Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 250-263

High level expression of birch pollen profilin (Bet v 2) in *Escherichia coli*: purification and characterization of the recombinant allergen

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Received August 17, 1995

SUMMARY: Up to 20% of the population in industrialized countries suffer from type I allergic symptoms (rhinitis, conjunctivitis, and bronchial asthma). The cDNA coding for birch pollen profilin, a highly conserved cross-reactive allergen and actin-binding protein was expressed in *Escherichia coli*. Upon induction with IPTG up to 30 mg recombinant profilin per liter culture could be obtained. A single step purification protocol based on the high affinity of profilin to poly-(L-proline) Sepharose was used to obtain large amounts of soluble and pure recombinant birch profilin. Recombinant birch pollen profilin specifically bound IgE, elicited dose dependent histamine release from patients basophils and could be used for skin prick testing without toxic effects. The results indicate that by using purified recombinant profilin, specific diagnosis of type I allergy might be improved.

Type I allergy is mostly caused by formation of IgE-antibodies against airborne antigens which upon cross-link of mastcell and basophil bound IgE (1), release biolgical active mediators such as histamine and then lead to allergic rhinitis, conjunctivitis and bronchial asthma in up to 20% of the population (2). Current diagnosis of allergy uses assays which are based on the binding of IgE-antibodies to mostly crude allergen extracts. The accuracy of diagnosis therefore depends on the presence of sufficient amounts of non-degraded allergens in the natural extracts.

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0006-291X/95 $12.00

In the last years cDNAs coding for a considerable number of allergens were isolated (3, 4). In contrast to crude allergen extracts which contain a number of different allergens and non-allergenic components, purified recombinant allergens allow to specifically determine the reactivity pattern of allergic patients. For tree and grass pollen allergy it was already shown that *in vitro* diagnosis can be achieved with a defined panel of recombinant allergens (5, 6). The characterization of the individual IgE-reactivity pattern may consecutively lead to the development of a patient tailored immunotherapy. The use of recombinant allergens for diagnosis and therapy of allergic diseases may be an advantage especially in those cases where the desired allergens are less represented in the natural allergen extracts. This applies for profilin, an actin-binding protein and ubiquitous allergen (7-13). The experiments with purified recombinant birch profilin promised an improvement of diagnosis and therapy of type I allergy.

MATERIALS AND METHODS

Characterization of allergic patients-Allergic patients and control individuals were characterized by case history, RAST, skin prick test and by testing for IgE-reactivity with natural and recombinant tree pollen allergens as was described (5).

Expression of recombinant birch profilin in *E. coli*-The birch profilin cDNA was transfered from pKK223-3 (Pharmacia, Uppsala, Sweden) to pMW175 resulting in pMW175Prof. pMW175 is derived from pMW172 (14) by changing the multiple cloning site (mcs) (Fig. 1). pMW172 is a derivative of pRK172 (15) which consists of approximately 2300 bp pBR322 (*Pvu*II 2066 to *Eco*RI 4361) and an insert of app. 280 bp containing 28 bp of the gene 10 promoter of bacteriophage T7 and an appropriately located mcs for genes to be expressed. The copy number control site of pBR322 is therefore removed from the plasmid and it is maintained at high copy numbers. Profilin was expressed from pMW175Prof in *E. coli* strain BL21(DE3) (16). This strain contains a copy of the T7 RNA polymerase gene under control of the *lac* UV5 promoter, inducible by isopropyl-β-thio-galactopyranoside (IPTG). The T7 RNA polymerase is integrated into the genome by the lysogenic phage lamda DE3.

Fermentation of cells-Precultures were grown over night on Lurea broth or Terrific broth (17) in the presence of 50µg/ml ampicillin. Cultures for production of birch profilin were inoculated to a concentration of approximatly $4x10^7$ cells/ml (oD_{600}=0,1). Medium used for production of profilin was an enriched variation of Lurea broth, containing 1% Peptone 140, 0,5% Yeast extract, 1% NaCl, 10 mM $MgSO_4$, 0,25% glycerol, 8,5 mM KH_2PO_4, 36 mM K_2HPO_4 pH 7.4. Phosphate buffer was autoclaved together with glycerol separately in tenfold concentration and

added to the sterilised basic medium. The growing cells were induced with 0,4 mM IPTG at oD_{600}=1.0 and 6 h later oD_{600}=3.5 was reached, cells harvested and frozen at -20°C. A yield of 5.5 g (wet weight) per l medium was achieved.

Purification of highly soluble recombinant birch profilin-Five g of frozen cells were thawed at room temperature and suspended in 5 ml/g wet weight of homogenisation buffer (HB: 25 mM imidazole, pH 7.4, 10 mg/l phenylmethylsulfonylfluoride (PMSF), 5 mM ß-mercapto ethanol (Et-SH), 0.1% Triton X-100) by pressing the suspension several times through a yellow Gilson tip mounted on a 50 ml syringe. This suspension was frozen and thawed two more times and diluted to 10 ml/g cells with HB. The extract was cleared by centrifugation (40 min. 18 000 rpm, Sorvall), treated for 15 min with Bioacryl-gel BPA-1000 (Toshiba) and centrifuged. The resulting extract was dialysed over night against buffer A (25 mM imidazole, pH 7.4, 10 mg/l PMSF, 5 mM Et-SH, 150 mM NaCl). The dialysate was applied to a 1x15 cm column of poly (L)-proline Sepharose. The gel was prepared by coupling 1g poly (L)-proline (Sigma, St. Louis, USA) to 15 g (dry weight) of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) as recommended by the manufacturer. The column was washed with buffer A until the baseline was reached, then washed with 2M urea in buffer A and finally the profilin was eluted with 6M urea in buffer A (18). Protein containing fractions were pooled, dialysed over night against 5 mM Phospate buffer pH 7.4, 0.5 mM Et-SH, concentrated in a vacuum evaporator, redialysed and - after determination of protein concentration - aliquoted and lyophylized. The yield of the preparation was approximately 30mg per 5g (wet weight) of cells.

Concentration of purified profilin was determined by measuring the extincton at 288 nm (mol. ext. coefficent 16 938, Mol. Wt. 14270) (19). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to (20).

Proteinextracts and IgE-immunoblots-Aequous birch pollen protein extracts were prepared from pollen of white birch (*Betula verrucosa*) as described (21) and stored lyophilized at -20°C until use. Pollen was purchased from Allergon, AB, Välinge, Sweden. Purified recombinant birch profilin was prepared as described above. Proteins were separated by SDS-polyacrylamide gel electrophoresis (20) and transferred to nitrocellulose (22). A 100 µg/cm gel total birch pollen proteins and 0.5µg/cm gel recombinant birch pollen profilin was applied to the gels as determined by Coomassie brilliant blue staining of gel sections using a rainbow protein marker (Amersham, Buckinghamshire, UK) as a standard. Nitrocellulose strips containing blotted proteins were incubated with 1:10 dilutions of allergic patients sera and bound IgE was detected using ^{125}I labelled anti-human IgE antibodies (Pharmacia, Uppsala, Sweden) as described (23).

IgE-inhibition studies-For the determination of the specific IgE-binding capacity of recombinant birch profilin, non-denatured allergens were used. Serial dilutions of allergic patients sera were tested for IgE-binding to non-denatured dot blotted birch pollen extracts, to determine a dilution where antigens were in excess to IgE

antibodies. 1:10-1:20 diluted sera were preabsorbed with 100 µg natural birch pollen proteins, with 5 µg recombinant birch profilin or 5 µg dog albumin (negative control) overnight at 4°C. The preabsorbed serum dilutions were then used to bind to natural birch pollen extracts which had been dot blotted to nitrocellulose strips cut to exactly the same size 10x5 mm. Bound IgE was detected with ^{125}I labelled rabbit anti-human IgE as described for the immunoblots. After washing strips were dried on Whatmann 3MM paper (Maidstone, USA) and counted in a γ-counter LKB, Uppsala, Sweden. After counting strips were subjected to autoradiography for control purposes. All determinations were done as duplicates and the results which are displayed in Table 1 reflect mean values.

Histamine release from patients basophils-Heparinized blood samples were taken from profilin allergic patients, from patients with different allergies and healthy individuals after informed consent was given. Granulocytes were prepared by dextran sedimentation (24). Cells were incubated with different concentrations of recombinant birch profilin, without additional protein and anti-human IgE mAB as described (25). Histamine was determined in the cell free supernatants using a RIA (Immunotech, Marseille, France).

Skin testing-For skin prick testing 10µl of each test solution was placed on the patients forearms at least 3cm apart to avoid false positive results. The skin was then pricked with a sterile lanzette and the reactions were recorded after 20 minutes (26). Recombinant birch profilin was resuspended in sterile 0.9% NaCl, sterile 0.9% NaCl was used as a negative control, histaminehydrochloride as a positive control. Commercial skin prick solutions (grass-mix, birch) were from ALK, Copenhagen, Denmark. The skin-prick tests were performed by the same investigator at the same day.

RESULTS

Construction of the plasmid pMW175Prof for high level expression of birch profilin in *E.coli*

It was found in several experiments that redundant sequences in cDNAs could reduce expression of the gene product in bacteria, even when these sequences were downstream of the coding region and did not influence the distance of the start codon to the ribosome binding site. The coding region of the cDNA coding for birch profilin (7) was transcribed by PCR to a fragment suitable to be inserted into the expression vector (Fig.1). Primers were used which contained an *Afl*III site at the start ATG of the cDNA and an *Eco*RI site 30 bp 3' of the termination codon; the *Afl*III restriction site is compatible with the profilin sequence and the enzyme creates ends, compatible with the *Nco*I end of pMW175. The resulting PCR fragment was cut with *Afl*III and *Eco*RI and inserted into the vector.

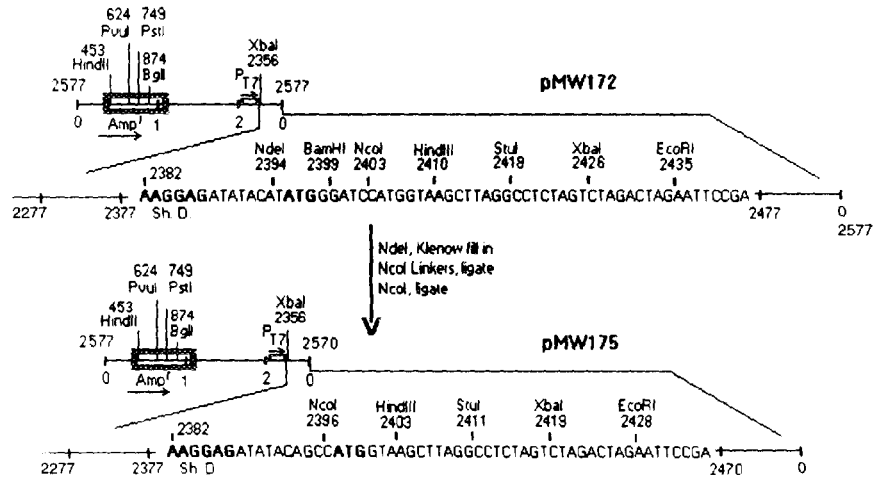

Figure 1. Construction of the expression plasmid pMW175Prof. Abbreviations: P_{tac}, *tac* promoter; $rrnBT_1T_2$, ribosomal transcription terminator (Brosius, J. and Holy, A., 1984. Proc. Natl. Acad. Sci. USA **81**: 6929); P_{T7}, gene 10 promoter of bacteriophage T_7.

Expression of recombinant birch profilin in *E. coli*

Induction of profilin expression by IPTG was about 4 fold, resulting in approximatly 3-5% of total protein. Induction was complete at 4h; further growth of the cells (up to 6h) did not result in a significant change of the ratio of profilin to background, but enhanced the yield of cells by 30%. Growth of cells over night in presence of IPTG did clearly reduce the percentage of profilin in total protein (data not shown).

Purification of highly soluble recombinant birch profilin

Extraction of cells resulted in a cleared extract containing approximately 15-20% profilin (of total protein) (Fig.2, lane 1). The pelleted cell debries (Fig.2, lane 2 represented double the amount of cells compared to lane 1) did not contain large amounts of profilin, and a second extraction did not recover substantial amounts. Fig.2, lane 3 shows purified profilin (5µg). The purity of the product was estimated to be >99%. The protein was lyophylized to get a stable formulation for shipment and extended storage. After half a year of storage in the lyophilized form no degradation was observed and IgE-reactivity was fully retained. The solubility of the lyophilized

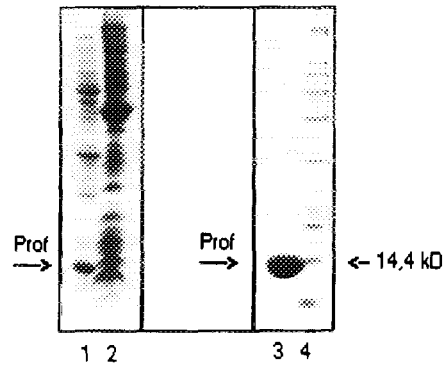

Figure 2. Analysis of *E. coli* for profilin content.

SDS-PAGE: lane 1, soluble proteins; lane 2, pelleted cell debries; lane 3, 6M urea eluate from poly-(L) proline sepharose (5µg); lane 4, mol. weight marker.

preparation was estimated approximatly 99% by analyzing high speed supernatants and pellets of the dissolved protein by SDS-PAGE and Coomassie Blue staining.

Comparison of allergic patients IgE-reactivity with natural birch pollen proteins and recombinant birch profilin by immunoblotting

Sera from birch pollen allergic individuals (Figure 3: lanes 1-7), a non-allergic control individual (lane 8) and buffer were tested for IgE reactivity to nitrocellulose blotted natural birch pollen allergens (Figure 3a) and purified recombinant birch profilin (Figure 3b). Sera with specificity for birch pollen profilin (lanes 3-6) showed less pronounced signals at 14kD with natural birch pollen proteins than with purified recombinant birch profilin, indicating that the natural birch pollen extract contains insufficient amounts and possibly in part degraded profilin. Strong IgE-reactivity is observed to the major birch pollen allergen, Bet v 1 (27) at 17 kD, which is abundantly present in natural birch pollen extracts. Patients 5 and 6 show additional IgE-binding to birch pollen allergens of more than 30kD molecular weight. IgE-reactivity to recombinant birch pollen profilin is observed exclusively in patients which gave binding at 14kD with natural birch pollen proteins but not in patients with strong and exclusive IgE-reactivity to Bet v 1 at 17kD (patient 1 and 7). IgE-binding to natural birch pollen profilin could be completely abolished by preadsorption of the sera with recombinant birch profilin (data not shown).

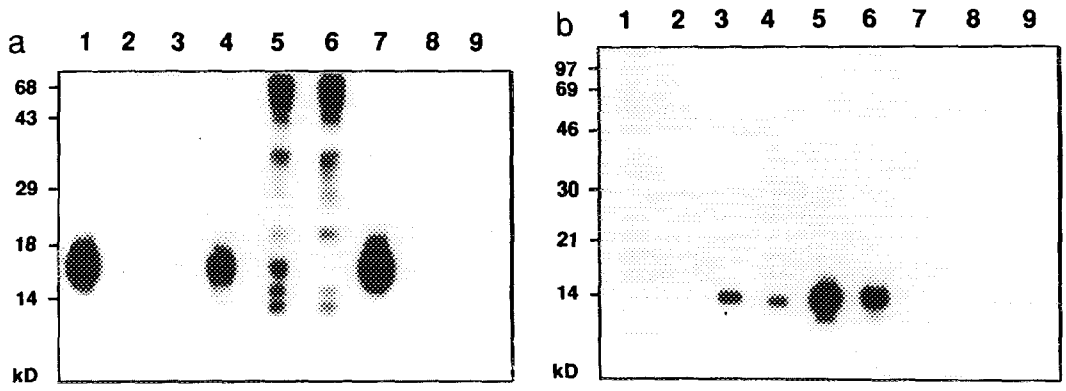

Figure 3.

Immunoblot-comparison of the IgE-reactivity of birch pollen allergic patients with natural birch pollen proteins (a) and recombinant birch profilin (b).

Sera from 7 birch pollen allergic individuals (lanes 1-7), a healthy nonallergic individual (lane 8) and buffer without addition of serum (lane 9) were tested for IgE-reactivity with nitrocellulose blotted natural birch pollen allergens (a) and purified recombinant birch profilin (b).

IgE-adsorption studies using non-denatured natural and recombinant allergens

To investigate the percentage of specific IgE directed against non-denatured recombinant birch profilin, adsorption studies were done. Serum dilutions were determined, where antigen was in excess to IgE antibodies. The diluted sera were then incubated with natural birch pollen extracts, recombinant birch profilin or dog albumin (28) as a control protein. The same panel of sera as in the IgE-immunoblots was used for the adsorption studies. Table 1 shows the percentage of profilin specific IgE in the sera of profilin allergic patients. The percentage of inhibition is in rough agreement with the immunoblot pattern of the individual patients. Patients with exclusive profilin reactivity such as patient 3 and patients with strong IgE-reactivity against the blotted profilin (#3, 5, and 6) showed a high percentage of profilin specific IgE (> 25%) whereas the serum of the patient with weak profilin reactivity (#4) contained little profilin specific IgE. Almost no birch pollen specific IgE could be absorbed from sera without reactivity to immunoblotted birch profilin (patients #1, 2, and 7). The non-allergic individual (#8) showed no IgE-reactivity with dot blotted birch pollen allergens.

Table 1

Percentage inhibition of IgE-binding to natural birch pollen allergens upon preincubation of sera from profilin allergic individuals with natural birch pollen extract or recombinant birch profilin

PATIENT	3	4	5	6
birch pollen extract	83%	99%	97%	95%
recombinant birch profilin	69%	9%	36%	26%

Patients are numbered as in the immunoblots in Figure 3. All results reflect means of duplicate determinations. The percentage of inhibition was calculated to the binding which was obtained when a control protein (dog albumin) which does not share IgE epitopes with birch pollen allergens was used for preincubation. Non-allergic patients gave no IgE-reactivity to natural birch pollen allergens. No significant inhibition of IgE-reactivity to natural birch pollen allergens was observed with four sera from birch pollen allergic patients without specificity for birch profilin, whereas with natural birch pollen extract > 98% inhibition was obtained.

Induction of specific histamine release from patients basophils with recombinant birch profilin

The recombinant birch profilin preparation was tested by *in vitro* histamine release for its capacity to elicit dose dependent specific histamine release from allergic patients blood basophils. Basophils from a profilin allergic patient and a control individual were incubated with increasing amounts of recombinant birch profilin, dog albumin (negative control) or anti-human IgE mAb (positive control). Figure 4 shows that recombinant birch profilin induces specific dose dependent histamine release from basophils of the profilin sensitized patient but not in the non-allergic control individual.

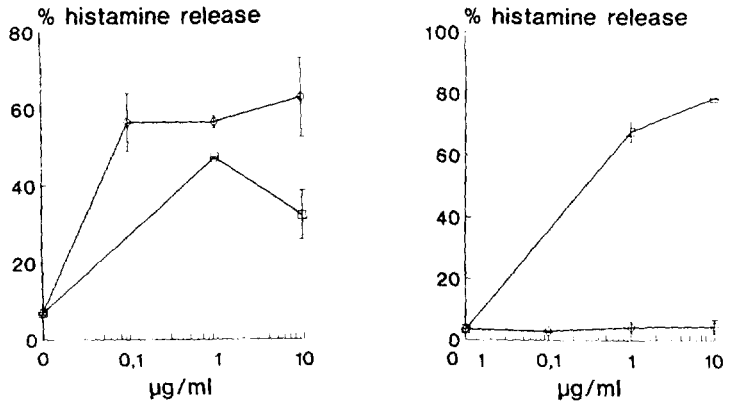

Figure 4. Dose-dependency of histamine release upon stimulation of human basophils with purified recombinant birch profilin.

Basophils from a profilin allergic individual (left) and a nonallergic individual (right) were stimulated with different doses of recombinant birch profilin (ϕ) and a monoclonal anti-human IgE antibody (□) (positive control). Results reflect means of triplicate determinations.

Skin-prick testing with purified recombinant birch profilin

Seven individuals belonging to the laboratory staff were tested by skin prick with commercial available test solutions (ALK, Copenhagen, Denmark), histamine, and recombinant birch profilin dissolved in 0.9% sodium chloride. In none of the six control individuals (non-allergic individuals and patients who were sensitized against allergens other than birch profilin) reactivity was found with recombinant birch profilin up to doses of 1µg/prick test. The profilin allergic patient (patient #6) gave a dose dependent reaction from 1µg/prick test as far as 10ng/prick test with recombinant birch profilin. The reaction obtained with the commercial birch pollen extract was equivalent to the reaction observed with 10ng recombinant birch profilin. Table 2 summarizes the skin-prick data. No toxic effects were observed in any of the individuals using up to 1µg recombinant birch profilin/skin prick test.

DISCUSSION

In vitro and *in vivo* diagnosis of type I allergy is currently performed with crude allergen extracts. A satisfying diagnosis is obtained in those cases where large amounts of stable major allergens are well represented in the extracts used for testing. Some allergen sources such as plant derived food (apples (29), nuts(30),

Table 2. Skin prick testing with recombinant birch profilin.

Summary of the results obtained by skin testing of allergic patients and nonallergic controls with different concentrations of recombinant birch profilin.

Individual:	1	2	3	4	5	6	7
1µg rbprof	-	-	-	-	-	+++	-
10^{-1}µg rbprof	-	-	-	-	-	++	-
10^{-2}µg rbprof	-	-	-	-	-	+	-
10^{-3}µg rbprof	-	-	-	-	-	+	-
10^{-4}µg rbprof	-	-	-	-	-	+/-	-
10^{-5}µg rbprof	-	-	-	-	-	-	-
10^{-6}µg rbprof	-	-	-	-	-	-	-
10^{-7}µg rbprof	-	-	-	-	-	-	-
10^{-8}µg rbprof	-	-	-	-	-	-	-
10^{-9}µg rbprof	-	-	-	-	-	-	-
grassmix	++	-	-	-	-	+++	-
birch	-	-	-	-	-	+	++
0	-	-	-	-	-	-	-
histamine	++	++	++	++	++	++	++

Intensity of reactivity: -: no reactivity, +/-: red without swelling, +: swelling, diameter <3mm, ++: swelling, diameter <8mm, +++: swelling, diameter>8mm and/or satellites. Patient 1 represents a grass pollen allergic without Bet v 2 specific IgE, patient 6 had Bet v 2 specific IgE antibodies and patient 7 was sensitized exclusively against Bet v 1, the major birch pollen allergen. Individuals 2, 3 and 4 were nonallergic healthy controls.

celery (31)) contain varying amounts of partly degraded allergens. Although monoclonal antibodies can be used to estimate the allergen content in natural extracts, such a standardization suffers from the drawback that monoclonal antibodies react mostly with sequential epitopes (32) which are mostly not conserved within isoallergenic variants and homologous allergens found in related species (33).

The production of recombinant allergens in heterologous expression systems may provide defined proteins for diagnostic or therapeutic purposes. In the field of tree and grass pollen allergy relevant recombinant allergens could be defined which are sufficient to diagnoze tree and grass pollen allergy and bear most IgE-epitopes (5, 6, 34, 35). The large scale expression in *E. coli* and the purification of birch pollen profilin, an important cross-reactive allergen (7, 8, 9, 10, 30, 31, 37) and cytoskeletal protein (11-13) is now reported. Expression of the recombinant allergen could be induced up to 30mg allergen/l culture and a single step affinity purification based on the affinity of profilins to poly-proline Sepharose (37) was used to obtain highly soluble (>99%) homogenous recombinant birch profilin. It is demonstrated that the purified recombinant allergen strongly bound patients IgE, elicited dose dependent histamine release and could be used to specifically identify profilin allergic patients by skin-prick testing without toxic side effects. The comparison of patients IgE binding to natural birch pollen extracts and purified recombinant birch profilin even indicated that assays based on recombinant profilin are more sensitive in detecting specific IgE-antibodies than natural allergen extracts. The lyophilized recombinant birch profilin could be stored for more than half a year without significant loss of IgE-reactivity and could be reconstituted in physiological solutions up to concentrations of 4mg/ml in a completely soluble form. The described recombinant allergen could be used to equip already established diagnostic tests such as RAST, ELISA tests or skin-prick tests which allowed to identify profilin allergic individuals who mostly belong to a group of patients sensitized against different unrelated plants and plant derived food. Using defined recombinant allergens, such as profilin it is hence possible to determine precisely the individual pattern of IgE-reactivity for each patient. According to such allergograms specific forms of a patient tailored immunotherapy could be developed.

ACKNOWLEDGMENTS

This study was supported by grants S06703-Med, P-7891, S6002Bio of the Fonds zur Förderung der wissenschaftlichen Forschung, Vienna, Austria and by grant 3/8603 of the Fonds zur Förderung der gewerblichen Wirtschaft, Austria.

Plasmid pMW172 and *E.coli* strain BL21(DE3) was kindly provided by Dr. P. Strasser, Inst. f. Mol. Biol., Austrian Acad. Sci., Salzburg. We thank M. A. Matzke for critically reading the manuscript.

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