

Monoclonal Antibodies against Birch Pollen Allergens: Characterization by Immunoblotting and Use for Single-Step Affinity Purification of the Major Allergen *Bet v I*¹

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Abstract. Two monoclonal antibodies against birch pollen proteins were produced by immunizing BALB/c mice with birch pollen extract. In immunoblotting experiments, antibody BIP 1 reacted with a 17-kilodalton (kD) protein considered to represent the major birch pollen allergen *Bet v I*. A second monoclonal antibody, BIP 3, reacted with 3 different birch pollen proteins of molecular weights 32, 36 and 68 kD of which the 36- and 68-kD proteins corresponded to minor allergens of birch pollen. Two-dimensional electrophoresis/immunoblotting experiments revealed that BIP 1 reacted with all *Bet v I* isoallergens, also identified by human IgE antibodies. Using BIP 1 coupled to Sepharose 4B as reverse immunosorbent, *Bet v I* was obtained in a single-step procedure and characterized as single band by SDS-PAGE.

Introduction

Monoclonal antibodies (MoAbs) are considered to be useful tools for characterization and standardization of allergen extracts and for purification of allergenic molecules. They have been raised against *Alternaria tenuis* [1], grass [2-5] and ragweed [6, 7] pollen or mite allergens [8-10], which are predominant causes of type I allergic reactions in humans.

Among tree pollen allergens, birch pollen (BP) represents the most prominent cause for allergic diseases. About 22% of allergic patients in central Europe suffer from birch pollinosis [11]. BP allergens share allergenic determinants with other tree pollen, e.g. pollen from hazel or alder [12, 13], or with vegetables and fruits [14-17]. In this context, MoAbs could help to gain more information about cross-reacting structures.

The allergen profile of BP is well known. Using various techniques it was possible to identify a 17-kilodalton (kD) protein as the major allergen [18]. According to the present WHO nomenclature this protein was designated *Bet v I* [19]. In addition, new

studies reported the existence of independent minor allergens [20, 21]. Efforts have been made to purify and characterize BP antigens/allergens by applying several chromatographic techniques [18, 22-25].

In this study, a single-step purification of *Bet v I* is presented using MoAb BIP 1 for affinity chromatography. Furthermore, another MoAb, BIP 3, is described which is directed against distinct BP proteins of 32, 36 and 68 kD.

Materials and Methods

BP Extraction Procedure

Betula verrucosa pollen (BP) was obtained from Allergon AB (Engelholm, Sweden) and was extracted essentially according to Ipsen and Loewenstein [18] with slight modifications [36].

Protein Assay

The protein concentration was measured by the method of Bradford [26].

Production of MoAbs against BP Proteins

Immunization. Inbred Balb/c mice were immunized subcutaneously with 100 µg of BP extract emulsified in Freund's complete adjuvant on day 0, and in Freund's incomplete adjuvant on days 21 and 42-48. Mouse sera were collected 10 days after the second booster. Mice exhibiting serum antibodies against BP proteins (BIPs) as tested by enzyme-linked immunosorbent assay (ELISA)

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and immunoblotting (IB) were chosen for the fusion. Spleens were removed for the fusion 3 days after the final intraperitoneal injection [100 µg BP extract in phosphate-buffered saline (PBS)].

Cell Fusion. Hybridization was carried out as described by Koehler and Milstein [27], using the nonsecreting myeloma cell line P3-X-Ag8.653X, with polyethyleneglycol 4000 GK (Merck, Darmstadt, FRG) as fusing agent. Hybrid cells were placed in 96-well Costar tissue plates (Costar, Cambridge, Mass., USA) containing peritoneal macrophages as feeder cells, and fed 3 weeks in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum (Flow Laboratories, Irvine, Scotland) and hypoxanthine, thymidine and aminopterin (HAT medium; Sigma, St. Louis, Mo., USA). Hybridoma supernatants were harvested after 3 weeks, screened for antibody production against BP proteins by ELISA, and cells from positive wells were cloned at least 3 times by the limiting dilution method. Individual clones were tested for anti-BP activity by IB.

Gel Precipitation Test

IgG subclass was determined by double immunodiffusion.

Elisa

Monoclonal culture supernatants and mouse sera were screened by ELISA. ELISA plates (Nunc, Copenhagen, Denmark) were coated with 50 µg BP extract per milliliter borate-buffered saline for 6 h at room temperature (RT). Remaining binding sites were blocked by incubation with borate-buffered saline containing 1% bovine serum albumin (BSA) at RT for at least 30 min. Bound mouse IgG was detected by POX-conjugated rabbit antimouse IgG (Fc) (Dianova, Hamburg, FRG) and 2,2'-azino-bis(3-ethylbenzthiazoline)sulfonic acid as substrate (Sigma). Optical density was measured at 490–405 nm with a Dynatech microplate reader.

Purification of BIP 1

BIP 1 was purified from ascites using an r-protein A purification kit (Beckman Instruments, Fullerton, Calif., USA) according to the manufacturer's instructions.

Affinity Chromatography

Preparation of Reversed Immunosorbent. Purified MoAb BIP 1 (1.1 mg/ml) was dialyzed against 1,000 ml of coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.8). 2 g CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) were washed extensively with 1 mM HCl. The gel was washed with 20 ml coupling buffer and immediately thereafter mixed with 6 ml BIP 1. This suspension was rotated end-over-end for 2 h at RT. Excess antibody was removed by washing with coupling buffer. Any remaining active groups on the gel were blocked by incubating with 1 M ethanolamine, pH 9, for 2 h at RT. The obtained immunosorbent was then washed alternately 3 times each with 0.1 M acetate, 0.5 M NaCl, pH 4, and 0.1 M tris(hydroxymethyl)-aminomethane (Tris), 0.5 M NaCl, pH 8.

Affinity Chromatography. The immunosorbent was degassed, packed into a glass column and extensively washed with PBS. 2 mg extract was then applied to the immunosorbent column (0.5 mg/ml PBS). The gel was again washed extensively first with PBS and then with 5 mM phosphate buffer, pH 7.2. Protein still adsorbed was eluted in a gradient (0–3 M KSCN in 5 mM phosphate buffer, pH 7.2). The eluted material was dialyzed against distilled water, lyophilized, and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and IB.

Sera

Sera from patients with established BP allergy (allergic rhinoconjunctivitis, asthma bronchiale) were selected according to typical case history, positive skin prick tests and positive radioallergosorbent test (RAST) to BP extract. 3 patients' sera (A, B and C) were selected according to different patterns in IB studies. In addition, we selected 13 healthy volunteers with a negative case history concerning any type I allergy, negative skin prick test and negative RAST to BP extracts. Sera were collected, pooled in equal parts and used as negative control serum pool.

SDS-PAGE

SDS-PAGE was performed in a vertical slab gel apparatus (Hoefer, San Francisco, Calif., USA) according to Laemmli [28]. 12% gels (180 × 150 × 5 mm) with 5% stacking gels were used. Electrophoresis was carried out in a discontinuous buffer system (0.025 M Tris, 0.2 M glycine, 0.1% SDS). Samples were boiled at 95°C for 5 min in a 4-fold Laemmli sample buffer. 2.4 mg BP extract was loaded onto one gel. Molecular weights (MWs) were determined using prestained protein standards of 14.3–200 kD from Amersham International (Amersham, UK). Gels were stained using silver stain kits from Bio-Rad (Richmond, Calif., USA).

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis (2DE) was performed on an ISO-DALT electrophoresis system from Anderson and Anderson [29] (Electro Nucleonics Incorp., Oak Ridge, Tenn., USA) as described previously [30].

Sample Preparation. 300 µg of BP extract was boiled at 95°C for 5 min in 100 µl sample buffer [2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 50 mM cyclohexylaminosulfonic acid (Bio-Rad), 10% glycerol, pH 9.5]. 10 µl of the sample was loaded onto one ISO gel.

Electrophoresis. The first or ISO dimension is isoelectric focusing in polyacrylamide (35 g/l) with 9 M urea, 20 g surfactant NP-40, 19 g 3–10 ampholytes and 5 g 5–7 ampholytes (Ampholines, LKB Instruments), 1.6 g arginine and 1.0 g lysine per liter. Isoelectric focusing was performed at 14,000 Vh after prefocusing for 1 h at 200 V with 0.85 g/l phosphoric acid and 1 M sodium hydroxide as anolyte. ISO gels were immediately fused onto second-dimension slab gels (10–23% polyacrylamide gradient SDS gel). SDS-PAGE was carried out in a DALT tank with buffer (3.5 mM SDS, 24 mM Tris, 0.2 mM glycine). MWs were determined by use of prestained MW markers from BRL (Gaithersburg, Md., USA). The pH gradient of isoelectric focusing was determined by cutting a pair from each patch into 15 equal parts. Each part was put into 0.5 ml degassed distilled water. After incubation for at least 1 h on a shaker, the pH was measured [31].

Electroblotting

Electroblotting was performed according to Towbin et al. [32] using a Bio-Rad blotting tank. Proteins were transferred to nitrocellulose (NC; pore size 0.2 µm, Schleicher & Schuell, Dassel, FRG) at 159 mA for 6 h in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3. In case of 2DE gels, transfer was performed for 2.5 h at 1.2 A.

Immunoblotting

NC sheets were cut into 5-mm strips and treated with 1 ml as follows.

Detection of MoAbs. Strips were saturated for 30 min in Tris-

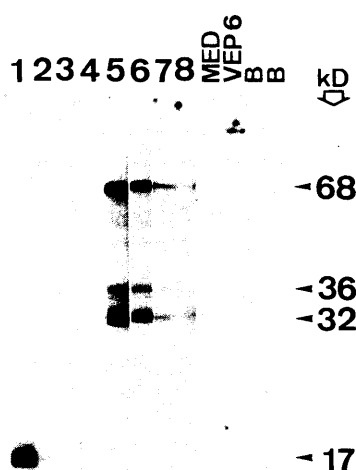


Fig. 1. BP extract was separated by SDS-PAGE and binding of MoAbs in immunoblots inhibited using native BP extract. Lane 1 = BIP 1; lanes 2-4 = BIP 1 inhibited: 250 µg (lane 2); 500 µg (lane 3) or 1,000 µg (lane 4) BP extract per milliliter culture supernatant; lane 5 = BIP 3; lanes 6-8 = BIP 3 inhibited: 1,000 µg (lane 6), 2,500 µg (lane 7) or 5,000 µg (lane 8) BP extract per milliliter culture supernatant; lanes MED, VEP6, B = control strips; kD = MW of antigens recognized by BIPs.



Fig. 2. BP extract was separated by SDS-PAGE. BIP binding was compared to binding of human IgE in IB. Lane 1 = Patient A IgE; lane 2 = BIP 1; lane 3 = patient B IgE; lane 4 = BIP 3; lane 5 = patient C IgE; lane N = normal human serum pool; lanes MED, VEP6, B = control strips; kD = MW of antigens recognized by BIPs.

buffered saline (TBS; 50 mmol Tris, 150 mmol NaCl, pH 7.4), 0.5% Tween 20 (TW), 3% powdered milk (PM), and incubated with hybridoma supernatants diluted 1:64 in TBS/TW/PM overnight at 4°C. Strips were washed for 30 min at RT in TBS/TW and bound IgG was incubated with rabbit antimouse IgG (Jackson Labs, Bar Harbor, Me., USA) diluted 1:3,000 in TBS/TW/PM for 1 h at RT. Strips were washed in TBS/TW (30 min, RT) and incubated with ^{125} I-goat antirabbit immunoglobulin (300,000 cpm per strip; Kirkegaard & Perry, London, UK). Labeling was performed by use of the chloramine T method [33]. Control strips were processed with TBS/TW/PM, HAT medium and MoAb VEP6 [34], respectively.

BIP Inhibition. Culture supernatants diluted 1:64 in TBS/TW/PM were incubated with BP extract (500–5,000 µg/ml supernatant) and then applied onto saturated NC strips. Strips were further processed as described above.

Detection of Human IgE. Strips were processed by saturation in sodium phosphate buffer (PB; 50 mM, pH 7.5), 0.5% (v/v) TW, 0.5% (w/v) BSA, for 30 min at RT. Sera were diluted 1:4 in PB/TW/BSA containing 1% (v/v) normal goat serum. NC strips were incubated with the sera overnight at 4°C and subsequently washed in PB/TW/BSA for 30 min at RT. Bound IgE was detected by ^{125} I-rabbit antihuman IgE, taken from Phadebas RAST kits (Pharmacia) [300,000 cpm/ml PB/TW/BSA/0.4% (w/v) gelatine]. Control strips were processed with normal serum pool (1:4) and buffer.

For 2DE, NC sheets were incubated with patients' sera diluted 1:10. ^{125} I-rabbit antihuman IgE was used in a concentration of 7,500,000 cpm per NC sheet.

Autoradiography. Blots were investigated by autoradiography at -70°C for 48–72 h, using hyperfilm MP (RPN 6, Amersham) in exposure cassettes (X-omatic, Kodak) containing intensifying screens (X-omatic regular, Kodak).

Results

Characterization of MoAbs against BP Antigens

Two MoAbs, BIP 1 and BIP 3, reacting with BP proteins in ELISA were obtained. They were found to be IgG1 by means of double immunodiffusion tests. Using IB, 1 MoAb (BIP 1) exhibited strong binding to a 17-kD protein. The other MoAb (BIP 3) reacted with BP antigens of MW 32, 36 and 68 kD in IB experiments (fig. 1, lanes 1 and 5).

To answer the question whether or not BIPs react with native BP proteins, inhibition experiments were performed using BP extract in aqueous solution. The binding of both antibodies to BP proteins in IB was inhibited in a dose-dependent manner with BP extract dissolved in aqua bidest. In case of BIP 1 antibody, 1 mg lyophilized BP extract per milliliter culture supernatant was sufficient for inhibition (fig. 1, lanes 2–4), whereas in case of BIP 3 antibody 5 mg BP extract per milliliter culture supernatant was needed to reduce binding in IB experiments (fig. 1, lanes 6–8).

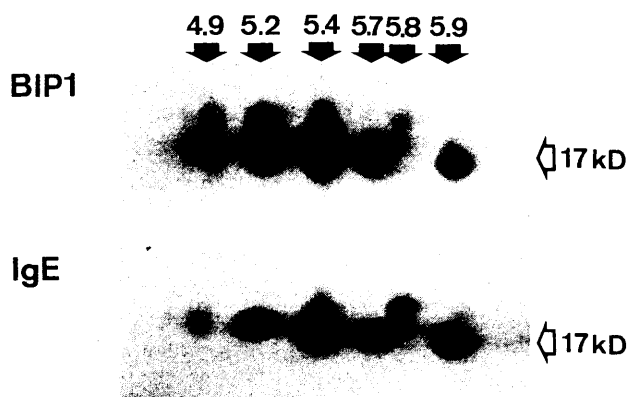


Fig. 3. 2DE/IB. Comparison of binding patterns of BIP 1 and human IgE (patient A) to the major BP allergen, *Bet v I*. White arrows indicate MW, black arrows isoelectric points of *Bet v I* isoallergens.

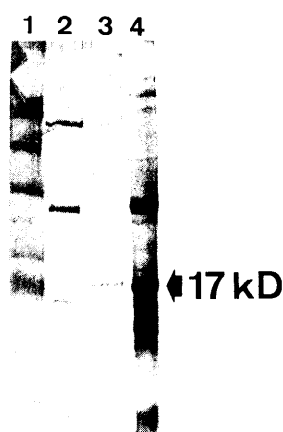


Fig. 4. Affinity chromatography. Affinity-purified material was investigated by analytical SDS-PAGE (silver stain). Lane 1 = MW markers; lane 2 = nonadsorbed material; lane 3 = eluted material; lane 4 = BP extract.

Correlation between BP Proteins Recognized by MoAbs and Human IgE Antibodies

Selected sera of BP-allergic patients were tested in IB experiments and the binding patterns of IgE and MoAbs were compared. Serum from patient A showed binding of IgE antibodies exclusively directed against *Bet v I* (17-kD protein). BIP 1 bound to a BP protein of identical MW (fig. 2). The 36- and 68-kD proteins, recognized by BIP 3, corresponded to proteins recognized by IgE of patients B and C. The 32-kD protein bound only BIP 3, but not IgE of patient B or C.

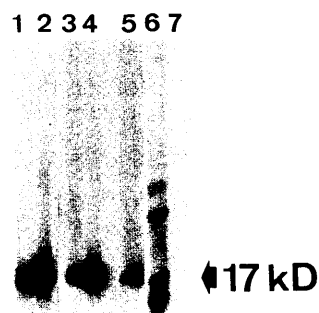


Fig. 5. Immunological properties of affinity-purified material as tested by IB. Lanes 1, 3, 5, 7 = Eluted material; lanes 2, 4, 6 = BP extract; lanes 1, 2 = reactivity with BIP 1; lanes 3, 4 = reactivity with patient A IgE; lanes 5, 6 = reactivity with patient C IgE; lane 7 = negative control serum pool (normal human serum).

Two-Dimensional Electrophoresis

BP proteins were further separated by 2DE, transferred to NC and studied by IB. BIP 1 and IgE of patients showed binding to the same cluster consisting of 10 protein spots with MW of 17 kD and pIs ranging from 4.9 to 5.9 (fig. 3).

*Affinity Purification of *Bet v I* Using BIP 1*

BIP 1 was coupled to Sepharose 4B and used as reversed immunosorbent for purification of *Bet v I* by affinity chromatography. 90% of the protein applied was recovered and represented a single band in SDS-PAGE (fig. 4, lane 3). In IB experiments, the antibody-binding pattern of this affinity-purified material was compared with that of BP extract (fig. 5). BIP 1 bound to the affinity-purified material (fig. 5, lane 1) as well as to BP extract (fig. 5, lane 2). In addition, patients' IgE bound to both the affinity-purified material [fig. 5, lane 3 (patient A) and lane 5 (patient C)] and to BP extract [fig. 5, lane 4 (patient A) and lane 6 (patient C)]. No reactivity of the purified material with normal human serum (fig. 5, lane 7) was observed.

Discussion

The standardization of allergen extracts is essential for the diagnosis and therapy of type I allergic reactions. The content of major and minor allergens in extracts may vary from batch to batch, thus influencing

diagnostic tests and the outcome of hyposensitization. MoAbs may help with this problem, as they allow the set up of ELISA or other tests for the detection and quantitative determination of various allergens. Furthermore, they can be used as immunosorbents for purification purposes.

In this study, we describe two IgG1 MoAbs against BP proteins, BIP 1 and BIP 3. In IB experiments, BIP 1 antibody bound to a 17-kD protein which was also recognized by human IgE as the major allergen (*Bet v I*). Previous studies by Vik et al. [35] suggested the existence of *Bet v I* isoallergens (pI 5.8 and Bv 45). Precipitation in tandem crossed immunoelectrophoresis/crossed radioimmuno-electrophoresis of both fractions by monospecific rabbit antibody was observed, and thus allergenic identity of those BP fractions was concluded [36]. However, the reaction of polyclonal antibody to more than one component of an extract is not unlikely and immunization of rabbits with material containing impurities has to be discussed. Recent work performed in our laboratory, employing 2DE combined with IB, resolved the 17-kD protein into a cluster of approximately 10 IgE-binding protein spots [21]. In 2DE/IB experiments, BIP 1 MoAb was found to bind exactly to the same spots as IgE from patient A (fig. 3), thus providing additional evidence for *Bet v I* isoallergens. Using in vitro translated *Bet v I* protein, which is devoid of carbohydrates, we recently showed that its capability to bind human IgE in IB experiments is comparable with that of pollen-derived *Bet v I* [34]. Similar results were obtained with BIP 1 [in preparation]. We assume that the heterogeneity of *Bet v I* observed in 2DE/IB experiments is due to differential glycosylation and that both human IgE and BIP 1 are directed against the protein moiety of *Bet v I*.

A second MoAb, BIP 3, reacted with two proteins of MW 36 and 68 kD (fig. 2), which corresponded to IgE-binding minor allergens as described in a previous paper [21]. However, in that study certain individual patients' sera recognized those proteins [22]. Furthermore, BIP 3 identifies a 32-kD protein not recognized by human IgE. Apparently, BIP 3 is directed against an epitope which may not be essential for IgE binding.

Possible denaturation of allergens is a main point of criticism concerning IB techniques. Binding of MoAbs to epitopes present on native BP allergens was therefore investigated by means of inhibition

experiments. The reaction of BIP 1 and BIP 3 could be completely quenched by incubating the antibodies with different amounts of native BP extract before application to blot strips, thus indicating that the epitopes recognized by BIP 1 and BIP 3 are present both on the native molecules as well as on the electroblotted proteins. The finding that higher amounts of BP extract needed to inhibit the binding of BIP 3 is considered to be due to the lower content of minor allergens in BP extract.

Successful immunotherapy using extracts containing exclusively allergen components against which the patient's IgE are directed has been discussed [3, 21, 35] and hyposensitization with extracts composed according to IgE-binding patterns in IB seems to be desirable [37, 38]. Prerequisite hereto is the availability of pure components of allergen extracts. So far, chromatographic procedures like gel filtration, ion-exchange chromatography and related techniques have to be used in sequence to obtain preparations of sufficient purity. These methods have to be considered rather consumptuous on a preparative scale. Therefore, we attempted to use MoAb BIP 1 for affinity purification of *Bet v I*, using BIP 1 coupled to Sepharose 4B as a reversed immunosorbent. A 17-kD protein (*Bet v I*) could be purified from crude extract in a single-step purification under standard conditions used for immunoaffinity chromatography. The IgE-binding capacity of this affinity-purified protein was maintained when investigated by means of IB. Purification of allergens by this method seems to be worthwhile (fig. 4).

The antigenic relationship within the family of Betulaceae has been studied by Ipsen et al. [12], applying crossed immunoelectrophoretic techniques, using polyclonal monospecific antibodies. The results emphasize the strong immunochemical relationships of the major allergens of birch, hazel, alder and hornbeam, and species specificity of some of the minor allergens. We consider BIP 1 and BIP 3 to be useful reagents to further study this issue.

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