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Characterization of Recombinant *Bet v* I, the Major Pollen Allergen of *Betula verrucosa* (White Birch), Produced by Fed-Batch Fermentation

Key Words

Betula verrucosa
Escherichia coli
Fed-batch fermentation
Pollen allergen
Recombinant *Bet v* I
characterization

Abstract

The gene encoding the major allergen, *Bet v* I, from *Betula verrucosa* (white birch) pollen was cloned by application of the polymerase chain reaction with double-stranded cDNA as template and specific primers based on the published nucleotide sequence of the gene. The gene was inserted into plasmid pKK223-3 and expressed in *Escherichia coli* K-12 strain JM105 grown to high cell density in a fermenter using a fed-batch procedure. Ample material was provided for a thorough characterization of the epitope structure of recombinant *Bet v* I contained in an unpurified soluble lysate. The antibody-binding characteristics of recombinant *Bet v* I was compared with that of the natural allergen using polyclonal rabbit antibodies raised against *Bet v* I in crossed immunoelectrophoresis, tandem crossed immunoelectrophoresis, and Western blotting. IgE from a pool of 16 allergic patients' serum was applied in crossed radioimmunoelectrophoresis, Western blotting, and a quantitative luminescence inhibition immunoassay. Well-defined epitopes were assayed by the application of a panel of six murine monoclonal antibodies in a quantitative radio inhibition immunoassay. In all assays the activity of recombinant *Bet v* I was comparable to that of natural *Bet v* I, and it is concluded that the epitope structure of recombinant *Bet v* I closely resembles that of natural *Bet v* I. This result has important implications for the future use of recombinant tree pollen allergens as a model system for the study of allergenic B and T cell epitopes aiming at improvements in reagents used for the management of allergic disease.

Introduction

During spring in the temperate climate zone of the northern hemisphere pollens from the trees of the *Fagales* constitute a major source of airborne allergens [1].

Clinical observations on the reactivity of patients allergic to the pollens of the *Betulaceae* indicate a high degree of cross-reactivity among the genera *Alnus* (alder), *Betula* (birch), and *Corylus* (hazel) [2–5].

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The immunochemical cross-reactivity has been confirmed by analyses applying aqueous pollen extracts of selected species including *Alnus glutinosa*, *Betula verrucosa*, *Corylus avellana*, and *Carpinus betulus* [6, 7]. Molecular studies have demonstrated, that most of the allergenicity, i.e., the IgE-binding capacity, of these extracts can be accounted for by one major allergen [8]. The major allergens share physicochemical properties and display ~80% identity within the amino-terminal sequences [7, 9–11].

Studies applying molecular cloning have emphasized the structural similarities of the major allergens by cross-hybridization experiments [12] and sequence homology. Genes encoding *Ain g I*, *Bet v I*, *Car b I*, and *Cor a I* have been cloned and sequenced [13–16], and the deduced amino acid sequences are identical in over 70% of the positions, indicating identical three-dimensional folding patterns of the molecules [17].

Even though the structural similarities are obvious, notable immunochemical differences have been documented [7]. These differences in epitope structure may be accounted for by local perturbations of the surface structure mediated by ten, but significant amino acid substitutions. Precise establishment of epitope structures based on major allergens derived from pollen extracts, however, is hindered by the presence of numerous isoallergens [18–21] with individual primary structures, deviating from one another in up to 10% of the positions [15]. Optimal management of allergic disease, and the forecast for the use of recombinant allergens herein, is dependent upon an evaluation of the significance of identities/differences in the structures of T and B cell epitopes of the major allergens – not only among genera and species of the tree pollen families, but also between individual isoallergens within each species. Since isoallergens cannot at the present be separated quantitatively by biochemical techniques, the method of choice for the generation of homogenous allergen preparations is molecular cloning. However, recombinant allergens are not always folded correctly when produced in bacteria. An important aspect of this methodology is, therefore, to perform a comparison between conformational epitopes of the recombinant and natural allergens.

In this communication we describe the production and characterization of recombinant *Bet v I*, the major allergen of *B. verrucosa*, white birch.

Materials and Methods

Pollen extract and rabbit antibodies raised against purified *Bet v I* were prepared as described [7]. Monoclonal hybridoma antibodies were derived from Balb/c mice immunized with purified tree pollen allergen [22]. All monoclonal antibodies were of the IgG1 isotype, and supernatants from cells grown in the absence of serum were used. Coupling to amine-terminated magnetic iron oxide particles (Advanced Magnetics Inc.) was performed using glutaraldehyde. Tree pollen allergic patient serum pool was a mixture of equal volumes of serum from 16 donors. The patients were selected on the basis of case history, nasal or bronchial provocation tests, skin prick tests, and radioallergosorbent test, as described [3]. Other materials were from commercial suppliers. Methods that are not described in detail below were performed according to standard procedures [23, 24].

Preparation of mRNA from Pollen

B. verrucosa pollen (Allergon) was used as source material for the preparation of mRNA. The procedures used were as described for the cloning of the major allergen from the related species, *C. betulus* [15]. In brief, RNA was extracted by applying a phenol extraction procedure, followed by LiCl precipitation. Oligo(dT)-cellulose (Boehringer Mannheim) affinity chromatography was performed batch-wise in Eppendorf tubes.

cDNA Synthesis

Double-stranded cDNA synthesis was performed using a commercially available kit (Amersham) following the recommendations of the supplier.

Gene Cloning

The gene encoding *Bet v I* was specifically amplified in a polymerase chain reaction (PCR) using primer sequences derived from the published nucleotide sequence of the gene [14]. The primers were purchased from Biotechnological Institute (Copenhagen) and desalted using a Sep-Pak C₁₈ cartridge (Waters) following the recommendations of the supplier.

The sequences of the 33-mer primers were: 5'-CGC GAA TTC ACC ATG GGT GTT TTC AAT TAC GAA (primer No. 1) and 5'-CGC GAA TTC AAA GCT TCA GCT AGC AAG CAG ATG (primer No. 2). The promoter proximal primer No. 1 contains an artificial 'ATG' start codon inserted in front of the GGT codon, corresponding to the amino-terminal glycine, as determined by Edman degradation of the protein purified from pollen extract [7]. The 'ATG' start codon is incorporated into an *NcoI* site for cloning into pKK233-2 [25], and the primer also contains an *EcoRI* site for cloning into pKK233-3 [26]. The promoter distal primer No. 2 is designed to match the sequence in the 3'-untranslated region and contains an *EcoRI* and a *HindIII* site. PCR were performed in a volume of 100 µl containing 50 mM KCl, 2.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.5), 0.2 mM of each dNTP, 0.5 µM of each primer, 0.01% (w/v) gelatine, 2 µg/ml cDNA, and 25 U/ml AmpliTaq polymerase (Cetus). Thirty temperature cycles of 90 s at 94 °C, 90 s at 42, and 5 min at 72 °C were performed in a Hybaid Thermal Reactor. Agarose gel electrophoretic analysis of the PCR products revealed only one band, (fig. 1), and it was of the expected molecular size of 626 bp. The DNA fragment was cloned without purification. *Escherichia coli* K-12 strain JM105 [27] was transformed with the ligation mixture and plated on LB [24] containing 0.1 g/l ampicillin.

Nucleotide Sequencing

EcoRI-BamHI and *BamHI-HindIII* subclones were constructed in pUC19 [28]. Nucleotide sequencing was performed by the dideoxynucleotide chain termination method [29] using commercially available 17-mer universal sequencing primers. Two sets of specific 20-mer oligonucleotide primers, matching the inserts approximately in the middle, were synthesized on an Applied Biosystems 380A synthesizer according to the manufacturer's instructions. These primers were also used for verification of the sequence in the *BamHI* site which was used for subcloning. Full sequence was obtained on both DNA strands.

Fermentation Media

The initial medium contained the following per liter: NH_4Cl 7.0 g (131 mM), KH_2PO_4 7.5 (55), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 7.5 (42), K_2SO_4 0.85 (4.9), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.17 (0.7), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.4 g (0.3 mM), trace element solution 0.8 ml, glucose 1.0 g, thiamine 0.001, and ampicillin 0.1 g. Glucose, MgSO_4 , and CaCl_2 were autoclaved separately. Thiamine and ampicillin were sterile filtered. The trace element solution was not sterilized, whereas the other components were autoclaved with the fermenter. Trace element solution contained the following per liter of 5 M HCl: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 40.0 g (144 mM), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 10.0 (45), $\text{Al}_2(\text{SO}_4)_3 \cdot 16\text{H}_2\text{O}$ 51.0 (81), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 4.0 (17), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 2.0 (7.0), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 2.0 (9.7), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 1.0 (5.9), and H_2BO_3 0.5 g (6 mM).

Feed No. 1 contained the following per liter: glucose 20 g (0.11 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 (1.0 mM), trace element solution 4 ml, thiamine 0.001 g, and ampicillin 0.1 g. Feed No. 2 contained the following per liter: glucose 200 g (1.1 M), NH_4Cl 28 (523 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.51 (3.5), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2.22 g (9.0 mM), trace element solution 20 ml, thiamine 0.001 g, and ampicillin 0.1 g.

Solutions were sterilized as above, except that NH_4Cl , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were autoclaved separately.

Fermentation Equipment and Routine Measurements

All fermentations were performed in a 3-liter Applikon fermenter, equipped with a turbine impeller and automatic temperature and pH control (37 °C and pH 7.0, respectively). The pH was monitored by use of an autoclavable pH probe (Ingold) and adjusted by automatic addition of 10 M NH_3 . Dissolved oxygen was monitored by use of a polarographic dissolved oxygen probe (Ingold) and controlled by increasing agitation from 700 to 1,280 rpm in order to maintain relative dissolved oxygen above 20% saturation. Air was supplied at a flow rate of 2.5 vol/vol/min. Foam was controlled by automatic addition of silicone oil.

Samples were taken every 30 or 60 min and analyzed as follows: The concentrations of glucose and acetic acid were measured by high-performance liquid chromatography. To 1 ml sample was added 1.7 ml of 4 N H_2SO_4 containing 2 mM EDTA. Cell debris was subsequently removed by centrifugation at 5,000g for 20 min, and 25 μl of the supernatant was injected into the column. The system comprised the following: all apparatuses from Hitachi Merck; autosampler AS-4000 intelligent, L-6000 interface, variable wavelength monitor at 220 nm (ch I) and a differential refractometer (ch II); the detectors connected in series, and D-6000 high-performance liquid chromatography manager software connected to an IBM PS2 model 30 286 computer. An organic acid analysis Aminex ion-exchange column (300 mm, model HPX-87H; Bio-Rad Laboratories) was used, and organic acids were separated by using 8 mM H_2SO_4 and 0.01% (w/v) EDTA at a flow rate of 0.6 ml $\cdot$ min⁻¹. Standards (10 mg/ml) were in-

jected in parallel, and the results, based on retention time estimates and external standard calculations, were expressed in milligrams per milliliter.

The optical density was measured spectrophotometrically at 436 nm using a Hitachi U-1100 spectrophotometer. One absorbance unit corresponded to 0.25 ± 0.05 g dry weight per liter (depending on growth phase and cell size). Culture dry weight was measured by the filtration method. Twenty milliliters of appropriately diluted sample was filtered using a preweighed 47-mm (diameter), 0.22- μm Millipore membrane filter and washed with an equal volume of distilled water. The samples were subsequently dried for 24 h at 90 °C in an oven.

Preparation of Soluble Lysate

The cells were harvested by centrifugation (1,000g for 30 min), resuspended in 2 ml of water per gram cell wet weight, and stored frozen.

Upon thawing, the cells were broken either in a French press (SLM Aminco), two passes at 900 psi ($6.2 \times 10^6 \text{ N m}^{-2}$), or by ultrasonic treatment in a Branson sonicator (model 250, 2 min, 50% duty cycle at setting 7, corresponding to approximately 50 W). Cell debris was removed by centrifugation (28,000g for 30 min). The soluble lysate was subsequently dialyzed against 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl_2 , treated for 1 h with 10 $\mu\text{g/ml}$ DNase I (Boehringer Mannheim) at ambient temperature, and finally dialyzed against 125 mM ammoniumbicarbonate. Bacterial lysates were stored at 4 °C.

Quantitation of Bet v I

Quantitative measurement of Bet v I was performed by rocket immunoelectrophoresis [30] using a monospecific antiserum raised against purified Bet v I [7].

Immunoelectrophoresis

Crossed immunoelectrophoresis, tandem immunoelectrophoresis, and crossed radioimmunoelectrophoresis were performed essentially as previously described [7].

Immunoblotting

Sodiums dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and semidry electroblotting were performed as previously described [9]. The nitrocellulose was subsequently immunostained as described [15].

Radio Inhibition Immunoassay

Parallel dilution series in duplicate were mixed with a constant amount (25 nM final concentration) of ¹²⁵I-labelled, purified Bet v I [7] (specific activity 11.4×10^3 cpm/ng). Murine monoclonal antibodies coupled to paramagnetic particles [22] were added to a final concentration of 1 ± 0.5 nM antibody-binding sites (corresponding to maximal binding values of 22,000–55,000 cpm) in a total volume of 0.2 ml 0.2 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.4) with 0.1% (v/v) Tween 20. Following incubation for 1 h the magnetic particles were collected on a magnet, the samples were washed twice in the above-mentioned buffer, suspended in 0.1 ml water, and finally counted in a gamma counter. The inhibition percentage was calculated using the buffer value as 0% inhibition and 0 cpm corresponding to 100% inhibition. Statistical evaluation of linearity and parallelity of the inhibition curves ($10 \leq \text{nM} \leq 200$) were performed using standard statistics [31]. Curves were considered parallel when any two-sided stochastic probability was less than 0.05.

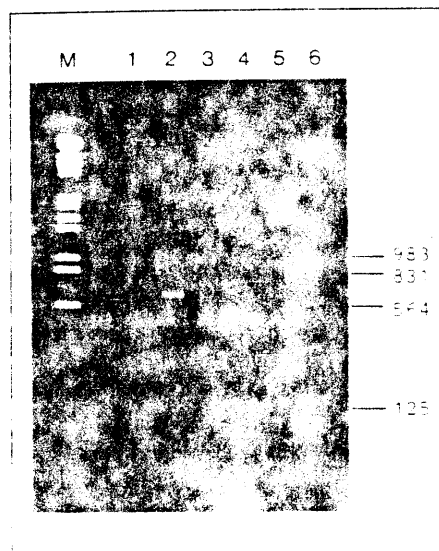


Fig. 1. Crude PCR products analyzed by agarose gel electrophoresis. Reaction conditions as described in Materials and Methods with the following deviations: lanes 1–3: 2.5 mM $MgCl_2$; lanes 4–6: 3.5 mM $MgCl_2$. Lanes 1 and 4: 128 cycles; lanes 2 and 5: 140 cycles; lanes 3 and 6: 160 cycles. 1.2 μ g RNA/lanes 2 and 5; cDNA pool 2.2 μ g/ml; lanes 3 and 6: cDNA pool 3.2 μ g/ml. Cloning was performed using the product from lane 1. λ wt HindIII (10 kb): 21,226, 5,148, 4,973, 4,277, 3,330, 2,627, 1,904, 1,584, 1,330, 983, 831, 564, and 125 bp.

Luminescence Inhibition Immunoassay

Parallel dilution series in triplicate were mixed with 25 μ g of paramagnetic particles coupled to a *Bet v* pollen extract in 0.2 M KH_2PO_4 /K₂HPO₄ buffer (pH 7.4) with 0.1% (v/v) Tween 20. Patient serum IgE pool ($n = 16$) was added to the antigen mixture and incubated for 2 h. The magnetic particles were subsequently collected on a magnet, washed once in the above-mentioned buffer, and the bound IgE was quantitated using luminescence-labeled murine monoclonal anti-IgE according to the Magic Lite procedure (ALK). Statistical evaluation of linearity and parallelity of the inhibition curves ($2 \leq nM \leq 100$) was performed as described above.

Results

Gene Cloning

mRNA was prepared from *B. verrucosa* pollen by a phenol extraction procedure followed by oligo-(dT) cellulose affinity purification, essentially as described [15]. 2 μ g/ml of double-stranded cDNA was amplified by PCR using specific primers derived from the nucleotide sequence of the gene [14]. Both primers were extended in the 5'-ends to accommodate restriction sites and 'ATG' start codon for the directed cloning into expression plasmids pKK223-3 and pKK223-2. When analyzed by agarose gel electropho-

resis and ethidium bromide staining, the crude PCR mixture revealed only one band, which was approximately 630 bp and neither smearing nor primer artefacts were prevalent (fig. 1). Thus, the identity of the DNA fragment could be established by restriction analysis of the crude PCR products. *Bam*HI, *Bgl*II, *Bst*XII, *Bst*NI, *Nhe*I, and *Sst*I, all of which cut the fragment, once according to the nucleotide sequence, were applied and yielded the expected fragments. The gene encoding *Bet v* I was subsequently cut with *Eco*RI and *Hind*III with subsequent purification and ligated with alkaline phosphatase treated pKK223-3. The ligation mixture was used to transform *E. coli* K-12 strain JM105. 17% of randomly picked transformants contained inserts as demonstrated by agarose gel electrophoresis of uncut plasmid minipreparations. A selection of apparently positive clones were analyzed for the ability to produce recombinant *Bet v* I. Soluble lysates were prepared as described [15] and subjected to SDS-PAGE followed by immunoblotting using a monoclonal antibody (BV03) raised against *Bet v* I purified from pollen. Lysates of most clones contained a protein component reactive with BV03 having an apparent molecular weight of 17.5 kD, in agreement with natural *Bet v* I from pollen. One clone was arbitrarily selected for further characterization and named pBvI.

Nucleotide Sequence

The recombinant DNA inserted in pBvI is 608 bp in length, of which 603 bp represent the *Bet v* I gene, including an open reading frame of 477 nucleotides coding for 159 amino acids excluding the terminal methionine. When compared to the published sequence of the *Bet v* I gene, only one nucleotide differs. This deviation in position 112 (A $\leftrightarrow$ G) – numbering according to Breiteneder et al. [14] – has no effect on the deduced amino acid sequence. When *Bet v* I is compared with the related tree pollen major allergens, the following percentages of identity between the deduced amino acid sequences are calculated: *Ahn g* I [13] from *A. glutinosa* 81%, *Car b* I [15] from *C. betulus* 74% (average of three described isoallergens), and *Cor a* I [16] from *C. avellana* 72% (average of four described isoallergens).

Fed-Batch Fermentation

E. coli K-12 strain JM105 containing pBvI was grown in minimal medium in a 3-liter Applikon fermenter for the production of recombinant *Bet v* I. The course of a typical fermentation ($n = 9$) is schematically outlined in figure 2. The equipment was provided with automatic temperature and pH control, set at 37 °C and pH 7.0, respectively. Dissolved oxygen was maintained above 20% of saturation by

manually increasing the speed of the agitator. Feed No. 1 was added immediately after inoculation and adjusted stepwise to maintain the glucose concentration at approximately 1 g/l. Calculation of the increase in feed rate was performed immediately after optical density measurements according to standard fermentation modelling [32]. At a cell density of 1 g dry weight per liter, the stronger feed No. 2 replaced feed No. 1 to accommodate high cell density. In this phase the culture grew exponentially with a specific growth rate of 0.58 h^{-1} , slowly accumulating low levels (up to $\sim 1 \text{ g/l}$) of acetate in the medium. After approximately 10 h ($\sim 5 \text{ g dry weight per liter}$) the level of dissolved oxygen could no longer be maintained, since the consumption rate exceeded the transfer rate even at maximum speed of the impeller. Thus, the culture was switched to carbon-limited linear growth in order to maintain the relative dissolved oxygen concentration above 20% of saturation throughout the fermentation. During the linear growth phase a constant feed rate of 9.3 g/l/h of glucose was used, giving rise to an average cell productivity of $3.4 \text{ g l}^{-1} \text{ h}^{-1}$. During this phase the acetate produced in the exponential phase was reassimilated. At a cell dry weight of $25\text{--}30 \text{ g/l}$, the culture entered the stationary phase.

Production of Recombinant Bet v I

Induction of the synthesis of *Bet v I* by the addition of Isopropyl- β -D-thiogalactopyranoside (IPTG) had no detectable effect on the growth rate. Induction during the stationary phase produced no recombinant *Bet v I*. During the linear growth phase a prolonged period of induction increased the overall yield; however, optimal yield relative to the cell mass was achieved after 1 h. As a consequence the culture was induced 1–2 h before harvest. Typically induction was performed late during the linear growth phase.

Using this procedure, yields obtained were 1.5 ± 0.4 ($n = 9$) mg/g dry cell weight or 30 mg/l culture medium at a typical cell mass of 20 g dry cell weight per liter, corresponding to an overall yield of approximately 75 mg recombinant *Bet v I*/per fermentation.

Characterization of Recombinant Bet v I

Recombinant *Bet v I* was shown by crossed immunoelectrophoresis to precipitate in gel with polyclonal rabbit antiserum, raised against purified *Bet v I* from pollen. The morphology of the precipitate (fig. 3B) closely resembles that of natural *Bet v I* (fig. 3C). The precipitation arc of natural *Bet v I* is a little wider and displays a tiny shoulder, indicating the presence of isoallergenic variants. The perfect lack of spurs in tandem immunoelectrophoresis

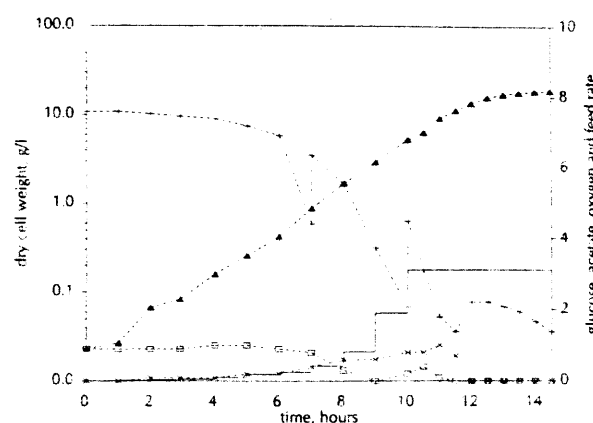


Fig. 2. Course of a typical fed-batch fermentation. Dry cell weight (logarithmic scale: $\blacktriangle$), glucose ($\square$), and acetate ($\times$), in grams per liter, oxygen in milligrams per liter ($+$), and feed rate $\times 0.1 \text{ g of glucose/min}$ (—).

(fig. 3D) is a strong indication of similarity between the epitope structures. The same results were obtained when rabbit antibirch pollen extract antibodies were used (data not shown).

The binding of allergic patient serum IgE was investigated using crossed radioimmuno-electrophoresis. The intensity of the staining of the autoradiographic film was comparable between natural *Bet v I* (fig. 3G) and recombinant *Bet v I* (fig. 3F).

SDS-PAGE was performed using birch pollen extract, lysate devoid of recombinant protein, and lysate containing recombinant *Bet v I*. It is apparent from the gel stained with Coomassie brilliant blue (fig. 4A) that the bacterial lysates contain multiple protein components, but comparison of the two lysates reveal the presence of a $\sim 17.5\text{-kD}$ component corresponding to *Bet v I* in the recombinant lysate that is not present in the lysate of pKK223-3 in JM105. Electroblots of the same samples were stained with colloidal gold (fig. 4B) rabbit anti *Bet v I* antibodies followed by alkaline phosphatase conjugated secondary antibodies and BCIP/nitroblue tetrazolium stain (fig. 4C) and serum IgE followed by radiolabelled antihuman IgE and autoradiography (fig. 4D), respectively. The gold staining of electroblots is merely a more sensitive staining technique than Coomassie brilliant blue staining of gels, and the same observations as stated above hold true. The Western blots (fig. 4C, D) confirm the results obtained by immunoelectrophoresis in that recombinant *Bet v I* binds rabbit antibodies and human serum IgE directed against natural

Fig. 3. Crossed immunoelectrophoresis (upper panel) and crossed radioimmuno-electrophoresis (lower panel). From left to right: 4.4 μ l bacterial lysate of pKK223-3 JM105 (**A, E**), 4.4 μ l lysate of pBV1 in JM105 containing 2 mg/ml recombinant *Bet v 1* (**F**), 40 μ g birch pollen extract (**C, G**), and 4.4 μ l immunoelectrophoresis of the same amounts of natural *Bet v 1* (left wells) or recombinant *Bet v 1* (right wells) (**D, H**). Plates were incubated with 150,000 cpm of plate of antihuman IgE antibodies (Pharmacia) followed by 4 days of autoradiography.

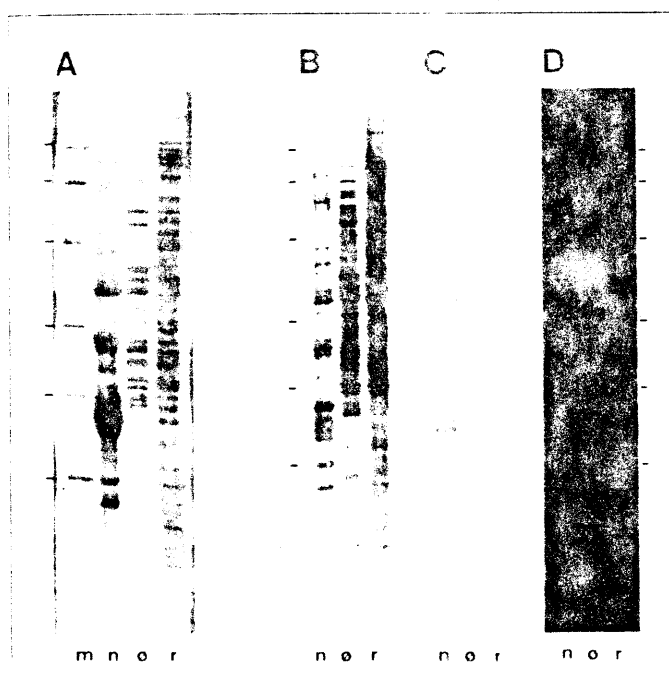


Fig. 4. SDS-PAGE and electroblots. Molecular weight markers (m), birch pollen extract (n), lysate of pKK223-3 in JM105 (o), and lysate of pBV1 in JM105 containing recombinant *Bet v 1* (r) were subjected to SDS-PAGE. The gels were stained with Coomassie brilliant blue (**A**) or electroblotted onto nitrocellulose. Electroblots were stained with colloidal gold (**B**), incubated with monospecific rabbit antibodies raised against natural *Bet v 1* followed by alkaline phosphatase conjugated secondary antibodies and a BCIP/Nitroblue tetrazolium stain (**C**) or incubated with serum IgE followed by 240,000 cpm of radiolabelled antihuman IgE (Pharmacia) and 4 days of autoradiography (**D**). Molecular weight markers (Bio-Rad) were the following: rabbit muscle phosphorylase b (97.4 kD), bovine serum albumin (66.2), hen's egg white (ovalbumin (45.0), bovine carbonic anhydrase (31.0), soybean trypsin inhibitor (21.5), and hen's egg white lysozyme (14.4 kD).

Bet v 1. Furthermore, the immunoblots show the presence of isoallergenic variants in natural *Bet v 1* which appears as a triplet and indicates that the recombinant allergen is homogeneous, as expected.

The binding of IgE was further evaluated in a quantitative inhibition assay. There was no significant difference between the capacity of a bacterial lysate containing recombinant *Bet v 1* and a pollen extract to inhibit the bind-

ing of IgE to paramagnetic particles coated with a birch pollen extract (fig. 5).

The integrity of well-defined epitopes was measured in a radioimmuno-inhibition assay using a panel of murine monoclonal antibodies. The monoclonal antibodies have been shown to represent three separate determinants in such a way that pairwise combinations of BV07, BV08, BV09, and BV10 inhibit the binding of each other compet-

itively, whereas BV16 and CB11 do not inhibit in a competitive manner the binding of any other monoclonal antibody within this panel. The assay was designed to measure quantitatively the degree of inhibition obtained when a mixture of a dilution series of bacterial lysate containing recombinant *Bet v I* or a birch pollen extract and a small amount of highly purified radiolabelled *Bet v I* was allowed to compete for the binding to a solid phase coated with monoclonal antibodies. The concentration of radiolabelled *Bet v I* was balanced in such a way as to achieve optimal sensitivity of the assay. Radiolabelled *Bet v I* was present in all assays at a concentration of 25 nM which is slightly above the K_d for complex formation. For the antibodies used the K_d values are in the range 3–20 nM [22]. The concentration of antibody-binding sites was in all assays 1 nM to achieve saturation, and the inhibitor concentration in the range of 0.01–1,000 nM.

Statistical evaluation of the inhibition curves (fig. 6) revealed that all curves were linear and parallel and that no statistically significant differences could be observed when the inhibitory capacities of recombinant *Bet v I* and natural *Bet v I* were compared.

Discussion

The gene encoding *Bet v I* was amplified from cDNA by PCR using primers derived from the published sequence of the gene. The identity of the cloned gene was verified by the agreement in amino-terminal sequence, amino acid composition, and molecular mass between the deduced amino acid sequence and data obtained by analysis of the allergen purified from pollen [7]. In addition, the resemblance of the nucleotide sequence to the published sequence and the immunochemical data presented support the assignment.

The gene was cloned in plasmid pKK223-3 and expressed in *E. coli* K-12 strain JM105 at low levels. Strain JM105 was chosen to promote high cell density, since it has a low glucose consumption rate, a high yield, and a low acetate production [34]. Accumulation of acetate and an accompanying lowering of the productivity seem to be a general problem in the production of recombinant proteins by batch fermentation of *E. coli* which may be overcome by the design of a fed-batch fermentation in which the glucose concentration can be kept low throughout the fermentation [35]. The presented strategy may be divided into two phases: an initial phase of exponential growth during which acetate is accumulated to approximately 1 g/l, followed by glucose-limited linear growth during

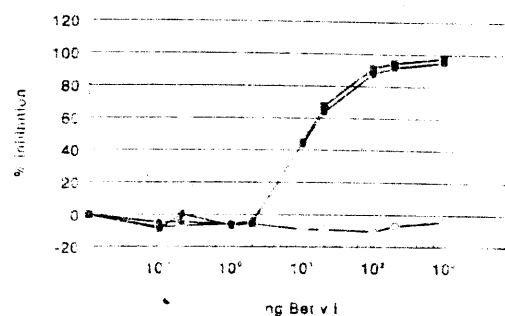


Fig. 5. Luminescence inhibition immunoassay. Inhibition of serum IgE binding to a solid phase coated birch pollen extract with dilution series of birch pollen extract (■), bacterial lysate containing recombinant *Bet v I* (●), and natural lysate (▲) (KK223-3, JM105). The bacterial lysates contained comparable amounts of total protein. Bound IgE was detected with luminescence-labeled anti-IgE.

which the acetate is reassimilated. Induction of the synthesis of recombinant *Bet v I* was performed during the late linear growth phase to achieve yields of approximately 1.5 mg per gram dry cell weight.

Apart from the physiological characteristics of the growing *E. coli* culture, the yield of recombinant protein is governed by features of the expression system, such as the efficiency of the transcription and translation processes, and the level of proteolytic degradation of the product.

pKK223-3 [26] is a relaxed intermediate-to-high copy number plasmid with pBR322 origin, and the recombinant gene is inserted under transcriptional control of the strongly inducible *tac* promoter. The plasmid is stabilized by the strong *mm* terminators. These features are combined to facilitate high levels of mRNA upon induction. Furthermore, proteolytic degradation does not seem to be a severe problem, since the soluble lysates are stable for months (data not shown). The limiting step, however, was not determined experimentally.

An important aspect of the present study was to compare the epitope structure of recombinant *Bet v I* with that of the natural allergen.

When analyzed with crossed immunoelectrophoresis using polyclonal rabbit antibodies, the morphology of the precipitation arcs of recombinant *Bet v I* and natural *Bet v I* was identical. In addition, there was no sign of spurs in tandem immunoelectrophoresis. These results are strong indications that the epitope structures are similar.

IgE binding was evaluated qualitatively using crossed radioimmuno-electrophoresis and Western blotting, and

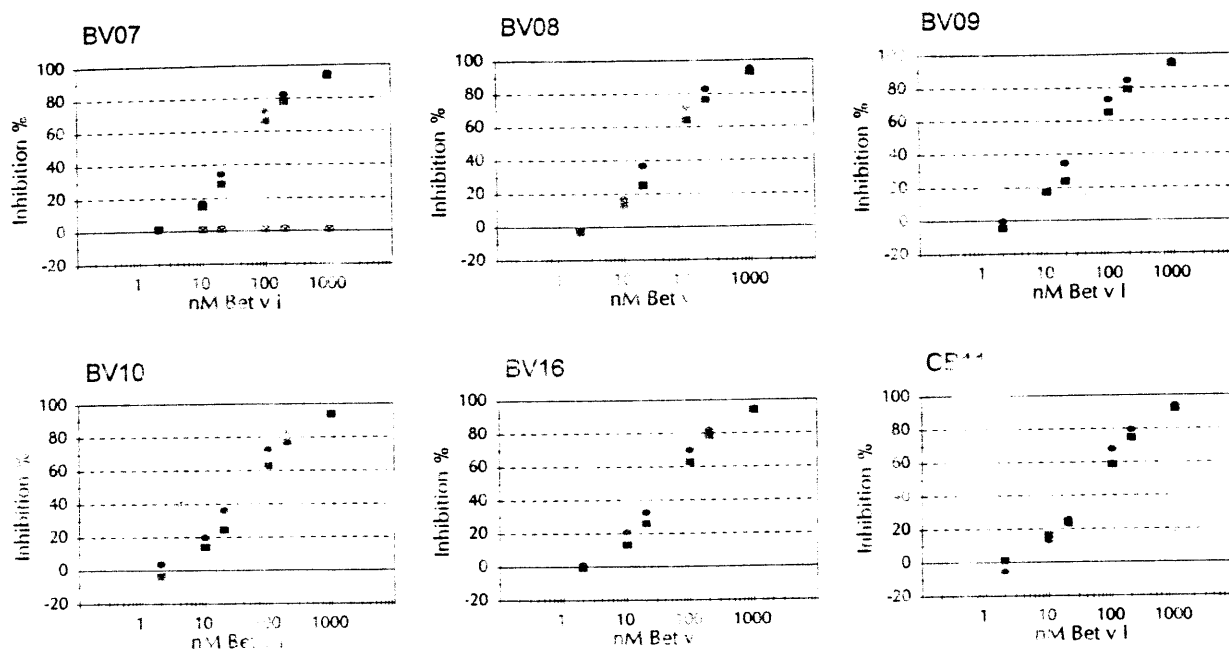


Fig. 6. Radioinhibition immunoassay. Inhibition of the binding of iodinated purified *Bet v I* to a solid phase coupled monoclonal antibody, as indicated, by dilution series of birch pollen extract (■) and bacterial lysate containing recombinant *Bet v I* (●). Negative control experiment included in upper left panel using a monoclonal antibody raised against *Cyn d I* [33], the major allergen from the grass species *Cynodon dactylon* (x and o, respectively).

quantitatively by applying a luminescence inhibition assay. There was no statistically significant difference between the capacity of the bacterial lysate and a pollen extract to inhibit the binding of IgE to a solid-phase pollen extract. This result is in agreement with earlier findings obtained by RAST inhibition, demonstrating that most IgE specificities of Scandinavian patients are directed towards the major allergen and that the inhibitory potency of purified *Bet v I* is identical to that of the pollen extract [8].

Epitopes defined by six murine monoclonal antibodies were investigated using a radioinhibition immunoassay. All inhibition curves were linear and parallel, and again there were no significant differences between the inhibitory potency of recombinant and natural *Bet v I*. The results indicate that substantial parts of the molecular surfaces have similar contours, since the antibody-binding epitopes cover relatively large areas. From X-ray crystallographic studies of hen's egg white lysozyme, it appears that the area buried by the antigen-antibody interaction is $\sim 700 \text{ \AA}^2$ [36]. Recent studies applying hydrogen-exchange labeling and two-dimensional nuclear magnetic resonance suggest even larger epitopes, since areas of $\sim 1200 \text{ \AA}^2$ seem

to be affected by the interaction. Extrapolated to *Bet v I*, it may be anticipated that the six epitopes in this study cover over one third of the *Bet v I* surface, since they represent three determinants to which the pairwise binding of antibodies do not inhibit each other competitively.

Bet v I from pollen is known to be moderately glycosylated, containing $\sim 7.5\%$ (w/w) carbohydrate [Paulsen, personal commun.], whereas recombinant proteins of *E. coli* origin are not. The results presented indicate, that the carbohydrate moiety is not involved in antibody binding.

Apart from glycosylation, the biochemical characterization of *Bet v I* had not indicated the presence of post-translational modifications that would complicate its production in *E. coli*. Neither does the molecule contain cysteine residues. Theoretical considerations, therefore, point to the feasibility of producing this particular allergen in its native conformation in bacteria. The immunochemical results presented are in agreement herewith.

The close resemblance of the epitope structure of recombinant *Bet v I* with that of the natural allergen makes the tree pollen system an ideal model system for the study

of allergenic epitopes. Specific amino acid substitutions may be tailored into the recombinant allergen for optimal preservation of the structure of complex epitopes. Much of the current excitement in the characterization of allergenic epitopes relies on the prospect of conveying regulatory function via specific T cell epitopes. The present model system offers the possibility of tailoring such epitopes into the recombinant allergen for the purpose of exploring therapeutic agents that reflect the dualism of the immune system.

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The recombinant *Bet v* I described in this communication is available for research use.

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