

PCR BASED CLONING AND SEQUENCING OF ISOGENES
ENCODING THE TREE POLLEN MAJOR ALLERGEN *Car b 1*
FROM *CARPINUS BETULUS*, HORNBEAM*

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Abstract—Cloning of the gene encoding the major allergen, *Car b 1*, from *Carpinus betulus* (hornbeam) pollen was performed using the Polymerase Chain Reaction (PCR) to specifically amplify the gene of interest using single stranded cDNA as template. Specific primers, deduced from the aminoterminal sequence of the purified protein, were tailored to facilitate direct expression of plasmic clones, and the large fraction of positive clones obtained, revealed the presence of isogenic variation.

Three clones were characterized in detail by antibody based assays and nucleotide sequencing. The recombinant allergens were shown by crossed immunoelectrophoresis (CIE) to precipitate with monospecific polyclonal rabbit antibodies raised against purified *Bet v 1*, by crossed radioimmuno-electrophoresis (CRIE) to bind tree pollen allergic patient serum IgE, and by immunoblotting to bind murine monoclonal antibodies, raised against purified *Car b 1* from pollen.

Car b 1 is encoded by a 159-triplets open reading frame. The molecular masses (M_r = 17272, 17355 and 17217 Da, respectively), the amino acid composition, and the aminoterminal sequence of the predicted polypeptides agree well with data obtained by analysis of the protein purified from pollen.

The deduced amino acid sequences show pronounced homology (73, 75 and 74% identities respectively) to *Bet v 1*, the major allergen from *Betula verrucosa* (white birch) pollen.

Soluble recombinant *Car b 1*, without a fusion partner, was produced in *Escherichia coli* with an immunochemical reactivity closely resembling that of the native pollen allergen. The tree pollen major allergens therefore constitute an ideal system for the study of allergenic epitopes.

INTRODUCTION

On a global basis the most abundant sources of airborne allergens are house dust mite derived particles, fungal spores and grass pollens. Equally important on a local basis are pollens from weeds or trees such as pollens of the Fagales tree order including alder, *Alnus*, birch, *Betula*, hazel, *Corylus*, hornbeam, *Carpinus*, oak, *Quercus* and beech, *Fagus*, which dominate the temperate climate zone of the Northern hemisphere (D'Amato *et al.*, 1991).

Patients sensitive to one of the species most often also react to one or more of the other species within the birch, alder, hazel and hornbeam group (Dirksen and Østerballe, 1978; Ipsen *et al.*, 1988; Nüchel Petersen *et al.*, 1988; Wihl *et al.*, 1988). Studies of pollen extracts of these four species have shown that only a few of the extractable proteins are IgE binding, i.e. allergens (Ipsen and Löwenstein, 1983; Ipsen and Larsen, 1988). One allergen from each of the species accounts for most of the allergenicity of the extracts.

The major allergens, *Aln g 1*, *Bet v 1*, *Cor a 1* and *Car b 1* are relatively well characterized (Ipsen and Hansen, 1991). They seem to represent homologous proteins as they share physical/chemical properties. They are all acidic glycoproteins with pI values in the range of 4.0–6.0, have molecular masses of approx. 17 kDa, and display highly correlated amino acid compositions. They have been purified to near homogeneity applying identical biochemical purification procedures and the respective aminoterminal amino acid sequences display approx. 80% sequence identity.

The first complete sequence of a tree pollen major allergen has been obtained from a cDNA clone encoding *Bet v 1* (Breiteneder *et al.*, 1989). Cross-hybridization experiments between the four species using derivatives of this clone have demonstrated the presence of polynucleotides homologous in sequence both at the DNA and at the mRNA level (Valenta *et al.*, 1991).

The relatedness of the major allergens is further substantiated by the immunochemical cross-reactivity. When analysed by immunoelectrophoretic methods applying polyspecific rabbit antibodies raised against purified *Bet v 1*, reactions towards all four major allergens are evident, although differences in affinities are also noted (Ipsen and Hansen, 1991).

Analysis of commercially available pollen extracts by two-dimensional gel electrophoresis/immunoblotting indicate microheterogeneous variation among the major

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allergens (Florvaag *et al.*, 1986; Florvaag *et al.*, 1988; Jarolim *et al.*, 1989; Rohac *et al.*, 1991). When analysed by Western blotting applying monospecific rabbit antibodies raised against purified *Bet v* 1, even highly purified preparations of major allergen reveal a small cluster of bands, demonstrating the presence of variants having distinct apparent molecular masses (Ipsen and Larsen, 1988; Ipsen and Hansen, 1991).

Indications that this variation may be genetically based comes from the recent isolation of two *Bet v* 1 fractions, displaying different aminoterminal sequences (Elsayed and Vik, 1991). In this communication we describe the molecular cloning of isoallergic variants of the gene encoding the major allergen from *Carpinus betulus*.

MATERIALS AND METHODS

Materials

Pollen extract and rabbit antibodies were prepared as described (Ipsen and Hansen, 1991). Tree pollen allergic serum pool was a mixture of equal volumes of serum from 30 donors. Patients were selected as described (Nüchel Petersen *et al.*, 1988). Monoclonal hybridoma antibodies were derived from Balb/c mice immunized with purified *Car b* 1 as described (Ipsen and Schou, 1992).

Methods

Methods that are not described in detail below were performed according to standard procedures (Maniatis *et al.*, 1982; Ausubel *et al.*, 1987).

Preparation of mRNA from pollen

Frozen pollen (2 g) were suspended in 10 ml buffer (250 mM sucrose, 200 mM Tris-HCl pH = 8.5, 200 mM KCl, 30 mM MgCl₂, 2% (w/v) polyvinylpyrrolidone-40 and 5 mM β -mercaptoethanol) and 10 ml freshly equilibrated phenol at 0°C. The suspension was immediately sonicated for 5 min, duty cycle 50% ultrasonic exposure, at setting 7, approx. 50 W, in a Branson sonifier model 250. Chloroform (10 ml) was added and the suspension was incubated with shaking for at least 15 min at ambient temperature. Phases were separated by centrifugation, 10,000 g for 20 min at 4°C. The aqueous phase was transferred to a clean tube and extracted two more times with an equal (v/v) mixture of phenol and chloroform. Nucleic acids were precipitated with sodium acetate and ethanol at -20°C overnight. The precipitate was collected by centrifugation, 13,000 g for 20 min at 4°C, washed with 70% ethanol and dried. The samples, usually four run in parallel, were pooled in 20 ml water, made 3 M in LiCl, and RNA selectively precipitated for 4 hr at -20°C. The precipitate was collected as described above, resuspended in water and again precipitated with sodium acetate and ethanol.

Oligo (dT)-cellulose selection was performed batch-wise as a series of washings, centrifugation for 20 sec, 15,000 g, removal of the supernatant and resuspension of the oligo (dT)-cellulose, in an Eppendorf tube at ambient temp.

RNA (100–200 OD₂₆₀) were dissolved in 0.5 ml water and denatured at 65°C for 5 min. 0.5 ml 2 $\times$ loading buffer [0.1 M citrate buffer pH = 7.6, 1 M NaCl, 2 mM EDTA, 0.2% (w/v) SDS] was added, and the sample was mixed with 0.2 g oligo (dT)-cellulose (Boehringer Mannheim), prepared as recommended by the supplier. After addition of the sample the oligo (dT)-cellulose was washed with 8 $\times$ 0.5 ml 1 $\times$ loading buffer and the poly(A)⁺ RNA eluted with 10 $\times$ 0.5 ml elution buffer [25 mM citrate buffer pH = 7.6, 1 mM EDTA, 0.05% (w/v) SDS]. Fifty microlitres of each fraction was withdrawn for OD₂₆₀ measurement and fractions containing RNA, usually 5 or 6, were precipitated with lithium chloride and ethanol at -20°C overnight. The precipitate was collected by centrifugation at room temp., washed, dried and pooled in water, and again precipitated with sodium acetate and ethanol.

cDNA synthesis

cDNA synthesis was performed as described (Klickstein and Neve, 1987) with the following modifications: cDNA synthesis was primed with PCR primer #1, see below, at a concn of 13 μ M, and only one cDNA strand was synthesized, which was precipitated with ethanol after phenol extraction, step 6 (Klickstein and Neve, 1987).

Design of PCR primers

Primer #1, which was designed to prime the cDNA in a location corresponding to the poly(A) tract of the mRNA, was composed of 15 dT residues, extended in the 5' end with restriction sites appropriate for directed cloning. The degenerate sequence of primer #2 was deduced from the amino terminal amino acid sequence of the purified protein: GVFN^{YEAET}PSVIPAARL^{FK}... (Ipsen and Hansen, 1991). A stretch of seven amino acids with minimum degeneracy (underlined above) was selected to produce a 20mer oligonucleotide covering 128 combinations of codons. One of the codons (GCG) was left out of account on the basis of rare occurrence (Murray *et al.*, 1989) resulting in a lowering of the number of oligonucleotides in the mixture to 96. The sequences were extended in the 5' end to accommodate an appropriately positioned ATG start codon and restriction sites for directed cloning.

The sequences of the primers were:

#1: (37mer) 5'-GCG GAA TTC CAG CTG CAG

AAG CTT TTT TTT TTT TTT T-3'

#2: (41mer) 5'-GCG GAA TTC ACC ATG GGT GTT

TT(C/T) AA(C/T) TA(C/T)

GA(A/G) GC(A/C/T) GA(A/G) AC-3'

Polymerase chain reaction

An optimized PCR reaction contained 0.33 μ g of single stranded cDNA in a 0.1 ml reaction with 50 mM KCl, 10 mM Tris-HCl pH = 8.3, 2.5 mM MgCl₂, 0.01%

gelatine, 0.2 μ M primer #1, 1 μ M degenerate primer #2, 0.2 mM each of dATP, cCTP, dGTP and dTTP and 2.5 units AMPLI-Taq polymerase (Cetus).

Thirty cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C were performed in a Hybaid Thermal Reactor with the final 72°C incubation step extended to 8 min. The yield was optimized by varying the Mg²⁺ concn, the primer #1 concn, the cDNA concn and the annealing temp.

Nucleotide sequencing

Nucleotide sequencing was performed by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using commercially available 17-mer universal sequencing primers and specific 20-mer oligonucleotide primers synthesized on an Applied Biosystems 380A synthesizer using the manufacturers instructions.

Expression

Escherichia coli K-12 strain JM105 (Yanisch-Perron *et al.*, 1985) containing recombinant plasmids was grown in the (A + B) minimal medium of Clark and Maaløe (Clark and Maaløe, 1967) supplemented with 0.2% (w/v) glucose, 0.01% (w/v) thiamine, and 0.1 g/l ampicillin. Exponentially growing cultures were induced by addition of isopropyl β -D-thiogalactoside (IPTG) to 0.5 mM at an OD₄₃₆ of 0.8, corresponding to approx. 10⁷ cells/ml. After 2 hr of induction cells were harvested, washed and resuspended in 3 ml (6% of the original volume) lysis buffer (50 mM Tris-HCl pH = 8.0, 0.1 M NaCl, 1 mM EDTA) and disrupted by ultrasonic treatment for 2 times 15 sec. Cell debris was removed by centrifugation at 28,000 g for 15 min. After extensive dialysis against 25 mM ammonium bicarbonate the bacterial extract was freeze-dried and stored at -20°C for subsequent analysis.

Crossed (radio)immuno-electrophoresis

Crossed immuno-electrophoresis, CIE, and crossed radioimmuno-electrophoresis, CRIE, were performed essentially as described previously (Ipsen and Hansen, 1991).

Western blotting

SDS-PAGE and semi-dry electroblotting were performed as previously described (Ipsen and Larsen, 1988). Following blotting the nitrocellulose sheet was blocked by incubation for 2 min in [50 mM Tris pH = 10.3, 150 mM NaCl and 2% (v/v) Tween 20]. The following incubations and washings were performed in buffer containing 50 mM Tris pH = 10.3, 150 mM NaCl and 0.05% (v/v) Tween 20 at ambient temp. Following blocking, the filter was washed for 5 min and subsequently incubated with monoclonal antibody diluted 1:100 for 16 hr. Following three washings of 5 min each, the filter was incubated with affinity purified, alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (DAKO) diluted 1:1000 for 5 hr. Another three washings of 5 min each were performed and the filter was

stained with 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue tetrazolium in ethanolamine buffer as described (Ipsen and Larsen, 1988).

RESULTS

The yield of RNA in a typical experiment was 1 mg per g of pollen, as calculated from OD₂₆₀ measurement. The integrity of the RNA was assessed by formaldehyde denatured agarose gel electrophoresis. The RNA pellet appeared light brown in color and tended to impede the flow of an oligo (dT)-cellulose column. For this reason the oligo (dT) selection was performed batch wise. The yield of poly (A)⁺ RNA was typically 15 μ g per g of pollen as calculated from OD₂₆₀ measurement.

The efficiency of cDNA synthesis was assayed by trichloroacetic acid precipitation. Yields were typically 6% of the amount of input RNA, resulting in a net yield of 1 μ g single stranded cDNA per g of pollen.

PCR was performed using single stranded cDNA as template. In a similar system we have shown a decrease in specificity when double stranded cDNA was substituted for single stranded cDNA. Eight distinct bands appeared as opposed to a single band, although weaker, when single stranded cDNA was used (data not shown).

Theoretically, the distal primer is unspecifically priming all double stranded cDNAs, whereas no single stranded cDNAs are likely to contain a poly(A) stretch. Thus, in the latter case, the distal primer can act only on molecules that have previously been primed by the proximal primer, which conveys the specificity of the reaction.

A rather fuzzy band approx. 800 basepairs in length, Fig. 1, was electroeluted from a preparative 1% agarose gel in an analytical unidirectional electroelutor (IBI) according to the manufacturers instructions. The DNA fragment was cut with *Eco*RI and *Hind*III, and ligated to expression vector pKK223 (Brosius and Holy, 1984) cut with the same restriction enzymes. pKK223 contains the inducible *tac* promoter (de Boer *et al.*, 1983). The vector was treated to minimize self ligation by inclusion of *Pst*I in the restriction reaction and by subsequent alkaline phosphatase treatment. Ligation was allowed to proceed overnight at 16°C and the ligation mixture was used for transformation of *Escherichia coli* K-12 strain JM105 (Yanisch-Perron *et al.*, 1985). Transformation efficiency was 2 $\times$ 10⁴ transformants per μ g of vector DNA in the ligation reaction. In one experiment 42 individual transformants were picked at random, of which 13 contained inserts, 23 did not, and 6 represented dimerized plasmids as judged by uncut plasmid mini preparations.

Clones containing inserts were cut with *Eco*RI and *Hind*III to determine the sizes of the cloned DNA. Insert sizes varied significantly between 600 and 900 base-pairs, indicating that the origin of different genes were individual cDNA's rather than identical DNA fragments generated in the PCR. Furthermore, when the clones were cut with *Bam*HI, a fragment of fixed length was generated by most clones, confining the variability to the distal part

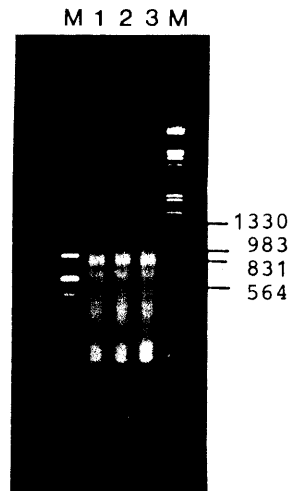


Fig. 1. Crude PCR products analysed by agarose gel electrophoresis. Reaction conditions as described in *Materials and Methods* with the following exception: lane 1: 2.3 mM $MgCl_2$, lane 2: 2.5 mM $MgCl_2$, lane 3: 2.7 mM $MgCl_2$. Lanes M: molecular weight markers, left: pBR322/*A**lu*1910, 659/655, 521, 403, 281, 257 and 226 basepairs. Right: λ wt/*Hind*III/*Eco*RI 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831 and 564 base-pairs.

of the genes, since the vector contains a *Bam*HI site 257 base-pairs upstream for the *Eco*RI site and most clones contain another *Bam*HI site 343 base-pairs downstream from the *Eco*RI site.

Potentially positive clones were expressed in a volume of 50 ml. Soluble lysates were analysed by Western blotting using a monoclonal antibody raised against *Carb* I purified from pollen, Fig. 2.

The apparent molecular mass of the recombinant allergens differ within the range defined by the native allergen from a pollen extract, indicating the presence of isoforms.

Three clones, showing different mobility of the recombinant allergens in SDS-PAGE, were selected for sequencing. *Eco*RI-*Bam*HI and *Bam*HI-*Hind*III subclones were constructed in pUC19 (Vieira and Messing, 1982), and sequencing was performed using the dideoxy chain termination method. Two sets of primers were synthesized to match the inserts approximately in the middle. These primers were also used to verify the sequence in the *Bam*HI site, which was used for subcloning. On one clone full sequence on both DNA strands was obtained on the *Eco*RI-*Bam*HI subclone only. This subclone covers 70% of the open reading frame. The remaining 141 nucleotides of the open reading frame was sequenced on one DNA strand without the occurrence of ambiguities.

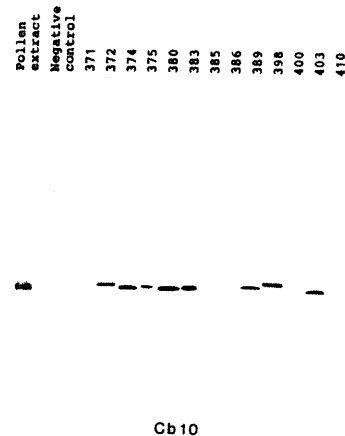


Fig. 2. Western blot using monoclonal antibody Cb10, raised against purified *Carb* I from pollen, of arbitrarily numbered soluble bacterial lysates. From left: *Carpinus betulus*, horn-beam, pollen extract 5 μ g, soluble lysate of pK223 in JM105 (negative control), and 13 soluble lysates from transformants containing recombinant plasmids, 20 μ l.

Internationally approved nomenclature rules (Marsh *et al.*, 1987) are not clear for isoallergens obtained by gene cloning. In agreement with the designations of the *Amb* a1 variants (Rafnar *et al.*, 1991) the following nomenclature will be used: Group 1, *Carb* I, 1a is derived from plasmid pCB1a which is identical to clone 375, Fig. 2, *Carb* I, 1b is derived from plasmid pCB1b which is identical to clone 403, Fig. 2. Group 2, *Carb* I, 2 is derived from plasmid pCB2 which is identical to clone 398, Fig. 2.

The nucleotide sequences are shown in Fig. 3. The three sequences have open reading frames 477 nucleotides in length, coding for 159 amino acids. This is the same length as the open reading frame of the gene encoding *Bet* v 1 (Breiteneder *et al.*, 1989).

In all three sequences A:T and G:C base-pairs are equally dispersed and AT and CG dinucleotides are under-represented. In agreement with other plant genes usage of codons NCG and NTA are rare (Murray *et al.*, 1989).

The deduced amino acid sequences have the following characteristics: The amino acid compositions correlate with the amino acid composition of the protein purified from pollen. The calculated molecular masses (M_r = 17272 Da, M_r = 17355 Da and M_r = 17217 Da, respectively) are in good agreement with that of the pollen protein, 17.5 kDa as determined by SDS-PAGE (Ipsen and Larsen, 1988). The three sequences display a consensus glucosylation site (N-X-S/T) in position 82. Fig. 4. Group 1 sequences have an additional potential glucosylation site in position 55.

The hydrophobic profiles of the deduced amino acid sequences contain no dominating hydrophobic or

1b	GGTGTGTTTCAATTACGAGGCGAGAAACCCCTCCGTTATCCACGGCTAGGCTGTTCAAG	60
1a	-----t-----a-c-----a-----a-----a-----a-----	
2	-----t-c-t-a-c-----A-----a-----a-----a-----a-----	
1b	TCCTATGTCCTTGATGGCGATAAGCTGATCCCAAAGTTGCACCTCAAGCTATTAGCAGC	120
1a	-----c-----T-----c-----T-----c-----T-----c-----	
2	G--T-A-----A-----c-----T-----c-----G-----t-----	
1b	GTTGAAACGTTGGAGGAAATGGAGGGCCTGGAACCATCAAGAATATCACCTTTGCCGAA	180
1a	-----A-----a-----a-----a-----a-----a-----a-----	
2	-----A-----a-----a-----a-----a-----a-----a-----	
1b	GGCAGCCCTTTCAAGTTCGTGAAGGAGAGGGTTGATGAGTTGACAAACGAACTTCAAA	240
1a	-----T-----a-----a-----a-----a-----a-----a-----	
2	-----G-----A-----a-----a-----a-----a-----a-----	
1b	TACAACTACACCGTGATCGAGGGTGATGCTCGGGCGACAAGCTGGAGAAGTTCAGCCAC	300
1a	-----a-----a-----a-----a-----a-----a-----a-----	
2	-----a-----a-----a-----a-----a-----a-----a-----	
1b	GAGCTGAAGATAGTGGCAGCCCTGGTGGTGGATCCATCGTGAAGATCAGCAGAGTTTC	360
1a	-----a-----a-----a-----a-----a-----a-----a-----	
2	-----a-----a-----a-----a-----a-----a-----a-----	
1b	CACGCCAAAGGCTACCATGAGGTTAACGCGAGGAGATGAAGGGTGCAGAAAGTGGCC	420
1a	-----t-----A-----a-----a-----a-----a-----a-----	
2	-----t-----A-----a-----a-----a-----a-----a-----	
1b	GAGAACTTTTAAAGGCGGTTGAGAGCTACCTCTGGCACACACTGCTGAATACAATAA	480
1a	-----a-----a-----a-----a-----a-----a-----a-----	
2	-----a-----a-----a-----a-----a-----a-----a-----	
1b	ACCTCGTCTTGTCCTTCGCCCAATAATGACTTGTCCTTGAATTTTTTTCTTTTTC	540
1b	TTTTTCTTTCTTAAATTTGTTTACTGCTTAATTTGGCTTGCTAATAAGGAGCTCCG	600
1b	GGTTGTGTTTCATCTGCTGCTGAAGATCGATGTTGTAACTCGGAAGATGCAAACTGAAT	660
1b	GTTGTATTACTTTTTCATATATACAAATGATGGAAGATAACATCAATTGAAGTTCAA	720
1b	GTTTGAATGAATGAATTTTACTGAAGGATTATGATAAAAAAAAAAAAAAAAAA	775

Fig. 3. Nucleotide sequences of the full insert of pCB1b (1b) and of the open reading frames of pCB1a (1a) and pCB2 (2). TAA stop codon underlined. Bold face: Nucleotides covered by the PCR primers. In pCB1a and pCB2 sequences lower case letters represent silent deviations, while upper case denotes deviations giving rise to amino acid substitutions. Horizontal bars (---): Identity to pCB1b.

hydrophilic clusters, Fig. 5. Average hydrophobic indices are -0.21, -0.29 and -0.26 respectively in agreement with other globular proteins (Kyte and Doolittle, 1982).

When comparing the nucleotide sequences the leading 26 nucleotides should be excluded, since substitutions in this region could originate from mismatching of the PCR primer. The remaining 451 nucleotides show 6 deviations (99% identities) between pCB1a and pCB1b, 24 deviations (95% identities) between pCB1a and pCB2, and 20 deviations (96% identities) between pCB1b and pCB2. 22 of the 25 deviating positions are located in the first half of the structural genes.

The deduced amino acid sequences, Fig. 4, show 3 deviations (98% identities) between *Carb* I, 1a and *Carb* I, 1b, 19 deviations (88% identities) between *Carb* I, 1a and *Carb* I, 2, and 16 deviations (90% identities) between *Carb* I, 1b and *Carb* I, 2. On this basis the variants may be distributed in two groups: group 1 represented by *Carb* I, 1a and *Carb* I, 1b, and group 2 represented by *Carb* I, 2.

When compared to the sequence of the related major allergen from *Betula verrucosa*, *Bet* v 1, a pronounced homology is noted. The three *Carb* I deduced amino acid sequences show 73, 75 and 74% identities respectively, to the *Bet* v 1 sequence, Fig. 4.

The immunochemical activity of the recombinant allergens was assessed by crossed immunoelectrophoresis (CIE) and crossed radioimmuno-electrophoresis (CRIE), Fig. 6. One well-defined precipitate was formed against monospecific rabbit anti-*Bet* v I, and it was shown that this precipitate binds tree pollen allergic patients serum IgE.

DISCUSSION

Utilization of the Polymerase Chain Reaction (PCR) in gene cloning is simple, rapid and efficient. The specific amplification of the gene of interest results in a dramatic increase in the fraction of positive clones. The method is, in theory, generally applicable wherever the amino terminal sequence of a protein is known. Also facilitated are virtually unlimited possibilities for extension of the primers to accommodate appropriately positioned restriction sites and translational start codon for the directed cloning into given expression vectors.

The error incorporation rate of the TAQ polymerase has been studied by two groups, who find an error incorporation rate of 2×10^{-4} (Saiki *et al.*, 1988) and 1.1×10^{-4} (Tindall and Kunkel, 1988; Eckert and Kunkel, 1990) respectively. There is good agreement between the two determinations in spite of the fact

native pollen allergen, indicating that the predominant conformation in solution is more or less the same. This conclusion is supported by the observed spontaneous renaturation of *Car b* I following denaturation, and the lack of cysteines. Furthermore, the tree pollen major allergens have not been observed to contain modified amino acids or other post-translational modification which would abrogate their production in *E. coli*. The birch major allergen, *Bet v* I, is known to be moderately glucosylated, 7.5% (w/w) (Smestad Paulsen, personal communication), whereas recombinant polypeptides produced in *E. coli* are not. The lack of glucosylation of recombinant *Bet v* I does not affect the reactivity of the allergen with patient serum IgE (Breiteneder *et al.*, 1988, 1989), and if native *Car b* I is glucosylated, this conclusion seems to hold also for this tree pollen major allergen.

On this background the tree pollen major allergens seem to constitute an ideal model system for the study of allergenic epitopes. Mapping and characterization of epitopes is a prerequisite for the detailed study of the interaction between allergenic molecules and the specific T and B lymphocytes, respectively, which govern the immune response and the pathogenesis of type I allergic disease. Molecular characterization of epitopes will facilitate improvement of the specificity of diagnosis and the standardization of extracts for treatment, and thus a general improvement of the positive outcome of specific immunotherapy.

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