



Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01

D. Lüttkopf^a, U. Müller^a, P.S. Skov^b, B.K. Ballmer-Weber^c, B. Wüthrich^c,
K. Skamstrup Hansen^d, L.K. Poulsen^d, M. Kästner^a, D. Haustein^a, S. Vieths^{a,*}

^a Department of Allergology, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, D-63225 Langen, Germany

^b Reference Laboratory, Copenhagen, Denmark

^c Allergy Unit, Department of Dermatology, University Hospital, Zurich, Switzerland

^d Allergy Unit, National University Hospital, Copenhagen, Denmark

Received 9 May 2001; accepted 19 September 2001

Abstract

The aim of this study was to produce the Bet v 1-related major hazelnut allergen Cor a 1.0401 and variants thereof as recombinant allergens, and to compare their immuno-reactivity with the major hazel pollen allergen using sera of patients whose hazelnut allergy recently was confirmed by double-blind placebo-controlled food challenges (DBPCFC) in a multicenter study.

Total RNA was isolated from immature hazelnuts and transcribed into cDNA. Full length coding DNA obtained by PCR-strategy was subcloned into pTYB11 vector and expressed in *E. coli* ER2566 cells. Native non-fusion target proteins were purified by DTT-induced self-cleavage of the intein-tagged N-terminal fusion proteins. IgE reactivity of the recombinant allergens was tested by enzyme allergosorbent test (EAST), EAST-inhibition, immunoblot-inhibition and histamine release assays. Four recombinant allergens were produced showing deduced amino acid sequence identities among each other of 97–99%, and were considered as variants Cor a 1.0401 (GenBank Accession no.: AF136945), Cor a 1.0402 (AF323973), Cor a 1.0403 (AF323974) and Cor a 1.0404 (AF323975). Cor a 1.0402 and 03 only differed in a C4S exchange. Cor a 1.0404 had a unique proline residue in position 99. Surprisingly, only 63% identity was revealed with hazel pollen Cor a 1. EAST with 43 sera of patients with positive DBPCFC to hazelnut indicated IgE reactivity to Cor a 1.0401 in 95% of the sera, to Cor a 1.0402 in 93%, to Cor a 1.0403 in 91%, and in only 74% of the sera to the proline variant Cor a 1.0404. The allergenic activity of the four variants was confirmed by histamine release assays in 15 hazelnut-allergic patients stimulated with the four variants and controls. Eleven sera were positive with extract from native hazelnut, 13 with rCor a 1.0401, 12 with rCor a 1.0402, 11 with rCor a 1.0403, and only two with rCor a 1.0404 containing the proline exchange. The high IgE binding variant Cor a 1.0401 showed only partial IgE cross-reactivity with pollen Cor a 1. IgE-binding and histamine release capacity led to a concordant ranking of the allergenic activity of the recombinant variants: Cor a 1.0401 > Cor a 1.0402 and 03 > Cor a 1.0404 (the proline variant). Similar results for Cor a 1.0402 and 03 suggest a minor influence in IgE binding of cysteine in position 4, whereas proline in position 99 appears to be responsible for the decrease in IgE reactivity in Cor a 1.0404. It appears that the epitopes of hazelnut Cor a 1.04 are less related to pollen Cor a 1 than to Bet v 1 from birch pollen. Low IgE binding variants or mutants of Cor a 1.04 are candidate compounds for developing a novel and safe approach of specific immunotherapy of hazelnut allergy. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Variants; Allergen; Hazelnut allergy; Cor a 1.04; Cor a 1.01

1. Introduction

Hazelnut is one of the most frequent causes of food allergy (Ortolani et al., 1988; Eriksson et al., 1982; de Groot

et al., 1996). Two studies may be cited in which hazelnut was revealed as the most common source of allergy to foodstuffs with 37% among 383 food allergic patients (Etesamifar and Wüthrich, 1998), and 53% among 380 birch pollen allergic patients (Eriksson et al., 1982). Recently, allergy to hazelnut was, for the first time, confirmed in a multicenter study by DBPCFC (Ortolani et al., 2000). Allergenic structures cross-reacting with birch pollen have been identified in hazelnut extract (Belin, 1972; Andersen and Løwenstein, 1978; Roeser et al., 1987; Rohac et al., 1991). Also, cross-reactive counterparts in hazelnut and

Abbreviations: DBPCFC, double-blind placebo-controlled food challenge; SPT, skin prick test; EAST, enzyme allergosorbent test; OAS, oral allergy syndrome; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focussing

*Corresponding author. Tel.: +49-6103-772256;
fax: +49-6103-771258.

E-mail address: viest@pei.de (S. Vieths).

hazel pollen were reported (Belin, 1972; Herkenrath et al., 1982). Hirschwehr et al. (1992) identified major allergens in hazelnut (18 kDa) and hazel pollen (17 kDa) related to the birch pollen allergen Bet v 1 (Breiteneder et al., 1989) as well as a minor 14 kDa hazelnut allergen related to birch profilin Bet v 2 (Ebner et al., 1995). Recently, a heat-resistant 48 kDa glycoprotein was identified as a minor hazelnut allergen with cross-reactive carbohydrate determinants. Partial N-terminal sequencing demonstrated similarity to legume storage proteins (Müller et al., 2000). Apart from the above-described more frequent tree pollen-related allergens a subgroup of hazelnut allergens shows no relationship to pollen allergens. Lately, such pollen-independent allergens with molecular masses below 12 kDa were identified in hazelnut (Schocker et al., 1999, 2000; Akkerdaas et al., 2000). These low molecular weight proteins may represent lipid transfer proteins (Akkerdaas et al., 2000) or 2S albumins (Teuber et al., 1998). Furthermore, IgE binding structures above 18 kDa were described at molecular masses of 20, 23, 31, 37, 50–55, 62, and 69 kDa (Calkhoven et al., 1987; Hirschwehr et al., 1992; Müller et al., 2000; Schocker et al., 2000). So far, more effort has been undertaken in the characterization of hazel pollen allergens, especially the major allergen Cor a 1.01 (Breiteneder et al., 1993) and a comparison to Bet v 1 (Valenta et al., 1991; Ebner et al., 1993) or birch pollen in general (Rohac et al., 1991; Florvaag et al., 1988; Ipsen et al., 1985).

Yet, no molecular characterization of recombinant allergens from hazelnut has been published so far. In this study, we focussed on the cloning, expression and characterization of the Bet v 1-related major allergen from hazelnut Cor a 1.0401 and variants thereof, and a comparison with the homologue in hazel pollen, Cor a 1.01. The characterization was performed with sera from patients with confirmed hazelnut allergy (Ortolani et al., 2000).

2. Materials and methods

2.1. Patients' sera

Altogether, 43 sera of patients with a positive DBPCFC to hazelnut were available for this study. They represent a subgroup of patients described in a multicenter study challenged according to a single protocol (Ortolani et al., 2000). Nineteen patients were selected at the University Hospital in Zurich (Ballmer-Weber et al., 2000), and 24 patients at the National University Hospital in Copenhagen (Table 1). Placebo- and non-responders were excluded from this study. Control serum from a nonallergic donor (N) was taken from the serum collection of the Paul-Ehrlich-Institut, Langen, Germany.

2.2. Protein extracts and recombinant pollen allergens

Recombinant (r) birch pollen allergens rBet v 1 and rBet v 2, and hazel pollen rCor a 1 were purchased from BIOMAY

(Vienna, Austria). Recombinant major timothy grass pollen allergen Phl p 1 (Petersen et al., 1995) was kindly provided by Dr. A. Petersen (Research Center Borstel, Borstel, Germany), and was used as negative control. Birch pollen extract was obtained from MAST Diagnostica (Reinhold, Germany), hazel pollen extract from Allergopharma (Reinbek, Germany).

Immature, mature raw and roasted (140 °C, 40 min) hazelnuts (*Corylus avellana* var. Piemonte) were provided by Dr G. Malgarini (Sorematec, Arlon-Schoppach, Belgium). Hazelnut extracts were prepared as described (Müller et al., 2000).

The amount of protein was estimated by a commercial dye-binding assay (Roti Quant, Roth, Karlsruhe, Germany) based on the method according to Bradford (Bradford, 1976). All antigens were stored freeze-dried at –20 °C until used.

2.3. RNA isolation, cDNA synthesis and PCR-amplification

Immature hazelnuts were ground to a fine powder with pestle and mortar under liquid nitrogen. Total RNA was extracted from 170 mg powder using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) yielding 34 µg RNA per gram powdered hazelnut.

The First-Strand cDNA Synthesis Kit (amersham pharmacia, Freiburg, Germany) was applied for transcribing 1.5 µg (total) RNA into cDNA according to the manufacturer's instructions using a degenerated oligo dT-primer (dTdeg: 5'-T₁₆NN-3') (Eurogentec, Searing, Belgium) for hybridization with the poly(A)⁺-tracts of mRNAs. Amplification of first strand cDNA was carried out with the same primer used as the reverse primer, and a degenerated primer Cora5 (5'-CCY GGA ACC ATY AAG AAK ATC-3'; Y = (C/T); K = (G/T)) as the forward primer derived as follows: a completely conserved region of DNA from four known sequences of the major allergen Cor a 1.0101-04 from hazel pollens (Accession nos.: X70997, X70998, X70999, X71000) (Breiteneder et al., 1993) was aligned with a region highly conserved among food allergens of the Bet v 1 family (Mal d 1 (X83672) (Schöning et al., 1996), Pru av 1 (U66076) (Scheurer et al., 1997)) and Bet v 1a (X15877) (Breiteneder et al., 1989) itself. From a 21 bp fragment, primer Cor a 5 was derived with degenerated nucleotides in 3 positions. The degenerated primer also matched with a DNA sequence of a hazel leaf allergen Cor a 1.0301 (Z72440) (Hoffmann-Sommergruber et al., 1997).

The unknown 5'-end was amplified using the 5'-RACE technique according to the manufacturer's instructions (5'-RACE System, GIBCO BRL, Eggenstein, Germany). RNA (0.34 µg) was primed with an antisense gene-specific primer (GSP1(81–100): 5'-AGC AGT ATT TGA AGT TTG CG-3') and transcribed into cDNA. A homopolymeric dC-tail was added to the 3'-end of the purified cDNA by terminal desoxynucleotidyltransferase. The dC-tailed cDNA was amplified by PCR using an antisense nested second

Table 1
Clinical and serological data of patients with positive DBPCFC to hazelnut^a

Patient no.	Symptoms in DBPCFC	SPT hazelnut	CAP			EAST HN extracts		EAST rCor a 1.04-variants				EAST rCor a 1.01 Hazel pollen
			Hazelnut	Bet v 1	Bet v 2	Native	Roasted	1.0401	1.0402	1.0403	1.0404	
1-43	Σ positive results		70%	100%	16%	72%	12%	95%	93%	91%	74%	86%
Zurich												
1	OAS	+	3	4	0	3	0	3	3	3	3	3
2	OAS	++	3	4	0	3	0	3	3	3	3	3
3	OAS	+	3	6	3	4	1	4	4	4	4	4
4	OAS/SR	+	2	3	0	2	0	3	3	3	2	2
5	OAS	++	2	6	0	2	0	4	3	3	3	3
6	OAS	++	1	4	0	2	0	3	2	2	2	2
7	OAS	+	2	4	0	3	1	3	3	3	3	3
8	OAS	+++	2	4	2	2	1	3	3	3	2	2
9	OAS	+	3	5	2	2	0	3	3	3	3	3
10	OAS	+	3	5	0	3	0	3	3	3	3	3
11	OAS	0	3	6	3	3	0	3	3	3	3	4
12	OAS	+	1	4	0	2	0	2	2	2	1	3
13	OAS	+	2	4	0	2	0	3	3	3	2	3
14	OAS	+	3	6	0	3	1	4	3	3	3	4
15	OAS	+	1	2	0	0	0	0	0	0	0	1
16	OAS	++	3	5	2	3	0	3	3	3	3	3
17	OAS	++	3	4	0	3	0	3	3	3	3	2
18	OAS	++	2	4	0	2	0	3	2	2	2	2
19	OAS	+	0	3	0	0	0	1	1	0	0	0
Copenhagen												
20	OAS	+	2	5	0	3	0	3	3	3	3	3
21	OAS	++	0	3	0	1	0	3	2	2	1	2
22	OAS	+	3	4	0	2	0	3	2	2	2	2
23	OAS	+	2	5	3	2	0	3	3	3	2	3
24	OAS	+	0	3	0	0	0	2	2	2	0	2
25	OAS	+	2	3	0	2	0	2	2	2	2	2
26	OAS	++	0	3	0	0	0	1	0	0	0	0
27	OAS	±	2	4	0	1	0	3	3	2	2	2
28	OAS	+	0	3	0	0	0	2	1	1	0	0
29	OAS	+	2	4	0	2	0	2	2	2	2	2
30	OAS	+++	0	3	0	0	0	1	1	1	0	0
31	OAS	+	0	3	0	0	0	2	2	2	0	2
32	OAS	±	0	2	1	0	0	0	0	0	0	0
33	OAS	++	2	4	0	3	0	3	3	3	3	3

Table 1 (continued)

Patient no.	Symptoms in DBPCFC	SPT hazelnut	CAP			EAST HN extracts		EAST rCor a 1.04-variants				EAST rCor a 1.01 Hazel pollen
			Hazelnut	Bet v 1	Bet v 2	Native	Roasted	1.0401	1.0402	1.0403	1.0404	
34	OAS	++	2	6	0	3	0	3	3	4	3	2
35	OAS	++	0	3	0	0	0	1	1	1	0	0
36	OAS	+	1	4	0	2	0	2	2	2	2	2
37	OAS	++	1	5	0	2	0	3	3	3	3	3
38	OAS	+	3	6	0	4	0	4	4	4	4	3
39	OAS	+	0	4	0	2	0	3	3	3	2	2
40	OAS	+	0	3	0	0	0	2	2	2	1	2
41	OAS	+	0	3	0	0	0	2	2	2	0	2
42	OAS	+++	3	6	0	3	1	3	3	4	3	3
43	OAS	+	0	3	0	0	0	2	2	2	0	1
Summary	EAST/CAP results	class 0	13	0	36	12	37	2	3	4	11	6
		class 1	5	0	1	2	5	4	4	3	3	2
		class 2	13	2	3	15	0	10	13	14	12	17
		class 3	12	13	3	12	0	23	21	18	15	15
		class 4	0	15	0	2	0	4	2	4	2	3
		class 5	0	6	0							
		class 6	0	7	0							
		>class 0	30/43 (70%)	43/43 (100%)	7/43 (16%)	31/43 (72%)	5/43 (12%)	41/43 (95%)	40/43 (93%)	39/43 (91%)	32/43 (74%)	37/43 (86%)

^a SR: systemic reactions; HN: Hazelnut.

gene-specific primer (GSP2(57–76): 5'-CGA TCT CCT CAA CCT TGT CG-3') and an abridged anchor primer provided with the kit for hybridization with the dC-tail.

2.4. Cloning and sequencing

PCR-products were purified from agarose gels using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany), were ligated into vector pGEM-T (Promega, Madison, USA) and transformed in *E. coli* strain XL-1 Blue (Stratagene, Heidelberg, Germany) by electroporation. Plasmids from positive clones were subjected to sequence analysis by using an ABI 377 sequencer (Applied Biosystems, Weiterstadt, Germany) and vector-specific primers T7 (5'-TAA TAC GAC TCA CTA TAG G-3') and SP6 (5'-GAT TTA GGT GAC ACT ATA GA-3'). Full-length cDNA was obtained by PCR with terminal 5'-primers 4Cys (5'-GGT GTT TTC TGC TAC GAG G-3') and 4Ser (5'-GGT GTT TTC AGC TAC GAG G-3'), and primer dTdeg for hybridization with the poly-A tract.

For recombinant expression *Sap* I- and *Xho* I-restriction sites were added to the corresponding full-length cDNA by PCR using *Sap*S (5'-GCT CTT CCA AC*G GTG TTT TCA GCT ACG AGG-3') or *Sap*C (5'-GCT CTT CCA AC*G GTG TTT TCT GCT ACG AGG-3') as terminal 5'-primers and *Xho*A (5'-CCG CTC GAG CGG CTA ACA GTA GGC ATC AGG-3') or *Xho*T (5'-CCG CTC GAG CGG CTA ACA GTA GGT ATC AGG-3') as terminal 3'-primers (recognition sites for restriction enzymes are underlined, stop-codons in italics, and the (after expression) on amino acid level induced cleavage site of the intein fusion protein is marked by * followed by the codon for the first amino acid of the target protein, i.e. glycine). The digested constructs were ligated into plasmid pTYB11 (New England Biolabs, Frankfurt, M., Germany), amplified in *E. coli* Nova Blue Singles Competent cells (Novagen, CN-Biosciences, Schwalbach/Ts, Germany), purified, and transformed into expression cells *E. coli* ER2566 (New England Biolabs, Frankfurt, M., Germany).

2.5. Expression and purification of recombinant allergens

Plasmid pTYB11 encodes for a 55 kDa N-terminal intein fusion protein with an additional chitin binding domain (CBD). The modified intein of the vector system (IMPACT-CN, New England Biolabs, Frankfurt, M., Germany), originally a protein splicing element from the yeast VMA1 gene, undergoes self-cleavage at low temperatures in the presence of thiols such as DTT (Perler et al., 1994; Chong et al., 1996, 1997).

Protein synthesis was induced with 0.3 mmol/l isopropyl-D-thiogalactopyranoside at 15 °C overnight, and cells were harvested by centrifugation. The cell pellet was suspended in 750 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 8.0, 0.1% Tween 20, and sonicated for lysis. After centrifugation the supernatant was loaded onto a chitin column and washed with buffers decreasing in concentration until 150 mM NaCl,

20 mM Tris-HCl pH 8.0. To the last flow-through, 30 mM DTT was added and incubated at 5 °C overnight. Non-fusion target protein was eluted while the intein-CBD-tag remained bound to the column. Fractions containing protein were pooled and dialysed against 150 mM NaCl, 20 mM Tris-HCl pH 7.4.

2.6. Electrophoresis and immunoblotting

Protein samples were separated by SDS-PAGE (Laemmli, 1970) (T = 12.5%, C = 2.7%) as described (Vieths et al., 1992). Proteins were visualized either by Coomassie Brilliant Blue G250 staining or immunoblot-detection after transfer to nitrocellulose (NC) membranes by semi-dry blotting (Khyse-Andersen, 1984) performed as described previously (Vieths et al., 1992).

For an immunoblot inhibition experiment 0.5 µg rCor a 1.0401 per cm was blotted onto NC membranes and probed with patient's serum no. 3 (diluted 1:11) simultaneously incubated with inhibitors or controls as indicated in the figure legends. Immunostaining of bound IgE antibodies was performed with a cascade of rabbit anti-human-IgE (1:4000, 1 h; DAKO, Hamburg, Germany), biotinylated goat anti-rabbit-IgG (1:6000, 1 h; DAKO), alkaline phosphatase (AP) conjugated streptavidin (1:3000, 30 min; Medac, Hamburg, Germany) and an AP staining kit (Biorad, Munich, Germany).

2.7. 2D-PAGE and N-terminal amino acid sequencing

Two-dimensional PAGE with hazelnut extract (500 µg) was performed with IEF as the first and SDS-PAGE as the second dimension. The extract was transferred to NC membranes for the detection of hazelnut-specific IgE from a hazelnut allergic patient as well as to PVDF membranes for excising the corresponding Coomassie-stained spots for N-terminal amino acid sequencing. Microsequencing was performed using a Procise 492 protein sequencer in pulsed liquid mode (Applied Biosystems, Weiterstadt, Germany).

2.8. CAP and EAST

Allergen-specific IgE was measured with the CAP System FEIA (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Enzyme allergosorbent test (EAST) was performed for additional IgE determinations as described for celery (Lüttkopf et al., 2000) with 50 µg of extract from raw or heated hazelnut immobilized per disk and 0.25 µg of recombinant allergens per disk. Dose-related EAST-inhibition studies with extract from raw hazelnut or with rCor a 1.0401 (0.1 µg per disk) immobilized on disks were performed with serum no. 20 as described (Schocker et al., 2000; Vieths et al., 1995). Serial dilutions of inhibitors were prepared from rCor a 1.0401–04, pollen rCor a 1, rPhl p 1, extract from raw hazelnut, hazel pollen, birch pollen

and milk. Inhibitor concentrations are given in the abscissa of corresponding figures. When rCor a 1.0402 and 04 were used as inhibitors maximal concentrations deviate from 100 $\mu\text{g/ml}$ since their concentrations at disposal only were 75 and 10 $\mu\text{g/ml}$, respectively. Non-allergic control sera did not show any unspecific reactivities to the allergen-disks.

2.9. Histamine release assays

With sera from seven patients of this study umbilical cord blood basophils were passively sensitized and stimulated with extract from raw hazelnut, and the four Cor a 1.04-variants, respectively (Ebbesen et al., 1986). Whole blood was drawn from eight patients, and the basophils were directly stimulated with the allergens (Stahl Skov et al., 1985). Histamine release (HR) was measured following exposure to recombinant allergens and hazelnut extracts at decreasing concentrations starting with (C1) 100 $\mu\text{g/ml}$ in case of hazelnut extract and 10 $\mu\text{g/ml}$ in case of recombinant allergens. Dilution factor was 1:3.5 for subsequent concentrations C2–C4. Maximal HR is given at the corresponding concentration of stimulators.

3. Results

3.1. Cloning of cDNA

The 3'-end of the target cDNA was generated by reverse transcription of hazelnut mRNA using an oligo dT-primer and a PCR with the oligo dT-primer and degenerated primer Cora5.

PCR-products were ligated into vector pGEM-T, sequenced and gene-specific primers GSP1 and GSP2 were

designed for 5'-RACE. Both partial sequences matched in an overlap of 35 bp (excluding primers). Sequences of all 5'-RACE clones were strictly conserved within the region of previously applied primer Cora5 underlining the specificity of the "degenerated" primer.

Next to the sequenced 5'-terminus, the fourth codon (without the start codon) was either TGC (coding for cysteine) or AGC (coding for serine), with about equal frequency among 5'-RACE-yielded sequences. Terminal 5'-primers 4Cys and 4Ser were applied in PCR with primer dTdeg which resulted in five different full-length sequences.

3.2. Sequence analysis of Cor a 1.04-variants cDNA

Twelve clones were sequenced and showed five amino acid exchanges in highly conserved positions (C4S, G40S, S62N, S130N and A158T) sharing amino acid sequence identities of 97–99%. One additional variant with a S99P exchange was obtained with Cor a 1.0404. Four clones were selected for expression and were considered as variants Cor a 1.0401 (GenBank Accession no.: AF136945), Cor a 1.0402 (AF323973), Cor a 1.0403 (AF323974) and Cor a 1.0404 (AF323975). Cor a 1.0402 and 03 only differed in one cysteine exchange C4S (Fig. 1).

Full-length-cDNA comprised 480 bp (without start and stop codon). The deduced proteins contained 160 amino acids, with a theoretical molecular mass of 17.4 kDa and a calculated pI of 6.1.

Amino acid sequences were subjected to a FASTA (Pearson and Lipman, 1988) similarity search. The highest degree of 85% amino acid identity was revealed with the inducible stress protein Bet v 1 Sc3. Cor a 1.04-variants showed 66–67% identity to Bet v 1a, and 61–65% to

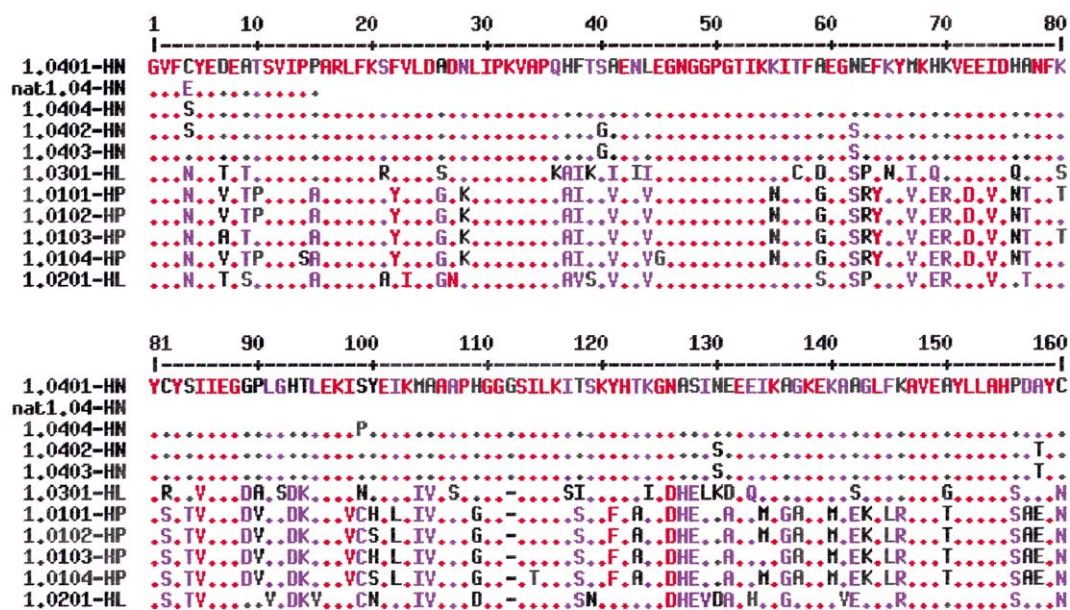


Fig. 1. Multiple sequence alignment of amino acid sequences of rCor a 1 from hazelnut (HN), hazel pollen (HP), hazel leaf (HL) and natural hazelnut Cor a 1 (nat).

Table 2
Comparison of amino acid sequence identity of allergens and pathogenesis related proteins (PRP) with hazelnut Cor a 1.0401

Name	Origin	Function	Accession no.	Identity %
Bet v 1-Sc3	Birch pollen	PRP	X77601	85
	Beech	PRP	AJ130889	74
Cor a 1.0301	Hazel leaf	Allergen	Z72440	71
Bet v 1a	Birch pollen	Allergen	X15877	67
Car b 1	Hornbeam pollen	Allergen	Z80170	68
Pru av 1	Cherry	Allergen	U66076	65
Cor a 1.0101	Hazel pollen	Allergen	X70999	63
Mal d 1	Apple	Allergen	X83672	62
SAM 22	Soybean	PRP	X60043	57
PcPR 1-1	Parsley	PRP	X12574	43
Api g 1.0101	Celery	Allergen	Z48967	38

Cor a 1.01-variants from hazel pollen and therefore were considered as variants of an isoform Cor a 1.04 (Table 2).

3.3. Microsequencing of natural hazelnut allergen nCor a 1

Extract from raw mature hazelnuts was separated by 2D-PAGE and semi-dry blotted. Transferred to NC membranes, natural (n) Cor a 1.04 was detected by IgE (from a hazelnut-allergic patient) directed against a distinct 17–18 kDa band on immunoblots with raw hazelnut extract. Two corresponding spots at 17.5 kDa/pI ~ 6.9 and 17.0 kDa/pI ~ 7.3 were excised from PVDF membranes and microsequenced (2D separation was shown previously (Vieths et al., 2000)). Both spots had identical 15 N-terminal amino acids (GVFEYEDEATSVIPP) which were the same as those of the recombinant variants except position 4, in which the natural protein contained an E and the recombinant molecules either C or S. Remarkably, there were 4–6 amino acid exchanges to the isoforms of hazel pollen Cor a 1.

A multiple sequence alignment (Corpet, 1988) of all known Cor a 1 isoforms and variants from recombinant hazelnut, hazel pollen, leaves and the partial N-terminal sequence of the natural hazelnut allergen is shown in Fig. 1.

3.4. Expression and purification strategy

For recombinant expression, *Sap* I and *Xho* I restriction sites were added to the full-length cDNA by PCR and the construct was ligated into plasmid pTYB11. Via the *Sap* I restriction site, the target product was cloned adjacent to the cleavage site so that the intein spliced exactly ahead of the first amino acid (glycine) of the target protein. Plasmids pTYB11 containing the inserts were amplified in *E. coli* Nova Blue cells, restriction sites were checked by sequencing and purified pTYB11 was subcloned into expression cells *E. coli* ER2566. During induction of protein expression, aliquots were taken and separated by SDS-PAGE (Fig. 2). The fusion protein showed an appar-

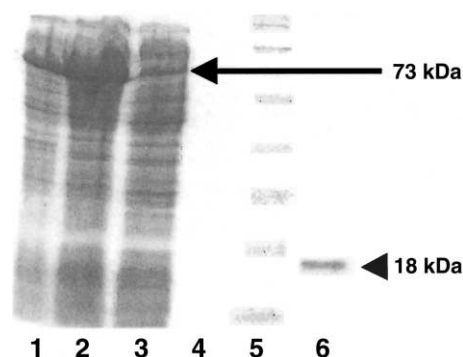


Fig. 2. Expression of Cor a 1.0401 as intein fusion protein (73 kDa) in *E. coli* ER2566, and elution of soluble non-fusion target protein (18 kDa). Lane: (1) bacterial pellet after induction o/n at 15 °C, (2) supernatant after centrifugation, (3) flow-through after binding to column, (4) DTT flow-through, (5) MW marker (16, 20, 25, 32, 47, 83, 175 kDa), (6) elution of Cor a 1.0401.

ent mass of about 73 kDa. For purification, the cell pellet was sonicated for lysis and the supernatant was loaded onto a chitin column, washed, and treated with 30 mM DTT at 15 °C overnight for the cleavage reaction. Non-fusion protein (18 kDa) was eluted from the column (lane 6). The yields of the recombinant variants were between 0.6 and 1.3 mg/l of bacterial cultures.

3.5. Patients with a positive DBPCFC to hazelnut

Patients' characteristics are summarized in Table 1. All the 43 patients of this study reacted with OAS during the DBPCFC-procedure with hazelnut. One patient (no. 4) additionally suffered from rhinoconjunctivitis with a delay of 4 h. In SPT with self-prepared hazelnut extract 90% of the patients displayed positive skin responses. All the patients were sensitized to Bet v 1 according to CAP classes ranging from 2 to 6. Sensitivity of EAST with hazelnut extract was slightly better than with CAP (72% versus 70% of the sera with class ≥ 1).

3.6. Common epitopes of natural and recombinant Cor a 1.04

Recombinant Cor a 1.0401 was blotted to nitrocellulose membranes, incubated with patient serum no. 3 and different inhibitors. Recombinant Cor a 1.0401, rBet v 1 and hazelnut extract inhibited IgE binding to rCor a 1.0401 on solid phase completely, whereas rBet v 2, rPhl p 1, ovalbumin and buffer controls showed no influence in IgE binding. Interestingly, hazel pollen rCor a 1 quenched IgE binding only partially (Fig. 3).

3.7. Epitope differences between Cor a 1 from hazelnut and pollen

For dose-dependent EAST-inhibition experiments serial dilutions of inhibitors were prepared from rCor a 1.0401–04,

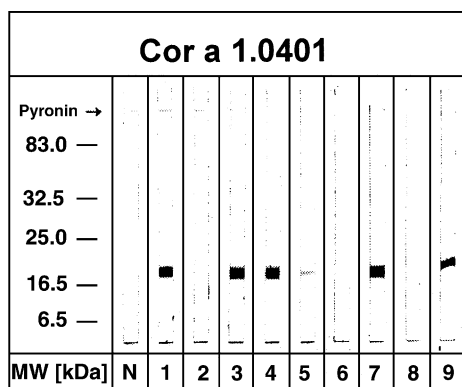


Fig. 3. Immunoblot-inhibition of rCor a 1.0401 (0.5 $\mu\text{g}/\text{cm}$) transferred to NC membranes with serum no. 3. Lane (N) non-allergic control serum, (1) no inhibitor, (2) 15 μg rBet v 1, (3) 15 μg Bet v 2, (4) 15 μg rPhl p 1, (5) 15 μg hazel pollen Cor a 1.01, (6) 5 μg rCor a 1.0401, (7) elution buffer of Cor a 1.04-variants, (8) 100 μg hazelnut extract, (9) 100 μg ovalbumin.

pollen rCor a 1, rPhl p 1, extract from raw hazelnut, hazel pollen, birch pollen and milk. They were examined for their ability to inhibit IgE binding to rCor a 1.0401 and hazelnut extract coupled to paper filter disks. As illustrated in Fig. 4 with both sorts of disks inhibitors rCor a 1.0401 and birch pollen extract totally blocked IgE binding whereas the inhibition capacity of pollen rCor a 1 was clearly reduced showing only 35% maximal inhibition with Cor a 1.0401-disks and 60% with hazelnut-disks. Cor a 1.0402 and 03 reached a total inhibition with hazelnut extract coupled to disks, and about 80% with variant-01-disks. Extract of raw hazelnut led to 75% inhibition with Cor a 1.0401-disks at an inhibitor concentration of 1000 $\mu\text{g}/\text{ml}$, with hazelnut extract coupled to disks it showed a complete self-inhibition at this concentration. The shallow slopes of the inhibition curves of hazel pollen Cor a 1 and extract were significantly different from those of the other inhibitors, and concentrations at 50% inhibition (c_{50}) were achieved at higher values if attained at all.

3.8. Immunological characterization of recombinant Cor a 1.04-variants

The four recombinant variants of Cor a 1.04 were coupled to cyanogen bromide activated paper filter disks and subsequently investigated in EAST for the IgE reactivity of sera of 43 patients with a positive DBPCFC to hazelnut. The highest EAST classes were achieved with Cor a 1.0401 which showed a positive EAST ($\geq$ class 1) in 95% of the sera. Cor a 1.0402 showed a positive EAST in 93% of the sera and Cor a 1.0403 in 91%. With Cor a 1.0404 the most frequent class zero results were found, only 74% of the sera were positive. EAST was also performed with recombinant pollen Cor a 1 coupled to disks. IgE binding with 86% positive results was weaker compared to the three variants Cor a 1.0401–03 (summary in Table 1).

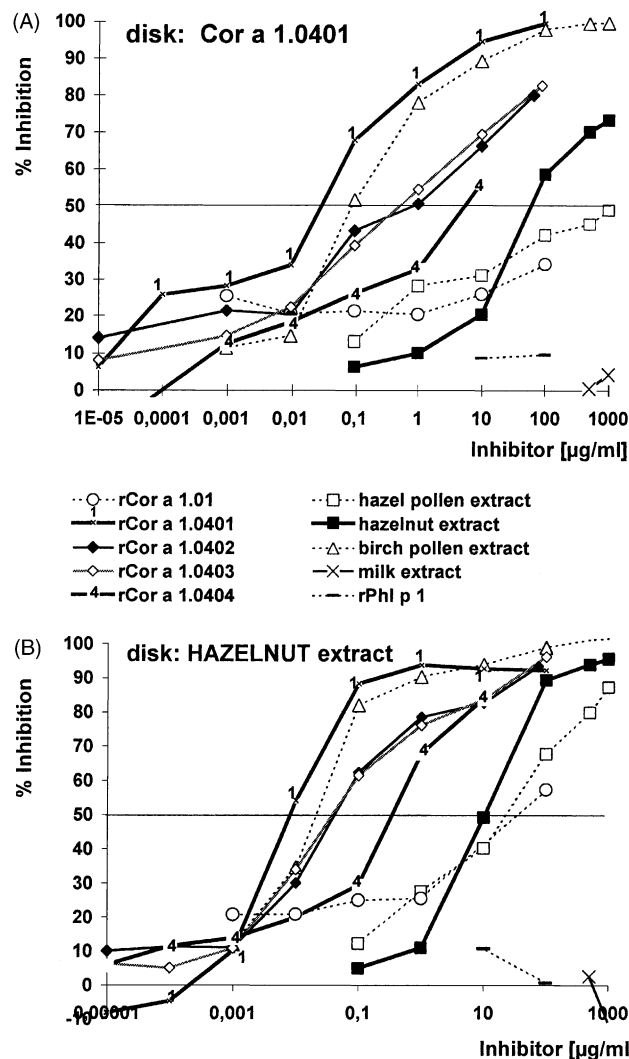


Fig. 4. Dose-related EAST-inhibitions with (A) rCor a 1.0401 (0.1 μg per disk) and (B) hazelnut extract (50 μg per disk) coated to paper filter disks and serum no. 20 (in a final dilution of 1:5 (A) and 1:12 (B)).

3.9. Histamine release assays

The four recombinant Cor a 1.04-variants were investigated for their capability of inducing histamine release. The results of these experiments are summarized in Table 3. In short, 11 sera of 15 sera under investigation were positive with extract from raw hazelnut, 13 with rCor a 1.0401, 12 with rCor a 1.0402, 11 with rCor a 1.0403, and only 4 with rCor a 1.0404, the variant containing the proline exchange. In general, no significant release was obtained with milk protein as the negative control.

4. Discussion

In this study, we focussed on the cloning, expression and characterization of the Bet v 1-related major allergen from hazelnut and the immunological properties of four different

Table 3

Results of histamine release (HR) assays with hazelnut extract and the Cor a 1.04 variants with *P* passively sensitized cord blood basophils or *D* directly stimulated whole blood basophils^a

Patient no.	Extract from raw hazelnut		rCor a 1.0401		rCor a 1.0402		rCor a 1.0403		rCor a 1.0404	
Maximum HR	ng/ml	at	ng/ml	at	ng/ml	at	ng/ml	at	ng/ml	at
20 D	45	C1	66	C3	60	C3	51	C2	28	C2
22 D	94	C2	101	C3	96	C4	94	C3	65	C3
24 D	66	C1	173	C1	120	C1	83	C1	0	
25 P	0		9	C1	11	C2	9	C1	0	
26 P	0		0		0		0		0	
27 D	22	C1	29	C1	29	C2	19	C1	0	
29 P	18	C1	38	C1	22	C2	15	C1	0	
30 D	52	C2	48	C1	56	C2	48	C2	35	C2
31 D	27	C2	30	C3	31	C3	27	C2	0	
33 P	62	C1	128	C1	91	C1	66	C1	18	C1
34 P	56	C1	122	C1	84	C1	47	C1	0	
35 P	11	C1	0		0		0		0	
40 P	0		24	C1	12	C1	0		0	
41 D	0		39	C2	17	C2	12	C1	0	
XX D	36	C3	23	C1	0		0		0	

^a Maximal histamine release (ng/ml) is given at the corresponding concentrations of stimulators at C1, C2, C3 or C4 with a serial dilution (1:3.5) of the maximal concentration C1 (100 µg/ml for extract and 10 µg/ml for recombinant allergens).

variants Cor a 1.0401 to 1.0404 in comparison to pollen Cor a 1.

The four hazelnut variants Cor a 1.0401–04 were expressed as recombinant allergens with a N-terminal intein chitin binding fusion protein (which facilitated purification via a chitin column and after DTT-induced intein-mediated cleavage of the fusion partner elution of soluble target protein without any fusion-tag). The target proteins were expressed without methionine derived from the start codon.

All four variants comprised 160 amino acids, had a calculated molecular mass of 17.4 kDa and an estimated isoelectric point of 6.1. Among each other they showed 97–99% amino acid sequence identity with six amino acid exchanges obtained in five highly conserved positions. Referring to Cor a 1.0401, these were C4S, S40G, N62S, N130S and A158T which were all replaced in variant Cor a 1.0402. Cor a 1.0403 corresponded to Cor a 1.0402 except for the fourth amino acid displaying a cysteine residue similar to Cor a 1.0401. Variant Cor a 1.0404 showed only two exchanges in comparison to Cor a 1.0401 which were a serine residue at position 4 (like Cor a 1.0402) and an additional unique exchange with a proline at position 99 instead of a serine obtained with all the other variants.

All deduced amino acid exchanges were restricted to one nucleotide exchange each. Since five of them were highly conserved within the sequenced plasmids it is unlikely that they result from errors of Taq DNA polymerase. However, this cannot be totally excluded for the unique S99P exchange of Cor a 1.0404. Yet, also this variant seemed worth to be examined as proline is known for its ability in destroying secondary structures which could have an influence on IgE-reactivity.

Protein alignments with rCor a 1.0401 revealed the highest degree of 85% amino acid sequence identity with a pathogenesis related protein of the PrP10 family Bet v 1 Sc3, a protein inducible in callus cultures from birch pollen under stress conditions. Cor a 1.0401 showed 67% identity to Bet v 1a, 71% identity to Cor a 1.0301 from hazel leaves, and 63% to Cor a 1.01 from hazel pollen and therefore was considered as an isoform Cor a 1.04.

Microsequencing of natural nCor a 1.04 resulted in 15 N-terminal amino acid residues identical with the recombinant allergens except for amino acid in position 4 in which the natural partial sequence showed glutamic acid while the recombinant allergen showed either a cysteine or serine residue. Remarkably, there were four to six amino acid exchanges compared with the different hazel pollen Cor a 1.01 isoforms, indicating that indeed different expression products of Cor a 1 exist in pollen and nuts.

Moreover, our inhibition data indicate epitope identity of the natural and the recombinant hazelnut allergen Cor a 1.0401 whereas only partial epitope identity was revealed between Cor a 1 from hazelnut and hazel pollen (Figs. 3 and 4). In the EAST, inhibition curves of Cor a 1.0401 and birch pollen extract looked very much the same pointing to a high similarity between epitopes existing in birch pollen and Cor a 1.0401. By contrast, pollen Cor a 1 showed a clearly weaker inhibitory activity with a maximum of 35% with rCor a 1.0401 disks and of 60% with hazelnut extract disks. The inhibition graph of commercially available pollen Cor a 1 was simulated by native hazel pollen extract, indicating that the recombinant protein shares a similar structure with the natural pollen allergen. Furthermore, the shallow slopes of their graphs indicated a much weaker reactivity than hazelnut Cor a 1, possibly resulting from a differing recognition

of epitopes. The inhibition graphs of Cor a 1.0402 and 03 proceeded closely parallel. Thus, indicating a comparable reactivity, the amino acid exchange C4S seemed to be of minor influence for protein folding or epitope reactivity. Compared to variant-01, c_{50} -values of variants-02 and -03 were achieved at higher inhibitor concentrations with variant-04 at the highest among the four variants, complying with the ranking obtained in EAST.

From a multicenter study (Ortolani et al., 2000), a subgroup of 43 sera of patients with a positive DBPCFC to hazelnut was available for this study. Since all our 43 patients were sensitized to birch pollen allergens (Table 1) it is very likely that most of them acquired hazelnut allergy via cross-reactive sensitization by birch pollen. At least for our patient group, the high sensitivity of the SPT as an in vivo test for hazelnut-allergy was not achieved for in vitro tests so far (Ballmer-Weber et al., 2000). By introducing recombinant hazelnut Cor a 1.0401 for EAST an in vitro test could be established yielding 95% sensitivity, similar to the skin prick test.

Differences in IgE-reactivity of the 43 sera to the four Cor a 1.04-variants were demonstrated by EAST performed on paper disks coupled to each variant, respectively. The number of positive results to each variant reflected a concordant ranking of the immunological activity of the variants: Cor a 1.0401 > 02 > 03 >> 04. Cor a 1.0404 showed the strongest reduction in IgE reactivity. On the basis of single sera this loss of activity among the variants was confirmed for the majority of patients. With Cor a 1.0404 not a single serum showed a higher EAST class than with the other variants and in 30% of the sera Cor a 1.0404 was the only variant showing a lower class than the others including reductions from class 2 to 0 in four cases. On the other hand, in 42% of the sera their general activity regarding EAST classes remained unchanged among the four variants. Thus, it may be concluded that at least one IgE epitope is destroyed or altered in Cor a 1.0404 resulting in a decline of IgE reactivity for one part of the sera whilst other sera are not affected due to recognition of other epitopes.

The allergenic activity of the four Cor a 1.04-variants was confirmed by histamine release assays which resulted in the same ranking of decreasing activity as obtained with IgE binding in EAST experiments: Cor a 1.0401 had the highest allergenic potency, followed by variant 02 and 03, and Cor a 1.0404 which contained a proline at position 99 had a drastically reduced allergenic potential, stimulating histamine release in only 30% (4 of 13) of the patients. Compared to the loss of activity obtained in EAST experiments this decrease in histamine release capacity is even more pronounced. Hence, variant Cor a 1.0404 may still be capable of binding IgE to some extent but its ability in cross-linking cell-bound IgE antibodies appeared to be abolished.

Similar features of Cor a 1.0402 and 03 in EAST and histamine release assays suggest a minor influence in IgE binding of cysteine in position 4 as this exchange was the only difference between both variants. Therefore, proline

in position 99 presumably is responsible for the vast decrease in IgE reactivity of many sera towards Cor a 1.0404. A further reduction of allergenicity by site-directed mutagenesis may lead to low IgE binding hazelnut proteins with preserved T-cell reactivity which may represent candidates for developing novel and safe strategies for specific immunotherapy of hazelnut allergy.

Acknowledgements

This study was funded by the EU (EC-FAIR CT97-3224), and by the Swiss Federal Office for Education and Science, BBW 97.0334. We are grateful to Dr. G. Malgarini for providing us with lots of tasty hazelnuts.

References

- Akkerdaas, J., Hafle, S., Aalberse, R., van Ree, R., 2000. Characterization of non-pollen-related hazelnut allergens. *J. Allergy Clin. Immunol.* 105, S136.
- Andersen, K.E., Løwenstein, H., 1978. An investigation of the possible immunological relationship between allergen extracts from birch pollen, hazelnut, potato and apple. *Cont. Derm.* 4, 73–79.
- Ballmer-Weber, B.K., Vieths, S., Bucher, C., Lüttkopf, D., Wüthrich, B., 2000. Haselnußallergie. Validierung der diagnostischen Verfahren anhand der doppelblinden, placebokontrollierten Nahrungsmittelprovokation—Hazelnut allergy. Validation of diagnostic procedures on the basis of double-blind placebo-controlled food challenges. *Allergologie* 23, 285–291.
- Belin, E., 1972. Immunological analyses of birch pollen allergens with special reference to the allergenic components. *Int. Arch. Allergy* 42, 300–322.
- Bradford, M.M., 1976. Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O., Breitenbach, M., 1989. The gene coding for the major birch pollen allergen Bet v 1, is highly homologous to a pea disease resistance response gene. *EMBO J.* 8, 1935–1938.
- Breiteneder, H., Ferreira, F., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Rumpold, H., Kraft, D., Scheiner, O., 1993. Four recombinant isoforms of Cor a I, the major allergen of hazel pollen, show different IgE-binding properties. *Eur. J. Biochem.* 212, 355–362.
- Calkhoven, P.G., Aalbers, M., Koshte, V.L., Pos, O., Oei, H.D., Aalberse, R.C., 1987. Cross-reactivity among birch pollen vegetables and fruits as detected by IgE antibodies is due to at least three distinct cross-reactive structures. *Allergy* 42, 382–390.
- Chong, S., Mersha, F.B., Comb, D.G., Scott, M.E., Landry, D., Vence, L.M., Perler, F.B., Benner, J., Kucera, R.B., Hirvonen, C.A., Pelletier, J.J., Paulus, H., Xu, M.-Q., 1997. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* 192, 271–281.
- Chong, S., Shao, Y., Paulus, H., Benner, J., Perler, F.B., Xu, M.-Q., 1996. Protein splicing involving the *Saccharomyces cerevisiae* VMA intein. *J. Biol. Chem.* 271, 22159–22168.
- Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. *Nucl. Acids Res.* 16, 10881–10890.
- de Groot, H., de Jong, N.W., Vuijk, M.H., Gerth Van Wijk, R., 1996. Birch pollinosis and atopy caused by apple, peach, and hazelnut; comparison of three extraction procedures with two apple strains. *Allergy* 51, 712–718.

- Ebbesen, F., Skov, P.S., Jensen, C., Norn, S., Sondergaard, I., 1986. Basophil histamine release in cord blood. Regulatory role of IgE. *Allergy* 41, 562–567.
- Ebner, C., Ferreira, F., Hoffmann, K., Hirschwehr, R., Schenk, S., Szépfalusi, Z., Breiteneder, H., Parronchi, P., Romagnani, S., Scheiner, O., 1993. T cell clones specific for Bet v I the major birch pollen allergen cross-react with the major allergens of hazel Cor a I and alder Aln g I. *Mol. Immunol.* 30, 1323–1329.
- Ebner, C., Hirschwehr, R., Bauer, L., Breiteneder, H., Valenta, R., Ebner, H., Kraft, D., Scheiner, O., 1995. Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J. Allergy Clin. Immunol.* 95, 962–969.
- Eriksson, N.E., Formgren, H., Svenonius, E., 1982. Food hypersensitivity in patients with pollen allergy. *Allergy* 37, 437–443.
- Etesamifar, M., Wüthrich, B., 1998. IgE-vermittelte Nahrungsmittelallergien bei 383 Patienten unter Berücksichtigung des oralen Allergie-Syndroms. *Allergologie* 21, 451–457.
- Florvaag, E., Holen, E., Vik, H., Elsayed, S., 1988. Comparative studies on tree pollen allergens. XIV. Characterization of the birch (*Betula verrucosa*) and hazel (*Corylus avellana*) pollen extracts by horizontal 2-D SDS-PAGE combined with electrophoretic transfer and IgE immunoautoradiography. *Ann. Allergy* 61, 392–400.
- Herkenrath, C., Gottmann-Lückerrath, I., Steigleder, G.K., 1982. Gemeinsames Vorkommen von Allergien gegen Haselpollen und gegen Haselnüsse—combined allergy against hazel pollen and hazel nuts. *Z. Hautkr.* 57, 1399–1405.
- Hirschwehr, R., Valenta, R., Ebner, C., Ferreira, F., Sperr, W.R., Valent, P., Rohac, M., Rumpold, H., Scheiner, O., Kraft, D., 1992. Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol.* 90, 927–936.
- Hoffmann-Sommergruber, K., Vanek-Krebitz, M., Radauer, C., Wen, J., Ferreira, F., Scheiner, O., Breiteneder, H., 1997. Genomic characterization of members of the Bet v 1 family: genes coding for allergens and pathogenesis-related proteins share intron positions. *Gene* 197, 91–100.
- Ipsen, H., Bowadt, H., Janniche, H., Nüchel Petersen, B., Munch, E.P., Wihl, J.A., Löwenstein, H., 1985. Immunochemical characterization of reference alder (*Alnus glutinosa*) and hazel (*Corylus avellana*) pollen extracts and the partial immunochemical identity between the major allergens of alder birch and hazel pollens. *Allergy* 40, 510–518.
- Khyse-Andersen, J., 1984. Electrophoretic blotting of multiple gels: a simple apparatus without a buffer tank for rapid transfer of proteins from polyacrylamide gels to nitocellulose. *J. Biochem. Biophys. Methods* 10, 203–209.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lüttkopf, D., Ballmer-Weber, B.K., Wüthrich, B., Vieths, S., 2000. Celery allergens in patients with positive double-blind placebo-controlled food challenge. *J. Allergy Clin. Immunol.* 106, 390–399.
- Müller, U., Lüttkopf, D., Hoffmann, A., Petersen, A., Becker, W.M., Schocker, F., Niggemann, B., Altmann, F., Kolarich, D., Hausteine, D., Vieths, S., 2000. Allergens in raw and roasted hazelnuts (*Corylus avellana*) and their cross-reactivity to pollen. *Eur. Food Res. Technol.* 212, 2–12.
- Ortolani, C., Ispano, M., Pastorello, E., Bigi, A., Ansaloni, R., 1988. The oral allergy syndrome. *Ann. Allergy Asthma Immunol.* 61, 47–52.
- Ortolani, C., Ballmer-Weber, B.K., Skamstrup Hansen, K., Ispano, M., Wüthrich, B., Bindslev-Jensen, C., Ansaloni, R., Vannucci, L., Pravettoni, V., Scibilia, J., Poulsen, L.K., Pastorello, E.A., 2000. Hazelnut allergy: a double-blind, placebo-controlled food challenge multicenter study. *J. Allergy Clin. Immunol.* 105, 577–581.
- Pearson, W.R., Lipman, D.J., 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. U.S.A.* 85, 2444–2448.
- Perler, F.B., Davies, E.O., Dean, G.E., Gimble, F.S., Jack, E.W., Neff, N., Noren, C.J., Thorner, J., Belfort, M., 1994. Protein splicing elements: inteins and exteins—a definition of terms and recommended nomenclature. *Nucl. Acids Res.* 22, 1125–1127.
- Petersen, A., Schramm, G., Bufer, A., Schlaak, M., Becker, W.M., 1995. Structural investigations of the major allergen Phl p I on the complementary DNA and protein level. *J. Allergy Clin. Immunol.* 95, 1–8.
- Roeser, A., Debelic, M., Kalveram, K.J., 1987. Untersuchungen zur Kreuzreaktivität bei Patienten mit Birkenpollen- und Haselnußallergie. *Allergologie* 10, 190–193.
- Rohac, M., Birkner, T., Reimitzer, I., Bohle, B., Steiner, R., Breitenbach, M., Kraft, D., Scheiner, O., Gabl, F., Rumpold, H., 1991. The immunological relationship of epitopes on major tree pollen allergens. *Mol. Immunol.* 28, 897–906.
- Scheurer, S., Metzner, K., Hausteine, D., Vieths, S., 1997. Molecular cloning expression and characterization of Pru a 1, the major cherry allergen. *Mol. Immunol.* 34, 619–629.
- Schocker, F., Lepp, U., Thomas, P., Schlaak, M., Becker, W.M., 1999. Identifizierung und Charakterisierung nicht-pollenassoziierter Allergene der Haselnuß. *Allergo J.* 8, 32.
- Schocker, F., Lüttkopf, D., Müller, U., Thomas, P., Vieths, S., Becker, M.W., 2000. IgE binding to unique hazelnut allergens: identification of non pollen-related and heat-stable hazelnut allergens eliciting severe allergic reactions. *Eur. J. Nutr.* 39, 172–180.
- Schöning, B., Ziegler, W.H., Vieths, S., Baltes, W., 1996. Apple allergy: the cDNA sequence determined by performing PCR with a primer based on the N-terminal amino acid sequence of the major allergen of apple is highly homologous to the sequence of the major birch pollen allergen. *J. Sci. Food Agric.* 71, 475–482.
- Stahl Skov, P., Mosbech, H., Nom, S., Weeke, B., 1985. Sensitive glass microfibre-based histamine analysis for allergy testing in washed blood cells. *Allergy* 40, 213–218.
- Teuber, S.S., Dandekar, A.M., Peterson, W.R., Sellers, C.L., 1998. Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (*Juglans regia*), a major food allergen. *J. Allergy Clin. Immunol.* 101, 807–814.
- Valenta, R., Breiteneder, H., Pettenburger, K., Breitenbach, M., Rumpold, H., Kraft, D., Scheiner, O., 1991. Homology of the major birch-pollen allergen, Bet v I, with the major allergens of alder, hazel, and hornbeam at the nucleic acid level as determined by cross-hybridization. *J. Allergy Clin. Immunol.* 87, 677–682.
- Vieths, S., Schoening, B., Baltes, W., 1992. Allergy to fruits and vegetables in pollen-sensitive patients: allergen characterization by IgE immunoblotting and peroxidase staining. *Food Agric. Immunol.* 4, 181–197.
- Vieths, S., Janek, K., Aulepp, H., Petersen, A., 1995. Isolation and characterization of the 18 kDa major apple allergen and comparison with the major birch pollen allergen (Bet v I). *Allergy* 50, 421–430.
- Vieths, S., Karamloo, F., Lüttkopf, D., Scheurer, S., Fötisch, K., May, S., Altmann, F., Skov, P.S., Hausteine, D., 2000. Recombinant allergens expressed in *E. coli*: benefits and drawbacks in the diagnosis of food allergies. In: Kurth, R., Hausteine, D. (Eds.), *Proceedings of the 9th International Paul-Ehrlich-Seminar on Regulatory Control and Standardization of Allergenic Extracts (93)*, GIT Verlag, Darmstadt, Germany, pp. 159–168.