

## Original article

Identification of an obeche (*Triplochiton scleroxylon*) wood allergen as a class I chitinase

**Background:** Wood dust is known to cause allergic occupational asthma and obeche (*Triplochiton scleroxylon*) is a prominent exponent in this field. However, the knowledge about wood allergens is still limited. The aim of this study was to identify and characterize obeche wood allergens.

**Methods:** Obeche extracts were prepared from freshly ground in comparison to 7 years stored wood dust and investigated by Sodium dodecyl sulfate-polyacrylamid gel electrophoresis, enzyme-linked allergosorbent test and immunoglobulin (Ig)E-immunoblot. Allergens were detected by specific IgE of seven obeche allergic patients' sera and protein analysis was performed by mass spectrometry. Cross-reactivity was demonstrated by ImmunoCAP-inhibition with sera of seven obeche and four latex-allergic patients.

**Results:** Obeche extracts showed different protein pattern and IgE-binding capacities depend on the age of the wood dust. A 38 kDa protein was identified as major obeche wood allergen, detected by six of seven (85%) obeche allergic patients' sera and was entitled as Trip s 1. Trip s 1 is homologous to plant class I chitinases and exhibited enzyme activity demonstrated by chitinolysis.

Co-recognition or cross-reactivity of Trip s 1 according to structural similarity was seen in sera of latex allergic patients. IgE inhibition studies with obeche as solid phase and Trip s 1 and latex hevein as inhibitor demonstrated that Trip s 1 was a more effective inhibitor in obeche as well as in latex allergic patients' sera.

**Conclusions:** Trip s 1 is a new obeche wood allergen of the plant class I chitinase family. This finding may explain the dominant role of obeche in sensitization against wood dust.

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Among the woods causing immunoglobulin (Ig)E mediated allergy, obeche (*Triplochiton scleroxylon*) is frequently described (1–4). Typical allergic symptoms of wood dust sensitization including asthma, rhinitis and conjunctivitis are based on a type I immune response (5–7). The panel of woods responsible for allergic reactions is manifold and include tropical trees like antiaris (*Antiaris toxicaria*) (8), tali (*Erythrophleum guineense*) and jatoba (*Hymenaea* sp.) (9) and Pau marfin (*Balfourodendron riedeliamum*) (10), as well as trees growing in the temperate zones like ash (*Fraxinus exelsior*) (11), oak (*Quercus robur*) and beech (*Fagus sylvatica*) (12), pine (*Pinus sylvestris*) (7), spindle tree (*Euonymus atropureus*)

(13) and western red cedar (*Thuja plicata*) (6). The diagnosis of wood dust sensitization is often difficult because of lacking or nonstandardized wood extracts, especially in subjects with sensitization to cross-reactive environmental allergens. In fact there are no major protein allergens described so far. The low molecular substance plicatic acid from western red cedar was mentioned to be involved in a type I sensitization against wood dust (6) but no publication during the last decade supported the finding that IgE against plicatic acid is an important factor in the development of red cedar asthma (7). In contrast several case reports of sensitization to obeche wood showed IgE-immunoblots of obeche wood extracts with IgE-binding to specific proteins, indeed without further allergen identification (1, 3). Cross-reactivity between different wood species was described for obeche and ramin, but again without further classifying the responsible allergens (2). Venturini et al. (14) demonstrated cross-reactivity between obeche wood and

**Abbreviations:** CBP, chitin-binding protein; EAST, enzyme-linked allergosorbent test; MBP, maltose binding protein; MWCO, molecular weight cut off; NRL, natural rubber latex; PR, pathogenesis-related protein; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamid gel electrophoresis.

natural rubber latex (NRL) by a 38 kDa allergen from obeche. It was the aim of our study to characterize obeche wood extracts and to identify potential allergens.

## Material and methods

### Patients

Ten male subjects exposed to obeche wood in the wood processing industry with work-related asthma were examined by external physicians. Seven subjects were allergic to obeche wood (P1–P7) and showed positive skin prick tests (SPTs), and obeche-specific IgE concentrations in the serum. Three obeche wood dust exposed subjects were not obeche sensitized (C1–C3) (Table 1A). Four sera of latex allergic subjects (L1–L4) were included for cross-reactivity studies. All latex allergic patients were female health care workers (Table 1B) with occupational asthma, rhinitis, conjunctivitis and contact urticaria. They had high specific IgE concentrations to obeche wood in their serum without being exposed to wood dust.

### Obeche extract preparation

Both freshly ground obeche (*T. scleroxylon*) wood dust from Ghana (Africa) without preservatives and obeche wood dust stored for approximately 7 years at  $-40^{\circ}\text{C}$  were used. Wood dusts were mixed 1 : 10 (w/v) with phosphate buffered saline (PBS; pH 7.4) containing 0.5 mM Pefablock (Serva, Heidelberg, Germany) and 2% polyvinylpyrrolidone (PVPP; Sigma, Deisenhofen, Germany) and were gently stirred for 16 h at  $4^{\circ}\text{C}$ . After centrifugation (15 000 g,  $4^{\circ}\text{C}$ , 45 min) the wood dust pellet was solubilized in 0.1% CHAPS-PBS and stirred again for 4 h at  $4^{\circ}\text{C}$ . Protein fraction was separated again by centrifugation and wood material was finally

dissolved in 50% ethanol-PBS, extracted for further 4 h at  $4^{\circ}\text{C}$  and separated as above. All wood protein supernatants were dialyzed against distilled water [Spektra/Por<sup>®</sup>, 3.5 kDa molecular weight cut off (MWCO), Serva] and lyophilized afterwards. Protein concentration was quantified according to Bradford (15).

### Measurement of specific IgE

Different wood extracts were coupled to bromcyan activated (BrCN)-paper discs (Allergopharma, Reinbeck, Germany) as described elsewhere (16). IgE-binding capacity was verified by measuring specific IgE responses by enzyme-linked allergosorbent test. Allergen-coated discs were incubated with 50  $\mu\text{l}$  of patient serum for 3 h at room temperature. A Phadezym RAST test kit (Pharmacia Diagnostics, Upsalla, Sweden) was used to detect specific IgE antibodies.

Serological studies and inhibition experiments were performed with the UniCAP-System<sup>TM</sup> (Pharmacia Diagnostics) using commercial obeche wood ImmunoCAPs (k212) and latex ImmunoCAPs (k82). Hevein (rHev b 6.02) was expressed recombinantly as fusion protein with maltose binding protein (MBP) in *Escherichia coli* as described previously (17, 18). Specific IgE concentrations against rHev b 6.02 and MBP (as control) were coupled to ImmunoCAP (MIAB, Upsalla, Sweden) for specific IgE testing. Specific IgE concentrations greater than 0.35 kU/l were considered positive. For inhibition studies, sera with specific IgE concentrations between 3.0 and 5.0 kU/l were preincubated with 10  $\mu\text{l}$  of specific inhibitor solution (1 mg/ml). As control, PBS instead of inhibitor was applied.

Skin tests for wood specific IgE were performed as intracutan skin test with allergen solution of Allergopharma by external physicians. The SPT for latex type I reactions were performed with latex extract prepared in our laboratory with a total protein concentration of 10  $\mu\text{g}/\text{ml}$  in saline solution containing 0.4% phenol.

Table 1. Characteristics of (A) wood dust allergic subjects and controls and (B) latex allergic subjects

|     | Age (years) | OET (years) | Symptoms   | Total IgE (kU/l) | Obeche (kU/l) | Specific IgE to other woods (by EAST <sup>1</sup> /ICST <sup>2</sup> )                                                                                                                                         | Sensitization to other allergens (by CAP <sup>1</sup> /SPT <sup>2</sup> ) |
|-----|-------------|-------------|------------|------------------|---------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| (A) |             |             |            |                  |               |                                                                                                                                                                                                                |                                                                           |
| P1  | 40          | 15          | A, R, C    | 189.0            | 15.7          | sipo ( <i>Entenderophragma utile</i> ) <sup>1,2</sup> , pine ( <i>Pinus sylvestris</i> ) <sup>1,2</sup> , teak ( <i>Tectona grandis</i> ) <sup>1,2</sup>                                                       | b <sup>1</sup>                                                            |
| P2  | 48          | 8           | A, R       | 135              | 9.5           | oak ( <i>Quercus robur</i> ) <sup>1,2</sup> , pine ( <i>Pinus sylvestris</i> ) <sup>1,2</sup>                                                                                                                  | hdm <sup>1,2</sup> , stm <sup>1,2</sup>                                   |
| P3  | 24          | 2           | A, R, U    | 474.0            | 20.2          | –                                                                                                                                                                                                              | c <sup>2</sup> , gp <sup>2</sup> , hdm <sup>2</sup> , tp <sup>2</sup>     |
| P4  | 25          | 0.5         | A, R       | 84.30            | 29.6          | ofram ( <i>Terminalia superba</i> ) <sup>1</sup> , sipo ( <i>Entenderophragma utile</i> ) <sup>1</sup> , beech ( <i>Fagus sylvestris</i> ) <sup>1</sup> , teak ( <i>Tectona grandis</i> ) <sup>1</sup>         | gp <sup>2</sup> , hdm <sup>2</sup>                                        |
| P5  | 38          | 9           | A, R       | 197.0            | 20.3          | aprono ( <i>Mansonia altissima</i> ) <sup>1,2</sup> , elm ( <i>Ulmus carpinifolia</i> ) <sup>1,2</sup> , ash ( <i>Fraxinus excelsior</i> ) <sup>1,2</sup> , iroko ( <i>Chlorophora exelsa</i> ) <sup>1,2</sup> | f <sup>1,2</sup> , hdm <sup>2</sup>                                       |
| P6  | 30          | 4           | A, R, C    | 70.3             | 1.6           | –                                                                                                                                                                                                              | tp <sup>2</sup>                                                           |
| P7  | 36          | 15          | A, R       | 1360.0           | 20.4          | oak ( <i>Quercus robur</i> ) <sup>1,2</sup> , ramin ( <i>Gonystylus bancanus</i> ) <sup>1,2</sup> , teak ( <i>Tectona grandis</i> ) <sup>1,2</sup> , pine ( <i>Pinus sylvestris</i> ) <sup>1,2</sup>           | hdm <sup>2</sup> , gp <sup>2</sup> , m <sup>2</sup> , tp <sup>2</sup>     |
| C1  | 35          | 13          | A, R       | 26.0             | <0.35         | ash ( <i>Fraxinus exelsior</i> ) <sup>1,2</sup>                                                                                                                                                                | –                                                                         |
| C2  | 53          | 39          | A, R, U    | 290.0            | <0.35         | pine ( <i>Pinus sylvestris</i> ) <sup>1,2</sup> , meranti ( <i>Shorea pauciflora</i> ) <sup>1,2</sup>                                                                                                          | gp <sup>2</sup> , tp <sup>2</sup>                                         |
| C3  | 52          | 8           | A, R       | 82.0             | <0.35         | beech ( <i>Fagus sylvestris</i> ) <sup>1,2</sup> , teak ( <i>Tectona grandis</i> ) <sup>1,2</sup>                                                                                                              | c <sup>2</sup> , tp <sup>2</sup>                                          |
| (B) |             |             |            |                  |               |                                                                                                                                                                                                                |                                                                           |
| L1  | 42          | 7           | A, R, C, U | 267.0            | 12.2          | –                                                                                                                                                                                                              | gp <sup>2</sup> , hdm <sup>2</sup> , lfs <sup>1,2</sup>                   |
| L2  | 33          | 8           | A, R, C, U | 831.0            | 3.1           | –                                                                                                                                                                                                              | d <sup>2</sup> , hdm <sup>2</sup> , lfs <sup>1,2</sup>                    |
| L3  | 33          | 7           | A, R, C, U | >2000.0          | 24.4          | –                                                                                                                                                                                                              | d <sup>2</sup> , gp <sup>2</sup> , hdm <sup>2</sup> , lfs <sup>1,2</sup>  |
| L4  | 34          | 4           | A, R, C, U | 824.0            | 8.9           | –                                                                                                                                                                                                              | lfs <sup>1,2</sup>                                                        |

OET, occupational exposure time; A, asthma; R, rhinitis; C, conjunctivitis; U, urticaria; EAST, enzyme-linked allergosorbent test; ICST, intracutan skin test; SPT, skin prick test; b, banana; c, cat; d, dog; f, *Ficus benjamina*; gp, grass pollen; hdm, house dust mite; LFS, latex-fruit-syndrome; m, mould; stm, storage mite; tp, tree pollen.

## Electrophoresis and IgE-immunoblot

Protein extracts were resolved in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using precasted NuPAGE gels (Novex, San Diego, CA) with Xcell II mini-cell unit of Novex according to manufacturers' instructions. For immunostaining obeche wood extract from freshly ground material, extracted at pH 7.4 without additives, was used. Resolved proteins were blotted on polyvinylidene difluoride (PVDF) membrane (Millipore, Eschborn, Germany) and IgE-immunoblots were performed as described previously (16).

## Purification of chitin-binding proteins from obeche

Lyophilized obeche proteins were dissolved in 25 mM Tris/HCl, pH 7.3 with a protein concentration of 0.5 mg/ml. The sample was centrifuged for 5 min, 15 000 g, 4°C and the supernatant was then applied to a Pharmacia XK 16/20 column packed with chitin beads (New BioLab, Beverly, CA). Chitin-binding proteins (CBPs) were eluted by reversible protein denaturation with 6 M guanidium hydrochloride (Roth, Karlsruhe, Germany) pH 6.3. Eluted material was dialyzed (Spektra/Por®, 3.5 kDa MWCO) against distilled water and then concentrated by lyophilization.

## Detection of chitinase activity after SDS-PAGE

Chitinase activity was visualized by affinity of chitin to Calcofluor white M2R (Sigma) as described (19). Different amounts (0.5, 1.0, 2.5, 3.5 µg) of CBP from obeche wood were dissolved in SDS-sample buffer. The SDS-PAGE was performed in 10% polyacrylamide gels including 0.01% (w/v) glycol chitin (Sigma) as described previously (20). After electrophoresis chitinolysis was demonstrated by staining chitin with 0.01% (w/v) Calcofluor White M2R for 10 min. Lytic zones representing chitinase activity were visualized on a UV-desk as clear zones and images were scanned with elumination system V02 7325 (Vilber Lourmat, France).

## Mass-spectrometry

A coomassie stained protein band was excised from SDS-PAGE and tryptically digested at 37°C overnight. The peptides were extracted according to Schaefer et al. (21) and separated by reversed-phase HPLC using a 300 µm × 150 mm column. Fractions

of 5 µl were collected. Aliquots of each fraction (5 µl) were measured by MALDI-MS and/or MS/MS using an Ultraflex™ instrument from Bruker Daltonics (Faellanden, Switzerland).

## N-terminal sequence analysis

Obeche proteins were blotted onto PVDF membrane and visualized by staining with 0.2% Coomassie brilliant Blue R-250 (Serva) in 50% methanol. Protein bands of interest were excised from the blotting membrane and analyzed on a 476A protein sequencer (Applied Biosystems, Foster City, CA). Routinely, seven to ten Edman degradation cycles were performed.

## Results

## Obeche allergic patients

In all subjects with physician-diagnosed obeche allergy were confirmed by elevated concentrations of specific IgE to obeche (1.6–29.6 kU/l, Table 1A). Five of seven patients' sera showed specific IgE to other wood species. Sensitization to environmental aeroallergens were found in all seven subjects and one of them (P1) was sensitized to banana. Work-related asthma and rhinitis were reported by all subjects. In latex allergic patients (L1–L4) conjunctivitis and contact urticaria were more frequently observed. There was no correlation between total IgE and obeche-specific IgE concentrations.

## Obeche wood dust extracts

The extractable protein pattern was in the range of 5–70 kDa with dominant bands at 7, 12, 26, 28, 29, 30, 38 and 62–65 kDa depending on the age of the wood dust and the extraction buffer (Fig. 1A). Freshly ground wood dust (lanes 1–9) had a protein content up to 5% in the lyophilized extract, exhibiting clearly shaped protein bands in the SDS-PAGE. In contrast, the stored wood dust extract with a protein content of about 0.5% did not display single protein bands, but abundant degradation

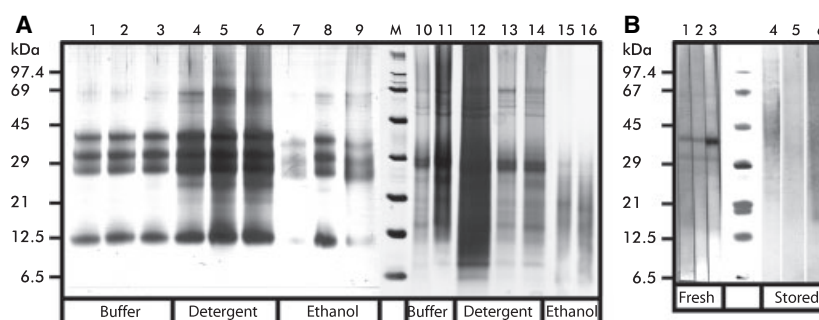


**Figure 1.** (A) Silver staining of obeche wood protein profile. Lanes 1–9 freshly ground wood dust protein pattern extracted with: 1–3: buffer (pH 6.4, pH 7.4, pH 8.4); 4–6: buffer (pH 6.4, pH 7.4, pH 8.4) plus detergent; 7–9: buffer (pH 6.4, pH 7.4, pH 8.4) plus ethanol. Lanes 10–16 stored wood dust protein pattern extracted with: 10–12: buffer (pH 7.4, pH 8.4); 12–13: buffer (pH 6.4, pH 7.4, pH 8.4) plus detergent; 14–16: buffer (pH 7.4, pH 8.4) plus ethanol. (B) IgE Western blot with obeche allergic patients' sera on freshly ground wood material (lanes 1–3) in comparison to stored wood dust (lanes 4–6), both extracted in buffer pH 7.4.

products (lanes 10–16). Using aqueous extraction conditions, sufficient protein amounts were obtained from obeche wood as shown in lanes 1–3. Other conditions like detergent (lanes 4–6) or ethanol (lanes 7–9) had only minor influence on the protein release. IgE binding activities of both freshly ground and stored wood dust extracts were compared by IgE immunoblots (Fig. 1B). Sera of three obeche allergic patients were tested exemplarily and all of them showed specific IgE against a 38 kDa protein in the freshly ground obeche wood material (Fig. 1B, lanes 1–3) followed by a moderate labeling of a 30 kDa protein. The same sera did not show any distinct IgE labeling on the obeche wood extract prepared from the 7 years stored wood dust material (Fig. 1B, lanes 4–6). This finding together with SDS-PAGE (Fig. 1A, lanes 10–16) suggest protein degradation during wood dust storage. For the IgE immunoassays and following allergen characterization freshly ground wood dust extracted at pH 7.4 was used.

#### N-terminal protein sequencing and mass fingerprinting

Amino acid sequences of the 38 kDa obeche wood allergen, deduced from mass fingerprint and N-terminal peptide sequence were matched with known proteins by BLAST (<http://www.ncbi.nlm.nih.gov/blast>). The four peptide fragments of 6, 9, 11 and 12 amino acids revealed high identity to class I chitinase from different plants as shown in Table 2. Comparing the N-terminal amino acid sequence of the 38 kDa allergen with class I chitinases from rice (*Oryza sativa*), banana (*Musa acuminata*), avocado (*Persea americana*) and hevein from latex (*Hevea brasiliensis*) an identity of 81% was observed. Considering further amino acids, which triplet-code differed only in one base the similarity increased up to 90%. The three tryptic fragments of the 38 kDa obeche allergen matched furthermore with 50–75% identity to class I chitinases from banana and avocado.

#### Purification and enzyme activity of the 38 kDa obeche allergen

Proceeding from the results of sequence analysis, 38 kDa protein was purified by chitin-affinity chromatography. Comparison of protein components before and after passing the chitin matrix demonstrated a reduction of the 38 kDa protein band (Fig. 2A, lanes 1 and 2). The CBP fraction consists of two major protein bands obeche wood allergen of approximately 38 kDa and a 14 kDa protein (lane 4).

Enzyme activity within varying amounts of purified CBP was proven after SDS-PAGE (Fig. 2B). Even the smallest amount of CBP obeche (0.5 µg) showed an explicit enzyme activity displayed in a clear zone of chitin degradation at 38 kDa (Fig. 2B, lane 1). Higher CBP protein amounts exhibited proportional higher enzyme activity generated by 38 kDa chitinase (Fig. 2B lanes 2–4). The second protein band of 14 kDa which was enriched after chitin affinity chromatography exhibited just weak chitinase activity and was visible only at the highest protein concentration of 3.5 µg (Fig. 2B, lane 4).

#### IgE-binding capacity of 38 kDa obeche allergen

Seven obeche wood allergic patients' sera (P1–P7) were tested with the CBP enriched obeche extract (Fig. 3, lane 1–7). The 38 kDa protein was the most prominent allergen, detected by six of seven sera (85%) of obeche allergic patients (lanes 1–5, and 7). Four of them demonstrated strong labeling (lanes 1, 2, 3, and 7), whereas two showed weaker binding (lanes 4 and 5). With one patient's serum no clear IgE binding to the 38 kDa protein was observed (lane 6). In three of the seven positive sera an additional IgE binding to the 14 kDa CBP was observed (lanes 4, 5, and 7). Specificity of IgE binding to obeche blots was documented by sera of three control subjects (C1–C3) as seen in lanes 8–10. Based on the identification of the 38 kDa protein as a chitinase,

Table 2. Alignment of peptide fragments from 38 kDa obeche protein with class I chitinases

| Peptid              | Protein/organism                                                          | % Homology         |
|---------------------|---------------------------------------------------------------------------|--------------------|
| <b>EQGGSQAGGSL</b>  | 38 kDa protein from <i>Triplochiton scleroxylon</i> , <u>N-terminal</u>   |                    |
| <b>EQGGSQAGGAL</b>  | 1–11 CAA39535 chitinase ( <i>Oryza sativa</i> )                           | ID: 81%; Pos: 90%  |
| <b>EQGGRQAGGAL</b>  | 1–11 AF001524 Mus a 1.1 ( <i>Musa acuminata</i> , part clone)             | ID: 81%; Pos: 90%  |
| <b>EQGGRQAGGAL</b>  | 1–11 Z78202 Prs a 1 ( <i>Persea americana</i> )                           | ID: 81%; Pos: 90%  |
| <b>EQGGRQAGGKL</b>  | 1–11 P02877 Prohevein ( <i>Hevea brasiliensis</i> )                       | ID: 81%; Pos: 82%  |
| <b>VAGQYFGR</b>     | 38 kDa protein from <i>Triplochiton scleroxylon</i> , <u>tryptic frag</u> |                    |
| <b>AAGKKYYGR</b>    | 91–99 AAL05885 endochitinase ( <i>Musa acuminata</i> )                    | ID: 56%; Pos: 100% |
| <b>APGKKYYGR</b>    | 178–186 CAB01591 endochitinase ( <i>Persea americana</i> )                | ID: 45%; Pos: 100% |
| <b>LPGRGVTTDID</b>  | 38 kDa protein from <i>Triplochiton scleroxylon</i> , <u>tryptic frag</u> |                    |
| <b>LPGYGVTTNIIN</b> | 174–185 AAL05885 endochitinase ( <i>Musa acuminata</i> )                  | ID: 75%; Pos: 91%  |
| <b>LPGYGVITNIIN</b> | 261–272 CAB01591 endochitinase ( <i>Persea americana</i> )                | ID: 66%; Pos: 91%  |
| <b>LAFYER</b>       | 38 kDa protein from <i>Triplochiton scleroxylon</i> , <u>tryptic frag</u> |                    |
| <b>IGFYKR</b>       | 202–208 AAL05885 endochitinase ( <i>Musa acuminata</i> )                  | ID: 50%; Pos: 100% |
| <b>IGFYKR</b>       | 288–296 CAB01591 endochitinase ( <i>Persea americana</i> )                | ID: 50%; Pos: 100% |

ID, identical amino acids (bold); Pos, amino acids with one base exchanged in codon (underlined).

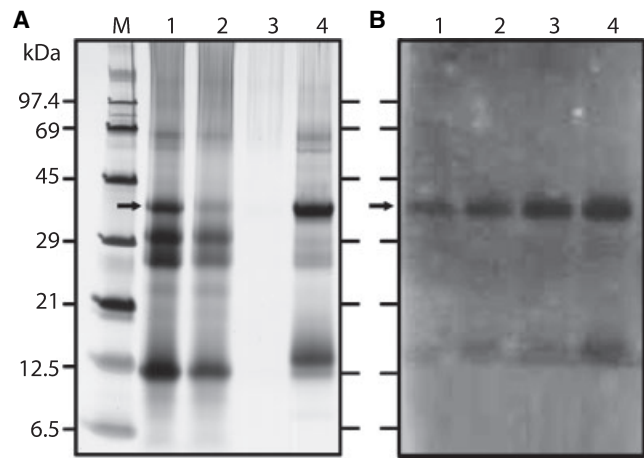


Figure 2. (A) Silver stained protein profile of obeche chitin-binding protein (CBP). Lane 1: obeche wood extract, lane 2: obeche wood extract after passing the chitin matrix, lane 3: wash fraction before elution, lane 4: CBP fraction. (B) Chitinase activity of CBP after Sodium dodecyl sulfate-polyacrylamid gel electrophoresis. Chitinolysis of different amounts of CBP obeche. Lane 1: 0.5 µg, lane 2: 1.0 µg, lane 3: 2.5 µg and lane 4: 3.5 µg.

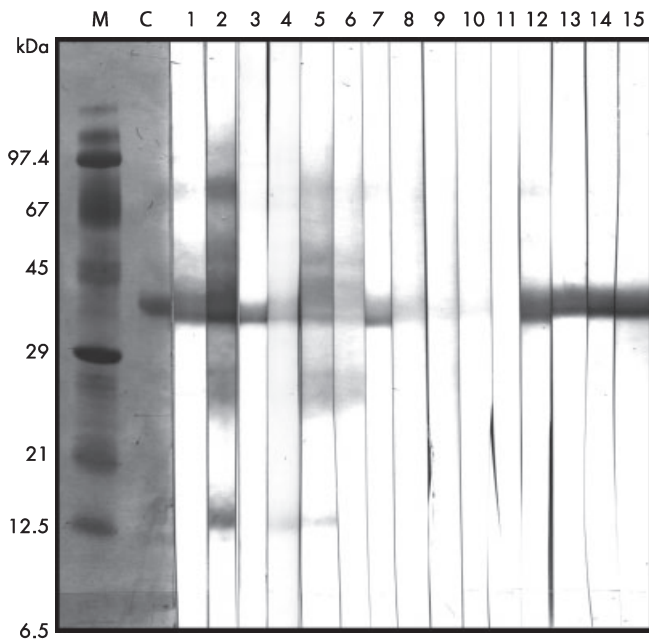


Figure 3. Immunoglobulin (IgE) Western blot of Trip s 1 in chitin-binding protein (CBP) enriched obeche extract. M: marker proteins, C: Coomassie stain of obeche CBP, lanes 1–7: IgE-immunostain with sera of obeche allergic subjects (P1–P7), lanes 8–10: control sera of obeche wood exposed, but nonallergic subjects (C1–C3), lanes 11–14: IgE-immunostain with sera of latex allergic subjects (L1–L4).

four latex allergic patients' sera (L1–L4) were also tested with the CBP obeche fraction (lanes 12–15). All four sera recognized the 38 kDa obeche protein which underlines

Table 3. Cross-reactivity of CBP-obeche, NRL and Hev b 6.02 in obeche and latex allergic subjects

|    | NRL specific IgE (kU/l) | rHev b 6.02 specific IgE (kU/l) | Inhibition of obeche specific IgE with |         |         |
|----|-------------------------|---------------------------------|----------------------------------------|---------|---------|
|    |                         |                                 | rHev b 6.02 (%)                        | CBP (%) | NRL (%) |
| P1 | 8.0                     | 7.0                             | 4                                      | 98      | 2       |
| P2 | 51.7                    | 55.3                            | 100                                    | 100     | 100     |
| P3 | <0.35                   | <0.35                           | 0                                      | 93      | 1       |
| P4 | <0.35                   | <0.35                           | 3                                      | 100     | 11      |
| P5 | 6.6                     | 6.2                             | 1                                      | 98      | 2       |
| P6 | <0.35                   | <0.35                           | 0                                      | 100     | 0       |
| P7 | 2.3                     | <0.35                           | 3                                      | 100     | 4       |
| L1 | 35.8                    | 24.2                            | 100                                    | 100     | 100     |
| L2 | 10.20                   | 10.80                           | 16                                     | 100     | 100     |
| L3 | >100                    | >100                            | 73                                     | 94      | 100     |
| L4 | 49.7                    | >100                            | 90                                     | 96      | 100     |

NRL, natural rubber latex; CBP, chitin-binding protein.

the homology of the allergen to class I chitinases of other plant sources. The 14 kDa CBP was not recognized by latex allergic patients' serum just as any other proteins in obeche wood extract. The 38 kDa allergen of obeche wood was entitled by the Allergen Nomenclature Sub-Committee as Trip s 1.

To estimate the importance of Trip s 1 in elucidating allergic reactions to obeche wood dust all patients' sera were incubated with 10 µg of CBP before measuring obeche specific IgE values by UniCAP. Specific IgE binding to k212 (obeche) was almost completely (>93%) inhibited by CBP in both obeche and latex sera (Table 3). The CBPs and particularly Trip s 1 seemed to be the main IgE binding protein group and thus may be considered as major allergens in obeche wood allergy.

#### Cross-reactivity between obeche wood chitinase and hevein

Sera of the obeche allergic patients were tested for specific IgE-binding to NRL and rHev b 6.02 using ImmunoCAPs. Latex-specific IgE was detected in four of seven sera (Table 3, P1, P2, P5, and P7), among them three sera (P1, P2, and P5) with specific IgE to rHev b 6.02. Three sera (P1, P5, and P7) demonstrated lower specific IgE concentrations to NRL or rHev b 6.02 than to obeche. However, the serum of one carpenter (P2) exhibited fivefold higher specific IgE concentrations to NRL (51.7 kU/l) and rHev b 6.02 (55.3 kU/l) than to obeche (9.5 kU/l).

Inhibition experiments with obeche as solid phase by NRL or rHev b 6.02 in obeche allergic patients' sera (P1, P5, and P7) showed no inhibition, whereas serum P2 was inhibited completely. In contrast, inhibition experiments with obeche as solid phase using NRL in latex allergic patients' sera (L1–L4) showed complete inhibition in all four sera. Inhibition with rHev b 6.02 was complete in two sera (L1 and L4), about 70% in L3, and only minimal (16%) in L2.

## Discussion

Nonstandardized allergen extracts can be a serious source of errors for *in vitro* diagnosis of IgE mediated wood allergy (5, 22). Both wood quality and protein preparation procedure have a great influence on extractable allergens and thereby on the sensitivity and specificity of the test system. As shown in our study obeche wood dust did not need complex and extensive extraction methods. However, the quality of the wood extract is dependent on the freshness of the wood dust material. A difference in extractable protein amount depending on the age of the wood was also reported for pine wood extract (7), where protein content and IgE-binding capacity decreased logarithmically with the age of the used wood material.

The protein pattern of obeche is characterized by one dominant protein in the lower molecular weight range < 20 kDa followed by a triplet of protein bands of about 30–40 kDa. Similar results were described recently (1–3). The calculated molecular masses of the single protein bands did not coincide exactly in all studies, which may be explained by differences in gel systems, running conditions or sample preparations.

In IgE-immunoblot studies with obeche allergic patients' sera we identified a 38 kDa protein as major allergen (Trip s 1). Others described a 28 kDa obeche allergen as major IgE-binding component (2, 3). Looking at the protein pattern itself, the 38 kDa protein constituted the protein band of the triplet with the highest molecular weight, whereas the 28 kDa protein described by others displayed the protein band of the triplet with the lowest molecular weight. This argues for two different IgE binding obeche proteins. Differences in IgE binding proteins of obeche allergic patients in studies from Spain and Germany might be explained by geographic factors like nutrition and life style as it is described for the food allergen lipid transfer protein (23) or by concomitant sensitization, especially to fruits (24).

Trip s 1 was identified as class I chitinase by N-terminal sequencing and mass fingerprint analysis. Although the examined peptide fragments covered only a small part of the whole protein, the identification as a chitinase is reliable because of the conformity in the conserved N-terminal hevein domain and the homology over the whole range of the catalytic region of the enzyme. This finding was corroborated by chitinase activity of Trip s 1.

Chitinases are pathogenesis-related (PR) proteins expressed in a wide range of plants. They are not only

induced by pathogen attack but are also under developmental control by the plant hormone ethylen (25), which regulates also the thickening growth in woody plants (26). The presence of class I chitinase in wood material is further supported by the finding of a cDNA clone in a mRNA bank from early and late wood of *Pinus pinaster* [BX249659] and by an active 28 kDa chitinase in cucumber xylem sap (27). Very small CBPs (< 5 kDa) with antifungal/antimicrobial activity were characterized in ginkgo (*Ginkgo biloba*) (28) and spindle tree (*Euonymus europaeus* L.) (29) with cystein-rich chitin-binding domains and strong sequence similarity to latex hevein. Although occupational type I allergy by spindle tree was reported (13), no CBPs were identified as allergens yet.

Next to their biological function class I chitinases are extensively characterized as cross-reactive allergens in plants (18, 30, 31). A strong similarity of the small N-terminal chitin-binding domain (hevein-like domain) is an important factor in latex-fruit-syndrome (31, 32). Comparison of peptide fragments from Trip s 1 displayed also sequence similarity of more than 80% to other plant chitinases (Table 2), stressing the existence of strongly conserved regions. Structural similarity among proteins from diverse sources is the molecular basis of allergic cross-reactivity (33) and this structural cross-reactivity or at least the co-recognition of the hevein domain was demonstrated by the present inhibition studies. The phenomena was further described for latex-fruit allergic patients (34, 35) as well as for other related proteins (33).

The finding that Trip s 1 is a more effective inhibitor in obeche and latex allergic patients' sera than rHev b 6.02 indicated additional IgE epitopes on the regulatory domain of the chitinase. Studies with class II chitinase from banana (34) and a hevein deleted version of Cas s 5 (class I chitinase) from chestnut (35) demonstrated that relevant IgE epitopes are located in the catalytic domain of class I chitinase. In conclusion our data suggest that the obeche chitinase Trip s 1 is a strongly cross-reactive allergen sharing some, but not all IgE binding epitopes with rHev b 6.02.

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## References

1. Ferrer A, Maranon F, Casanovas M, Fernandez-Caldas E. Asthma from inhalation of *Triplochiton scleroxylon* (Samba) wood dust. *J Investig Allergol Clin Immunol* 2001;**11**:199–203.
2. Hinojosa M, Losada E, Moneo I, Dominguez J, Carrillo T, Sanchez-Cano M. Occupational asthma caused by African maple (Obeche) and Ramin: evidence of cross reactivity between these two woods. *Clin Allergy* 1986;**16**:145–153.
3. Quirce S, Hinojosa M, Maranon F, Ferrer A, Fernandez-Caldas E, Sastre J. Identification of obeche wood (*Triplochiton scleroxylon*) allergens associated with occupational asthma. *J Allergy Clin Immunol* 2000;**106**:400–401.

4. Reijula K, Kujala V, Latvala J. Sauna builder's asthma caused by obeche (*Triplochiton scleroxylon*) dust. *Thorax* 1994;**49**:622–623.
5. Wirtz C, Chen Z, Raulf-Heimsoth M, van Kampen V, Papenfuss F, Baur X. Atemwegssensibilisierung durch Holzstaub. *Zbl Arbeitsmed* 1997;**47**:336–342.
6. Chan-Yeung M. Mechanism of occupational asthma due to western red cedar (*Thuja plicata*). *Am J Ind Med* 1994;**25**:13–18.
7. Skovsted TA, Schlunssen V, Schaumburg I, Wang P, Staun-Olsen P, Skov PS. Only few workers exposed to wood dust are detected with specific IgE against pine wood. *Allergy* 2003;**58**:772–779.
8. Higuero NC, Zabala BB, Villamuza YG, Gomez CM, de Gregorio AM, Sanchez CS. Occupational asthma caused by IgE-mediated reactivity to Antiaris wood dust. *J Allergy Clin Immunol* 2001;**107**:554–556.
9. Quirce S, Parra A, Anton E, Fernandez-Nieto M, Jerez J, Sastre J. Occupational asthma caused by tali and jatoba wood dusts. *J Allergy Clin Immunol* 2004;**113**:361–363.
10. Basomba A, Burches E, Almodovar A, de Rojas DH. Occupational rhinitis and asthma caused by inhalation of *Balfourodendron riedelianum* (Pau Marfim) wood dust. *Allergy* 1991;**46**:316–318.
11. Fernandez-Rivas M, Perez-Carral C, Senent CJ. Occupational asthma and rhinitis caused by ash (*Fraxinus excelsior*) wood dust. *Allergy* 1997;**52**:196–199.
12. Bohadana AB, Massin N, Wild P, Toamain JP, Engel S, Goutet P. Symptoms, airway responsiveness, and exposure to dust in beech and oak wood workers. *Occup Environ Med* 2000;**57**:268–273.
13. Herold DA, Wahl R, Maasch HJ, Hausen BM, Kunkel G. Occupational wood-dust sensitivity from *Euonymus europaeus* (spindle tree) and investigation of cross reactivity between E.e. wood and *Artemisia vulgaris* pollen (mugwort). *Allergy* 1991;**46**:186–190.
14. Venturini M, Gastaminza G, Kespohl S, Bernedo N, Garmendia M, Raulf-Heimsoth M et al. Cross-reactivity between obeche wood (*Triplochiton scleroxylon*) and natural rubber latex. *Allergy* 2004;**59**:225–228.
15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;**72**:248–254.
16. Sander I, Flagge A, Merget R, Halder TM, Meyer HE, Baur X. Identification of wheat flour allergens by means of 2-dimensional immunoblotting. *J Allergy Clin Immunol* 2001;**107**:907–913.
17. Raulf-Heimsoth M, Rozynek P, Bruening T, Rihs HP. Characterization of B- and T-cell responses and HLA-DR4 binding motifs of the latex allergen Hev b 6.01 (prohevein) and its post-transcriptionally formed proteins Hev b 6.02 and Hev b 6.03. *Allergy* 2004;**59**:724–733.
18. Rihs HP, Dumont B, Rozynek P, Lundberg M, Cremer R, Bruening T et al. Molecular cloning, purification, and IgE-binding of a recombinant class I chitinase from *Hevea brasiliensis* leaves (rHev b 11.0102). *Allergy* 2003;**58**:246–251.
19. Trudel J, Asselin A. Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal Biochem* 1989;**178**:362–366.
20. Leyer RJS, Staples RC, Roberts DW. Entomopathogenic isolates of *Metarhizium anisopliae*, *Beauveria bassiana*, and *Aspergillus flavus* produce multiple extracellular chitinase isozymes. *J Invertebr Pathol* 1993;**61**:81–84.
21. Schaefer H, Nau K, Sickmann A, Erdmann R, Meyer HE. Identification of peroxisomal membrane proteins of *Saccharomyces cerevisiae* by mass spectrometry. *Electrophoresis* 2001;**22**:2955–2968.
22. Dolen WK. IgE antibody in the serum-detection and diagnostic significance. *Allergy* 2003;**58**:717–723.
23. Ballmer-Weber BK, Scheurer S, Fritsche P, Enrique E, Cistero-Bahima A, Haase T et al. Component-resolved diagnosis with recombinant allergens in patients with cherry allergy. *J Allergy Clin Immunol* 2002;**110**:167–173.
24. Scheurer S, Pastorello EA, Wangorsch A, Kastner M, Haustein D, Vieths S. Recombinant allergens Pru av 1 and Pru av 4 and a newly identified lipid transfer protein in the *in vitro* diagnosis of cherry allergy. *J Allergy Clin Immunol* 2001;**107**:724–731.
25. Sanchez-Monge R, Blanco C, Perales AD, Collada C, Carrillo T, Aragoncillo C et al. Class I chitinases, the panallergens responsible for the latex-fruit syndrome, are induced by ethylene treatment and inactivated by heating. *J Allergy Clin Immunol* 2000;**106**:190–195.
26. Higuchi T. *Biochemistry and Molecular Biology of Wood*. New York: Springer Verlag Berlin Heidelberg New York, 2004.
27. Masuda S, Kamada H, Satoh S. Chitinase in cucumber xylem sap. *Biosci Biotechnol Biochem* 2001;**65**:1883–1885.
28. Huang X, Xie W, Gong Z. Characteristics and antifungal activity of a chitin binding protein from *Ginkgo biloba*. *FEBS Lett* 2000;**478**:123–126.
29. Van den Bergh KP, Proost P, Van Damme J, Coosemans J, Van Damme EJ, Peumans WJ. Five disulfide bridges stabilize a hevein-type antimicrobial peptide from the bark of spindle tree (*Euonymus europaeus* L.). *FEBS Lett* 2002;**530**:181–185.
30. Salcedo G, Diaz-Perales A, Sanchez-Monge R. The role of plant panallergens in sensitization to natural rubber latex. *Curr Opin Allergy Clin Immunol* 2001;**1**:177–183.
31. Posch A, Wheeler CH, Chen Z, Flagge A, Dunn MJ, Papenfuss F et al. Class I endochitinase containing a hevein domain is the causative allergen in latex-associated avocado allergy. *Clin Exp Allergy* 1999;**29**:667–672.
32. Chen Z, Posch A, Cremer R, Raulf-Heimsoth M, Baur X. Identification of hevein (Hev b 6.02) in *Hevea* latex as a major cross-reacting allergen with avocado fruit in patients with latex allergy. *J Allergy Clin Immunol* 1998;**102**:476–481.
33. Ferreira F, Hawranek T, Gruber P, Wopfner N, Mari A. Allergic cross-reactivity: from gene to the clinic. *Allergy* 2004;**59**:243–267.
34. Diaz-Perales A, Collada C, Blanco C, Sanchez-Monge R, Carrillo T, Aragoncillo C et al. Class I chitinases with hevein-like domain, but not class II enzymes, are relevant chestnut and avocado allergens. *J Allergy Clin Immunol* 1998;**102**:127–133.
35. Diaz-Perales A, Sanchez-Monge R, Blanco C, Lombardero M, Carrillo T, Salcedo G. What is the role of the hevein-like domain of fruit class I chitinases in their allergenic capacity? *Clin Exp All* 2002;**32**:448–454.