

Allergens from birch pollen and pollen of the European chestnut share common epitopes

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Summary

Type I allergy to pollen of the European chestnut (*Castanea sativa*) represents a major cause of pollinosis in (sub) Mediterranean areas. Using sera from 14 patients with established allergy to pollen of the European chestnut, 13/14 sera (92%) showed IgE-binding to a 22 kD protein, 2/14 (14%) displayed additional binding to a 14 kD protein and 1/14 (7%) bound only to the 14 kD protein of European chestnut pollen extract. Two monoclonal mouse antibodies, BIP 1 and BIP 4, directed against different epitopes of *Bet v I* (the major birch pollen allergen), and a rabbit antibody to recombinant birch profilin (*rBet v II*) were used to characterize the proteins of the European chestnut pollen. The recombinant birch pollen allergens, *rBet v I* and *rBet v II* (profilin) were employed to show common allergenic structures on proteins from both birch and European chestnut pollen by IgE-inhibition experiments. Despite the fact that the 22 kD protein displayed a higher molecular weight in comparison to the 17 kD major birch pollen allergen, *Bet v I*, we could demonstrate reactivity of both monoclonal antibodies, BIP 1 and BIP 4, with this protein. A complete inhibition of IgE-binding to this 22 kD protein was shown by pre-incubating sera with purified recombinant *Bet v I*. In addition, the 14 kD protein could be identified by IgE-inhibition studies with recombinant *Bet v II* and by using a rabbit anti-profilin antibody as the profilin from pollen of the European chestnut.

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Introduction

The European chestnut tree, *Castanea sativa*, belonging to the family Fagaceae (order Fagales) is native in the Mediterranean area, but is also cultivated outside this region. In Austria, *Castanea sativa* grows spontaneously in hilly areas of southern Burgenland, eastern and southern Styria and in southern Carinthia, but may also occasionally be found in Lower Austria and Vienna. The fresh pollen is covered with pollen kitt which is taken as food, thus collected and distributed by insects (beetles). In the afternoon, the pollen kitt dries and the pollen becomes airborne and is considered to be a significant source of allergens [1]. Long-term observations of annual totals of pollen count revealed an upward trend since 1977 which

has been significant ($P < 0.05$) for the period from 1977 until 1988 [2].

Since allergens of trees belonging to the order Fagales were shown to be highly crossreactive [3–6], the pollen of the European chestnut tree (*Castanea sativa*) is suspected to contribute in early summer to respiratory allergy in birch pollen-allergic individuals living in respective areas. Although a vast array of data has been published about allergic reactions to birch pollen [7–9], practically no studies on IgE-binding from patients' sera to extracts of *C. sativa* pollen (CSP) have been reported so far. In order to identify possible common allergenic structures in birch pollen (BVP) and European chestnut pollen (CSP), the IgE-binding patterns of BVP and CSP extracts were investigated by immunoblotting experiments using sera from birch pollen-allergic patients with or without a history of allergy to CSP.

The monoclonal antibodies, BIP 1 and BIP 4, directed against different epitopes of *Bet v I* (major birch pollen

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allergen) [10–12] and a rabbit antibody [13] raised against recombinant birch profilin (rBet v II) were investigated with regard to their capacity to bind to proteins in the CSP extract.

Purified recombinant birch pollen allergens, rBet v I and rBet v II (birch profilin) [14–18] were used in inhibition experiments to demonstrate shared epitopes on Bet v I and Bet v II and on the respective CSP allergens.

Materials and methods

Patients' sera

Sera from 22 patients suffering from established birch pollen allergy (case history, positive skin-prick tests, and RAST classes 3.5–5.0 to birch pollen) were divided into two groups. Group I comprised sera from 14 patients who displayed Type I-allergic symptoms from March until the end of July; these patients showed positive skin prick tests to CSP and BVP. Group II was formed using sera from eight patients suffering from Type I allergy in March and April. These patients showed positive skin-prick tests to BVP but showed negative skin-prick tests to CSP. All patients included in this study showed negative skin-prick tests to grass pollen. Sera from seven non-allergic persons with no indication for any Type I allergy (negative case history, negative skin-prick tests, negative birch pollen RAST) were pooled (NHS) and used for control experiments. All sera were stored at -20°C .

Protein extracts

Pollen of the European chestnut (85% pure, 15% plant parts) and the white birch (95% pure, 5% plant parts) were obtained from Allergon AB (Engelholm, Sweden) and extracted as described in detail [9]. Protein concentrations were determined according to Bradford [19].

SDS-PAGE, protein transfer and immunoblotting

SDS-PAGE (12% and 15% homogeneous gels, $140 \times 115 \times 1$ mm, 5% stacking gels) was performed according to Laemmli [20] in a vertical slab gel apparatus (Hoefer, San Francisco, U.S.A.) with modifications as previously described [9]. Pollen extract/cm ($14 \mu\text{g}$) were loaded onto gels. Prestained protein molecular weight standards (Amersham International, U.K.) were used to calibrate the gels. Proteins in the gels were visualized by the silver staining procedure according to Merrill [21]. Transfer of electrophoretically separated proteins to NC sheets was performed according to Towbin *et al.* [22]. The NC sheets were cut into strips of 0.5 cm width (corresponding to $7 \mu\text{g}$ transferred pollen extract) and IgE-immunoblotting was performed as described in detail previously [9].

Immunoblotting experiments using monoclonal antibodies BIP 1 and BIP 4, and rabbit anti-recombinant profilin antibody

MoAbs BIP 1 and BIP 4 [10] directed against different epitopes of Bet v I [11], crossreacting with the major allergens of pollen from hazel (*Cor a* I), alder (*Aln g* I), hornbeam (*Car b* I) [12] were used to investigate Bet v I-related proteins of the CSP extract. NC-strips containing CSP proteins and BVP proteins, respectively, were saturated in buffer A (TBS, 50 mmol/l of Tris, 150 mmol/l of NaCl, and 0.5% (v/v) of Tween 20, pH 7.4), supplemented with 3% powdered milk and subsequently probed with MoAbs BIP 1 and BIP 4 [10]. Thereafter, NC strips were incubated with undiluted hybridoma supernatants (1 ml/strip) while shaking continuously, at 4°C , overnight. After washing the strips with buffer A for 30 min the strips were incubated with rabbit anti-mouse IgG (Fc) antibody (Dianova, Hamburg, Germany) diluted 1:1000 in buffer A, supplemented with 3% powdered milk (1 ml/strip) for 2 hr at RT. After a wash with buffer A, strips were incubated with ^{125}I -labelled donkey anti-rabbit immunoglobulin (Amersham International, U.K.) diluted 1:1000 in buffer A, supplemented with 3% powdered milk (1 ml/strip) for 1 hr at RT. After washing the strips with buffer A for 30 min at RT, the strips were dried and subsequently subjected to autoradiography at -70°C . A rabbit anti-recombinant birch profilin antibody crossreacting with profilins from tree pollen (order Fagales), mugwort pollen, grass pollen, hazelnut, and apple [13] was used to investigate the presence of profilin in the CSP extract. NC-strips containing BVP proteins and proteins of the CSP extract, respectively, were probed with a 1:1000 dilution of the rabbit anti-recombinant profilin antibody in buffer A (1 ml/strip). Bound rabbit antibody was detected with ^{125}I -labelled donkey anti-rabbit antibody (Amersham, U.K., diluted 1:1000).

Expression and purification of recombinant Bet v I and birch profilin (rBet v II)

Expression and purification of recombinant non-fusion Bet v I and non-fusion birch profilin (rBet v II) was performed as described in detail previously [15,23]. Protein concentration of purified recombinant proteins was determined by the Micro-Kjeldahl method using glycine as standard [24].

Inhibition experiments with purified rBet v I or rBet v II

Proteins were separated by SDS-PAGE and transferred to NC. The NC was cut in 5 mm wide strips. Sera of following group I patients were used: sera 1–3 pooled, 8, 13 and 14. Sera were diluted 1:10 in buffer B and pre-

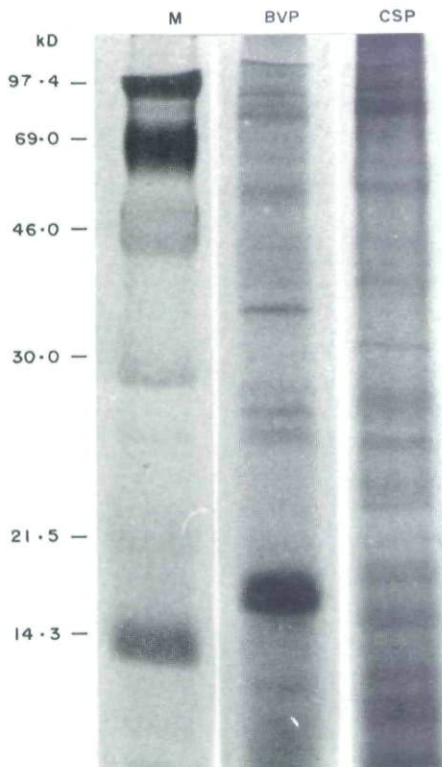


Fig. 1. Silver stained 15% SDS-polyacrylamide gel showing the separation of birch pollen (lane BVP) and *Castanea sativa* pollen (lane CSP) extract. Lane M: molecular weight marker.

incubated overnight at 4°C with 10 µg purified rBet v I or rBet v II. Thereafter, the NC strips were incubated with these dilutions (1 ml/NC strip) overnight at 4°C and bound IgE was detected as described [9].

Sugar detection

The electrophoretically separated proteins were transferred to NC and subsequently subjected to sugar detection by using a digoxigenin based immunodetection kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. The contrast of the stained glycosylated proteins was improved by using VIDAS 21 Image Analysis System (Kontron Electronic, Munich, Germany).

Results

SDS-PAGE

The silver staining procedure of the electrophoretically separated proteins extracted from the European chestnut pollen (lane CSP) and the white birch (lane BVP) revealed several proteins with a MW ranging from 13–70 kD in CSP and BVP extract, respectively (Fig. 1).

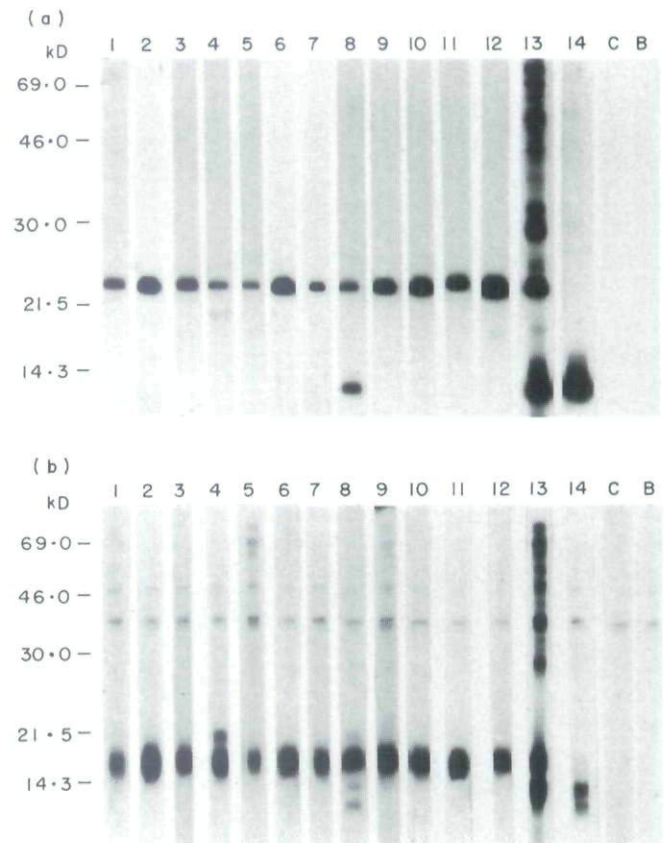


Fig. 2. IgE-immunoblot analysis of sera from individuals with tree pollinosis with symptoms from March to July (group I). (a) NC strips containing blotted proteins of European chestnut pollen extract were probed with sera of 14 patients suffering from tree pollinosis (group I), lanes 1–14, with a serum pool of non-allergic donors, lane C, and with buffer without addition of any serum, lane B. (b) NC strips containing blotted proteins of birch pollen extract were probed with sera of the same 14 patients (group I), lanes 1–14, with a serum pool of non-allergic donors, lane C, and buffer without addition of any serum, lane B.

Immunoblotting experiments

IgE-reactivity of sera from individuals suffering from tree pollinosis with symptoms from March to July (group I). NC-sheets containing electrophoretically separated proteins from the CSP extract were probed with 14 sera derived from patients suffering from allergic reactions from March to July. All sera tested recognized at least one protein of the CSP extract. Thirteen of fourteen sera (92.8%) showed IgE-binding to a protein band with a MW of 22 kD (Fig. 2, CSP, lanes 1–13). Two (14.2%) of those sera reacted in addition with a 14 kD protein (Fig. 2, CSP, lanes 8 and 13) and one with several other proteins of the CSP extract (Fig. 2, CSP, lane 13). One serum (7.2%) revealed exclusive binding to the 14 kD protein

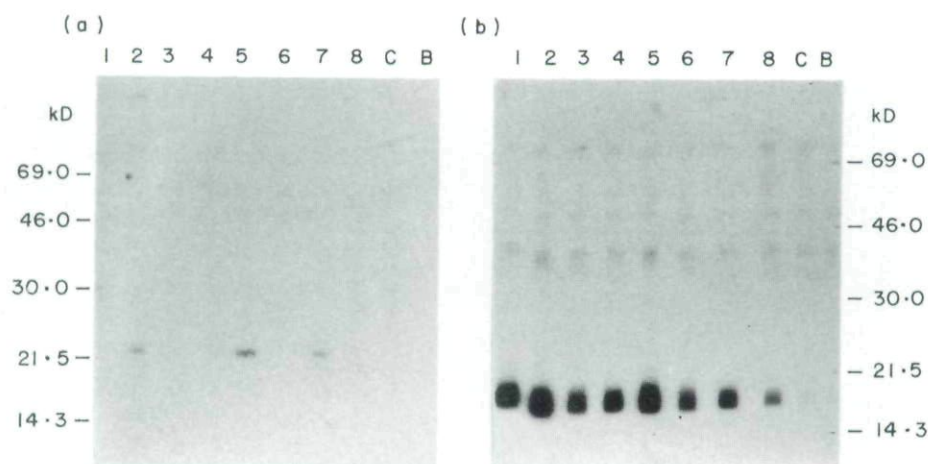


Fig. 3. IgE-reactivity of sera from individuals with tree pollinosis with symptoms during March and April (group II). NC-sheets containing electrophoretically separated proteins of extracts from pollen of the (a) European chestnut and (b) the white birch were probed with eight sera derived from patients suffering from allergic reactions to tree pollen in March and April (group II).

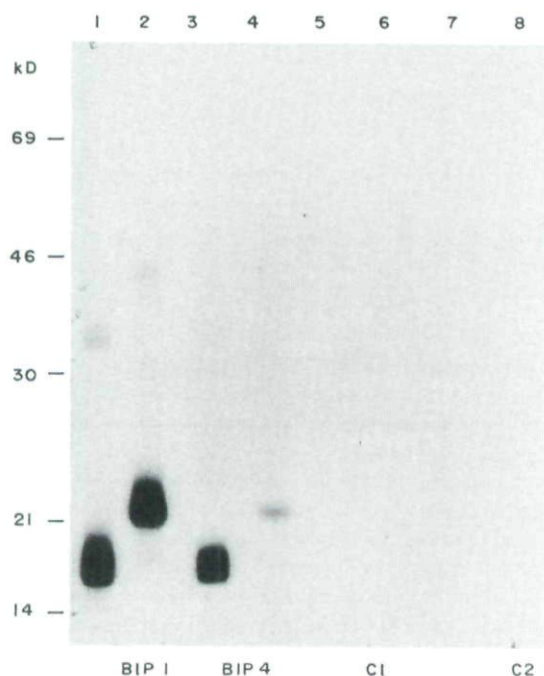


Fig. 4. Immunoblot with MoAbs BIP 1 and BIP 4. NC-sheets containing electrophoretically separated proteins of birch (lanes 1, 3, 5 and 7) and CSP extract (lanes 2, 4, 6 and 8), respectively, were probed with monoclonal anti-*Bet v* I antibodies BIP 1 and BIP 4. Controls: C₁ (BIP 1 or BIP 4 omitted), C₂ (rabbit anti-mouse IgG (Fc) antibody omitted).

(Fig. 2, CSP, lane 14). Based on this result, the CSP protein with an apparent MW of 22 kD is considered the major CSP allergen.

NC-sheets containing electrophoretically separated proteins from the BVP extract were probed with the same 14 sera. All sera tested recognized at least one protein of the BVP extract. IgE-binding to *Bet v* I (17 kD) was observed in 13 cases (Fig. 2, BVP, lanes 1–13), two sera



Fig. 5. Immunoblot with a rabbit anti-recombinant profilin antibody. A rabbit anti-recombinant profilin antibody, reacting with profilins from various plant sources, bound to a 14 kD protein of European chestnut pollen extract (Lane 1). Lane 2, pre-immune rabbit serum.

showed in addition binding to a 14 kD band (Fig. 2, BVP, lanes 8 and 13) and one serum displayed IgE-binding only to a 14 kD protein (Fig. 2, BVP, lane 14).

IgE-reactivity of sera from individuals suffering from tree pollinosis with symptoms from March to April (group II). NC-sheets containing electrophoretically separated proteins from the CSP extract were probed with eight sera derived from patients suffering from allergic reactions in March and April. All of these patients' sera contained IgE-antibodies reacting with *Bet v* I, but did not, or only very weakly show IgE-binding to CSP proteins (Fig. 3).

Fig. 6. Inhibition with purified rBet v I and purified rBet v II (birch profilin). Sera from patients (a) 1–3 (pooled) (b) 8, (c) 13 and (d) 14 were diluted 1:10 in buffer B (lanes 1) or diluted 1:10 in buffer B containing 10 µg purified rBet v I (lanes 2) or 10 µg purified rBet v II (lanes 3). Pre-incubation was carried out overnight at 4°C.

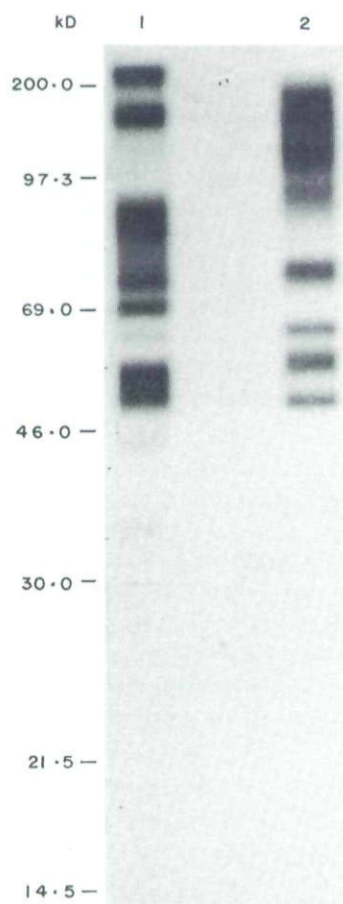
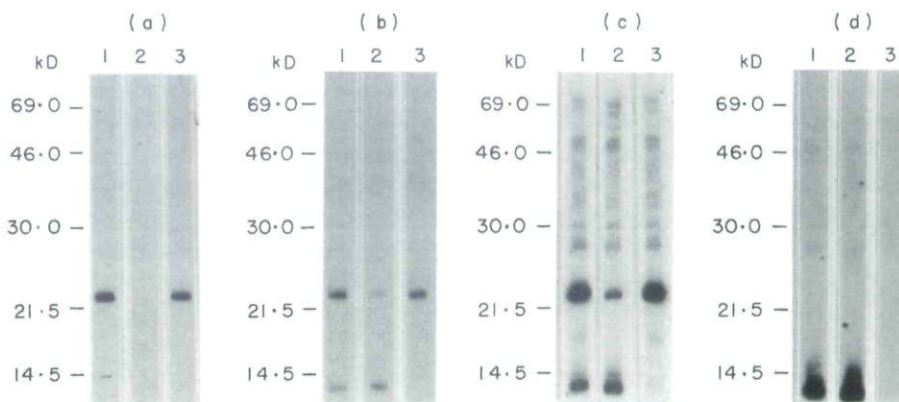


Fig. 7. Sugar detection. NC sheet containing electrophoretically separated proteins was subjected to sugar detection. Lane 1, transferrin as positive control. Lane 2, CSP extract.

Immunoblot with monoclonal anti Bet v I antibodies BIP 1 and BIP 4. Both MoAbs reacted strongly with Bet v I, the major allergen from BVP extract (17 kD) (Fig. 4, lanes 1 and 3). With CSP extract, BIP 1 displayed strong binding, whereas BIP 4 showed weak binding to the 22 kD protein (Fig. 4, lanes 2 and 4).

Immunoblot with a rabbit anti-recombinant birch profilin antibody. A rabbit anti-recombinant birch profilin antibody bound to the 14 kD protein of CSP extract (Fig. 5, lane 1).

Inhibition experiments

Inhibition with purified rBet v I. Pre-incubation of sera 1–3 (pooled), 8 and 13 (see Materials and Methods) diluted 1:10 in buffer B with 10 µg purified rBet v I abolished (Fig. 6(a), lane 2) or markedly reduced (Fig 6 (b) and (c), lanes 2) IgE-binding to the major allergen of the CSP extract.

Inhibition with purified rBet v II (birch profilin). Pre-incubation of sera 8, 13, 14 diluted 1:10 in buffer B with 10 µg purified rBet v II led to a complete blocking of IgE-binding to the 14 kD protein of the CSP extract (Fig. 6(b)–(d), lanes 3).

Sugar detection. No glycosylated proteins in CSP extract could be detected below 50 kD (Fig. 7).

Discussion

The clinical observation that birch pollen-allergic individuals living in areas where the European Chestnut tree (*Castanea sativa*) grows, suffer from symptoms of type I allergy from the beginning of March or earlier until the end of July, led to the assumption that pollen of *C. sativa* could be the causative agent for the prolongation of symptoms since they are not attributable to grass pollen allergens (negative skin-pricks tests). Allergic symptoms due to tree pollen normally stop at the end of April in birch pollen-allergic patients, at least in the southern parts of Central-Europe, like Austria. The phenomenon of cross-sensitivity to several tree pollen allergens due to their physicochemical properties like pI, MW, primary and tertiary structure has been well described for following major allergens from pollen of the Fagales order:

alder (*Aln g I*), birch (*Bet v I*), hazel (*Cor a I*), hornbeam (*Car b I*) [3–6,8,12]. Thus, it was of interest whether, and if, to what extent, allergens from CSP are related to the most potent allergens of tree pollen, *Bet v I* and *Bet v II* from *Betula verrucosa*. Using sera from patients allergic to CSP, an IgE-binding protein of CSP extract could be detected at 22 kD which was recognized by 13 of 14 sera tested. These 13 sera exhibited IgE-binding to the major birch pollen allergen *Bet v I* (17 kD) as well. This observation is remarkable since all major allergens from pollen of the Fagales order investigated so far possess a MW of 16.5–18 kD. It was of interest to test whether the anti-*Bet v I* MoAbs BIP 1 and BIP 4 would bind to the major CSP allergen. BIP 1 raised to an epitope on *Bet v I* which is shared by *Aln g I*, *Cor a I*, *Car b I*, whereas BIP 4 is reactive with a distinct epitope on *Bet v I* and *Aln g I*, but is missing on *Cor a I* and *Car b I* [12]. As shown in Fig. 4, BIP 1 reacted strongly with the major 22 kD CSP allergen. Surprisingly, also BIP 4, although markedly weaker, reacted with the major CSP allergen.

It seems likely that the major allergen from CSP belongs to the family of *Bet v I*-related pathogenesis-related proteins that are broadly distributed if not ubiquitous within the plant kingdom. However, all of the proteins of which the MW was determined, revealed an apparent MW of 16–18 kD, while the major CSP allergen possessed an MW of 22 kD. This additional 4–6 kD part of the molecule did not seem to consist of carbohydrates (Fig. 7) and may therefore be considered to be an extra peptide portion. It will be of interest whether this additional peptide is located on the N- or C- terminus or in the middle of the molecule. Experiments are in progress in our laboratory to purify this CSP allergen to homogeneity in order to sequence the molecule or proteolytic fragments thereof.

Recombinant allergens have been demonstrated to be one of the most sophisticated advances in defining allergenic epitopes on tree pollen proteins [14–18,23,25,26]. *rBet v I* and *rBet v II* (birch profilin) expressed in *E. coli* as non-fusion proteins were found to be similar to natural birch pollen-derived allergens regarding IgE-binding capacity [15,23] and capability to release histamine from basophils of allergic patients [27]. Pre-incubation of pooled (Fig 6(a), lane 2) or unpooled (Fig. 6(b) and (c) lanes 2) sera with IgE-reactivity to the 22 kD protein of CSP extract with purified *rBet v I* led to a complete blocking or marked reduction of IgE-binding to the 22 kD protein of CSP extract. Furthermore, IgE-antibodies from three of 14 sera displayed binding to a 14 kD component of CSP extract. It has been recently demonstrated that the plant pan-allergen profilin can be found at this MW range [16,17,28]. Pre-incubation of sera with IgE-reactivity to the 14 kD protein of CSP extract

with purified recombinant birch profilin (*rBet v II*) led to a complete blocking of IgE-binding to the 14 kD protein of CSP extract (Fig. 6(b)–(d), Lanes 3). Furthermore, we could show binding of a rabbit anti-profilin antibody to this protein. Thus, the availability of highly purified recombinant allergens (*rBet v I* and *rBet v II*) allowed the identification of the two prominent allergens from European chestnut pollen extract as proteins similar to already known pollen allergens of trees belonging to the Fagales order.

It is worth noting that sensitization to *Bet v I*, the major birch pollen allergen, does not necessarily lead to cross-reactivity of patient's IgE with the major CSP allergen (Fig. 3), which is in contrast with observations concerning the other major tree pollen allergens *Aln g I*, *Cor a I*, *Car b I*. This might be explained by the botanically more distant relationship of *B. verrucosa* and *C. sativa*. On the other hand, it is surprising that the anti-*Bet v I* MoAb BIP 4 bound to the major allergen from CSP extract (although weakly) although it neither reacted with *Cor a I* nor with *Car b I* [12]. Therefore, it seems of particular interest to us to investigate the chemical properties of purified *Cas s I* in terms of amino acid sequence and possible post-translational modifications. Moreover, we plan to investigate the IgE-binding to the major CSP allergen and *Bet v I* using sera from patients allergic to CSP living in areas without birches and who can be assumed not to be sensitized against *Bet v I*.

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