilin from BGP were separated by SDS-PAGE and transferred onto PVDF-membranes. Inhibition was performed by incubating patients' serum pool with recombinant BGP profilin (c) and with BSA (b) as control. Serum pool without preincubation (a) was also used as control. Lane M, molecular mass markers. Lane C, control immunoblot with serum pool from healthy individuals.

concentrations of natural and recombinant Cyn d 12. Both antigens were able to inhibit at a similar level rabbit IgG binding to immobilized nCyn d 12 (Fig. 5).

In immunoblot-inhibition assays, preincubation of a pool of the five profilin-reacting sera shown in Fig. 4 (patients # 2, 3, 5, 7, and 8) with purified rCyn d 12 (10 µg/mL) completely inhibited IgE binding to nCyn d 12, both in monomeric and dimeric forms, while preincubation with BSA did not (Fig. 6). This result showed that most of the allergenic determinants of natural BGP profilin are present in the recombinant BGP profilin. A 32 kDa protein in the purified natural profilin sample bound IgE antibodies for BGP allergic patient serum pool independently of if the serum was preadsorbed or not.

Discussion

Current forms of *in vitro* and *in vivo* diagnosis are based on the use of crude allergen extracts. However, it is rather difficult to obtain reproducible batches of allergens from crude extracts of natural sources, where some minor allergens, like profilin, are often under-represented [32]. For this reason, there is great interest in pure and reproducible preparations of allergens for diagnostic, therapeutic and experimental purposes. Cloning and production of recombinant allergens in heterologous expression systems may provide defined proteins for these purposes. A simple and efficient method has been employed to clone BGP cDNA molecules, encoding the translated region of the panallergen profilin from *Cynodon dactylon*, and to obtain high level expression of this recombinant allergen in *E. coli*.

The existence of profilin isoforms in pollen has been described in *Triticum aestivum* [26], *Zea mays* [30], *Nicotiana tabacum* [33] and *Olea europaea* [34], and might

represent a mechanism to protect cells from the loss of the important function that profilin plays in regulating the eukaryotic cytoskeleton. The unique polypeptide sequence found in all four cDNA clones sequenced and the abolition of all the IgE-binding to nCyn d 12 for its recombinant counterpart could indicate that only one profilin is expressed in *C. dactylon* pollen, or if there are more isoforms, they all have the same IgE-binding properties. The alignment of profilin amino acid sequences, and the existence of three-dimensional structures of related profilins [35] allow the identification of conserved domains, which could contain T- or B-cell epitopes. The new strategy for treatment of allergic diseases, based on synthetic peptides resembling T- and B-cell epitopes, has been successfully used in an experimental murine system with peptides derived from Fed d 1 allergen [36].

The yield of rCyn d 12 (45 mg/L culture) is higher than Cyn d 12 purified from BGP extract (9 mg/100 g BGP). Additionally, the purity of the recombinant preparation is > 95%, while the profilin purified from the extract contained insignificant amounts of contaminating Cyn d 1. This major allergen of BGP [3] co-purified with profilin by unspecific binding after one pass through PLP-Sepharose column, and could interfere in *in vivo* and *in vitro* diagnostics (Fig. 6). Improvement of the purification could be achieved using high resolution chromatography (SMART, Pharmacia Biotech) as described previously [37], or a second affinity column pass, but these procedures drastically reduce the yield of purified Cyn d 12.

Immunochemical assays performed in this study on recombinant Cyn d 12 showed that it is antigenically and allergenically similar to the natural allergen. Our results showed that this heterologous expression system and purification procedure are suitable for the production of large amounts of pure allergen which can be used for the characterization of allergenic epitopes recognized by T and B cells, and finally for possible diagnostic and therapeutic purposes.

Acknowledgements

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