

Recombinant allergens for immunoblot diagnosis of tree-pollen allergy

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Diagnosis of type I allergy essentially depends on the availability of defined allergens, which can be provided by recombinant deoxyribonucleic acid (DNA) technology. We have previously isolated the c(complementary)DNAs encoding the major birch-pollen allergen, Bet v I, and another allergen with a molecular weight of 14 kd that was identified as birch profilin and designated Bet v II. These cDNAs were isolated from a lambda gt11 expression library by screening with the serum IgE from allergic patients. To obtain expression in Escherichia coli of recombinant allergens without additional fused polypeptides, both cDNAs were inserted into the plasmid pKK223-3. E. coli cells expressing Bet v I and birch profilin (Bet v II) were used for the preparation of recombinant proteins. These proteins were tested for their IgE-binding properties on immunoblots with sera from 100 different birch pollen-allergic patients. All patients' sera, which reacted with the natural allergens, Bet v I and Bet v II, demonstrated an identical IgE-binding pattern to recombinant birch-pollen allergens. Recombinant allergens may therefore be useful for the setup of diagnostic tests that allow the discrimination of different IgE-binding patterns as well as for patient-tailored immunotherapy. (J ALLERGY CLIN IMMUNOL 1991;88:889-94.)

Key words: Pollen allergens, in vitro diagnosis, IgE, allergy, profilin

rDNA technology has proven to be a potent tool to characterize allergens.¹ Furthermore, this technology could provide cloned allergens for diagnosis and therapy of type I allergic diseases. So far, protein extracts containing allergenic proteins were difficult to standardize, and sometimes important allergens were missing. For example, mite extracts were found to lack a major mite allergen,² and a birch-pollen allergen with a molecular weight of 14 kd could not be detected in most birch-pollen extracts from commercial sources, even of standardized quality (Tejkl M, Jar-

Abbreviations used

cDNA:	Complementary deoxyribonucleic acid
r:	Recombinant
RNA:	Ribonucleic acid
pKK223-3:	Expression plasmid
Lambda gt11:	Phage-expression vector
Bet v I:	Major birch-pollen allergen
Bet v II:	Birch profilin
ATG:	Antithymocyte globulin
SPT:	Skin prick test
Ab:	Antibody

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olim E, Ebner Ch, et al. Unpublished results). Recombinant allergens produced in eukaryotic or prokaryotic expression systems could therefore be useful for diagnosis and possibly also therapy of allergic diseases.

For the production of recombinant pollen allergens, RNA is isolated from pollens and is then tested in an in vitro translation system.^{3,4} The RNA is then used to construct a cDNA library in a vector like lambda

TABLE I. Age and sex distribution of patients (N = 100)

Age (yr)	
<20	21%
21-30	19%
31-40	24%
41-50	15%
>50	21%
Sex	
M	49%
F	51%

TABLE II. Symptoms and duration of symptoms (patients, N = 100)

Symptoms	
Rhinitis	98%
Conjunctivitis	74%
Asthma	36%
Duration	
Spring	100%
Summer	43%
Perennial	36%

TABLE III. RAST scores of patients (N = 100)

	Allergens				
	Birch	Birch	Grasses	Weeds	Indoor
RAST scores	3-4	>4	>1	>1	>1
Percentage	72%	28%	48%	11%	25%

gt11⁵ that is capable of expressing the desired allergens. With serum IgE from allergic patients, clones can be identified and isolated, which are capable of producing IgE-binding fusion proteins. Although these fusion proteins allow the detection of allergen encoding cDNAs, they are not suited for clinical applications because they contain, in addition to the allergen, fused polypeptides, which may influence tertiary structure, solubility, and even immunologic properties of the original protein. Therefore, allergen-encoding cDNAs have to be inserted in eukaryotic or prokaryotic systems that express the recombinant allergenic proteins without fused peptides (nonfusion proteins²⁰).

Most in vitro diagnostic tests used in allergology like immunoblots, RAST, and ELISAs, are based on the binding of patients' IgE to allergens. Improvement of specificity and sensitivity of such diagnostic tests by use of recombinant or synthetic allergenic polypeptides requires therefore that natural and recombinant proteins possess identical IgE-binding capacity. This has to be demonstrated by comparing the IgE-binding properties of recombinant allergens with a representative number of sera from allergic patients who are well characterized by case history, SPTs, and serum IgE Abs against allergens derived from natural sources.

Recently, we cloned and sequenced cDNAs coding for the major birch-pollen allergen, *Bet v I*,⁶ and a 14

kd birch-pollen allergen that we identified as birch profilin and designated *Bet v II*.⁷ To obtain recombinant nonfusion proteins, both cDNAs were inserted into the plasmid pKK223-3⁸ and expressed in *Escherichia coli*. One hundred sera from well-defined (case history, RAST, and SPTs) birch pollen-allergic patients were tested for their ability to bind with their IgE Abs to natural and recombinant allergens on immunoblots.

MATERIAL AND METHODS

Characterization of patients

One hundred patients with positive SPTs to tree-pollen extracts and a birch pollen-specific RAST class of 3 and above as reported previously⁹ were included in the study to define a group of birch pollen-allergic patients. Patients are classified with respect to sex, age, symptoms, duration of symptoms, and RAST scores to birch, grasses, weeds, and perennial allergens in Tables I to III. In addition, we tested two patients who were negative in birch-pollen RAST and SPT but displayed allergic symptoms in early spring.

Expression of the cDNAs encoding *Bet v I* and *Bet v II*

The cDNAs encoding the major birch-pollen allergen, *Bet v I*,⁶ and *Bet v II*⁷ were subcloned into the *Eco* RI site of pKK223-3⁸ and transformed into *E. coli* JM105 (*thi*, *strA*, *endA*, *sbcB15*, *hsdR4* [*lac-proAB*], [*F'*, *traD36*, *proAB*, *lacIqZM15*]). Clones expressing the birch-pollen allergens were isolated with serum IgE from allergic patients. With

Bet v I, only low-level expression was obtained initially. Therefore, the noncoding DNA segment of 60 base pairs between the ribosomal binding site of pKK223-3 and the ATG initiation codon of the *Bet v I* cDNA insert was deleted by site-directed mutagenesis according to the method of Kunkel.¹⁰ The expression of *rBet v I* increased approximately tenfold.

Preparation and immunoblotting of natural and recombinant birch-pollen proteins

Birch-pollen proteins were extracted as described previously.⁹ Natural birch-pollen proteins were loaded onto each analytic gel (90 µg/cm of gel), subjected to electrophoresis,¹¹ and transferred to nitrocellulose.¹² The recombinant allergens were obtained from liquid cultures in Luria broth of *E. coli* JM105 transformed with the constructs illustrated in Fig. 1. The bacteria were grown at 37° C, induced at optical density 600, 0.4 with 1 mmol/L of final concentration of isopropylthio-β-galactoside and harvested after 3 hours, and washed three times with 0.9% sodium chloride. The pellet obtained from a 20 ml culture that contained approximately 4 mg of proteins from *E. coli* was boiled in sample buffer (125 mmol/L Tris, 20% glycerol, 2% sodium dodecyl sulfate, 2% (vol/vol) β-mercaptoethanol, and 0.001% bromophenol blue, pH 6.8) for 10 minutes before loading onto a 12% sodium dodecyl sulfate-polyacrylamide gel (onto a 12 cm wide analytic gel, 4 mg total proteins from *E. coli* were loaded).¹¹ Pilot immunoblot experiments demonstrated that one strip of nitrocellulose contained about 5 µg of the respective allergen (*Bet v I* or *Bet v II*), which compares with the amount of natural, pollen-derived allergens on the nitrocellulose. The same procedure was applied to *E. coli* JM105 transformed with pKK223-3 without insert. Strips of nitrocellulose containing either the recombinant allergens, proteins from *E. coli* JM105 transformed with the expression plasmid without insert (negative control), or natural birch-pollen proteins were incubated with 1:10 dilutions of patients' sera in buffer A (50 mmol/L of sodium phosphate, 0.5% bovine serum albumin, 0.05% sodium azide, and 0.5% Tween 20, pH 7.5) overnight at 4° C. Bound IgE was detected with ¹²⁵I-labeled rabbit antihuman IgE (1 to 2 µCi/ml) (Pharmacia, Uppsala, Sweden) that was diluted tenfold in buffer A.^{3,9} Blots were exposed for 48 hours to X-OMAT S films (Kodak, Heidelberg, Germany) at -70° C with intensifying screens.

RESULTS

Expression of *Bet v I* and *Bet v II*

The cDNAs coding for two birch-pollen allergens, *Bet v I*⁶ and *Bet v II*,⁷ were subcloned into the plasmid pKK223-3 and expressed in *E. coli* JM105 as IgE-binding nonfusion proteins (Fig. 1). In the case of *Bet v I*, the amount of the expressed recombinant protein could be increased approximately tenfold by deletion of noncoding DNA between the ribosomal binding site provided by the vector and the start ATG of the cDNA via site-directed mutagenesis.¹⁰ In the

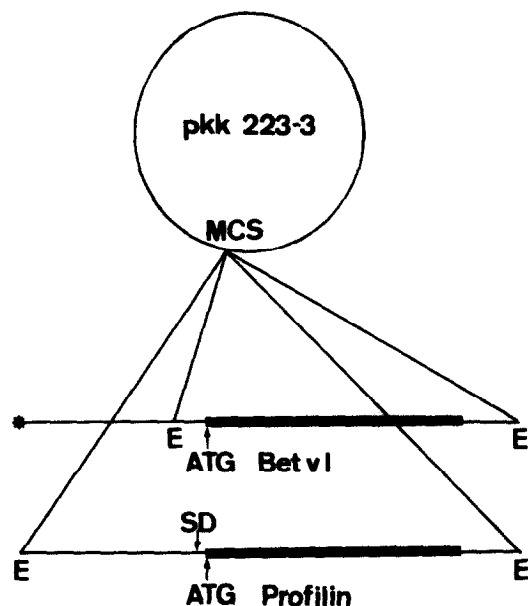


FIG. 1. Construction of plasmids expressing *Bet v I* and *Bet v II*. cDNAs encoding *Bet v I* and birch profilin (coding region, **bold line**) were subcloned into the *Eco* RI site (*E*) of the multiple cloning site of plasmid pKK223-3. With *Bet v I*, level of protein expression could be increased by deleting part of the 5' noncoding DNA (*thin line between asterisk and E*) between the ribosome binding site of the plasmid and the translation start (ATG) of cDNA. With birch profilin, Shine and Dalgarno-like¹³ motif (SD) close to translation start of cDNA allowed high-level expression of insert.

case of *Bet v II*, a sequence element similar to a procaryotic Shine and Dalgarno¹³ motif in the original birch sequence facilitated expression of the recombinant protein.

Thus, the two most important allergens of white birch were available as recombinant allergens, allowing direct comparison of their IgE-binding properties with those of natural birch-pollen proteins.

Immunoblot analyses

The recombinant proteins were tested for their ability to bind serum IgE from 100 birch pollen-allergic patients on IgE immunoblots. In all cases tested, we found comparable IgE binding to natural as well as to recombinant proteins. Each patient recognizing natural *Bet v I* and *Bet v II* also bound with comparable intensity to the recombinant proteins. Patients with high RAST scores demonstrated strong IgE binding to the nitrocellulose-bound allergens. In the case of two additional patients who suffered from allergic symptoms in early spring, although they had negative SPTs and RAST to birch pollen, we found IgE binding

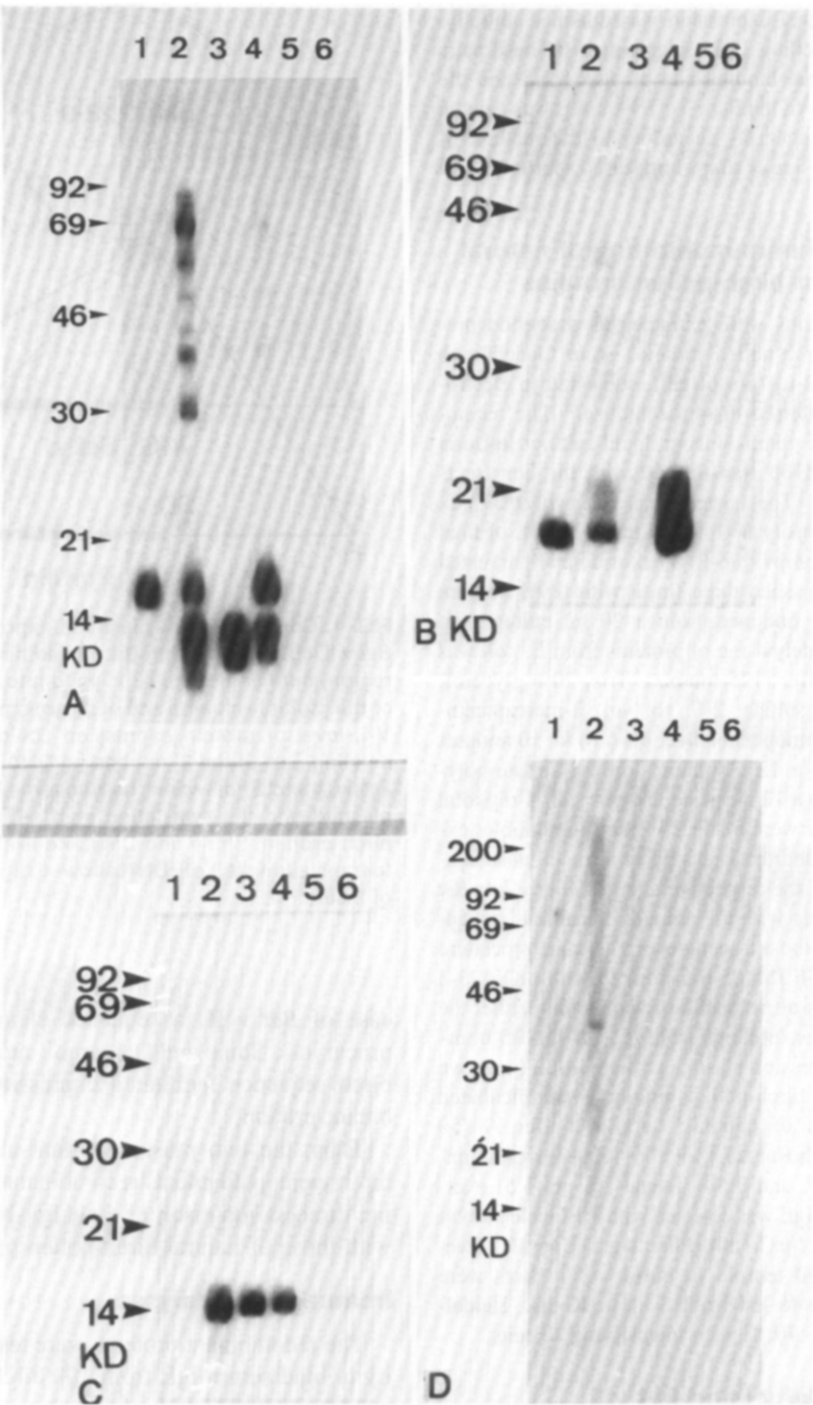


FIG. 2. A, IgE immunoblot. Typical IgE-binding pattern of selected birch pollen-allergic patients to natural birch-pollen proteins. Patient's serum in *lane 1* illustrates exclusive IgE binding to major birch-pollen allergen, *Bet v I*, at 17 kd; patient's serum in *lane 2* binds also to *Bet v II* at 14 kd and to proteins in the range between 30 to 90 kd; IgE from patient 3 binds exclusively to *Bet v II*, whereas IgE from patient 4 reacts with *Bet v I* as well as *Bet v II*. *Lane 5* illustrates a normal human serum pool of nonallergic individuals, and *lane 6* illustrates a buffer control without addition of serum. In both cases, no IgE binding was detected. **B,** IgE immunoblot. Proteins from *E. coli* JM105 transformed with pKK223-3 expressing *Bet v I* were loaded. Same panel of patient's sera as in **A** was tested for IgE binding to r*Bet v I*. **C,** IgE immunoblot. Proteins from *E. coli* JM105 transformed with pKK223-3 expressing birch profilin were loaded. Same panel of patients as in **A** was tested for IgE binding to recombinant birch profilin. **D,** IgE immunoblot. Proteins from *E. coli* JM105 transformed with pKK223-3 without insert were tested with same sera as in **A** to **C**.

to blotted *Bet v I* and *rBet v I*. This finding demonstrates higher sensitivity of the immunoblot.

Ninety-seven percent of the patients tested bound with their IgE to *rBet v I*, 9% reacted with *rBet v II*, and 6% with *rBet v I*, as well as *rBet v II*. Proteins from *E. coli* JM105 transformed with the expression plasmid without insert (negative control) were used to control the assay because some of the patients demonstrated IgE reactivity with proteins from *E. coli* (for example, Fig. 2, patient 2). The IgE-binding pattern is illustrated in Fig. 2 of a representative panel of birch pollen-allergic patients tested with natural (Fig. 2, A) and recombinant birch-pollen allergens, *Bet v I* (Fig. 2, B) and *Bet v II* (Fig. 2, C), as well as control proteins from *E. coli* (Fig. 2, D).

DISCUSSION

Purification, characterization, and standardization of allergens from pollen and other sources involve time-consuming protein chemistry as gel chromatography, isoelectric focusing, affinity chromatography, the preparation and use of monoclonal Abs, and other techniques. During protein extraction, important allergenic components may be lost for a number of reasons, like instability of proteins and protease activities in the extracts.² rDNA technology offers the possibility to isolate allergen-encoding cDNAs from expression libraries constructed from messenger RNA.¹ Screening such libraries with patients' IgE allows the isolation of these cDNAs, followed by determination of the DNA sequence and protein synthesis in suitable expression systems. After the cDNA sequence of allergens is obtained, mapping of B cell and T cell epitopes is substantially facilitated, and peptides can be synthesized according to the deduced amino acid sequences. Since, at this time, all data indicate that carbohydrates do not play an important role for IgE binding,^{2,4, 6, 7, 14, 15} cDNA cloning of allergens could be used to characterize allergens in the future in preference to standard biochemical techniques.

With serum IgE from allergic patients, we isolated the cDNAs encoding *Bet v I*⁶ and *Bet v II*⁷ from a lambda gt11 expression library that was constructed from birch-pollen messenger RNA. Both cDNAs were subcloned into pKK223-3 to express high levels of nonfusion proteins in *E. coli* JM105. A space of about 10 nucleotides between the ribosomal binding site of the expression plasmid and the start ATG of the cDNA insert allowed high-level expression of recombinant IgE-binding nonfusion proteins in *E. coli*.

To establish an allergen-specific test for the determination of the allergen profile of birch pollen-allergic patients, immunoblot analyses of recombinant allergens as well as proteins from *E. coli* JM105 trans-

TABLE IV. Percentage of birch pollen-allergic patients (N = 100)

With IgE binding to	
n/ <i>rBet v I</i>	97%
n/ <i>rBet v II</i> (profilin)	9%
n/ <i>rBet v II</i> (profilin)	6%

n, Natural; r, recombinant.

formed with pKK223-3 without insert (negative control) and natural birch-pollen proteins were performed. With these methods, IgE-binding patterns of 100 birch pollen-allergic individuals were determined. When the IgE binding was compared to natural as well as to recombinant proteins, no difference was observed with any serum tested, indicating that *rBet v I* and *rBet v II* are suited for diagnostic tests and possibly for treatment by hyposensitization.

Although IgE binding to proteins from *E. coli* was not frequently observed, it is necessary to control the assays with proteins from *E. coli* transformed with the expression plasmid without insert.

Since it was possible to identify 100% of the birch pollen-allergic patients by the use of only two proteins, *Bet v I* and *Bet v II*, it may be possible that these two allergens will prove to be sufficient for diagnosis of birch-pollen allergy.

High homology of the major tree-pollen allergens at the nucleic acid level was demonstrated recently.¹⁶ In addition, a series of data documents the close biochemical and immunologic relationship between the major tree-pollen allergens^{17, 18} and suggest strongly that *rBet v I* is suited not only for diagnosis and therapy of birch-pollen allergy but also for allergy against pollen of the closely related trees of the order Fagales (birch, alder, hazel, hornbeam, and oak).

With *rBet v II*, we could identify a subgroup of patients who suffered not only from tree-pollen allergy but also displayed allergic symptoms to pollens from distantly related plants (grasses, trees, and weeds). This finding can be explained by the fact that profilin, an essential, highly conserved component of the eukaryotic cytoskeleton, is likely to be expressed in virtually all pollens.¹⁹ In addition, IgE Abs of these patients demonstrated autoreactivity in vitro to human profilin.⁷

rBet v I and *rBet v II* fulfill criteria that are prerequisites for the setup of suitable in vitro allergy tests. Such tests, which combine the sensitivity of immunoblotting with the specificity obtained by the use of defined recombinant allergens, should significantly improve the diagnosis of type I allergies. Purification of these recombinant allergens from *E. coli* protein lysates may further improve this approach to in vitro

diagnosis, even including tests with basophils (histamine release assays with recombinant allergens), and therefore, recombinant proteins will possibly prove useful for in vivo tests (SPTs) and therapeutic applications (hyposensitization treatment).

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