

Molecular Cloning and Characterization of Hazel Pollen Protein (70 kD) as a Luminal Binding Protein (BiP): A Novel Cross-Reactive Plant Allergen

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Key Words

Cor a 10 · Pollen · Allergen · cDNA cloning · Human IgE · Chaperon · *Corylus avellana*

Abstract

Background: Tree pollen contains many allergens showing cross-reactivity to proteins from pollen, seeds, and fruits of different plant species. Amongst Fagales, responsible for several allergenic responses, hazel provides the best material to study pollen as well as food allergens in one species. The aim of this study was to identify and characterize the physiological function of an allergen from hazel pollen and to determine possible cross-reactivity to proteins from hazelnut. **Methods:** Monoclonal antibodies (mAbs) against hazel pollen crude extract were produced. On the basis of IgE binding, demonstrated by sera from patients allergic to hazel pollen, one mAb indicating the best correlation has been selected, and the putative allergen was purified by preparative gel electrophoresis. Isoforms were investigated by two-dimensional PAGE, and for molecular identification a hazel pollen cDNA library was constructed. In situ

localization of the allergen during pollen development was performed by immunofluorescence labelling. **Results:** Immunological staining of crude hazel pollen extract with specific IgE and mAb revealed a 70-kD protein. Immunoblot studies with mAb showed cross-reactive proteins of 70–72 kD in different plant tissues and species. After protein purification, the IgE-binding reactivity of the allergen has been reconfirmed, and two isoforms were detected. Molecular cloning identified the allergen as a luminal binding protein (BiP) of the Hsp70 family with 88–92% sequence identity in various plants. Further immunocytological studies indicated involvement of BiP during pollen development. **Conclusions:** Chaperons like BiP play an important role in protein synthesis and in the protection of cellular structures during stress-related processes. Because of their highly conserved protein sequences, we propose that such allergens could be responsible for at least a part of the allergenic cross-reactivity between proteins from different pollens and plant foods.

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Introduction

Tree pollen sensitization is a well-known trigger of hay fever and allergic asthma during winter to summer months of the northern hemisphere. Moreover, induction of cross-reactive IgE antibodies by pollen-sensitized patients can create allergy to plant foods during the whole year [1]. For example, several birch pollen allergens have been shown to contain cross-reactive IgE-binding epitopes present in fruits, vegetables, nuts, and spices [2–4]. The highly conserved protein structure/epitopes may be an important aspect of this cross-reactivity phenomenon [5]. On the other hand, the biological function of allergens may also play a role in sensitization of susceptible humans to allergy [6]. Production of such allergens in plants was observed in response to certain natural environmental conditions [7]. For example, the development of anemogamous tree pollen occurs only under cold and water stress conditions. The cellular adaptation to this abiotic stress conditions involves several different mechanisms, including membrane and protein protection, damage repair, stress avoidance, and defence against pathogen invasion. The maintenance of the structural integrity of pollen during development is essential for successful germination and fertilization. Similar mechanisms have also been observed during embryogenesis of different seeds. A correlation between biological function and allergenic capacity of proteins related to stress response is not clearly demonstrated, but can be speculated considering the known functions of recently cloned allergens like Bet v 1 exhibiting nuclease activity [8], Bet v 3, Bet v 4, and Cyn d 7 involved in cell signalling as calcium-binding proteins [9–11], and Bet v 2 as a profilin [12]. The identification and availability of potential cross-reactive allergens are described to simplify and improve allergy treatment by component-resolved diagnostics and therapy [13].

Until now, tree pollen allergy research has been predominantly focused on birch (*Betula verrucosa*). Other members of the *Betulaceae* include hazel, alder, and hornbeam which also contribute significantly to the air flora and allergy, but are less explored. In this study, we report the identification and characterization of a hazel pollen protein of 70 kD designated as Cor a 10 by the Allergen Nomenclature Committee (www.allergen.org). This protein shows cross-reactive homologues in not only hazelnut, but also in other plant tissues and species. Molecular cloning and sequencing of the cDNA encoding for the 70-kD protein revealed significant sequence identity with luminal binding protein (BiP) of the Hsp70 family, qualifying it as a new potential panallergen.

Materials and Methods

Plant Material and Extracts

Hazel pollen and birch pollen were collected in the Botanical Gardens of Bonn from February until May and stored at -80°C until use. Rye pollen and mugwort pollen were purchased from Greer Laboratories (Lenoir, N.C., USA). Pollen protein extracts were obtained as described elsewhere [14]. Hazelnuts (from California, USA) and apples (Granny Smith, Spain) were purchased from a local supermarket, and extracts were prepared as described previously [15, 16].

Human Sera and Monoclonal Antibodies (mAbs)

Sera from patients with a documented clinical history of tree pollen allergy with and without reported oral allergy symptoms after ingestion of apple and hazelnut were obtained from Prof. Bauer (Department of Dermatology and Allergology, University of Bonn Hospital, Bonn, Germany) and from Dr. Dr. Rosen (Dermatologist and Allergologist, Bonn, Germany). The diagnosis was based on positive skin prick tests (a wheal of at least 3×3 mm read after 15 min was considered positive) and serological data (RAST class >2). mAbs against hazel pollen crude extract were produced according to standard methods [17]. Hybridoma clones were selected producing IgG1 antibodies against a 70-kD hazel pollen protein correlating with an allergen demonstrated by the above-mentioned patient sera.

Gel Electrophoresis and Protein Purification

Protein extracts were resolved on SDS-PAGE according to the method of Lämmli [18] on the Miniprotean II gel system (Bio-Rad, Munich, Germany). Reducing SDS-PAGE was also used to separate the 70-kD protein from crude hazel pollen extract by preparative gel electrophoresis with a model 491 Prep Cell (Bio-Rad). The principle of this protein purification procedure is based on continuous electrophoresis elution of proteins with conventional gel electrophoresis buffer systems. Briefly, optimal conditions for purification of the 70-kD antigen from hazel pollen crude extract were obtained with 4% SDS stacking gel (2 cm long, 37 mm in diameter) and 8% SDS resolving gel (9 cm long, 37 mm in diameter) using acrylamide:bis-acrylamide at the ratio 37.5:1. A sample of 3 mg of pollen protein was separated by constant 12 W at 4°C . Proteins were eluted with Tris-buffered saline and collected in 2-ml fractions. The fractions were tested for purity by analytical SDS-PAGE followed by immunoblots with sera from patients allergic to hazel pollen as well as with a 70-kD-specific mAb. Isoelectric focusing (IEF) was performed with purified 70-kD protein on Immobiline DryStrips with a stable pH gradient from 3 to 10 (Amersham Pharmacia Biotech, Uppsala, Sweden), following the manufacturer's instructions. The second dimension was performed using SDS-PAGE as described above. Gels were either stained for total proteins with Coomassie brilliant blue or transferred onto nitrocellulose (NC) membranes for immunological studies as described below.

Immunoblot Studies

SDS-PAGE gels containing crude extract, purified proteins, or IEF-resolved protein isoforms were transferred to NC according to the method of Towbin et al. [19], using Transblot SD (Bio-Rad). Free protein-binding sites of NC blots were blocked in Tris-buffered saline containing 4% skim milk powder and then probed with either the 70 kD protein specific mAb (cell culture supernatant) or pooled human IgE serum (diluted 1:4 to 1:10 in 0.5% bovine serum albumin/Tris-buffered saline) following established protocols [14], with

slight modifications, by using alkaline phosphatase labeled secondary or tertiary antibodies 1:7,500 (Promega, Madison, Wisc., USA). Staining reaction was performed with Fast Red TR salt (Sigma-Aldrich, Munich, Germany) and naphthol AS-MX phosphate (Sigma-Aldrich) as the substrate.

cDNA Cloning and Sequencing

Molecular cloning and sequencing of the 70-kD hazel pollen protein was accomplished by the following standardized techniques [20]. Briefly, total RNA was extracted from hazel pollen (*Corylus avellana*) using the method of Chomczynski and Sacchi [21]. Polyadenylated RNA was purified using an oligo(dT) affinity column (Pharmacia, Uppsala, Sweden), and a cDNA library was constructed in the expression vector λ ZAP II (Stratagene, La Jolla, Calif., USA) using a Time Saver cDNA synthesis kit (Pharmacia) following the manufacturer's instructions. Screening of the cDNA library for the 70-kD protein was performed by taking plaque lifts and immunostaining with the specific mAb as described above. cDNA clones encoding the 70-kD protein were identified, excised from the master plate, and different dilutions of clones were replated and reprobed to isolate individual and, therefore, pure plaques. cDNA inserts from λ ZAP II clones were subcloned into pBluescript SK vectors (Stratagene) by co-infecting *Escherichia coli* SOLR cells with the λ ZAP II phage and the f1 helper phage, according to the manufacturer's instructions. Plasmid DNA was amplified and purified using affinity columns (Qiagen-tip 20; Qiagen, Hilden, Germany). The cDNA insert was sequenced using automated sequencing (Applied Biosystems, Foster City, Calif., USA), initially with T3/T7 vector sequencing primers and then by additional rounds with overlapping internal primer sequences. Analyses of nucleotide and amino acid sequences were performed using HUSAR (German Cancer Research Center, DKFZ, Heidelberg, Germany); comparisons with other sequences were carried out using BLAST (www.ncbi.nlm.nih.gov/blast). In addition, 5' RACE (Gibco BRL, Life Technologies, Eggenstein, Germany) of the 70-kD protein insert was necessary to obtain full-length cDNA. First-strand cDNA was synthesised from mRNA using the gene-specific primer GST1 (5'-AAT AGC TCT GTT GTC C-3'). Primers were designed from the putative N-terminal region of the known cDNA sequence of the target protein from the cDNA library. The dC-tailed cDNA was amplified by PCR with the nested second antisense primer GST 2 (5'-CAC CTC CAA GAT GAG TGT CTC C-3') and the bridged anchor primer provided within the 5' RACE system. PCR was performed under the following conditions: hot start at 94°C for 2 min, denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 90 s. After 35 cycles, the final extension was 7 min at 72°C. The amplified PCR product was purified (PCR purification kit; Boehringer Mannheim, Germany), and the 3' end was tailed with oligodA nucleotides, ligated into pCR2.1-vector (Invitrogen, Groningen, The Netherlands), and used for transformation of TOP10F' by heat shock, according to the manufacturer's instructions. Successfully transformed bacteria could be selected by blue-white screening. The PCR insert was sequenced with T3/T7 sequencing primers, and overlapping ends between 5' PCR product and 70-kD protein cDNA were determined.

Pollen Preparation for Microscopy

Pollen grains were fixed with 0.1% glutaraldehyde and 3.7% formaldehyde buffered in phosphate-buffered saline (PBS) at pH 7.6. For optimum infiltration, the samples were incubated under vacuum. The samples were then dehydrated in an alcohol series and

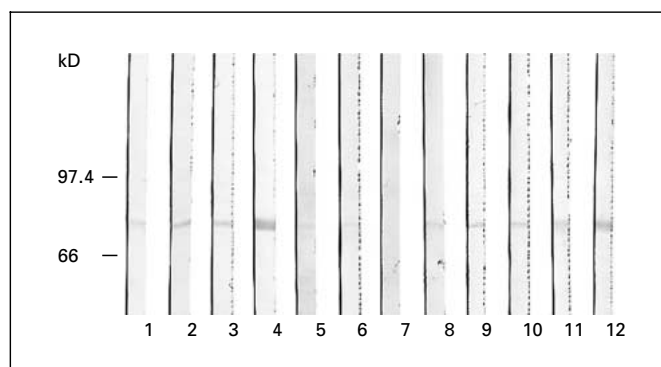


Fig. 1. Western blot analysis of different plant extracts with 70 kD protein specific mAb. Cross-reactive proteins were detected by the anti-70-kD protein mAb in maize root (lane 1), cress root (lane 2), apple (lane 3), hazelnut (lane 4), maize leaves (lane 5), parsley cell culture (lanes 6 and 7), *Arabidopsis* cell culture (lanes 8 and 9), cress hypocotyle (lane 10), maize coleoptile (lane 11), and birch pollen (lane 12). Dashes indicate 97- and 66-kD molecular mass markers, respectively.

embedded in LR White resin (Polysciences, Warrington, Pa., USA) according to the manufacturer's protocol. Immunofluorescence labelling was performed according to the method of Baluška et al. [22]. Semithin sections (0.5 μ m) were incubated first with the 70-kD protein mAb for 16 h, followed by antimouse fluorescein isothiocyanate conjugated antibodies for 1 h at room temperature. After immunostaining, sections were treated with an anti-fade mountant, and the 70-kD protein labelling was visualized using an Axiovert 405M inverse microscope (Zeiss, Oberkochen, Germany) equipped with epifluorescence and standard fluorescein isothiocyanate exciter and barrier filters (BP 450–490, LP 520). Photographs were taken using Kodak T-Max 400 ASA film.

Pollen Tube Preparation for Microscopy

Pollen tube preparation and fixation were performed according to the method of Gibbon et al. [23]. Protein structures were conserved after this treatment, and the cell walls of the pollen tubes could be permeabilized for immunostaining. Extracellular digestion of pollen tube walls was achieved by applying an enzyme cocktail containing 0.5% cellulase, 0.25% hemicellulase, 0.05% pectolyase, and 1 mM phenylmethylsulfonyl fluoride in PBS for 15 min at 37°C. The enzyme reaction was stopped by washing with 1% Tween 20 in PBS several times, and immunostaining was performed as described above.

Results

mAb Reactivity

Using hazel pollen crude extract as an immunogen, a mAb recognizing a protein of 70 kD as determined by Western blot studies was obtained. The antibody cross-reacted with 70- to 72-kD proteins of other tissues (root,

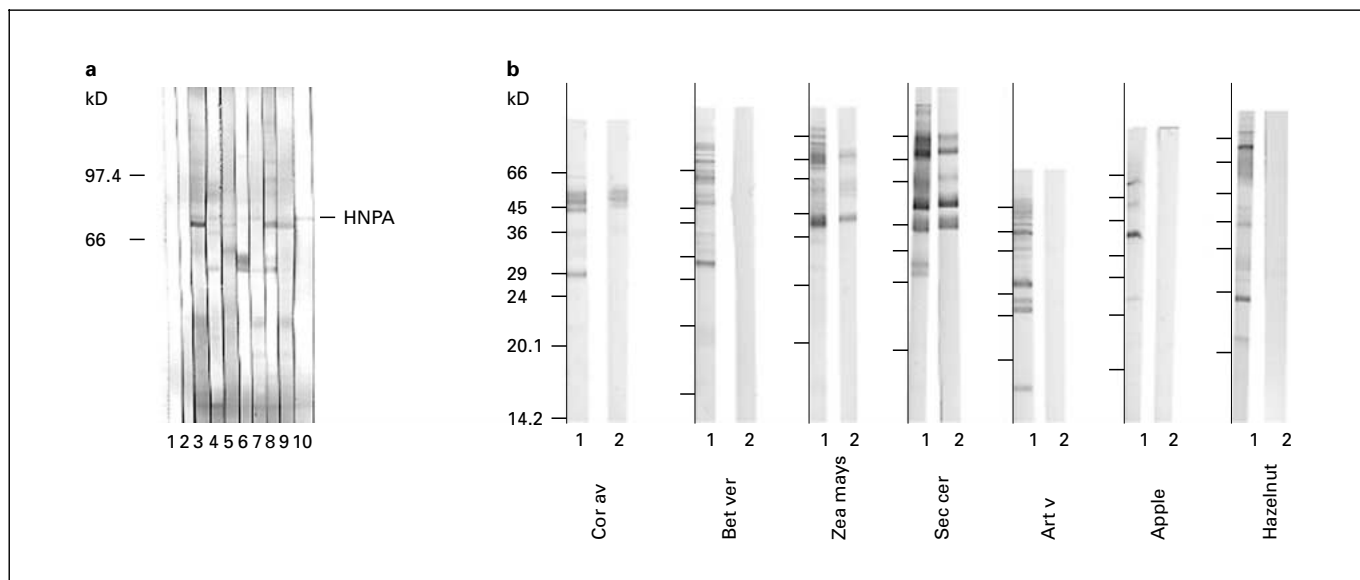


Fig. 2. Western blot analysis of different plant extracts with a panel of patient serum IgE. **a** Hazel pollen crude extract was separated by SDS-PAGE, transferred onto NC, and IgE-binding proteins were detected by incubation with non-allergic patient sera (lanes 1 and 2), allergic patient sera (lanes 3–9), and 70 kD protein specific mAb (lane 10). **b** IgE-binding patterns of two 70 kD protein reactive sera on different pollen and food extracts: patient 1 (lane 1) and patient 2 (lane 2). Extracts were from hazel pollen (Cor av), birch pollen (Bet ver), maize pollen (Zea mays), cultivated rye pollen (Sec cer), common mugwort pollen (Art v), Granny Smith apples, and hazelnuts.

coleoptile, leave, pollen, nut, and fruit) from different plant species like maize, apple, hazelnut, cress, and birch pollen (fig. 1). The signal intensity of mAb binding varied among the different types of tissues. For example, fast-growing material like maize coleoptile and root expressed higher levels of mAb-binding protein than maize leaves or cell cultures (fig. 1, lanes 1, 5, 6, 8, and 10). In potentially allergenic tissue material like apple fruit, hazelnut, and birch pollen (fig. 1, lanes 3, 4, and 12), the mAb-binding protein was also expressed in high amounts.

Human Serum IgE Reactivity

Using sera from patients with tree pollen allergy, crude hazel pollen extract revealed IgE-binding patterns, including proteins from 10 to 100 kD. Figure 2a illustrates the IgE-binding profile of seven different subjects on crude hazel pollen extract. Direct comparison with the anti-70-kD protein mAb reactivity showed a high correlation with the IgE binding of patient sera (fig. 2a, lanes 3–5, 7, and 9).

Additionally, two highly reactive patient sera were tested on different extracts from pollen, including hazel, birch, maize, rye, and mugwort, as well as from apple fruit and hazelnut. Patient serum 1 (fig. 2b, lane 1) exhibited

IgE antibody binding against all tested extracts, while patient serum 2 (fig. 2b, lane 2) reacted only with pollen extracts, but not with extracts from apple fruit or nut. Both sera recognized high molecular weight proteins in the different extracts which underlines the importance of proteins like the described 70-kD antigen with regard to IgE binding.

Protein Purification and Analysis

For further characterization of the 70-kD protein, we used preparative SDS-PAGE elution. The pollen protein mixture was separated into fractions according to molecular mass, ranging from 30 to 100 kD, as shown for every third fraction (fig. 3a). Subsequent Western blot analysis with 70 kD specific mAb showed a single protein band at 70 kD in the two elution fractions 39 and 40 (fig. 3b). The IgE-binding capacity of both fractions was tested with selected patient sera in Western blots, as shown in figure 3b. All sera contained serum IgE that bound to the 70-kD protein in both elution fractions. Individual tested sera (sera 1 and 2; see figure 2b) that reacted to pollen and other plant extracts indicated a more intensive reactivity than the pooled sera of tree pollen allergic patients.

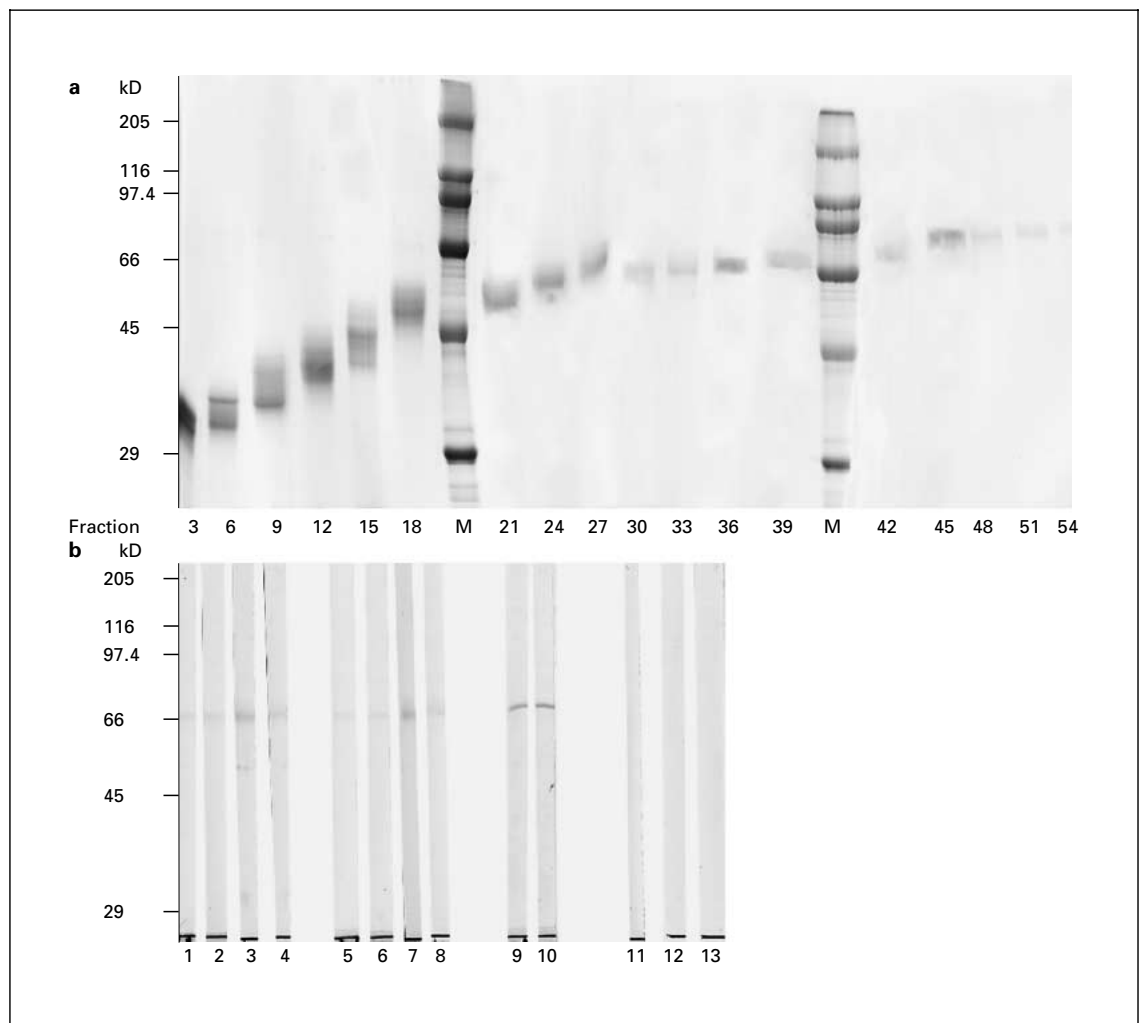


Fig. 3. Purification and analysis of the 70-kD protein from hazel pollen. **a** Coomassie brilliant blue profile of continuously eluted hazel pollen crude extract. Proteins with molecular masses between 50 and 90 kD could be detected in fractions 1–55. The 70-kD protein was labeled by specific mAb in fractions 39 and 40. **b** IgE reactivity of 70 kD protein containing elution fractions. PrepCell fractions 39 (lanes 1–4) and 40 (lanes 5–8) were separated in SDS-PAGE and blotted onto NC. Strips were incubated with pooled sera of patients 3, 4, and 5 (lanes 1 and 5), pooled sera of patients 6, 7, and 8 (lanes 2 and 6), serum 1 (lanes 3 and 7), serum 2 (lanes 4 + 8), 70 kD protein specific mAb on fraction 39 (lane 9), 70 kD protein specific mAb on fraction 40 (lane 10), and PBS probed as negative controls for secondary and tertiary antibodies (lanes 11–13).

Isoelectric Focusing

The elution fractions containing 70-kD protein were pooled and analyzed for the presence of possible isoforms by IEF using Immobiline DryStrips with a stable pH range from 3 to 10. The second dimension was resolved on SDS-PAGE, and proteins were transferred to NC. Subsequent Western blot analysis with 70-kD-specific mAb showed two acidic protein spots with pI values of 5.7 and 6.1 (fig. 4).

Molecular Cloning and Recombinant Expression of the 70-kD Protein

Since no N-terminal amino acid sequence can be obtained from the purified protein when subjected to peptide sequence analysis due to blocking, we constructed a cDNA expression library from mature hazel pollen in order to clone the 70 kD protein encoding gene. A specific mAb was used to screen the cDNA expression library to isolate cDNA clones encoding the allergen. Positive

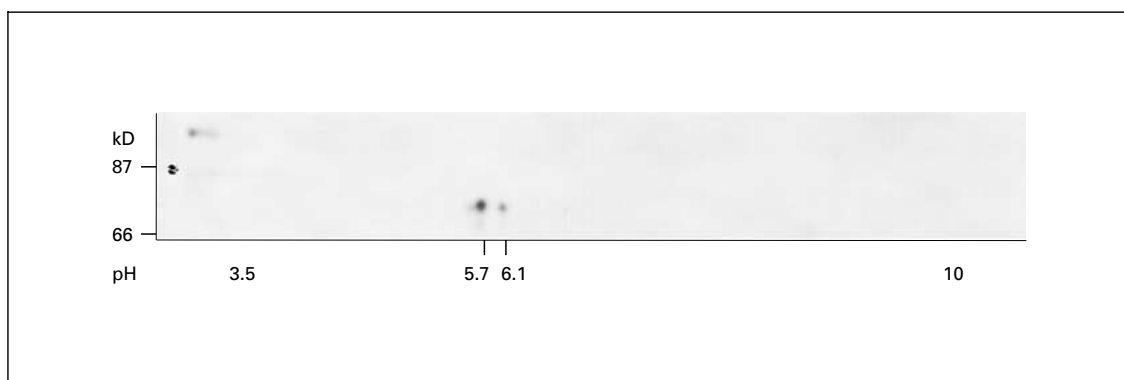


Fig. 4. Western two-dimensional analysis of the 70-kD protein with specific mAb. Two acidic isoforms of the pollen protein were detected, and the pH of each protein was determined as 5.7 and 6.1.

plaques were isolated and purified and the cDNA inserts subcloned into pBluescript by in vivo excision in *E. coli*. The nucleotide inserts were excised from plasmid by *Eco*-RI digestion and analyzed by agarose gel electrophoresis. The size was about 1,900 bp. In comparison to the native protein, the recombinant protein was about 10 kD smaller (data not shown). Sequencing of cDNA inserts displayed complete 3' ends with poly(A) tails. However, any missing nucleotides could only be of 5' origin and were synthesized by rapid amplification of cDNA ends (RACE) from hazel pollen mRNA. The resulting PCR product was transcribed to cDNA, and the amplified 5' cDNA fragment of the allergen encoding insert contained 800 bp. The fragment was subcloned and transformed in *E. coli*. Both allergen-encoding cDNAs were ligated into pBluescript, and the total construct was sequenced.

Sequence Analysis

The protein-encoding cDNA fragments were sequenced with T3/T7 primers plus an internal primer. The resulting nucleotide sequences were assembled according to their overlapping regions. The cDNA sequence (fig. 5) contains 2,007 bp of open reading frame (including the stop codon) that encodes a protein of 669 amino acid residues with a calculated molecular mass of 73.56 kD and an isoelectric point of pH 4.8. The deduced amino acid sequence of the protein (fig. 5) revealed 14 potential phosphorylation sites, one for tyrosine kinase, eight for casein kinase II, and five for protein kinase C. Two amidizing motifs are detected in the N-terminal part of the peptide. The C-terminal half shows a potential N-glycosylation site as well as a HDEL tetrapeptide which serve as a retention signal for soluble reticuloplasmans in plants [24].

Using the BLAST program, the protein demonstrates 90% identity with luminal binding proteins BLP-4 and BLP-5 from *Nicotiana tabacum* (swissprot: GR75-TOBAC Q03685 and GR74-TOBAC Q03684) and 90% identity with the dnaK-type molecular chaperon BiP precursor from *Glycine max* (pironly: T46574) and from *Ara-bidopsis thaliana* (pironly: s71171) and with several other molecular chaperons of the Hsp70 superfamily in plants and animals. All members of the Hsp70 family are described as stress-related proteins that bind to other peptides, thereby assisting the conformational protein-folding events. This protein interaction is ATP dependent, and Hsp70 chaperons contain an N-terminal ATPase region of about 45 kD. In our 70-kD protein this functional protein structure (fig. 5) is contained within the amino acid residues No. 1–435. The ATPase region is followed by a 141 amino acid peptide binding domain (436–577) and a C-terminal regulatory site of 75 amino acids (578–654).

The cDNA nucleotide sequence of the 70-kD protein has been submitted to the EMBL Data Bank (accession No. AJ295617).

Immunolocalization of the 70-kD Protein in Pollen

Hsp70 chaperon expression is known to be subject to temporal, developmental, and stress-induced regulation during plant differentiation. The connection between these physiological processes and allergy is less investigated. Therefore, we performed immunolocalization experiments of the 70-kD protein during hazel pollen development (fig. 6). Immature, bicellular pollen grains feature a vacuole in the vegetative cytoplasm at this stage. The pollen contained only a small amount of storage vesi-

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1  ATGGCTGGCT  CGTGGAGAGC  ACGCGGCTCC  TTGGTCGTAC  TAGCGATTCT
1  M A G S W R A R G S L V V L A I L
51  TCTCTTCGGA  TGCTTATTGG  CAATTTCAT  TCGGAAGAG  GAAGCCACAA
18  L F G C L F A I S I A K E E A T
101  AGTTGGGAAC  AGTCATTGGT  ATTGACCTTG  GCACAACTTA  TTCTTGTTGT
34  K L G T V I G D E L G T T Y S V
151  GGTGTCTACA  AGAACGGTCA  TGTTGAAATC  ATAGCCAACG  ACCAAGGAAA
51  G V Y K N G H V E I I A N D Q G N
201  CCGTATCACC  CCATCATGGG  TTGATTTCG  TGAAGGTGAG  AGATTGATCG
68  R I T P S W V G F T D G E R L I
251  GTGAGGCTGC  TAAGAACCG  GCAGCTGTCA  ACCCTGAGAG  GACCATCTTT
84  G E A A K N Q A A V N P E R T I F
301  GATGTCAAAA  GCTTATTGG  AAGAAAGTTT  GAAGACAAGG  AGTTTCAGAA
101  D V K R L I S R E F E D K E V Q K
351  GGACATGAAG  CTGTGTCCAT  ACAAGATTGT  GAATAAGGAT  GGAAAGCCIT
118  D M K L V P Y K I V N K D G K P
401  ACATTCAAGT  CAAGATTAAG  GATGGTGAGA  CCAAGGTTTT  CAGCCCTGAG
134  Y I Q V K I K D G E T K V F S P E
451  GAGATCAGTG  CTATGATTCT  GATTAAGATG  AAGGAAACAG  CTGAAGCATT
151  E I S A M I L I K M K E T A E A F
501  CCTTGGGAAG  AAAATCAAGG  ATGCTGTGCT  CACTGTTCCT  GCATACTTCA
168  L S K R I K D A V V T V P A Y F
551  ATGATGCCCA  GAGGCAGGCT  ACTAAGGATG  CAGTATTAT  TGCTGACTG
184  N D A Q R Q A T K D A G I I A G L
601  AATGTTGCCA  GAATTATCAA  TGAACCAACC  GCAGCCGCCA  TCGCATATGG
201  N V A R I I N E P T A A A I A Y G
651  TTTGGATAAA  AAGGCGGGG  AGAAGAACAT  CCTTGTTTTT  GACTTGGGTG
218  L D K K G G E K N I L V F D E G
701  GTGGGACATT  TGATGTCAGT  ATCTTGACCA  TTGATAACGG  TGTTTTTGAG
234  S G T F D V S I L T I D N G V F E
751  GTTCTCTCCA  CTAATGGAGA  CACTCATCTT  GGAGGTGAGG  ATTTTGATAT
251  V L S T N G D T H L G G E D F D M
801  GAGAATCATG  GAGTACTTCA  TCAAATTGAT  TAAGAAAAAG  CATGCAAGG
268  R I M E Y F I K L I K K K H G K
851  ACATCAGCAA  GGACACAGA  GCTATTGGAA  AGCTGAGGAG  GGAAGCTGAG
284  D I S K D N R A I G K L R R E A E
901  CGTGCCAAAG  GAGCTCTTAG  TAGCCAGCAC  CAAGTCGGTG  TGGAGATTGA
301  R A K R A L S S Q H Q V R V E I E
951  ATCACTTTTT  GATGGTGTG  ACTTTTCTGA  ACCACTGACC  CGAGCTAGGT
318  S L F D G V D F S E P L T R A R
1001  TCGAGGAGCT  GAACAATGAT  TTTGTTTCAG  AAGACCATGG  GACCCGTGAA
334  F E E L N G D F V K D H G T R E
1051  GAAGCTATGG  AAGATGCTGG  ATTGGCAAG  AACCAGATTG  ATGAGATTGT
351  E A M E D A G L A K N Q I D E F V
1101  TCTTGTGGT  GGAAGCACC  GGATTCACAA  GGTTCACAG  CTCTTAAGG
368  V V G G S T R I P R V Q Q L L K
1151  ACTACTTCA  TGGGAAGSAG  CCAACAAGG  GTGTGAACCC  AGATGAAGCA
384  D Y F D G K E P N K G V N P D E A
1201  GTTGCCATAG  GTGCTGCTGT  TCAAGGAAGC  ATCTTGAGTG  GAGAGGGTGG
401  V A Y G A A V Q G S I L S G E G G
1251  TGAAGAAACC  AAGATATCC  TTCTCTCGA  TGTGGCTCCT  CTGACCCTTG
418  E E T K D I L L L D V A P L T L
1301  GAATTGAAAC  TGTTGGTGA  GTGATGACCA  AGTTGATCCC  TAGAAACACT
434  G I E T V G G V M T K L I P R N T
1351  GTTATCCCAA  CAAAGAAATC  TCAGGTTTTC  ACTACTTACC  AGGATCAGCA
451  V I P T K K S Q V F T T Y Q D Q Q
1401  GACTACCGTA  TCATTTCAG  TCITTTGAAG  TGAGAGGAGT  CTCACAAAGG
468  T T V S I Q V F E G E R S L T K
1451  ATTGCAGGAA  TCTTGGGAAA  TTTGATCTAA  CTGGAGTTCC  ACCAGCTCCA
484  D C R N L G K F D L T G V P P A P
1501  AGGGGAACCC  CTCAAATTGA  AGTCACATTT  GAAGTTGATG  CAAATGGTAT
501  R G T P G I E V T F E V D A N G I
1551  CCTAAATGTT  AAGGCGGAAG  ACAAGGCGAC  TGGTAATCA  GAGAAGATCA

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518  L N V K A E D K G T G K S E K I
1601  CCATCACCAA  CGACAAGGGT  CGTTGAGGCC  AGGAGGAGAT  CGACCGCATG
534  T I T N D K G R L S Q E E I D R M
1651  GTTCAGGAGG  CGAAGAGATT  TGCTGAGGAA  GACAAGAAGG  TGAAGGAAAG
551  V Q E A E E F A E E D K K V K E R
1701  GATTGATGCT  CGCAACACCC  TTGAGACCTA  TGTGTATAAC  ATGAAGAACC
568  I D A R N T L E T Y V Y N M K N #
1751  AGGTCAACGA  CAAAGACAAG  CTTGCTGATA  AGCTTGAGTC  CGATGAGAAG
584  Q V N D K D K L A D K L E S D E K
1801  GACAAGATCG  AGTCCGCTGT  GAAGGACGCC  CTTGAATGSC  TCGACGACAA
601  D K I E S A V K D A L E W L D D N
1851  CCAGAGCGCT  GAGAAGGAGG  ACTACGACGA  GAAGCTGAAA  GAGGTGGAAG
618  Q S A E K E D Y D E K L K E V E
1901  CCGTTTGCAA  CCCAATCAIT  ACCGCCGTTT  ATCAGAGGTC  GGGTGGAGCC
634  A V C N P I I T A V Y Q R S G G A
1951  CCGGGTGGCG  GATCTGGGGA  GGAGGATGAG  GACTCAGAGT  CGCATGACGA
651  P G G G S G A E E D E D S E S H D E
2001  GCTGTAG
668  L *

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Fig. 5. Nucleotide and deduced amino acid sequences of 70-kD hazel pollen antigen. Phosphorylation sites are indicated in yellow, Hsp70 consensus sequences are marked in red, the amidizing pattern is labeled in blue, and Asp glycosylation sites are labeled in green. The 45-kD ATPase region of the 70-kD protein starts at the N-terminus and continues to amino acid 435 (*). The peptide-binding domain ends at amino acid 577 (#), and the regulatory domain terminates at position 652 (^). Underlined sequences mark functional ATPase domains. This sequence is available at EMBL Data Bank under the accession No. AJ295617.

cles, and the pollen coat structure was still incomplete. The immunolabelling of the protein at this stage was restricted to the carbohydrate-rich intine material (fig. 6a). In mature tricellular pollen, the cytoplasm contains many starch grains and lipid bodies. The pollen coat is completed, and three preformed germination pores can be detected as pyramidal peaks on the surface. Specific labelling is observed in cytoplasm and intine with remarkable accumulation underneath the germination pores (fig. 6b). The mature tricellular pollen stadium showed the strongest signal under normal environmental conditions. After germination, the signal was detected in the pollen tube tip (fig. 6c). Intensive endo- and exocytosis, transporting wall components and recycling plasma membrane, ensures pollen tube elongation. Proteins involved in pollen stigma adhesion or compatibility interaction are incorporated in the cell wall. The immunolocalization of the protein to this region of the pollen tube may indicate active participation in these processes.

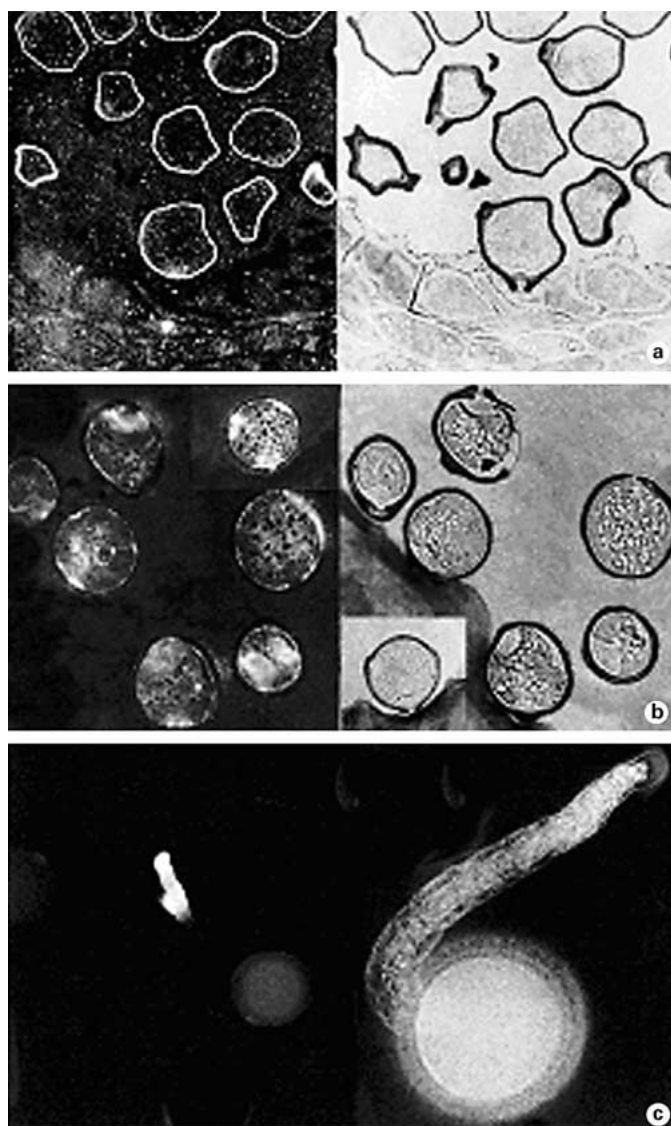


Fig. 6. Immunolocalization of the 70-kD protein in hazel pollen at different stages of development with the use of a specific mAb. Immunofluorescence labelling is shown on the upper left, while the corresponding light micrographs are shown on the upper right. **a** Pollen grains at the bicellular, immature stage surrounded by another tissue. Specific 70-kD protein labelling was detected exclusively in the intine. **b** Mature, tricellular hazel pollen. The 70-kD protein is localized in the cytoplasm without highlighting special structures. The boundary of cytoplasm and intine shows accumulation of antibody. Significant labelling also occurs in the region of the germinal apertures. **c** Freshly collected hazel pollen were germinated and labeled with specific mAb after an extracellular digest of the pollen tube wall. Immunolocalization of the 70-kD protein was focused to the tip region of the pollen tube.

Discussion

In this study, we have successfully identified a novel 70-kD hazel pollen allergen (designated Cor a 10 by the Allergen Nomenclature Committee under www.allergen.org), demonstrating IgE-binding capacity using Western blot analysis. Five of seven allergic patient sera demonstrated IgE binding to the 70-kD allergen Cor a 10 in the purified protein fraction as well as in the crude hazel pollen extract. Two separately tested human sera exhibiting IgE against high molecular weight allergens of different pollen and fruits showed also IgE binding to the 70-kD protein in hazel pollen. A causal correlation between IgE binding of these sera and glycosylation can be excluded because of the high sequence homology of the allergen to Hsp70 chaperons which are known to be unglycosylated. Moreover, biochemical characterization of the 70-kD hazel pollen allergen revealed physicochemical properties similar to those of other known allergens (i.e., polymorphism, acidic pI, IgE-binding capacity, and cross-reactivity) [25]. The high degree of sequence identity between the new allergen and Hsp70 chaperons may explain the ubiquitous expression of chaperon homologues and their association with protein synthesis rates in different tissues. Upregulation of Hsp70 expression in metabolically active cells as well as the protective function of BiP during desiccation periods are well-investigated and well-documented phenomena [26–28]. Nuts, fruits, and pollen excel in natural desiccation tolerance and, therefore, contain higher amounts of Hsp70 stress proteins, as shown in this study. A pollen-sensitized patient with specific IgE against the 70-kD allergen (Cor a 10) may, therefore, exhibit an oral allergic syndrome to plant foods as well.

Like Hsp70, we have shown that the 70-kD hazel pollen allergen occurs as acidic polymorphic forms. For example, polymorphism of BiP was detected in tobacco [29] and soy [30]. An isoelectric point of 4.8 was calculated from deduced amino acid sequence of Cor a 10, while the natural protein showed two isoforms at pI 5.7 and 6.1. The difference between the calculated and actual pI of Cor a 10 may be attributed to phosphorylation, since 14 potential phosphorylation sites for different kinases have been detected in the Cor a 10 sequence. Expression and phosphorylation levels of BiP isoforms have been described as regulatory mechanisms following abiotic stresses [31, 32]. For example, different phosphorylation levels of BiP have been shown to contribute to a shift from cationic to anionic net charge following osmotic stress in soybean. This shift was described as an effective mechanism for protein regulation [32].

There was no difference in IgE binding between the purified Cor a 10 isoforms, indicating that the two polymorphic forms may have resulted exclusively from phosphorylation of the allergen without any significant sequence difference.

The 70-kD protein displays sequence identity of up to 85–90% with plant molecular chaperons of the Hsp70 family like luminal binding proteins (BiP) or glucose-regulated proteins. The high degree of sequence identity between tobacco, soy, maize, tomato, and hazel pollen BiP implicates an effective phylogenetic protein conservation and combined with the IgE reactivity of the 70-kD allergen qualifies it as a possible panallergen. Significant differences were only observed in the N-terminal signal sequence region and in the C-terminal region adjacent to the HDEL retention sequence. Therefore, both regions may not be essential for protein function. In spite of the putative Asp glycosylation site of the 70-kD allergen in the C-terminal region, glycosylation is not likely, since the molecular mass estimated by SDS-PAGE (i.e., 70 kD) agreed well with the calculated size (i.e., 73.56 kD). Moreover, glycosylation was not detected among other BiPs identified previously [29].

Immunolocalization of the 70-kD allergen in hazel pollen differed in both intensity and location, depending on the stage of development. The development-dependent transcription and expression levels of Hsp70 have already been studied in detail with tomato and maize pollen [30, 33]. Both studies identified the late bicellular pollen stage to contain the highest mRNA transcription of BiP. The highest amount of Hsp70 protein translation occurred in the tricellular pollen stadium, accumulated for impending germination. Our results support these findings, since immunostaining was localized to the intine material in bicellular pollen, while tricellular pollen grains exhibited strong labelling of cytoplasm and intine, especially in the germination pore region. During pollen tube growth, the 70-kD protein was found in the tip region, where intensive exo- and endocytosis ensures optimal pollen tube elongation. Our immunolocalization studies with the hazel pollen antigen indicate a substantial involvement of the chaperon in pollen coat formation, synthesis of storage vesicles, and pollen tube growth. Furthermore, it was shown that ER-based proteins have a unique distribution in tip-growing systems like maize root hairs. Proteins with HDEL retention sequences have been shown to accumulate within the outgrowing bulges and remain enriched at the tip of emerging hairs [34].

Unlike for other allergens, where their enzymatic function can be hypothesized to play an active role in allergy

induction by helping the allergen to penetrate the cell barrier (i.e., chitinase or pectinase), the same is not clear for BiP. However, chaperon coexpression has been shown to improve expression levels and protein folding of recombinant Cry j 2, an allergen of Japanese cedar pollen [35]. Molecular chaperons play an important role in the expression and structural integrity of proteins in general and might thereby contribute to the structural integrity of allergens in different plant tissues as well.

In conclusion, the results presented here indicate that BiPs may be important for cell function and are, therefore, present in many plant tissues. Moreover, hazel pollen BiP (i.e., the 70-kD protein, Cor a 10) has been shown to possess IgE-binding activity, and its potential role to contribute and maintain the expression and structural integrity of other allergens makes this protein a potential novel panallergen. Due to the ubiquitous nature of molecular chaperons, their identification, biochemical characterization, and recombinant production might be important for effective allergy diagnosis and treatment.

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