

Biochemical and immunochemical characterization of hop-hornbeam (*Ostrya Carpinifolia* Scop.) pollen

A new allergenic pollen from the Corylaceae family

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Abstract

In the last few years *Ostrya carpinifolia* pollen is considered as an important cause of respiratory allergy in Mediterranean areas. The concentration of the pollen was measured over a period of fifteen years from 1981 to 1996 in an area around Genoa; the results of this study have clearly indicated an increasing trend that correlate with persons sensitization.

In this study we sought to define the immunochemical and biochemical properties of hop-hornbeam pollen. Soluble proteins extracted from *Ostrya carpinifolia* pollen and from the taxonomically related species *Corylus Avellana*, were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE), by horizontal isoelectrofocusing (IEF) and by two dimensions electrophoresis (2D-PAGE). Allergenic proteins were identified with sera of sensitized patients and cross-reactivity was evaluated by immunoblotting techniques. The electrophoretic analysis showed a partial identity between the proteins from *Ostrya* and *Corylus* extracts. The immunoblotting assay, developed with human IgE from subjects allergic to hop-hornbeam pollen, displayed the major IgE reactivity for a component with a molecular weight of 17 kDa expressed in both *Ostrya* and *Corylus* extracts. This reactivity is consistent with the presence of Bet v 1 that is described as the major pollen allergen in the Betulaceae and Corylaceae families. Sera from subjects allergic to *Ostrya* were then preadsorbed with recombinant Bet v 1 immobilized in the Pharmacia CAP System; a significant reduction of the IgE binding activity was observed after the treatment. We therefore suggest that Bet v 1 could be one of the allergenic proteins present in the *Ostrya* pollen possibly being responsible for cross-reactivity with other members of taxonomically related families.

Introduction

Hop-hornbeam tree (*Ostrya carpinifolia* Scop.) belonging to the Corylaceae family, is widely spread in the Mediterranean area, mostly in Italy and Yugoslavia (Charpin, 1974; Spieksma, 1980): however, no scientific data about its role in causing respiratory allergy is available. Airborne pollen sampling performed since 1981 clearly shows that *Ostrya* pollen diffusion accounts for most of the tree pollens detected in the atmosphere in Genoa, with a

significant increasing trend (Voltolini, 1996). Over the same period we observed an increasing number of patients with seasonal respiratory symptoms and cutaneous sensitization towards Betulaceae and/or Corylaceae pollen (from 6.2% to 44% of pollinosis patients). Clinical symptoms, registered by patients on daily diary cards, show a clear worsening in the period of *Ostrya* pollen airborne diffusion (from March to April), which follows *Corylus* and *Alnus* pollen season. Then, *Ostrya carpinifolia* pollen collected from different stages of inflorescence and years have

been analyzed for proteins and allergenic compounds. The aim of this study is to characterize allergens specific for *Ostrya carpinifolia* pollen or shared with other taxonomically related species.

In the last years clinical and laboratory observations have provided evidence of allergenic cross-reactivity between pollen from Betulaceae family (Florvaag, 1988; Niederberger, 1998). The major pollen allergen is a protein of 159 aminoacids with a molecular weight of 17 kDa named Bet v 1 (Ferreira, 1993): the complete amino acid sequence of the protein derived from *Betula*, *Alnus* and *Corylus* has been determined and large homologies have been observed between these allergenic molecules (Florvaag, 1988).

In this report we partially characterize the biochemical properties of *Ostrya carpinifolia* pollen and by means of immunoblotting and inhibition studies we indicate Bet v 1 as an allergenic component present in *Ostrya carpinifolia* pollen, causing cross-reactivity at the IgE level with other members of the related family.

Materials and methods

Pollen collection

Pollen from *Ostrya carpinifolia* was collected in March from plants growing on the area around Genoa. Source plants were identified by means of botanical criteria. Pollen was collected from catkins mature flowering plants and purity was checked by microscopic analysis.

Protein extraction

Proteins were extracted in 0.2 M phosphate buffer saline (pH 7.0–7.5) at 4 °C after shaking for 3 h. The extract was clarified by centrifugation at $15000 \times g$ for 30 min at 4 °C. The supernatant was filtered and extensively dialyzed against phosphate buffer saline.

Dialysates were stored at –20 °C. Protein concentrations were determined by the Micro BCA Protein Assay Reagent Kit (Pierce, USA).

Subjects

Serum samples from 20 patients allergic to Corylaceae pollen were investigated (provided by Allergologic Unit, St. Martin Hospital, Genoa, Italy). All patients suffered from inhalant allergy during the flowering

season and were selected on the basis of clinical record, results of skin prick tests and RAST value. Some 17/20 patients' sera displayed IgE binding to hop-hornbeam pollen proteins in immunoblotting. A serum pool from persons without indications of type I allergy was used for control experiments, showing no IgE binding to hop-hornbeam extract.

SDS-polyacrilamide gel electrophoresis (SDS-PAGE), protein transfer and immunoblotting

The protein sample was heated with an equal amount of sample buffer (15% Bromophenol blue, 15% glycerol, 0.25 M Tris-HCl pH 6.5, 10% sodium dodecylsulphate (SDS) and 20% b-mercaptoethanol) at 100 °C for 5 min. 46 µg pollen proteins were applied to a gradient gel with 10% and 18% SDS-polyacrilamide gel (SDS-PAGE), stained with 0.05% Coomassie Brilliant Blue R 250 (Imperial Chemical Industries, Ltd., Macclesfield, Cheshire, U.K.) and destained with methanol:acetic acid:water (4:1:5) mixture (Westermeier, 1997). Alternatively proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The filters are incubated with patients sera at 1:10 dilution and with a rabbit anti-human IgE (Dako, Glostrup, Denmark) as the secondary antibody.

Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Southern Biotechnology Bistotechnology Associates, USA) was added and the reaction was revealed using peroxidase ECL substrate and exposure to ECL-Hyperfilm (Amersham). Molecular weights were determined by using Sigma standards.

2D-electrophoresis (2D-PAGE)

Two dimensional polyacrilamide gel electrophoresis (2D-PAGE) was performed according to Westermeier (1997) First dimension is isoelectrofocusing (IEF) with pH gradient 3.0 to 10.0. Second dimension was run in a gradient SDS-PAGE from 10% to 18% polyacrilamide concentration.

IgE-inhibition experiments

Patients' sera reacting with the major allergens of hop-hornbeam in IgE immunoblot analysis were diluted as described above. A volume of 1 ml diluted serum was absorbed through immobilized recombinant Bet v 1 (Pharmacia CAP System) and subsequently used in

Table 1. Characteristics of patients selected for laboratory analysis.

Patient N	Initials	Sex/age	Symptoms	SPT <i>Ostrya</i>	PT betula	RAST	Food allergy
1	G.D.	M/25	Rhinitis	+++	++		None
2	LM.F.	F/30	Rhinitis	++			One
3	F.A.	M/38	Rhinitis	+++	+++		Apple, kiwi, peach
4	B.M.	F/42	Rhinitis/asthma	+++	+++		None
5	C.A.	F/62	Rhinitis/asthma	++++	+++		Hazelnut
6	F.A.	M/30	Rhinitis	+++	++		None
7	P.E.	M/19	Rhinitis	++++	++		Apple, pear, potato, kiwi
9	L.G.	M/42	Rhinitis/asthma	+++	+++		Hazelnut
10	C.F.	M/63	Rhinitis/asthma	++++	+++		None
11	C.F.	F/30	Rhinitis/asthma	++++	+++		None
12	B.L.	F/55	Rhinitis/asthma	++++	++	Bet 2Cor 4	Apple, kiwi
13	M.M.	F/40	Rhinitis/asthma	++++	+++	<i>Ostrya</i> 3(5,25U.rast)	Kiwi
14	B.A.	F/27	Rhinitis/asthma	+++	+++	<i>Ostrya</i> 2(1,62U.rast)	None
15	D.L.	F/41	Rhinitis/asthma	+++	+++	<i>Ostrya</i> 3(15,7U.rast)	None
16	P.G.	M/18	Rhinitis	+++	+++	<i>Ostrya</i> 2 (1,98U.rast)	None
17	M.M.	F/44	Rhinitis/asthma	++++	++	<i>Ostrya</i> 3(4,54U.rast)	Hazelnut Rosaceae

a western blot analysis. Detection of bound IgE was again performed as described above

Results

Clinical and environmental background

17 adult patients found to be hypersensitive to *Ostrya carpinifolia* by skin prick test were analyzed: this group is living in an area close to the pollen source (*Ostrya*) and exposed directly for most of the *Ostrya* pollen season.

All the patients had positive skin prick test for Corylaceae and Betulaceae families and high RAST values. In the Table 1 specific IgE, skin prick tests and RAST are shown. A respective study as well as a 2 years follow up, were carried out to assess the presence of symptoms during the *Ostrya* pollination period.

Phenological criteria were used to define the beginning, the magnitude and the end of the pollinating period of *Ostrya carpinifolia*. Daily observations were performed from March to the end of April over three years and the percent of pollinating plants in the study area was considered. Cumulative data of *Ostrya carpinifolia* phenological survey indicated the presence of mature pollinating aments from the beginning to the end of March (Montanari et al manuscript in preparation).

The concentration of hop-hornbeam pollen in the atmosphere of Genoa was measured over a period of 18 years and the results already reported by Voltolini et al. (1996) are summarized in Table 2.

The values of pollen concentration and the correlation with the percentage of sensitized patients indicate the increasing relevance of this pollen as allergen source in the area around Genoa.

Biochemical characterization of pollen from Ostrya carpinifolia

The hop-hornbeam pollen extract is analyzed by SDS-PAGE and compared with *Corylus avellana*. Extracts loaded on a SDS PAGE gradient gel and stained by Coomassie Brilliant Blue are shown in Figure 1A. The electrophoretic pattern consists of about 30 bands corresponding to proteins ranged between 14 and 80 kDa. The majority of the proteins are shared between *Ostrya carpinifolia* and *Corylus avellana* with three main bands having a MW of 17, 33 and 38 kDa. The pollen extracts, analyzed by IEF (pH range 3–10), are tested by a densitometric analysis of the Coomassie Brilliant Blue stained lanes. The electrophoretic pattern of the bands (Figure 1B) confirms the partial identity of the protein content in *Corylus* and *Ostrya* pollen.

2D electrophoretic analysis is then performed: the first dimension is settled between pH 3–10 and the second between molecular weights 10^4 – 10^5 Da. As

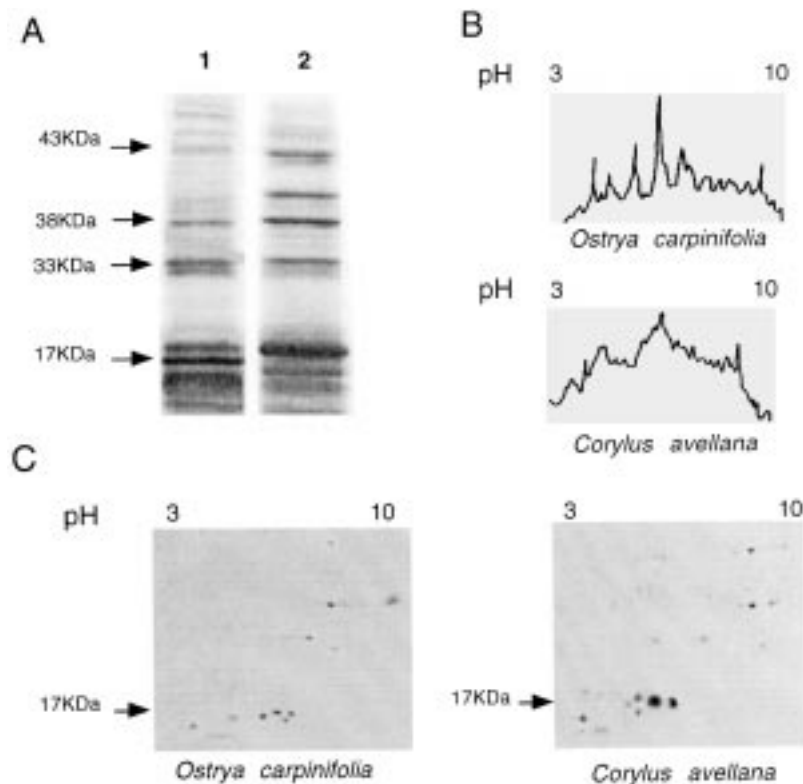


Figure 1. Panel A. SDS-PAGE of *Ostrya carpinifolia* and *Corylus avellana* (lanes 1 and 2 respectively). 46 μ g pollen proteins were applied to a gradient SDS-PAGE from 10% to 18% polyacrylamide concentration. Panel B. Representation of densitometer drawing of *Ostrya carpinifolia* and *Corylus avellana* pollen extracts analyzed by isoelectrofocusing (IEF) with the pH gradient ranging from 3.0 to 10. Panel C. Coomassie brilliant blue-stained two dimensional polyacrylamide gel electrophoresis (2D-PAGE); first dimension is isoelectrofocusing (IEF) with the pH gradient ranging from 3.0 to 10; second dimension run in gradient SDS-PAGE from 10% to 18% polyacrylamide concentration.

shown in Figure 1C, a comparable molecular pattern is observed in *Ostrya carpinifolia* and *Corylus avellana* suggesting the presence of a set of proteins with a high homology degree.

Immunochemical characterization of pollen from *Ostrya carpinifolia*

Twelve patients sensitized to hop-hornbeam pollen were studied in order to assess specific IgE reactivity to *Ostrya carpinifolia* and *Corylus avellana* pollen extracts. All the sera detected a 17 Kda band in the pollen extract of *Ostrya carpinifolia* and *Corylus avellana*. Other IgE-binding proteins were seen at about 20, 31, 35, 40, 46 and 60 kDa (Figure 2A) in hazel extracts.

One representative serum (n.10) out of the twelve used before, was probed in two dimensional polyacrylamide gel electrophoresis (2D-PAGE) against *Ostrya*

carpinifolia and *Corylus avellana* pollen. Again the same reactivity profile was obtained suggesting the presence of common epitopes shared within the two species (Figure 2C).

IgE inhibition studies

The IgE binding profiles obtained from *Ostrya* and *Corylus* pollen, suggest the presence of common epitopes shared between the two related species. The IgE specific reactivity to a 17 kDa component observed in the 95% of the sera, could be consistent with the presence of birch pollen allergen Bet v 1.

Bet v 1 is a protein with a molecular weight of 17 kDa described as the major allergen in the Betulaceae family. Variants of Bet v 1 with same allergenic properties and high homology of structure, were found also in *Alnus* and *Corylus* (Florvaag, 1988).

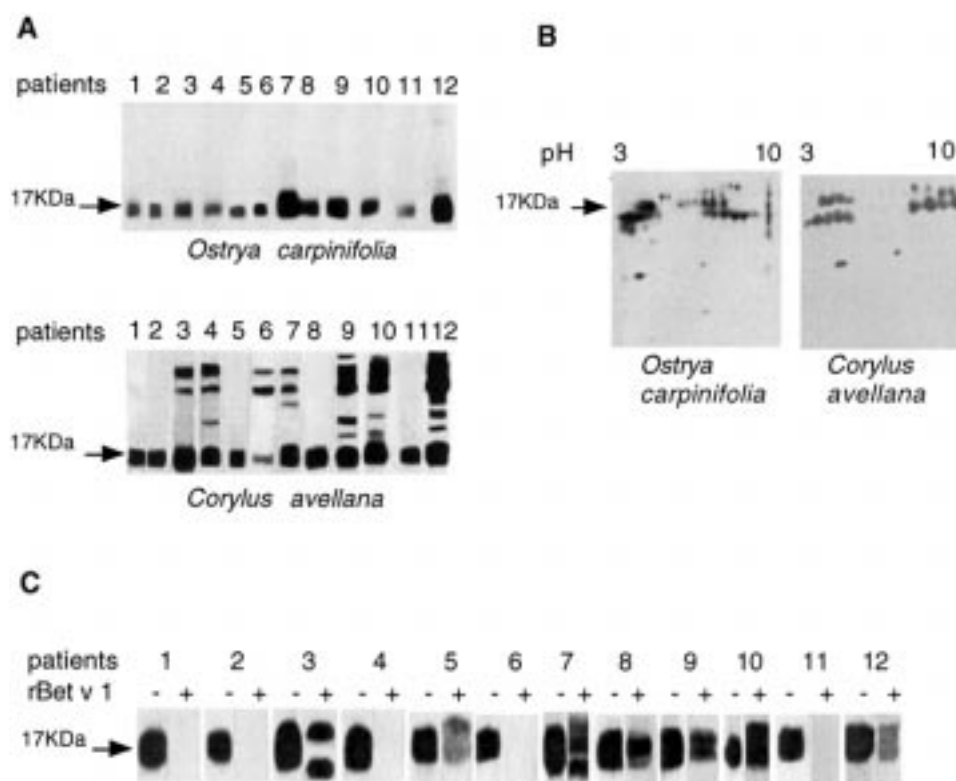


Figure 2. Panel A. IgE immunoblots where twelve sera from patients allergic to *Ostrya carpinifolia* pollen are used as probes. Pollen extracts of *Ostrya carpinifolia* and *Corylus avellana* are loaded on gradient SDS-PAGE performed as above.

Panel B. IgE immunoblots of one representative serum (n° 10) on *Ostrya carpinifolia* and *Corylus avellana* extracts in two dimensional polyacrylamide gel electrophoresis.

Panel C. Inhibition of IgE immunoblots: twelve sera from patients allergic to *Ostrya carpinifolia* were inhibited with recombinant Bet v 1.

(-) IgE binding to *Ostrya carpinifolia* extract without any treatment.

(+) IgE binding after incubation of sera with recombinant Bet v 1.

Then to determine the identity of the 17 kDa allergenic protein present in *Ostrya carpinifolia* and give some explanations for the cross-reactivity with *Corylus Avellana* pollen, we analyzed the IgE mediated reactions by competition experiments (Fuchs, 1997). Sera of allergic patients were absorbed on recombinant Bet v 1 allergen immobilized on an insoluble support (Pharmacia CAP System) and then probed in a western blot analysis against *Ostrya* and *Corylus* extracts. The treatment greatly reduces the IgE binding specific for Bet v 1 and as shown in Figure 2D, in 65% of the patients a significant inhibition of the reactivity to 17 kDa molecule was observed. Then this reactivity profile suggests the presence of Bet v 1 as an allergenic component in *Ostrya carpinifolia* pollen, causing cross-reactivity at the IgE level with other members of the related family.

Discussion and conclusions

Ostrya Carpinifolia is a tree growing in well defined areas of the South Europe. In Liguria the number of these trees have increased in the last few years: several explanations could be given but in our opinion the main reasons are the deserting of agricultural space and the use of this plant in reforestation programs in gardens and parks. Following that, *Ostrya carpinifolia* pollen became an important cause of early spring hay fever and allergic asthma.

Several reports have shown that pollen from Betulaceae family is highly allergenic mainly as respiratory sensitizer; nevertheless at the moment no data are available about *Ostrya carpinifolia* proteic content. This research deals with the characterization of the extract obtained from pollen of *Ostrya carpinifolia*, the aller-

Table 2. Pollen annual totals and allergic patients.

Year	Patients %*	<i>Ostrya Carpinifolia</i> pollen annual totals [§]
1981	6.2	3454
1982	10.1	3784
1983	7.8	3141
1984	13.5	3459
1985	19.3	4650
1986	20	4903
1987	20	4124
1988	21.9	5636
1989	13.9	3121
1990	24.5	11159
1991	26	7888
1992	32.4	7391
1993	28	2618
1994	44.7	23600
1995	36.1	3405

* Percent of patients with clinical symptoms during *Ostrya* pollination period

[§] Airborne *Ostrya* pollen annual totals in Genoa

genic relevance of which has not been investigated before.

The biochemical characterization revealed that the majority of the proteins extractable from pollen of *Ostrya* seems to be present at least as homologous variants in *Corylus avellana*. The SDS-PAGE analysis showed many components with approximately the same apparent molecular weight as detected in *Corylus* extract. The IEF studies again demonstrated a great qualitative and quantitative reciprocal similarities.

The binding pattern of patients IgE deduced from immunoblot experiments indicated a cross-reactivity towards pollen from *Ostrya* and *Corylus*, linked to the presence of epitopes common to their major allergens.

The majority of the sera contained IgE antibodies which reactivity was consistent with the expression of Bet v 1 (Niederberger, 1998). Preincubation with recombinant Bet v 1 significantly inhibited Ig E binding and confirmed the expression of Bet v 1 in *Ostrya* pollen.

In conclusion *Ostrya Carpinifolia* is a newly characterized allergenic pollen of the Corylaceae family which shares Bet v 1 epitopes with other members of related genera.

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