

**PURIFICATION, CHARACTERIZATION AND N-TERMINAL AMINO ACID
SEQUENCE OF A NEW MAJOR ALLERGEN FROM EUROPEAN
CHESTNUT POLLEN - CAS S 1**

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Pollens from trees of the order *Fagales* (e.g. birch, alder, hazel, and hornbeam) all contain one major allergen - the main cause for tree pollen allergy. So far the major allergens from birch (Bet v 1), alder (Aln g 1), hazel (Cor a 1), and hornbeam (Car b 1) have been characterized, showing high sequence similarity with each other (1-4). We present the molecular and immunologic characterization of Cas s 1, the major allergen from the European chestnut (*Castanea sativa*). From aqueous pollen extracts from European chestnut a protein was purified to homogeneity and was subjected to further investigation. The protein revealed a Mr of 22 kDa and was shown to represent the major allergen of the European chestnut (immunoblotting, histamine release) and designated Cas s 1. Despite a marked difference in Mr, Cas s 1 shows significant amino acid sequence similarity at the N-terminus and is antigenically closely related to the major birch pollen allergen Bet v 1 (17 kDa), as shown by binding to the anti-Bet v 1 monoclonal antibody BIP-1 and by IgE-inhibition tests using recombinant Bet v 1.

In industrialized countries, at least 15 - 20 % of the population suffer from Type I, i.e. IgE-mediated allergic diseases (rhinitis/rhinoconjunctivitis, pollen asthma; 5). In Northern and Central Europe, pollens from trees belonging to the family *Betulaceae*,

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ABBREVIATIONS:

Aln g 1	major allergen from alder pollen,
Bet v 1	major allergen from birch pollen,
BIP 1	monoclonal anti-Bet v 1 antibody,
Car b 1	major allergen from hornbeam pollen,
Cor a 1	major allergen from hazel pollen,
Cas s 1	major allergen from European chestnut pollen,
Que a 1	major allergen from oak pollen,
MA	major allergen,
moAb	monoclonal antibody,
rBet v 1	recombinant Bet v 1.

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in particular from white birch, but also from alder, hazel, and hornbeam, represent the major cause of Type I allergy in early or middle spring. The allergological importance of trees of the other family of the order *Fagales* (*Fagaceae*: oak, beech, European chestnut) is unclear and still a matter of controversy.

All of the pollens from these trees looked at so far possess a single major allergen (MA) or closely related isoforms thereof. These MAs are highly cross-reactive and were cloned from birch (1), alder (2), hornbeam (3), and hazel (4). Partial N-terminal amino acid sequences were published for beech and oak (6). All these proteins have a Mr of 17-18 kDa and are related by sequence comparisons to a family of plant pathogenesis-related proteins (1-3) with an as yet unknown biochemical function.

Recently, we showed in Western blot experiments that pollen from the European chestnut (*Castanea sativa*) also contains a single MA cross-reacting with Bet v 1, the MA from birch pollen (7). In those areas, where the European chestnut grows, its MA seems to be responsible for Type I allergic symptoms at the end of spring and in early summer, not due to grass pollen or house dust mite allergens (7).

It was surprising to find that - at marked difference to all MAs from the other trees of the order *Fagales*, and from the family of the plant pathogenesis-related proteins - the MA of *C. sativa* exhibited an apparent Mr of 22 kDa in SDS-Page (7). For this reason, it seemed of particular interest to gain information about the N-terminal amino acid sequence and to look at some immunochemical and biological properties of the protein, purified by its capacity to bind to the anti-Bet v 1 monoclonal antibody, BIP 1 (8).

MATERIALS AND METHODS

Pollen extracts:

Pollen from *Castanea sativa* and *Betula verrucosa* (Allergon AB, Sweden) was subjected to protein extraction as described previously (8).

Monoclonal antibody (moAb):

Murine anti-Bet v 1 moAb BIP 1 was purified from ascites using a MabTrap G purification kit (Pharmacia, Uppsala, Sweden; 9), following the manufacturer's instructions. The purified antibody was coupled covalently to Affi-Gel 10 (Pharmacia, Sweden) according to the manufacturer's protocol.

Sera:

Two different groups of sera from patients were established according to case history, skin prick test and RAST.

Group A: 8 sera derived from patients suffering from birch pollen allergy. Those patients showed Type I allergic (rhinitis) also in late spring and early summer. In skin prick tests they reacted with pollen extracts from *C. sativa* but not with allergens from grass pollen.

Group B: 8 sera from patients suffering exclusively from birch pollen allergy.

NHS-Pool (normal human serum pool): Sera from 20 non-allergic individuals were pooled and used as negative controls.

Furthermore one serum derived from a polyallergic patient (positive control) and one serum from a patient suffering from house-dust mite allergy exclusively (negative control) were used. All sera were stored at -20°C.

SDS-PAGE, Immunoblotting and inhibition of IgE-binding to Bet v 1 and to the MA from C. sativa respectively:

Protein extracts were separated by 12% SDS-PAGE and subsequently either silver-stained or transferred onto nitrocellulose (10). IgE-binding proteins were detected as previously described using patients' sera and ^{125}I -labelled rabbit anti-human IgE (8). Immunoblots performed with mAb BIP 1 and a rabbit anti-mouse antibody and detected by ^{125}I -labelled donkey anti-rabbit IgG (9).

Inhibition assays of IgE-binding to tree pollen allergens were performed as follows: different sera from each group were pooled and subsequently preincubated with purified MA from *C. sativa* and purified recombinant Bet v 1 (rBet v 1) for 24 hours (11), respectively, in the concentrations of 10 μg , 8 μg , 6 μg , 4 μg , 2 μg , and 0 $\mu\text{g/ml}$ for 24 hours and then subjected to immunoblots as described above.

N-terminal sequence analysis:

Hundredtwentyfive pmol of purified MA from *C. sativa* were subjected to SDS-Page and transferred onto PVDF membrane (Millipore). The protein band was stained with Coomassie blue R-250, excised, and applied on a 6*6mm Blott Cartridge of an Applied Biosystems 477 pulsed liquid phase sequencer. Amino acid derivatives were analyzed on-line by reversed phase HPLC (Applied Biosystems model 120A).

Histamine-release assay:

Basophils were isolated from peripheral blood as previously described (12). After incubation with various concentrations (0.0001 - 10 $\mu\text{g/ml}$) of purified MA from *C. sativa* or with Mab E-124-2-8anti-IgE (Immunotech, Marseille, France) as positive control, histamine was measured in the cell-free supernatant by radioimmunoassay (Immunotech).

RESULTS

Protein purification:

The MA from *C. sativa* was purified to homogeneity by affinity chromatography performed with an immobilized anti-Bet v 1 monoclonal antibody (9) and eluted with 0.1 M glycine/HCl, pH 2.5. followed by anion-exchange HPLC (DEAE-5WP 10 μm , 7.5 x 75mm; TOSO-HAAS, Japan; solvent A: 20mM Tris/HCl pH 8.3; solvent B: 20mM Tris/HCl /0.5 M NaCl pH 8.3; flow rate 1 ml/min;) using a linear gradient at room temperature while monitoring absorbance at 280nm. Protein concentration of purified Cas s 1 was determined by the Micro-Kjeldahl method using glycine as standard (13). (Fig. 1).

Immunoblot experiments:

Using sera derived from patients suffering from tree pollen allergy, the IgE-binding capacity of Cas s 1 was tested in immunoblot experiments (Fig. 2A, 2B). With the exception of serum no. 8 and the polyallergic patient's serum, IgE-antibodies reacted with the 22kDa protein exclusively (Fig. 2A).

Inhibition experiments with purified Cas s 1 and purified rBet v 1: A serum pool derived from patients allergic to birch and European chestnut pollen (group A) showed weak reduction of IgE-binding capacity to Bet v 1 after preincubation with different concentrations of purified Cas s 1 (Fig. 3A). A similar weak blocking reaction of Cas s 1 was demonstrated when testing a serum pool from exclusive birch pollen allergic patient's (Fig. 3B). Yet, when testing serum pool A with European chestnut pollen extracts after preincubation with the same range of concentrations of purified rBet v 1 the IgE-binding capacity of those sera was inhibited completely (Fig. 3C).

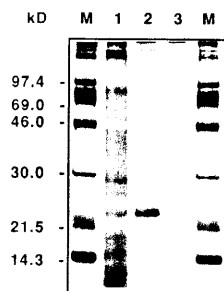


Fig. 1. SDS-PAGE of Cas s 1.

Aqueous pollen extract from *Castanea sativa* and purified Cas s 1 respectively were subjected to 12% SDS-PAGE and silver-stained. Lane 1: Total pollen extract from *C. sativa*; Lane 2: Cas s 1 purified by affinity chromatography; Lane 3: Cas s 1 after final purification step by HPLC anion exchange; M: molecular weight marker.

N-terminal sequence:

The first 24 amino acids were determined by Edman degradation (Fig. 4).

Histamine-release:

Purified Cas s 1 was capable to induce IgE-mediated histamine-release from tree pollen allergic patient's blood basophils in a dose dependent manner (Fig. 5A), whereas blood basophils derived from non-allergic individuals did not show histamine release upon stimulation with purified Cas s 1 (Fig. 5B).

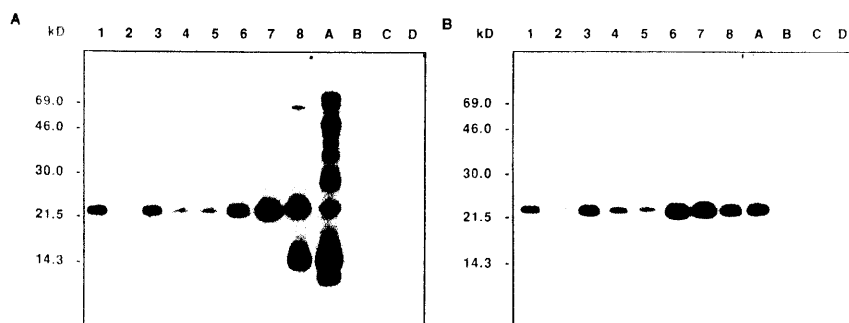


Fig. 2. Immunoblot analysis of *Castanea sativa* pollen (2A) and purified Cas s 1 (2B) respectively.

Total pollen extract and purified Cas s 1 respectively were separated by 12% SDS-PAGE and transferred onto nitrocellulose sheets. Bound IgE derived from patients' sera were detected by ^{125} I-labelled anti-human-IgE. Lanes 1-8: Sera from patients suffering from tree pollen allergy. Lane A: serum from a polyallergic patient; Lane B: serum derived from a patient suffering from house dust mite allergy; Lane C: NHS-pool; Lane D: buffer control.

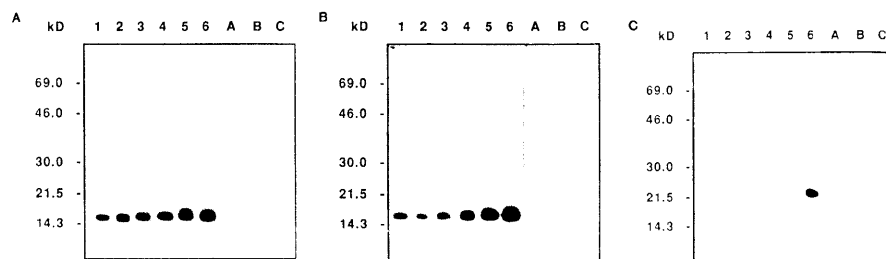


Fig 3. Inhibition of IgE-binding to Cas s 1 and Bet v 1.

3A: Immunoblot of birch pollen extract tested with a serum pool from *C. sativa* and birch pollen allergic patients (group A) preincubated with purified Cas s 1 (Lanes 1-6: 10 μ g, 8 μ g, 6 μ g, 4 μ g, 2 μ g, and 0 μ g/ml Cas s 1, respectively). Lanes A-C: NHS-pool previously incubated with 10 μ g, 5 μ g and 0 μ g Cas s 1.

3B: Immunoblot of birch pollen extract tested with serum pool group B after incubation with Cas s 1 in decreasing concentrations (10 μ g, 8 μ g, 6 μ g, 4 μ g, 2 μ g, and 0 μ g/ml).

3C: Immunoblot of chestnut pollen extract probed with serum pool group A preincubated with purified rBet v 1 in decreasing concentrations (10 μ g, 8 μ g, 6 μ g, 4 μ g, 2 μ g, and 0 μ g/ml).

DISCUSSION

Pollens from the early flowering trees of the order *Fagales* are known to be an important source causing Type I (pollen) allergies in early spring. *C. sativa*, the European chestnut (*Fagaceae*, *Fagales*) is native to the (sub) mediterranean area. It is also found in the temperate climate zones, where it flowers in late spring and early summer. Frequently, allergic patients from this area suffering from tree pollinosis showed a prolongation of their symptoms into early summer, which might be due to pollen from the European chestnut, in particular, since it was shown by us that pollen from *C. sativa* contains a single major allergen that cross-reacts with Bet v 1, the major allergen from birch (7).

In the present paper we could demonstrate that the 22kDa protein of *C. sativa*, in spite of higher molecular mass, which is not due to carbohydrate moieties (7), belongs to the group of major allergens from trees of the order *Fagales*, all of which possess a Mr of 17-18 kDa.

	1	10	20
<u>Cas s 1</u>	G V F T H E S Q E T S V I P P A R L F K A F V L		
	I I I	I I	I I I I I I I I I I I I I I I I I I
<u>Bet v 1</u>	G V F N Y E T E T T S V I P A A R L F K A F I L		

Fig. 4. N-terminal amino-acid sequence of Cas s 1 in comparison to the amino acid sequence of Bet v 1.

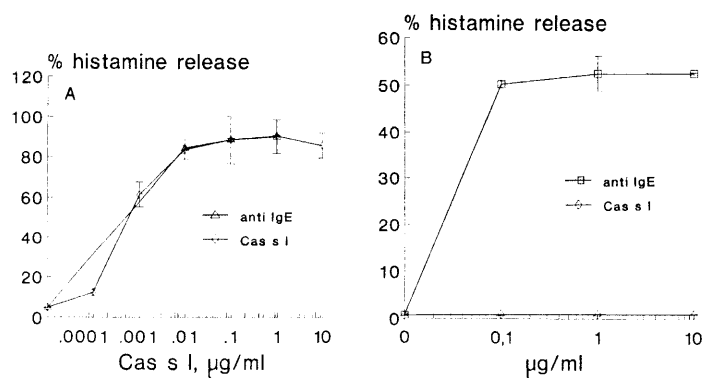


Fig 5. *In vitro* Histamine-release assay. Basophils from a Cas s I allergic patient (5A) and a normal individual (5B) were incubated with different concentrations of Cas s I (0.0001 - 10 µg/ml) and monoclonal anti-IgE-124-28.

The N-terminal amino acid sequence of Cas s I revealed high sequence similarities to the major allergens (Bet v 1, Aln g 1, Cor a 1, Car b 1, and Que a 1; 1-4, 6) ranging from 62 - 75%.

Purified Cas s I was able to reduce, but not to eliminate IgE-binding of birch and chestnut allergic patients' sera to Bet v 1. On the other hand purified rBet v 1 could inhibit IgE-binding to Cas s I, suggesting that Cas s I shares epitopes with Bet v 1 (IgE-binding, cross-reactivity of moAbs raised against Bet v 1) but probably due to differences in amino acid sequences and molecular mass, some epitopes present on Bet v 1 are missing in Cas s I. Therefore experiments are under way to obtain nucleotide sequences coding for Cas s I by molecular cloning techniques. These data will help us to characterize minimale sequences representing B-cell epitopes.

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