

Quantification in mass units of Bet v 1, the main allergen of *Betula verrucosa* pollen, by a monoclonal antibody based-ELISA

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Summary

Background Fagales pollens are considered among the main agents responsible for allergic diseases in many countries of the northern hemisphere and single major allergens have been shown to be responsible for these responses.

Objective To develop a solid phase immunoassay for the quantification of Bet v 1, the main allergen from *Betula verrucosa* (birch), and to assess its suitability for quantitating the equivalent major allergen in other Fagales species as well.

Methods The assay is based on the use of two different anti-Bet v 1 monoclonal antibodies which were immobilized on the solid phase and, as a primary standard, affinity purified Bet v 1, the protein content of which was determined by amino acid analysis.

Results The ELISA proved to measure less than 0.2 ng/mL of Bet v 1 with a practical range of 0.4–40 ng/mL and could be suitable to quantify the equivalent major allergen in other Fagales species such as *Corylus avellana* (hazel), *Carpinus betulus* (hornbeam) and *Alnus glutinosa* (alder). The method was compared with quantitative electrophoresis and rocket immuno-electrophoresis for the determination of the allergen content in several *Betula verrucosa* extracts, and a very good quantitative correlation was found. Likewise, the Bet v 1 content exhibited a good correlation ($r = 0.87$; $P < 0.005$) with the allergenic potency values obtained by RAST inhibition.

Conclusions The results indicate that the Bet v 1-assay could be useful for standardization purposes in Fagales pollen extracts intended for clinical use.

Keywords: allergen standardization, mass units, *Betula verrucosa*, Bet v 1, Fagales pollen, monoclonal antibody ELISA

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Introduction

The pollen of birch (*Betula verrucosa*) is one of the main causes of type I hypersensitivity during spring in many regions of the northern hemisphere. More than 20% of the total atopic subjects in these areas are sensitive to *Betula verrucosa* allergens and the IgE-prevalence among tree pollen allergic patients is >90% [1]. Bet v 1, the single major allergen of *Betula verrucosa* pollen has been extensively characterized as a 17 kDa protein with multiple

isomeric variants [2–5]. More than 90% of birch allergic patients display IgE antibodies to Bet v 1 and 60% react exclusively to this allergen [2,6], indicating the importance of this protein in birch pollen allergy. Bet v 1 shows allergenic crossreactivity and structural homology with the single major allergens from *Corylus avellana* (Cor a 1), *Carpinus betulus* (Car b 1) and *Alnus glutinosa* (Aln g 1) [7–9].

The use of monoclonal antibodies (MoAb) has become a powerful tool for the purification and characterization of allergenic structures as well as for the development of specific immunoassays for quantification of allergens in crude extracts [10]. This kind of assays enables the

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quantitative measurement of allergens in absolute units (μg protein/mL), which is very useful in the standardization of allergen extracts intended for clinical use.

In this study, a solid phase ELISA to measure Bet v 1, based on specific MoAb, was set up. The assay is sensitive to and specific for the major Fagales pollen allergens, and exhibits a very good correlation when compared with other analytical methods such as quantitative SDS-PAGE and rocket immunoelectrophoresis (RIE). In addition, the Bet v 1 ELISA showed a good correlation with allergenic potency as determined by RAST inhibition.

Material and methods

Pollen extracts

Pollen of *Betula verrucosa*, *Corylus avellana*, *Carpinus betulus* and *Alnus glutinosa* were purchased from Allergon (Engelholm, Sweden), Greer Laboratories (Lenoir, CA, USA) or collected by Dr J.A. Jimenez (Madrid, Spain). The pollen contained less than 1% of foreign particles and less than 0.5% of foreign pollen. Aqueous extracts were prepared by suspending the pollen material in 50 mM NH_4CO_3 buffer pH 7.7 (10% w/v) while continuously shaking, for 30 min at room temperature, and then centrifuging at 4°C, 12 000 *g*, for 45 min. The supernatant was filtered through 0.4 μm pore size membrane and dialysed against H_2O at 4°C, overnight. The samples were finally filtered through 0.2 μm pore size membrane and then stored in aliquots at -20°C. Total protein content of pollen extracts was determined by the Lowry method [11].

Antibodies

Two different anti Bet v 1 MoAb (BV16 and CB11) previously obtained [12] were purified from ascites by protein G sepharose affinity chromatography (Pharmacia, Uppsala, Sweden), as indicated by the manufacturer. An SDS-PAGE analysis was performed to control the purity of MoAb preparations and a specific mouse IgG ELISA was used to determine their concentrations [13].

The rabbit antisera against whole *Betula verrucosa* pollen extract as well as the monospecific anti Bet v 1 rabbit serum were obtained as described [7].

Purification of Bet v 1

Two different Bet v 1 preparations were purified by MoAb affinity chromatography using the MoAb CB11 or BV16, coupled to CNBr activated Sepharose CL4B gel (Pharmacia, Uppsala, Sweden). The purity of Bet v 1 preparations was checked by SDS-PAGE. Quantitative amino acid analysis was performed to measure the protein concentration,

using a Biochrom 20 analyser (Pharmacia, Uppsala, Sweden). Samples were previously hydrolysed in a vapor phase technique as described [14].

SDS-PAGE

Electrophoresis of whole extracts and purified preparations was performed according to the method of Laemmli [15] in a Hoeffer vertical slab system with 14% acrylamide separation gel and 5% stacking gel under reducing conditions. BioRad low molecular weight standards were used as molecular weight markers, and the protein bands were detected with Coomassie brilliant blue. The Bet v 1 bands were quantified with a Sharp JX-330 scanner, using a Pharmacia Image Master software.

Rocket immunoelectrophoresis

RIE of *Betula verrucosa* extracts was performed according to Laurell [16] in a 1% agarose gel containing anti Bet v 1 rabbit serum ($2.6 \mu\text{L}/\text{cm}^2$). Staining was performed with Coomassie brilliant blue and the Bet v 1 concentration was evaluated using purified Bet v 1 (range 0.1–0.8 μg , 1/2 dilutions) as standard.

RAST inhibition

The allergenic activity of *Betula verrucosa* extracts was determined by RAST inhibition. Paper discs were activated with CNBr and coupled with an in-house reference of *Betula verrucosa* pollen extract, basically as described by Ceska *et al.* [17]. Fifty microlitres of 1/4 dilutions of a human serum pool ($n = 20$) from patients allergic to *Betula verrucosa* (RAST classes 3 and 4) were added per well to a 96-well microtitre plate and incubated along with the same volume of threefold serial dilutions of samples and in-house reference for 30 min at 37°C. The *Betula* sensitized discs were then added and incubated for another 3 h at room temperature. After washing, 100 000 cpm of ^{125}I -anti-human IgE MoAb HE2 [18] was added and incubated overnight at room temperature. Finally, the discs were washed and bound radioactivity was measured in a gamma counter. Allergenic activity of extracts was calculated in biological units (BU)/mL by comparison with the in-house reference previously calibrated by skin-prick test [19].

Bet v 1 ELISA

Microtitre 96-well plates (EIA/RIA plates, Costar, Cambridge, MA, USA) were coated at 4°C, overnight, with 50 μL of a mixture of the anti-Bet v 1 MoAb, BV16 and CB11, at 2.5 $\mu\text{g}/\text{mL}$ each. This optimum MoAb concentration for coating had been determined in previous

experiments. Plates were then blocked with 1% BSA in phosphate buffered saline (PBS) (100 µL/well) for 30 min, at room temperature and incubated for 1 h with 50 µL/well of samples diluted with 0.1% BSA, 0.05% Tween 20 in phosphate buffered saline (PBS). Samples were assayed in duplicate at several threefold dilutions. After washing with 0.1% Tween 20 in PBS, 50 µL of a 1/20 000 dilution of the anti-*Betula verrucosa* rabbit serum was added per well and incubated for 1 h. Following four washes, 50 µL of peroxidase conjugate goat anti-rabbit IgG (Tago, Burlingame, CA, USA), diluted 1/20 000, was added per well and incubated for another hour. Finally, the colour reaction development was carried out by incubating with peroxidase substrate buffer (50 µL/well of 0.03% H₂O₂, 1 mg/mL orthophenilendiamine in 50 mM citrate-phosphate buffer pH 6.0). After 30 min the colour reaction was stopped by adding 50 µL/well of 2 M HCl and the optical density (OD) was read at 490 nm with a reference filter at 650 nm. The Bet v 1 concentration of samples was obtained by interpolating in standard curves obtained from different concentrations (range 100–0.1 ng/mL) of purified Bet v 1.

Results

Bet v 1 purification

Two different Bet v 1 preparations were obtained from *Betula verrucosa* pollen extracts by affinity chromatography using the MoAb BV16 and CB11, which recognized non-overlapping epitopes on the allergen. Both preparations exhibited the same molecular weight by SDS-PAGE (~17 kDa) and their amino acid analyses were very similar to the data reported by Ipsen *et al.* [7]. These two Bet v 1 preparations were mixed at equivalent protein concentration, as determined by quantitative amino acid analysis, and used as a primary standard throughout the study. Figure 1 shows an SDS-PAGE experiment of this preparation at different protein loads.

Bet v 1 ELISA

A solid phase ELISA was set up for mass units determination of Bet v 1. The MoAbs BV16 and CB 11 were used on the solid phase, to catch the Bet v 1 molecules in the samples and, as second antibody, a rabbit serum against *Betula verrucosa* pollen extract was used. Figure 2 shows the dose–response curves obtained with the affinity purified Bet v 1 and whole *Betula verrucosa*, *Corylus avellana*, *Carpinus betulus* and *Alnus glutinosa* pollen extracts. The assay was able to detect less than 0.2 ng Bet v 1/mL with a practical working range of about 0.4–40 ng/mL. The slopes obtained with Bet v 1 and whole *Betula verrucosa* extracts were very similar indicating that no alteration had occurred

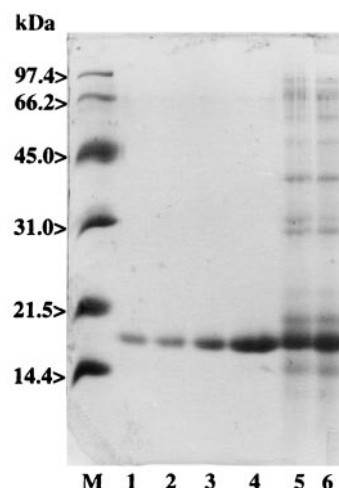


Fig. 1. SDS-PAGE analysis of purified Bet v 1 (lanes 1–4, with 1, 2, 4 and 8 µg protein applied, respectively) and *Betula verrucosa* pollen extract (lanes 5–6, 80 µg protein applied).

during the purification process of Bet v 1. In addition, similar slopes were also obtained with other Fagales pollen extracts such as *Corylus avellana* and *Alnus glutinosa*, but in the case of *Carpinus betulus* the slope and plateau level were slightly different with a lower relative allergen content.

The specificity of the ELISA was ascertained within a group of different allergenic extracts and the results are shown in Table 1. The Bet v 1 concentration of several *Betula verrucosa* pollen extracts was measured using the ELISA and the results are listed in Table 2. The relative Bet v 1 content varied between 5 and 14% in the extracts studied

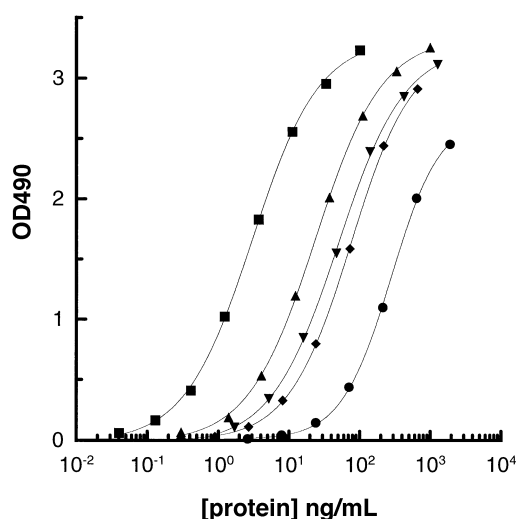


Fig. 2. Dose–response curves in ELISA of Bet v 1 and whole Fagales pollen extracts. ■, Bet v 1; ▲, *Betula v*; ◆, *Corylus a*; ●, *Carpinus b*; ▼, *Alnus g*.

Table 1. Specificity of the Bet v 1 MoAb-ELISA

Extract	OD490	Bet v 1/total protein (%)
<i>Betula verrucosa</i>	> 2.5	9.5
<i>Corylus avellana</i>	> 2.5	4
<i>Carpinus betulus</i>	> 2.5	0.7
<i>Alnus glutinosa</i>	> 2.5	6
<i>Olea europaea</i>	0.035	<0.0001
<i>Parietaria judaica</i>	0.030	<0.0001
<i>Dermatophagoides pteronyssinus</i>	0.027	<0.0001
<i>Alternaria alternata</i>	0.038	<0.0001
Cat dander	0.025	<0.0001
Egg white	0.029	<0.0001

The Bet v 1 of the extracts listed (1 µg/mL of protein) was determined by the MoAb ELISA.

and the mean interassay coefficient of variation was about 15%.

Comparison of Bet v 1 ELISA with quantitative SDS-PAGE, RIE and RAST inhibition

Table 2 shows the Bet v 1 content in eight different *Betula verrucosa* pollen extracts, determined by ELISA and two other methods: quantitative SDS-PAGE and RIE. In addition, the total allergenic activity of these extracts was determined by RAST inhibition and the results are expressed in BU/mL by comparison with an in-house reference previously calibrated by the skin-prick test (last column, Table 2). The ELISA data were compared with the other two analytical methods (SDS-PAGE and RIE) by linear regression test and the results are shown in Fig. 3.

A very good quantitative correlation can be observed among these methods. Figure 4 shows the comparison by linear regression of allergenic potency and Bet v 1 concentration. A good correlation can also be observed ($r=0.9$; $P=0.0021$) with 1BU corresponding, on the average, to 0.66 µg of allergen.

Discussion

In this study, a solid phase ELISA method to measure Bet v 1, the main *Betula verrucosa* pollen allergen, was set up. The immunoassay is based on the use, as adsorbing antibodies, of two different MoAb recognizing non-overlapping epitopes on the allergenic molecule. Bet v 1 displays a considerable degree of heterogeneity and several isoforms have been detected [3,5]. This is a remarkable fact to consider in the design of MoAb based immunoassays and the use of more than one MoAb is advisable. In this sense, the epitopes recognized by the two anti Bet v 1 MoAb used in the present study are widely distributed along several Bet v 1 isoforms [12], thus allowing to detect other close allergenic molecules sharing epitope characteristics. A mixture of two preparations of affinity purified Bet v 1 (obtained with MoAb BV16 and CB11, respectively) was used as primary standard of the assay. The protein content of the preparations was determined by quantitative amino acid analysis. The binding curves obtained in the assay with Bet v 1 turned out to be parallel to those obtained with *Betula verrucosa* pollen extract, as well as to recombinant Bet v 1 (data not shown), indicating that the allergen had not been antigenically altered during the purification process. Similar dose-response curves were obtained with other Fagales pollen extracts such as *Corylus avellana*, *Carpinus betulus* and *Alnus glutinosa*, although in the case of *Carpinus* the curve is somewhat different (Fig. 2) and the relative

Table 2. Bet v 1 content and allergenic activity in *Betula verrucosa* extracts

Extract	Total protein mg/mL	µg Bet v 1/mL ELISA	Bet v 1/total protein (%)	µg Bet v 1/mL SDS-PAGE	µg Bet v 1/mL RIE	BU/mL RAST inhibition
1	3.5	214	6.11	217	242	240
2	2.61	276	10.57	299	291	488
3	2.66	189	7.1	252	231	395
4	2.95	272	9.22	267	299	540
5	3.04	380	12.5	384	385	932
6	2.93	415	14.16	412	389	656
7	1.00	122	12.20	143	101	187
8	2.5	134	5.36	155	192	117

Average Bet v 1 content in 8 different *Betula verrucosa* pollen extracts determined by ELISA ($n = 8$), quantitative SDS-PAGE ($n = 3$) and RIE ($n = 4$). The allergenic activity of the extracts was determined by RAST inhibition and expressed in BU/mL ($n = 2$).

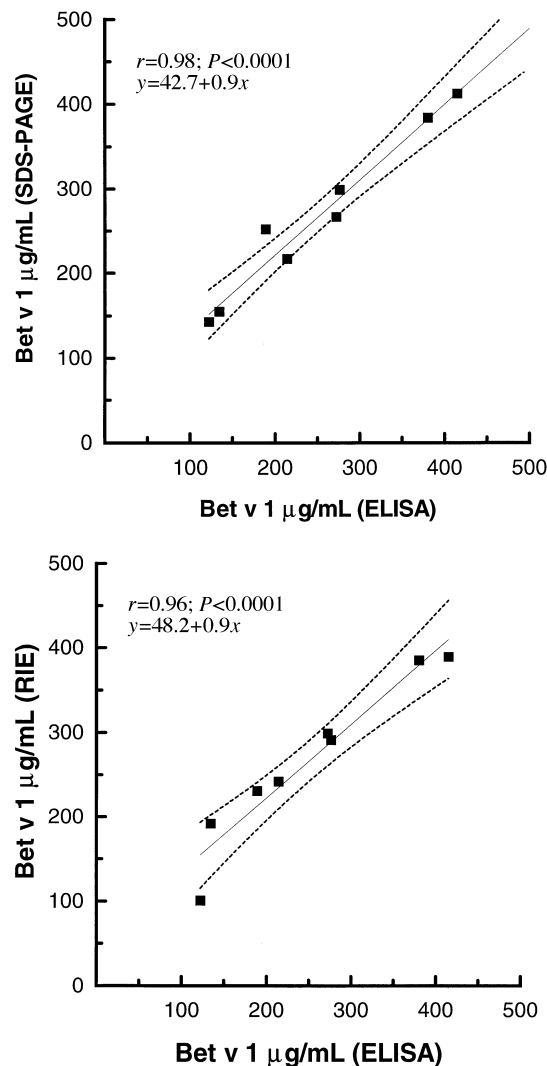


Fig. 3. Correlation between ELISA and quantitative SDS-PAGE or RIE. The Bet v 1 content in eight different *Betula verrucosa* pollen extracts was determined by ELISA (abcisa), quantitative SDS-PAGE (a) and RIE (b) and the results were analysed by linear regression.

allergen content is lower (Table 1). These preliminary results are in agreement with the high homology degree detected among the major allergens of Fagales species [7–9] and indicate that the Bet v 1 ELISA could also be useful to measure Cor a 1, Car b 1, and Aln g 1. The Bet v 1 measurements obtained by ELISA in a number of *Betula verrucosa* extracts were compared with data obtained with two other methods: quantitative SDS-PAGE and RIE. A very good quantitative correlation was found in all cases (Fig. 3), demonstrating that these techniques were measuring the same molecule. In addition, a good correlation was obtained between Bet v 1 concentration and allergenic potency, determined by RAST inhibition (Fig. 4), thus

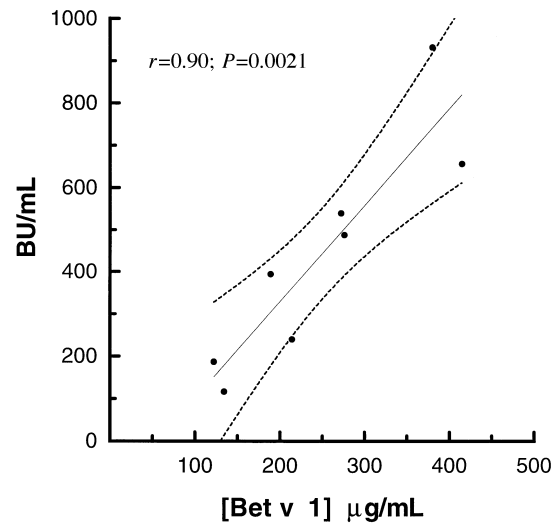


Fig. 4. Correlation between allergenic activity (BU/mL), determined by RAST inhibition and Bet v 1 concentration, determined by ELISA in eight different *Betula verrucosa* pollen extracts.

validating the use of the immunoassay for quality control. These results are in agreement with the fact that Bet v 1 is the most important birch allergen and more than 90% of birch sensitive patients have specific IgE against the Bet v 1 molecule [2,6]. On the average, 1 BU (~20 mm² area on skin-prick test) corresponded to about 0.7 µg of Bet v 1. Interestingly, very similar relations have been found in other allergenic materials with single major allergens such as cat dander [20] and olive pollen [21]. In conclusion, the Bet v 1 measurements by ELISA could be very useful for quality control of preparations intended for clinical use, including evaluation of allergenic potency, mass units standardization, as well as stability studies.

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