

Four recombinant isoforms of *Cor a* I, the major allergen of hazel pollen, show different IgE-binding properties

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Previous studies showed that pollens from trees of the order *Fagales* (e. g. birch, alder, hazel and hornbeam) all contain one major allergen. These proteins are cross-reactive between these tree species, and approximately 95% of tree-pollen-allergic patients display IgE binding to these allergens. Using the reported N-terminal amino acid sequence of the hazel pollen allergen *Cor a* I, it was possible to amplify *Cor a*-I cDNA by use of the polymerase chain reaction. Four clones with cDNA inserts were isolated. All four clones contained an open reading frame of 477 nucleotides (159 amino acids) but differed in length of their 3'-non-coding regions. Within the overlapping regions, the nucleotide sequence of the 3'-non-coding regions of the four clones were nearly identical. The open reading frames coded for different isoforms of the major hazel pollen allergen, *Cor a* I. The clones were designated *Cor a* I/5, 6, 11 and 16, respectively. Comparison of the deduced amino acid sequences of these *Cor a* I isoforms revealed identities of 96–99%. The sequence identities between the *Cor a* I isoforms and *Bet v* I, the major birch pollen allergen, were 71–73% (80.5–83% similarity). Comparing amino acid sequences of *Cor a* I isoforms with the published sequences of *Aln g* I, the major allergen from alder, and *Car b* I and isoforms, the major allergen from hornbeam, 75.5–76.7% identity (83.6–85% similarity) and 83.6–89.9% sequence identity (89.3–95% similarity), respectively, was found. The four *Cor a* I cDNAs were subcloned into plasmid pKK223-3 and expressed in *Escherichia coli* as non-fusion proteins; their capacity to bind serum IgE from tree-pollen-allergic patients was investigated. The four cloned isoforms showed an apparent molecular mass of 17 kDa in SDS/PAGE, identical to the natural, pollen-derived *Cor a* I. IgE antibodies from tree-pollen-allergic patients reacted with all four recombinant isoforms. However, we noted marked differences in the IgE-binding patterns of the distinct isoforms. Furthermore, *Cor a* I/11 was the only isoform recognized by the anti-(*Bet v* I) mAb, BIP 1. Our results demonstrate that *Cor a* I isoforms display different antigenic and allergenic properties, very likely due to few but significant changes in their amino acid sequences. These findings have implications for the development of reagents for diagnosis and immunotherapy of type I allergies.

At least 15% of the population of industrialized countries in the Northern hemisphere suffer from type I, i. e. IgE-mediated, allergies. In this respect, the pollens of early flowering trees of the order *Fagales* (in particular birch, alder, hazel and hornbeam) represent an important source of allergens. Numerous studies have been carried out to identify and characterize precisely the allergens of these pollens [1–6].

The major allergen from birch pollen, *Bet v* I, has been cloned, sequenced and expressed from a cDNA library [3].

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Abbreviations. *Aln g* I, major allergen from alder pollen; *Bet v* I, major allergen from birch pollen; BIP 1, anti-(*Bet v* I) mAb; *Car b* I, major allergen from hornbeam pollen; *Cor a* I, major allergen from hazel pollen; PCR, polymerase chain reaction; r, recombinant.

Enzymes. Restriction endonucleases *Eco*RI and *Hind*III (EC 3.1.21.4); reverse transcriptase (EC 2.7.7.49); *Taq* polymerase, DNA polymerase from *Thermophilus aquaticus* (EC 2.7.7.7).

It was shown that, not only pollens from birch, but also from other trees of the order *Fagales* contained one predominant major allergen showing high similarity with one another on the nucleic acid [4] and amino acid levels [1–3] and cross-reactivity with IgE from birch-pollen-allergic patients [2, 5]. This fits well with the observation that patients who display specific IgE to *Bet v* I also show symptoms during the flowering season of hazel and the other trees of the order *Fagales*, in particular of alder and hornbeam [7]. It was therefore of interest to obtain the complete amino acid sequence of *Cor a* I, the major allergen from hazel pollen, in order to gain information on the degree of homology or diversity between tree pollen major allergens, and on structural features which may be responsible for IgE binding.

Diagnostic methods routinely used for the measuring specific IgE level are the radioallergosorbent test, the enzyme-linked immunosorbent assay (ELISA) and histamine-release assays. The reliability of all these tests mainly depends on the availability of pure and well-defined allergens.

A		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
<i>Cor a I</i>	5	ATG	GGT	GTT	TTC	AAT	TAC	GAG	GTT	GAG	ACT	CCC	TCC	GTT	ATC	CCT	GCG	GCA	AGG	CTG	TTC	AAG	TCC	TAT	GTC	CTT	GAT	GGC	GAT	AAG	CTC
<i>Cor a I</i>	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	..A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I</i>	11	-	-	-	-	-	-	-	..C	-	..C	A..	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I</i>	16	-	-	-	-	-	-	-	-	-	..C	-	-	-	-	T..A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bet v I</i>		-	-	-	-	-	-	..A	AC..	-	..C	A..	..T	-	-	..A	..A	..T	C..A	-	-	-	G..	T..T	A..	-	-	-	-	..T	-
		30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
<i>Cor a I</i>	5	ATC	CCA	AAG	GTT	GCA	CCT	CAA	GCT	ATT	ACC	AGC	GTT	GAA	AAC	GTT	GAA	GGA	AAT	GGA	GGG	CCT	GGA	ACC	ATC	AAG	AAT	ATC	ACC	TTT	GGC
<i>Cor a I</i>	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I</i>	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I</i>	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	..G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bet v I</i>		T..T	-	-	-	-	..C	-	..C	-	..G	..T	-	-	-	A..	-	-	-	-	-	-	-	-	..T	-	..G	-	..G	-	CC..
		60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89
<i>Cor a I</i>	5	GAA	GGC	AGC	CGT	TAC	AAG	TAC	GTG	AAG	GAG	AGG	GTT	GAT	GAG	GTT	GAC	AAC	ACA	AAC	TTC	ACA	TAC	AGC	TAC	ACC	GTG	ATC	GAG	GGT	GAT
<i>Cor a I</i>	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	..A	..T	-	-	-	-	-	-	-	-
<i>Cor a I</i>	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I</i>	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	..A	..T	-	-	-	-	-	-	-	-
<i>Bet v I</i>		-	-	TT..	..C	..T	-	-	-	-	..C	..A	-	-	-	..G	-	C..	-	-	-	..A	-	..AT	-	..G	-	-	-	..C	..G
		90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119
<i>Cor a I</i>	5	GTC	CTG	GGT	GAC	AAG	CTG	GAG	AAG	GTC	TGC	CAC	GAG	CTG	AAG	ATA	GTG	GCA	GCC	CCT	GGT	GCA	GGA	TCC	ATC	TTG	AAG	ATC	AGC	AGC	AAG
<i>Cor a I</i>	6	-	-	-	-	-	-	-	-	-	-	AG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I</i>	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I</i>	16	-	-	-	-	-	-	-	-	..A	-	AG	-	-	-	-	-	-	-	-	-	..G	-	..C	-	-	-	-	-	-	-
<i>Bet v I</i>		CC..	A..A	..C	-	..CA	T..	-	-	A..	..C	A..	-	A..A	-	-	-	-	A..	-	..A	-	-	-	-	-	-	-	-	..A	-
		120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149
<i>Cor a I</i>	5	TTC	CAC	GCC	AAA	GGC	GAC	CAT	GAG	ATT	AAT	GCA	GAG	GAG	ATG	AAG	GGT	GCC	AAA	GAA	ATG	GCA	GAG	AAA	CTT	TTA	AGG	GCG	GTT	GAG	ACC
<i>Cor a I</i>	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	..C	-	-	-	-	-	-	-	-	-
<i>Cor a I</i>	11	-	-	-	-	..T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	..C	-	-	-	-	-	-	-	-	-
<i>Cor a I</i>	16	-	-	-	-	..T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	..C	-	-	-	-	-	-	-	-	-
<i>Bet v I</i>		..A	-	A..	-	..T	-	-	-	G..G	..G	-	-	C..	G..T	-	..CA	AGT	-	-	-	..CC	-	..C	-	..G	-	..C	-	-	..G
		150	151	152	153	154	155	156	157	158	159																				
<i>Cor a I</i>	5	TAC	CTA	TTG	GCA	CAC	TCT	GCT	GAA	TAC	AAC																				
<i>Cor a I</i>	6	-	-	-	-	-	-	-	-	-	-																				
<i>Cor a I</i>	11	-	-	-	-	-	-	-	-	-	-																				
<i>Cor a I</i>	16	-	-	-	-	-	-	-	-	-	-																				
<i>Bet v I</i>		-	..C	-	-	-	..C	..A	..CC	-	-																				

Protein extracts obtained from natural source are difficult to standardize and, therefore, preparations vary from batch to batch. Total pollen extracts (in the case of tree-pollen-allergic patients, usually a mixture of birch, alder and hazel pollen) are commonly used for diagnosis and immunotherapy (often the method of choice for treatment of type I allergies). These extracts contain, on the one hand, constituents to which the patient is not allergic; on the other hand, proteins may be lost during the extraction procedure and thus be missing in the final preparations. Allergen preparations containing a maximum range of well-defined and reproducibly prepared allergens would therefore be superior to raw pollen extracts consisting of an insufficiently defined mixture of allergens and other components.

Expression of allergenic proteins (or only allergenic or tolerogenic fragments thereof) by recombinant DNA techniques would allow reproducible preparation of allergens. Since the N-terminal protein sequence of *Cor a I* has been determined by Edman degradation [1], it was possible to directly amplify *Cor-a-I* cDNA using a sequence-specific 5'-terminal primer and a non-specific 3'-terminal primer by the polymerase chain reaction (PCR) as recently published for cDNA cloning of *Aln g I*, the major allergen from alder pollen [8] and *Car b I* and isoforms, the major allergen from hornbeam [9].

In this study, we report the complete cDNA sequences of four isoforms of *Cor a I*, showing more than 70% sequence identity with *Bet v I*. These *Cor a I* isoforms were expressed in *Escherichia coli* as non-fusion proteins, and differences in their capacity to bind serum IgE from tree-pollen-allergic patients were demonstrated.

MATERIALS AND METHODS

Extraction and isolation of poly(A)-enriched RNA

Total RNA was extracted from 4 g non-defatted pollen from hazel (*Corylus avellana*; Allergon AB, Engelholm, Sweden) according to the acidic guanidinium thiocyanate method [10]. The pollen grains were initially broken under liquid nitrogen with a mortar and pestle and further homogenized in extraction buffer [10] using an Ultraturrax blender. By passing the total RNA over an oligo(dT)-cellulose column, following standard procedures [11], 40 µg poly(A)-enriched RNA was isolated.

Synthesis of the first-strand cDNA

Poly(A)-enriched RNA (4 µg) was reverse-transcribed in a reaction volume of 20 µl using 50 U Maloney murine leu-

B		10	20	30	40
Cor a I 16	ATA*TCGCTCT	TGTCCTCTCG	CC*ATAATA	ACTTGACGT	
Cor a I 6	---	---	---	---	
Cor a I 11	CC*	---	---	G---	
Cor a I 5	---	---	---	---	
Bet v I	T--A-TAA--	---	G-T	*G--C*--G	T-CCTG-TCA
Cor a I 16	GGCTTTCATG	TTTTTTTTTA	AAAACTTTG	TTTACTTGCT	
Cor a I 6	---	---	T	*	A---
Cor a I 11	---	---	T	**	---
Cor a I 5	---	---	*	---	---
Bet v I	ATAATGG**	--GCAG-G-T	*C-TGG-G-T	---	TGGGT--
Cor a I 16	AATAAGGAG	CTTCGGTTG	TGTCATCTG	CTTGCTGAA*	
Cor a I 6	---	---	---	---	
Cor a I 11	---	---	---	---	G
Cor a I 5	---	---	---	---	A ₂₁
Bet v I	---	---	A	---	AGCT
Cor a I 16	ATCGATGTTG	TAATC***G	GAAGAATGCA	AACTGAATGT	
Cor a I 6	---	---	---	---	
Cor a I 11	---	---	---	---	T-T
Bet v I	GAA	---	T-TATT	-G-*	T-A-A
Cor a I 16	TGTATTACTT	TTTGCATATA	TACAAATAAT	GGAAAGGATA	
Cor a I 6	---	---	---	---	
Cor a I 11	---	A ₁₂	---	---	
Bet v I	-C-	A ₂₉	---	---	
Cor a I 16	ACATCATTTGA	AGTTCAAAAA	AAAGAAAAA	AAAAGCTTTT	
Cor a I 6	---	---	A ₅₀	---	
Cor a I 16	TTTTTTTTTT	TTTTTTTTTT	TTTTTTTTTT	TCAATTTTAA	
Cor a I 16	290	300	310	320	
Cor a I 16	CCCGATACTG	ATACTCAAAA	ATGCAAGAGA	GTTTCCGCAT	
Cor a I 16	330	340	350	360	
Cor a I 16	AAGCACAATT	TGTTTATGTT	GACTTAATAC	ATTATAAGCA ₂₄	

Fig. 1. (A) Open reading frames (cDNA sequences) and (B) 3'-non-coding regions (cDNA sequences) of four *Cor a I* isoforms and of *Bet v I* with reference to *Cor a I* 5. (A) Dashes (—) indicate identities of codons and dots (·) identities of nucleotides. (B) Dashes indicate identities of nucleotides, ★ indicates deletions and A_n indicates a poly(A) tail of *n* residues.

kemia virus reverse transcriptase (Boehringer Mannheim, FRG) according to the manufacturer's instructions. As a primer for the cDNA synthesis, a synthetic oligodeoxynucleotide, Cora 1, was used; this contained, besides a poly(dT) tract and a spacer sequence, a *Hind*III recognition site (underlined): 5'-GAGAGAGAGAGAAAGCTT-T₁₈-3'.

Priming oligodeoxynucleotides for DNA amplification

The oligonucleotides for DNA amplification were chosen from the region where the corresponding N-terminal amino acid sequences of *Cor a I* and *Bet v I* [1, 2] are identical, as determined by automated Edman degradation of the pollen proteins. The oligodeoxynucleotide designated Cora 2 corresponds to the amino-terminal coding-strand sequence encoding amino acids MGVFNY. The nucleotide sequence of Cora 2 was chosen following the codon usage of the *Bet-v I*-encoding cDNA [3] and it was also designed to contain an *Eco*RI site (underlined) at its 5' end: 5'-GGGAATTC-ATGGGTGTTTTCAATTACG-3'. Cora 3, which contained a *Hind*III site (underlined), was complementary to a part of Cora 1: 5'-GAGAGAGAGAGAAAGCTT-3'.

Amplification of *Cor-a-I* cDNA by PCR

The products of the first-strand cDNA synthesis were extracted once with phenol and once with chloroform/isoamyl

alcohol (24:1) and precipitated with ethanol. After centrifugation, the pellet was dried under vacuum and resuspended in 20 µl water. The single-stranded cDNA was then mixed with 20 pmol of each priming oligodeoxynucleotide Cora 2 and Cora 3, 10 µl of 10× reaction buffer (Boehringer Mannheim, FRG), 500 pmol of each dNTP, Mg²⁺ to a final concentration of 2.5 mM and 2.5 U *Taq* DNA polymerase (Boehringer). The mixture was brought to 100 µl with distilled water and overlaid with 60 µl mineral oil (Sigma). The sample was amplified with a programmable DNA thermal cycler from Perkin Elmer Cetus (Norwalk CT). Amplification for 30 rounds consisted of denaturation at 94 °C for 45 s, annealing of primers to template at 50 °C for 1 min and chain elongation at 72 °C for 3 min.

Amplified DNA was purified from an agarose gel by glass bead adherence with the GeneClean R II kit (BIO 101 Inc., La Jolla CA). The DNA was then digested simultaneously with *Eco*RI and *Hind*III, subjected to electrophoresis on a 1.2% agarose gel and recovered with the GeneClean R II kit. The digested DNA was ligated into *Eco*RI/*Hind*III-digested pUC18 plasmid.

The resulting plasmids were used to transform competent *E. coli* JM105 cells. Single colonies were picked and grown, plasmid DNA isolated and digested with *Eco*RI/*Hind*III. Clones bearing inserts of 600–850 nucleotides were chosen for dideoxy-sequencing [12]. Both strands were sequenced.

Expression of proteins and characterization by immunoblotting

The four *Cor a I* clones were expressed in pKK223-3 as non-fusion proteins. To release the inserts, the pUC18/*Cor a I* plasmids were simultaneously cut with *Eco*RI and *Hind*III and the cDNA fragments were purified from agarose gels with the GeneClean R II kit. Directional insertion of the cDNA fragments into the *Eco*RI and *Hind*III sites of the expression plasmid pKK223-3 (Pharmacia LKB Biotechnology, Uppsala, Sweden) enabled us to utilize the ribosomal binding site present in the plasmid.

Competent *E. coli* JM105 cells were transformed with the resulting recombinant plasmids. The cells were grown to an absorbance at 600 nm of 0.4 in 50-ml cultures. Isopropyl D-thiogalactopyranoside was then added to a final concentration of 2.5 mM and incubation continued for another 3 h at 37 °C. After expression of the recombinant proteins, cells were harvested by centrifugation. The pellets were resuspended in 750 µl 50 mM Tris/HCl pH 7.5 containing 220 mM NaCl. The cells were then disrupted by freezing in liquid nitrogen followed by thawing at 37 °C. This procedure was repeated twice. Insoluble material was then removed by centrifugation at 16 000×g for 10 min. Proteins in the supernatant were separated by electrophoresis on a 15% SDS/polyacrylamide gel [13] and either Coomassie-stained or equivalent amounts of the proteins (according to the staining) were transferred onto nitrocellulose membranes [14]. IgE-binding proteins were detected as previously described using allergic patients' sera (1:10 dilution) and ¹²⁵I-labeled rabbit anti-human IgE (Pharmacia) [6].

A cDNA encoding *Aln g I* [8] and *Bet v I* [3] was cloned into pKK223-3, expressed as *rAln g I* and *rBet v I* and subjected to immunoblotting as described above.

Patients and sera

Tree-pollen-allergic patients were chosen according to characteristic case history (type I allergic symptoms in March

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
<i>Cor a I 5</i>	Met	Gly	Val	Phe	Asn	Tyr	Glu	<u>Val</u>	Glu	Thr	<u>Pro</u>	Ser	Val	Ile	Pro	Ala	Ala	Arg	Leu	Phe	Lys	<u>Ser</u>	Tyr	Val	Leu	Asp	Gly	Asp	<u>Lys</u>	Leu
<i>Cor a I 6</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I 11</i>	-	-	-	-	-	-	-	<u>Ala</u>	-	-	Thr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I 16</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<u>Ser</u>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bet v I</i>	-	-	-	-	-	-	-	Thr	-	-	Thr	-	-	-	-	-	-	-	-	-	-	Ala	Phe	Ile	-	-	-	-	Asn	-
	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
<i>Cor a I 5</i>	<u>Ile</u>	Pro	Lys	Val	Ala	Pro	Gln	Ala	Ile	Thr	Ser	Val	Glu	Asn	Val	Glu	Gly	Asn	Gly	Gly	Pro	Gly	Thr	Ile	Lys	<u>Asn</u>	Ile	Thr	Phe	<u>Gly</u>
<i>Cor a I 6</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I 11</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I 16</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<u>Gly</u>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bet v I</i>	Phe	-	-	-	-	-	-	-	-	Ser	-	-	-	-	Ile	-	-	-	-	-	-	-	-	-	-	Lys	-	Ser	-	Pro
	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89
<i>Cor a I 5</i>	Glu	Gly	<u>Ser</u>	<u>Arg</u>	Tyr	Lys	Tyr	Val	Lys	Glu	Arg	Val	Asp	Glu	Val	Asp	<u>Asn</u>	Thr	Asn	Phe	<u>Thr</u>	Tyr	<u>Ser</u>	Tyr	Thr	Val	Ile	Glu	Gly	<u>Asp</u>
<i>Cor a I 6</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Lys	-	-	-	-	-	-	-	-	-
<i>Cor a I 11</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I 16</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Lys	-	-	-	-	-	-	-	-	-
<i>Bet v I</i>	-	-	Phe	Pro	Phe	-	-	-	-	Asp	-	-	-	-	-	-	His	-	-	-	Lys	-	Asn	-	Ser	-	-	-	-	Gly
	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119
<i>Cor a I 5</i>	<u>Val</u>	Leu	Gly	Asp	<u>Lys</u>	Leu	Glu	Lys	Val	<u>Cys</u>	<u>His</u>	Glu	Leu	Lys	Ile	Val	Ala	<u>Ala</u>	Pro	<u>Gly</u>	Gly	Gly	Ser	Ile	Leu	Lys	Ile	Ser	<u>Ser</u>	Lys
<i>Cor a I 6</i>	-	-	-	-	-	-	-	-	-	-	<u>Ser</u>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I 11</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I 16</i>	-	-	-	-	-	-	-	-	-	-	<u>Ser</u>	-	-	-	-	-	-	-	-	-	-	-	-	<u>Thr</u>	-	-	-	-	-	-
<i>Bet v I</i>	Pro	Ile	-	-	Thr	-	-	-	Ile	Ser	Asn	-	Ile	-	-	-	-	Thr	-	Asp	-	-	-	-	-	-	-	-	Asn	-
	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149
<i>Cor a I 5</i>	Phe	His	<u>Ala</u>	Lys	Gly	Asp	His	Glu	Ile	<u>Asn</u>	Ala	Glu	<u>Glu</u>	<u>Met</u>	Lys	Gly	<u>Ala</u>	Lys	Glu	Met	Ala	Glu	<u>Lys</u>	Leu	Leu	Arg	Ala	Val	Glu	Thr
<i>Cor a I 6</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I 11</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cor a I 16</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bet v I</i>	Tyr	-	Thr	-	-	-	-	-	Val	Lys	-	-	Gln	Val	-	Ala	Ser	-	-	-	Gly	-	Thr	-	-	-	-	-	-	Ser
	150	151	152	153	154	155	156	157	158	159																				
<i>Cor a I 5</i>	Tyr	Leu	Leu	Ala	His	Ser	<u>Ala</u>	<u>Glu</u>	Tyr	Asn																				
<i>Cor a I 6</i>	-	-	-	-	-	-	-	-	-	-																				
<i>Cor a I 11</i>	-	-	-	-	-	-	-	-	-	-																				
<i>Cor a I 16</i>	-	-	-	-	-	-	-	-	-	-																				
<i>Bet v I</i>	-	-	-	-	-	-	Asp	Ala	-	-																				

Fig. 2. Deduced amino acid sequences of four *Cor a I* isoforms and of *Bet v I* with reference to *Cor a I/5*. Amino acid replacements other than conservative are underlined.

and April), positive skin-prick tests and positive radioallergo-sorbent tests (classes > 3.5). Sera from 20 non-allergic individuals (case history, skin-prick and radioallergosorbent tests) were pooled and used for negative control experiments (normal human sera pool). Sera were stored at -20 °C.

Immunoblot analysis of pollen protein extracts

Total protein extracts from pollen and immunoblot analysis were made according to procedures described previously [6]. All reagents and cell lysates were from identical batches and were used in the same concentrations in all experiments. According to Coomassie staining, equivalent amounts of the four recombinant *Cor a I* isoforms were used for immunoblots. The *Cor a I* band was identified by comparison with immunoblots using identical gels. As a control, gels were run with *E. coli* lysates harboring the plasmid without a *Cor a I*-encoding sequence. Subsequently the radioactive signals of bound patient's IgE were analyzed after a 48-h exposure on an enhanced laser densitometer and peak areas were measured (Ultrosan XL, LKB; Bromma; software: gelscan XL, Pharmacia; Table 1).

Glycosylation analysis of proteins from hazel and birch pollen

Aqueous extracts from hazel and birch pollen were blotted onto nitrocellulose sheets and sugar detection was performed according to manufacturer's instructions (Boehringer Mannheim).

RESULTS

Amplification of *Cor a I* cDNAs with PCR and subcloning

By PCR, cDNA was amplified which migrated as two bands at an approximate length of 640 and 780 nucleotides in a 1.2% agarose gel (not shown). The cDNA fragments in these bands were used for the subsequent cloning procedures into pUC18. Four clones were chosen for their different length of cDNA inserts (690–860 bp) and subjected to sequence analysis.

Sequence analysis of *Cor a I* cDNAs

The four clones were designated *Cor a I/5*, 6, 11 and 16, respectively. Their coding regions were of identical length of

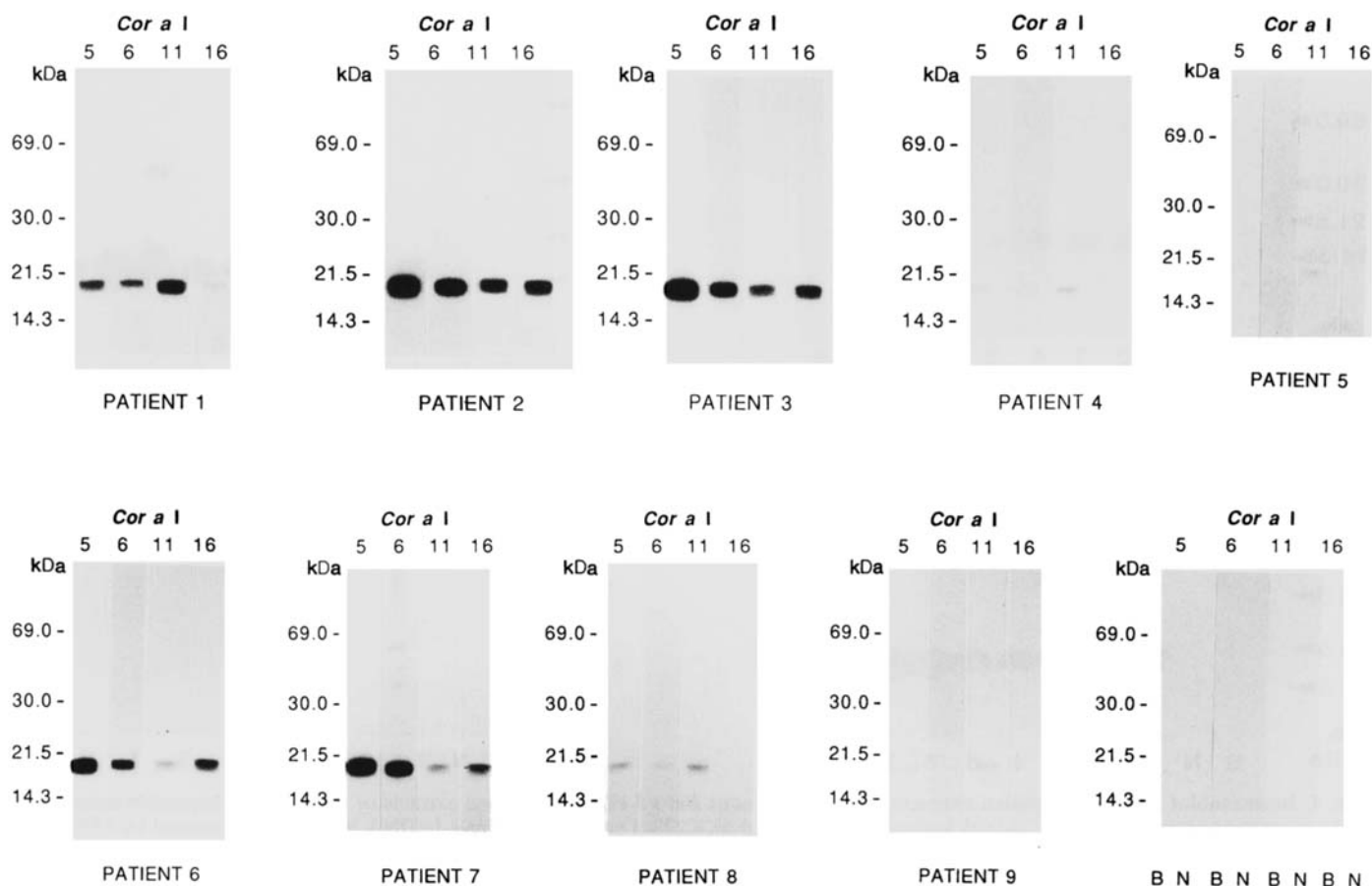


Fig. 3. Immunoblot analyses of r*Cor a I* isoforms performed with IgE derived from different allergic patient's sera. Soluble proteins from *E. coli* JM105, transformed with pKK223-3/*Cor a I*/5, 6, 11 or 16, were separated by 15% SDS/PAGE. The proteins were transferred onto nitrocellulose and probed with different tree-pollen-allergic patients' sera, as indicated, or a pool of sera from non-allergic individuals (lanes N), and serum-free buffer (B) as negative controls. Bound IgE antibodies were detected using ^{125} I-labeled rabbit anti-(human IgE).

477 nucleotides (excluding start and stop codons) and thus coded for proteins of 159 amino acids (Fig. 1). Using *Cor a I*/5 as reference, the following nucleotide exchanges were observed: *Cor a I*/6, six nucleotide exchanges, three of which lead to amino acid exchanges; *Cor a I*/11, five nucleotide and two amino acid exchanges; *Cor a I*/16, twelve nucleotide and six amino acid exchanges (Fig. 2). Comparison of the deduced amino acid sequences of the *Cor a I* isoforms with that of *Bet v I* [3] revealed the following identities and similarities (identities plus conservative replacements): *Cor a I*/5, 73% identity, 82.4% similarity; *Cor a I*/6, 73% and 83%; *Cor a I*/11, 73% and 83%; *Cor a I*/16, 71% and 80.5% (Fig. 2). The *Cor a I* isoforms also shared significant sequence similarities, 60–70%, with a group of pathogenesis-related proteins found in bean, parsley, pea and potato [15–18] as does *Bet v I* [3].

The 3'-non-coding regions of the four isoforms differed in length. Excluding the poly(A) tract, they consisted of 115 nucleotides for clone 5, 160 for clone 11, 209 for clone 6 and 353 for clone 16. The poly(A) tracts contained 21 adenylate residues in clone 5, 12 in clone 11, 50 in clone 6, and 24 in clone 16. A consensus polyadenylation signal AATAAA is present in the 3'-non-coding region of the four clones. Furthermore, significant nucleotide sequence identities could be observed in the overlapping 3'-non-coding regions of the *Cor a I* isoforms (Fig. 1 B).

Expression and immunoblot analysis of recombinant *Cor a I* isoforms

The four *Cor a I* isoforms expressed by pKK223-3 as non-fusion proteins were subjected to 12% PAGE and showed an apparent molecular mass of 17 kDa. According to Coomassie-stained gels (data not shown), equal amounts of recombinant protein of each isoform were tested for their capacity to bind IgE from allergic patients' sera in immunoblot experiments (Fig. 3). No IgE binding could be observed in control experiments (buffer and normal human serum control). Furthermore an identical set of experiments was performed for a protein extract from *E. coli* JM105 harboring the expression plasmid pKK223-3, but without cDNA insert. Again, no IgE binding could be detected (Fig. 4 D). There were marked differences in the ability of each r*Cor a I* isoform to bind IgE from the nine different patients' sera (Fig. 3).

Patients 1, 4, 5, 8 and 9 showed the most pronounced IgE reactivity with r*Cor a I*/11; on the other hand, patients 2, 3, 6 and 7 displayed the strongest IgE binding with r*Cor a I*/5 (Fig. 3). Comparing IgE binding to r*Cor a I*/11 with isoform r*Cor a I*/16, patients 1, 4, 5, 8 and 9 reacted more strongly with r*Cor a I*/11, whereas patients 3, 6 and 7 displayed stronger IgE binding to r*Cor a I*/16 (Fig. 3). Patients 1, 2, 3, 6 and 7 recognized every isoform, but with varying

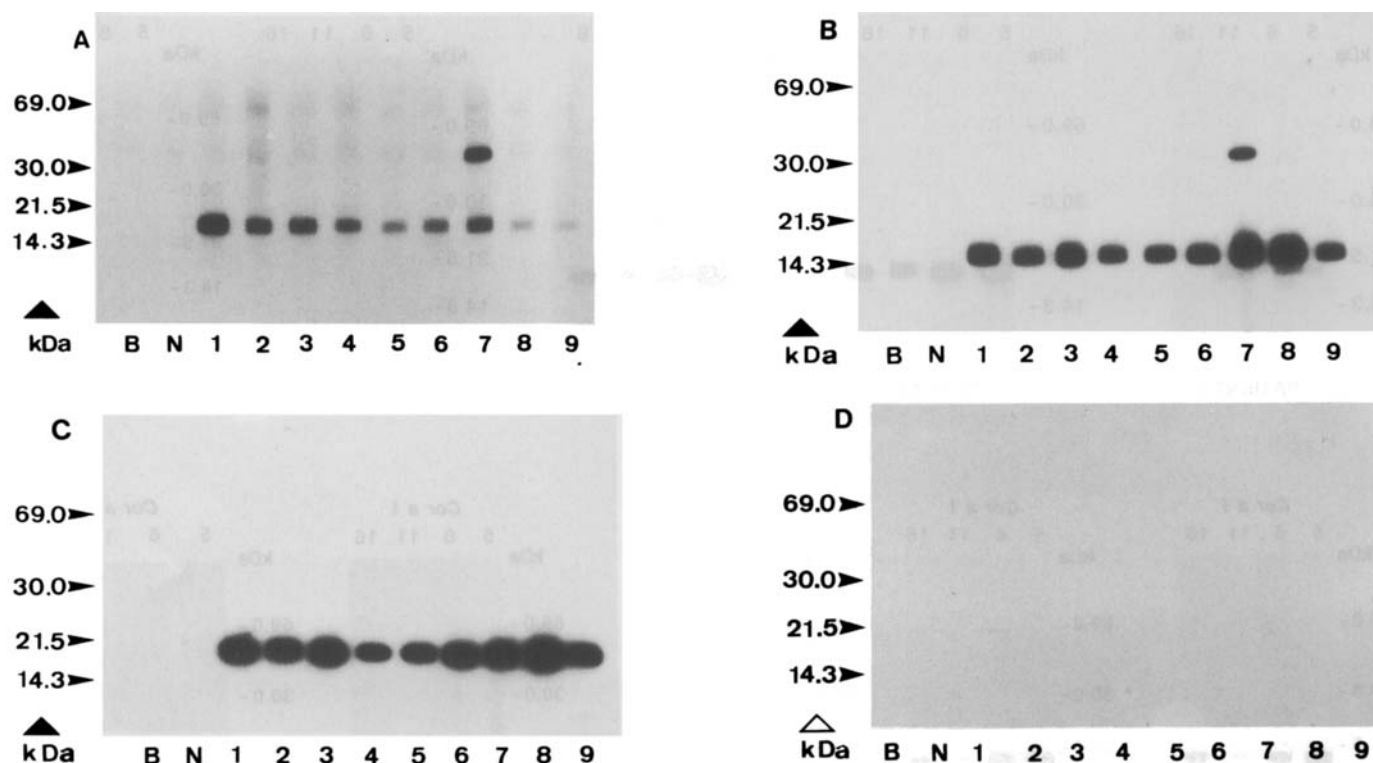


Fig. 4. Immunoblot analyses of pollen extracts and recombinant *Bet v I* cDNA. Aqueous extracts of pollen and comparable amounts of aqueous protein extracts of *E. coli* JM105 harboring the plasmid pKK223-3 carrying the *Bet-v-I* cDNA insert were separated by 15% SDS/PAGE, transferred onto nitrocellulose and probed with the same sera as in Fig. 3. For control experiments, the bacterial lysate of JM105 harboring the expression plasmid without any cDNA insert was used. The negative controls and the detection of bound IgE were performed as described in Fig. 3. (A) Hazel pollen; (B) birch pollen; (C) recombinant *Bet v I*; (D) *E. coli* lysate harbouring the plasmid without any insert.

Table 1. Scanning data of immunoblots. IgE binding of patient 2 to r*Cor a I/5* was considered 100%. Relative peak areas from Fig. 3 are listed.

Patient	Relative peak areas of			
Patient	<i>Cor a I/5</i>	<i>Cor a I/6</i>	<i>Cor a I/11</i>	<i>Cor a I/16</i>
	%			
1	21.0	14.6	54.6	4.3
2	100.0	77.3	43.2	46.1
3	94.0	58.8	28.2	34.8
4	2.3	<1	10.5	<1
5	<1	<1	4.7	<1
6	60.4	27.0	12.1	27.8
7	75.0	67.3	15.3	19.8
8	7.3	3.6	12.8	<1
9	2.8	<1	5.3	<1

degrees of intensity. IgE antibodies from patient 5 reacted with r*Cor a I/11* exclusively. Patients 4, 8 and 9 reacted with r*Cor a I/5*, 6, 11 but not with r*Cor a I/16*. Isoform r*Cor a I/11* was the only preparation which was recognized by IgE from all nine patients. These data were confirmed by scanning the bands on the autoradiographs using a gel scanner (Table 1). In Fig. 4 A–C, the same set of nine patients' sera was used in immunoblot experiments with hazel pollen extract (Fig. 4 A), birch pollen extract (Fig. 4 B) and r*Bet v I* (Fig. 4 C). Strong IgE binding to the major allergens, *Cor a I*,

Bet v I and r*Bet v I* was detected with each serum. Patients 7 and 8 reacted with additional proteins (Fig. 4 A–C, lanes 7 and 8).

Experiments with the mAb BIP 1

A mAb (BIP 1) raised against *Bet v I* [19], showing cross-reactivity with the major allergen of alder *Aln g I*, and with *Cor a I* and *Car b I* [5], was tested with both the hazel pollen extract and r*Cor a I* isoforms. The antibody bound with comparable intensity to the pollen extract of hazel, but only isoform r*Cor a I/11* gave a weak positive signal with this antibody. None of the other isoforms was recognized by BIP 1 (data not shown).

Detection of glycosylation

When performing the glycosylation detection analysis for hazel pollen extract as well as for birch pollen extract no sugar could be detected in the molecular mass region ranging over 30–14 kDa, suggesting no involvement of carbohydrate residues in antibody binding (data not shown).

DISCUSSION

There is great interest in pure and reproducible preparations of allergens for diagnostic as well as for therapeutic and experimental purposes. However, it is rather difficult to obtain reproducible batches of allergens from crude extracts

derived from natural sources. For these reasons, molecular cloning techniques have attracted allergologists' interest.

When partial amino acid sequences are known, the complete nucleotide sequence of the corresponding mature mRNA can be obtained by established PCR techniques [20–22]. These methods are particularly useful for cloning and sequencing members of a gene family which differ in only a few amino acids. This has been shown just recently for two allergens *Amb a I* [23] and *Car b I* and isoforms [9].

Analyzing the nucleotide sequences of the *rCor a I* isoforms, there is striking similarity with the conserved regions of other major tree pollen allergens of the order *Fagales* (*Aln g I* [2], *Bet v I* [3], *Car b I* [9]) as well as with pathogenesis-related proteins from distantly related species like bean, pea, parsley and potato expressed in somatic tissues upon chemical or microbial stress [15–17, 24]. One might speculate that the *Cor a I* protein possesses a similar biological function in the protection of the pollen grains from microbial or fungal attack [25].

It is unlikely that the sequence differences observed between the four isoforms of *Cor a I* reported here are due to PCR artefacts, for three reasons: (a) the frequency of nucleotide exchanges is much higher than the expected error frequency of the *Taq* polymerase ($1/10^{-5}$ nucleotides per cycle [26]); (b) about half of the nucleotide exchanges are silent, which is not expected for errors with the *Taq* polymerase; (c) the 3' extensions of the cDNAs certainly show that the four isoforms represent distinct molecular species.

Although all four isoforms of *Cor a I* contained a coding region of an identical length of 477 nucleotides, which agrees with the data obtained for related cloned allergens, *Aln g I* [8] and *Car b I* [9], they differed in their 3'-non-coding region ranging from 155 to 353 nucleotides plus adenylate residues ranging from 12 to 50. The 3'-non-coding regions of the *rCor a I* isoforms showed almost identical sequences where they overlap. Despite these identities, the different lengths of the 3'-non-coding regions may be responsible for completely different folding patterns of these regions, thus effecting different stability and capacity to bind to RNA- and poly(A)-binding proteins [27–30]. This could lead to a differential translation of the mRNAs coding for *Cor a I* isoforms. Nothing is known yet about whether different isoforms possess different functions in the plant.

Interestingly, at difference to the published sequences of *Bet v I* [3] and *Car b I* [9], the sequences of *Aln g I* [8] and the *Cor a I* isoforms contain a single Cys at position 99.

Although the *Cor a I* isoforms described in this paper all showed high amino acid sequence similarities (80.5–83%) compared with the sequence of *Bet v I* (Fig. 2) and 96–99% identity among each other, there were striking differences in their ability to bind IgE from allergic patients. All patients reacted with natural *Cor a I*, a mixture of isoforms. However, IgE from patients 1, 4, 5, 8 and 9, besides recognizing *Bet v I*, recognized the isoform *Cor a I/11* very much more than the other isoforms (Fig. 3). One explanation of this observation might be an amino acid exchange at position 10 from threonine in *Bet v I* and *Cor a I/11* to proline in isoform *rCor a I/5*, *rCor a I/6* and *rCor a I/16* (Fig. 2). On the other hand, IgE antibodies from patients 4, 5, 8 and 9 did not recognize isoform *rCor a I/16*; this correlates with an amino acid exchange from proline in *Bet v I* to serine in *rCor a I/16* at position 14. It can be assumed that the change of a threonine to proline, an amino acid which is known to strongly influence the three-dimensional structure of the protein [31], could change the antibody affinity to possible B cell epitopes.

Some allergens even might represent an epitope *in toto*, as described for *Der p II* [32] and as seems likely for *Bet v I*: in screening experiments [3], only complete cDNA clones of *Bet v I* reacted with patients' IgE, whereas expression of partial *Bet v I* sequences did not lead to the production of IgE-binding proteins (Valenta et al., unpublished). Furthermore, protease treatment of *Bet v I* abolished its IgE-binding capacity [33] (and data from our laboratory). Experiments are under way to investigate requirements for IgE binding by site-directed mutagenesis of *Cor a I* isoforms, leading to single amino acid exchanges at crucial positions, such as amino acid positions 10 and 14.

Summing up, it should be emphasized that the *rCor a I* isoforms are capable of binding allergic patients' IgE, yet with different intensities. This shows that the structure of the recombinant proteins must be identical or at least similar to their natural counterparts. This correlates with data published recently by Larsen et al. concerning *Car b I* isoforms [9] but, unfortunately, no quantitative or semiquantitative data on patients' IgE binding were presented.

A mAb raised against *Bet v I* (BIP 1 [19]), showing cross-reactivity with natural *Cor a I* [5], was tested with hazel pollen extract and isoforms *rCor a I/5*, 6, 11 and 16. BIP 1 reacted strongly with birch and hazel pollen extracts whereas only a weak signal was found with isoform *rCor a I/11* and no signal was seen with isoforms *rCor a I/5*, 6 and 16. This result, and the observation that some patients only react very weakly with the recombinant isoforms investigated in spite of strong reactivity with the natural *Cor a I* preparation, indicate the existence of additional *Cor a I* isoforms, not yet identified, with different antigenic properties.

The information obtained on the four *Cor a I* isoforms and also of closely related allergens, *Bet v I* and *Aln g I* [2–5, 8], will enable us to establish a model to define motifs crucial for binding of IgE antibodies and allergen-specific T cells. Moreover, information on IgE-binding (T-cell-activating) and putative non-IgE-binding (non-T-cell activating) isoforms of an allergen has implications for the design of hypo-sensitization vaccines. It is not unlikely that a better understanding of biochemical properties, structures and biological functions of plant allergens will provide some new ideas as to why these pollen proteins (amongst numerous other proteins) are capable of eliciting an IgE response in atopic individuals.

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