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Isolation and Characterization of Messenger RNA from Male Inflorescences and Pollen of the White Birch (*Betula Verrucosa*)

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Abstract. A glycoprotein with a molecular weight (MW) of 17 kilodaltons (kD), Bet v I, represents the major allergen of the white birch (*Betula verrucosa*, BV) and plays an important role in tree-pollen-induced type I allergic reactions. In order to characterize the major and also some minor allergens of BV, we investigated the IgE-binding properties of these allergens using immunoblot techniques. Normal and patients' sera were employed for this study. Furthermore, RNA from male inflorescences and from pollen of BV were isolated and purified by affinity chromatography on oligo-dT-cellulose. Poly(A)⁺-mRNA thus obtained was translated in vitro in a cell-free wheat germ system and the proteins synthesized were separated by SDS-PAGE and transferred to nitrocellulose. The blots were incubated with normal human sera and with sera from patients allergic to birch pollen. Bound IgE antibodies were detected with ¹²⁵I-labeled anti-IgE. We observed major IgE binding to a protein of an MW of 12.5 kD, and little IgE binding to a 17-kD protein, presumably Bet v I. Comparing the products of in vitro translation from mRNA preparations of mature pollen and of male inflorescences collected in June, October and February, little seasonal variations could be observed. As the in vitro translation system does not glycosylate proteins, our results show that the majority of IgE in patients' sera is not directed against the carbohydrate moieties of these allergens.

Introduction

Type I allergic reactions [1] to pollen proteins of the white birch (*Betula verrucosa*, BV) are widespread in central and northern Europe and in the northern and eastern part of North America. Therefore, major efforts have been undertaken to characterize the allergens responsible for IgE-induced mediator release. A 17-kilodalton (kD) glycoprotein was described by Ipsen and Loewenstein [2] to represent the major allergen of BV pollen. This protein has been designated Bet v I [3]. Since the reproducibility of extraction procedures and the exact quantitation of relevant allergens is essential for testing and treatment of allergic patients, much interest has been focused on aqueous protein extracts from pollen. However, the composition of different extracts following the various extraction procedures still varies. Therefore, quantitation of allergens and standardization of extracts can only be undertaken with difficulties.

Our aim was to get more information on BV allergens at the nucleic-acid level, an approach recently attracting interest [4], thus being independent of the various extraction methods of pollen proteins. Following this concept, we isolated RNA from pollen and from male inflorescences of various states of maturation, and translated the isolated messenger RNA (mRNA) in a cell-free wheat germ system in vitro. In this paper, we present the electrophoretic and immunological properties of BV proteins that were translated in vitro in comparison to proteins extracted conventionally.

Materials and Methods

Biological Material

Male inflorescences from BV (four selected trees, Maria Enzersdorf, Vienna) were collected at distinct stages of maturation (June, October, February) and immediately frozen in liquid nitrogen. The material was stored at -70°C until use. Pollen from BV was purchased from Allergon AB (Engelholm, Sweden).

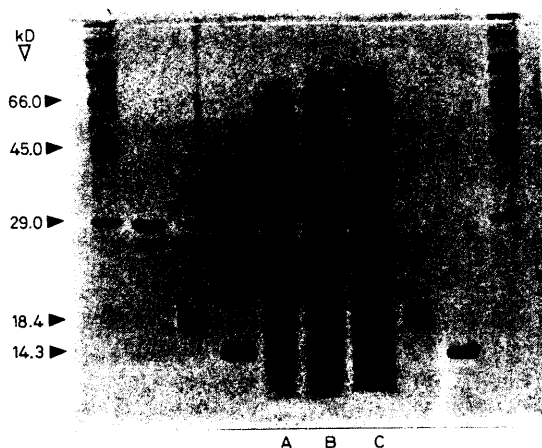


Fig. 1. Aqueous protein extracts from pollen of BV separated by SDS-PAGE (12%). Proteins were stained with Coomassie blue. 60 μ g (lane A), 80 μ g (lane B) and 120 μ g (lane C) of protein were applied to the gel.

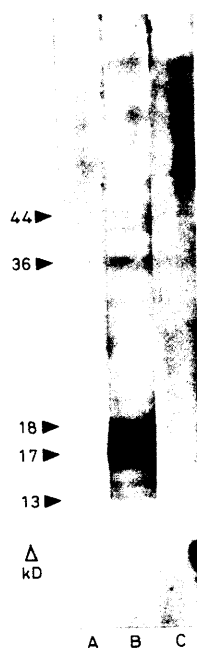


Fig. 2. Detection of IgE binding to proteins from aqueous BV pollen extracts. IgE from RAST 4 pool_a bound strongly to a 17-kD protein and weakly to proteins of 13, 14, 18, 29, 36, 40 and 44 kD (lane B). No reactivity was seen with NHS (lane C). Lane A = buffer control.

Preparation of mRNA

The biological material (10 g inflorescences or 3.5 g pollen) was ground in a mortar under liquid nitrogen. The resulting slurry was immediately shaken vigorously with sterilized glass beads (0.45–0.50 mm in diameter) in 12 ml of extraction buffer [150 mM NaCl, 50 mM Tris(hydroxymethyl)aminomethane (Tris), 5 mM ethylenediaminetetraacetic acid (EDTA), 5% sodium dodecyl sulfate

(SDS), pH 7.4] and 12 ml phenol:chloroform:isoamylalcohol, 24:24:1 (PCI), for 1 min at room temperature and subsequently cooled on ice for 1 min. This procedure was repeated 3 times. The mixture was then centrifuged at 10,000 g at 4°C for 3 min, and the aqueous phase was transferred to a new tube and then again extracted twice with an equal volume of PCI. The final aqueous phase was extracted with CHCl_3 and centrifuged at 10,000 g for 3 min at 4°C. Nucleic acids were precipitated with 2.5 vol of ethanol overnight at -20°C after addition of sodium acetate to a final concentration of 0.3 M.

The precipitate was collected by centrifugation at 10,000 g for 10 min at 4°C. The pellet was redissolved in sterile H_2O containing 0.1% diethylpyrocarbonate (DEP), and the RNA was precipitated with LiCl (final concentration, 2 M). The precipitate was collected by centrifugation as above, the pellet dissolved in H_2O + 0.1% DEP and the ethanol/sodium acetate precipitation was repeated twice. mRNA was enriched by passing the RNA twice over a column containing oligo-dT-cellulose (Sigma, St. Louis, Mo.). Care was taken to avoid enzymatic degradation of RNA during the preparation by using baked glassware and sterilized solutions.

In vitro Translation

mRNA from BV was translated in a wheat germ system (RPN.1, Amersham International, Amersham, UK) according to the manufacturer's instructions. The reaction mixture contained 15 μ l wheat germ system and 0.25 μ g mRNA. A concentration of 120 mM K^+ was found to be optimal. When labeling of the synthesized proteins was desired, 45 μCi ^{35}S -methionine was added. Reactions were allowed to proceed for 40 min at 25°C. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the in vitro synthesized proteins was performed immediately after in vitro translation.

Extraction of Pollen Proteins

Extraction of pollen proteins was performed essentially according to Ipsen and Loewenstein [2] with modifications (addition of 1 mM phenylmethylsulfonyl fluoride, 20 mM EDTA to the extraction buffer, pH 7.0). Extensive dialysis (3–4 days) followed the extraction procedure. Protein concentration was determined according to Bradford [5].

Sera

Sera from 16 untreated patients suffering from birch pollen allergy were tested in a radioallergosorbent test (RAST; Pharmacia, Uppsala, Sweden). These sera showing RAST class 4 to BV pollen allergens were pooled (RAST 4 pool_a) and used for experiments described in this paper. A second RAST 4 pool consisting of sera from 13 allergic individuals was also made (RAST 4 pool_b). Only sera from patients suffering exclusively from pollinosis, according to case history, skin tests and RAST, were selected for these pools. For control experiments, a normal human serum (NHS) pool was used, consisting of sera from 15 persons without case history of any type I allergy. In addition, a series of 30 human sera (RAST 4 to birch pollen allergens) was tested for their binding patterns to allergens extracted from birch pollen. Three serum types with different binding specificity to BV pollen proteins were selected among them. They were not subgroups of the above-mentioned RAST 4 pools. IgE from serum type B bound, in addition to Bet v I, mainly to proteins with higher molecular weights (MWs); IgE from serum type C to Bet v I and, in addition, mainly to proteins of MWs lower than 17 kD; IgE from serum type A solely recognized Bet v I [6].

SDS-PAGE

SDS-PAGE was performed as described by Laemmli [7] using a stacking gel (pH 6.8) and a 12% w/v polyacrylamide gel (0.06% bis-acrylamide). Electrophoresis was run at 12 mA per gel for 16 h. Proteins were stained with 0.06% w/v Coomassie blue R 250.

Electroblotting and Immunoblotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes essentially according to Towbin et al. [8] (for 2 h at 250 mA; Transblot cell, Bio-Rad, Richmond, Calif.). After blotting, unspecific protein-binding sites were blocked with 100 mM H_3BO_3 , 25 mM $Na_2B_4O_7$ and 75 mM NaCl, pH 8.2 (BBS), containing 3% dried milk (BBS-DM) for 15 h at room temperature. The nitrocellulose was cut into 5- or 10-mm strips representing the different lanes in electrophoresis and incubated overnight at room temperature with 500 μ l human serum (1:4, BBS-DM) and then washed 3 times with BBS-DM for 1 min. The strips were incubated with ^{125}I -labeled anti-IgE (3×10^5 cpm/ml BBS-DM) for 15 h at room temperature with gentle agitation. After washing (see above), the strips were dried and exposed to Kodak X-ray films in a Kodak X-omatic cassette for 10 h at $-70^\circ C$.

Autoradiography

Proteins synthesized in vitro and labeled with ^{35}S -methionine were detected by exposure of the SDS-PAGE to a Kodak X-ray film (exposure time, 72 h).

Results

SDS-PAGE Separation of Birch Pollen Proteins

An SDS-PAGE analysis of an aqueous protein extract of birch pollen is shown in figure 1. Major protein bands can be observed in the 12- to 18-kD range and 29-kD range.

Immunoblotting of Aqueous BV Pollen Extracts

Figure 2 shows the immunoblot of an aqueous pollen extract probed with the RAST 4 pool_a (lane B). IgE bound mainly to Bet v I; minor IgE binding could be seen to proteins with MWs of 13, 15, 18, 29, 36, 40 and 45 kD, respectively. No reactivity was seen with NHS (lane C) and buffer (lane A).

SDS-PAGE Analysis of ^{35}S -Methionine-Labeled Proteins of Male Inflorescences of White Birch

RNA extracted from male inflorescences collected in January 1986 was purified by affinity chromatography on oligo-dT-cellulose and translated in vitro using a cell-free wheat germ system with addition of ^{35}S -methionine. After separation by SDS-PAGE, autoradiography revealed major protein bands in the ranges of 12-14, 15-22, 30 and 40-60 kD (fig. 3; lane B). Only a few weak bands appeared in the MW



Fig. 3. Autoradiography of SDS-PAGE analysis of in vitro synthesized proteins from male inflorescences of BV (^{35}S -methionine-labeled). Identical in vitro translation assays were performed without addition of mRNA (lane A) and with addition of mRNA (lane B).

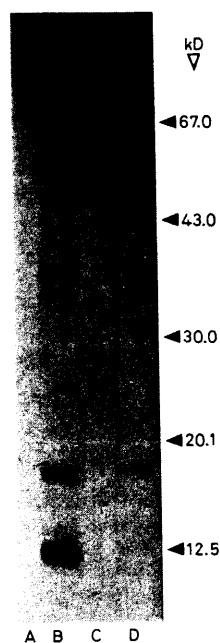


Fig. 4. Detection of IgE-binding proteins synthesized in vitro from mRNA isolated from male inflorescences collected in January. Autoradiography revealed major IgE binding to proteins of an MW of 12.5 kD and minor binding to proteins of an MW of 17 and 40 kD (RAST 4 pool_a, lane B). Weak staining of the same bands can be seen after incubation with NHS (RAST class 1.3, refer to text) in lane D. No IgE binding could be observed without addition of mRNA to in vitro translation assays (lane A = RAST 4 pool_a; lane C = NHS pool).

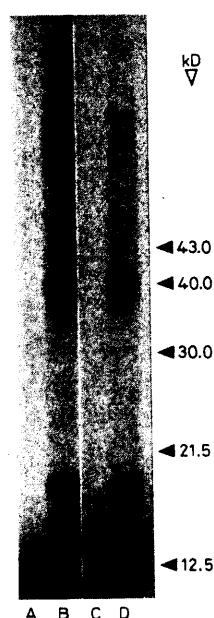


Fig. 5. Detection of IgE-binding proteins synthesized in vitro from mRNA isolated from male inflorescences collected in July. IgE bound strongly to proteins with MWs of 12.5 and 17 kD, and to several bands in the 40-kD region (lane B = RAST 4 pool_a; lane D = RAST 4 pool_b). Minor binding can be seen to bands with MWs of 14 and 16 kD (lanes B, D). No staining was observed in the negative controls without addition of mRNA (lanes A, C).

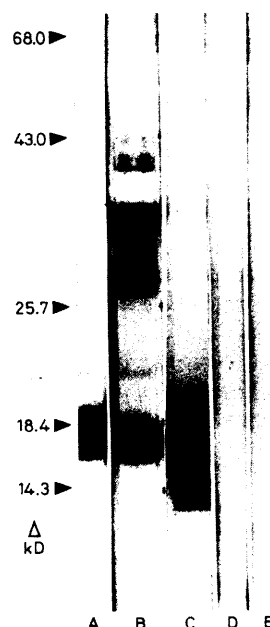


Fig. 6. Detection of IgE-binding proteins from aqueous extracts from BV pollen using distinct patients' sera A, B and C. Three distinct binding patterns were observed: IgE binding to a 17-kD protein only (pool A, lane A), IgE binding to a 17-kD protein and proteins with higher MWs (pool B, lane B), and IgE binding to a 17-kD band and proteins with lower MWs (pool C, lane C). No reactivity was seen in the NHS (lane D) and buffer controls (lane E).

range above 60 kD. No proteins could be detected in an identical assay without addition of mRNA (fig. 3; lane A).

Immunoblotting of Unlabeled BV Proteins Obtained by in vitro Translation

In vitro translation of RNA extracted from male inflorescences collected in January 1986 was performed using unlabeled amino acids. The synthesized proteins were separated by SDS-PAGE, transferred to nitrocellulose and incubated with RAST 4 pool_a, showing IgE binding to several birch pollen proteins (fig. 2) and ¹²⁵I-labeled anti-IgE. Autoradiography showed IgE binding to a band with an apparent MW of 12.5 kD and weaker binding to bands with MWs of 17 and 40 kD, respectively (fig. 4; lane B). No IgE binding could be observed when in vitro translation was performed without addition of mRNA (fig. 4; lane A). A slight binding of IgE to the 12.5-, 17- and 40-kD region was seen when the nitrocellulose strips were incubated with the NHS pool (fig. 4; lane D). However, this NHS pool showed RAST class 1.3

(birch pollen allergens) when reassessed. Again, no IgE binding could be observed in controls performed with NHS without addition of mRNA (fig. 4; lane C).

mRNA prepared from male inflorescences collected in June 1986 was purified and subjected to in vitro translation. After running on SDS gels and subsequent electroblotting, human IgE antibodies from two different RAST 4 pools bound strongly to proteins with apparent MWs of 12.5, 17 and 40 kD (fig. 5; lanes B, D). Minor binding could be observed in the 14- to 15-kD region and in the region of 60–70 kD (fig. 5; lanes B, D). Again, when in vitro translation was performed without addition of mRNA, no IgE binding could be observed (fig. 5; lanes A, C).

Immunoblot Analysis of in vitro Translated BV Proteins Using Patients' Sera with Different Binding Patterns

Three types of sera from patients allergic to birch pollen with distinct IgE-binding specificities to allergens in aqueous pollen extracts (see Materials and

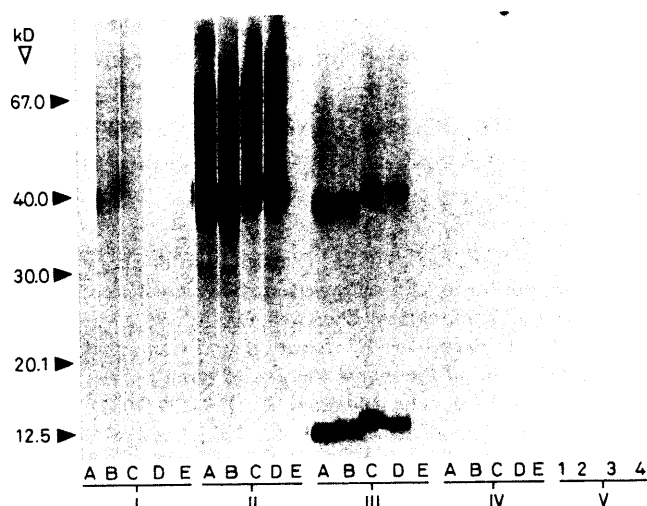


Fig. 7. Detection of IgE-binding proteins as resulting from in vitro translation of mRNA isolated from male inflorescences (July, October and January; lanes A, B and C, respectively) from BV pollen (lanes D), and, as a control, without addition of mRNA (lanes E). Serum type A was used to probe block I, B for II, C for III and NHS for IV. IgE from serum type B stained a protein with an MW of 40 kD (block II), and IgE from serum type C bound strongly to a 12.5-kD band (block III). IgE from serum type A exhibited no reactivity (block I) as did the NHS pool (block IV). mRNA isolated from mimosa (*A. catechu*, Mimosaceae) inflorescences was used for in vitro translations in block V; probing with serum type A, B and C (lanes 1, 2, 3) and NHS (lane 4) showed no IgE binding.

Methods) were chosen for the experiment: IgE from serum type A bound exclusively to the 17-kD protein (fig. 6; lane A). IgE from serum type B bound to the 17-kD protein and mainly to proteins with a higher MW (fig. 6; lane B). Serum type C was characterized by its IgE binding to the 17-kD protein and to proteins of lower MW (fig. 6; lane C). NHS pool (RAST class 0) and buffer showed no reactivity (fig. 6; lanes D and E, respectively).

Serum pools (A, B, C) with the above characteristics were used in probing an immunoblot of in vitro synthesized birch proteins. The mRNA for these experiments was isolated from male inflorescences collected in June, October and January, and from birch pollen, respectively. A blank (no addition of RNA) was included for each set of experiments using the three serum pools A, B and C. Serum pool B stained protein(s) with MW(s) of 40 kD and higher (fig. 7; block II); serum pool C showed IgE binding to the 12.5- and 40-kD proteins (fig. 7; block III), whereas serum pool A showed no reactivity (fig. 7;

block I). In negative controls (without addition of RNA to in vitro translation assays), no IgE binding was observed (fig. 7; lanes E). Probing in vitro translated proteins with NHS (RAST 0), no IgE binding could be detected with or without addition of RNA (fig. 7; block IV). Male inflorescences of a systematically quite unrelated plant (*Acacia catechu*, Mimosaceae) were worked up exactly as described above for BV. Isolated mRNA was translated in vitro and synthesized proteins were analyzed by immunoblotting also using serum types A, B and C (fig. 7; block V, lanes 1, 2 and 3) and NHS (fig. 7; block V, lane 4). In no case could immunological reactive material be observed.

Discussion

Using aqueous extracts from BV pollen, several authors described minor and major allergens [2, 9, 10, 11]. A glycoprotein with an apparent MW of 17 kD – antigen 23 according to Ipsen and Loewenstein [2] – is now considered to represent the major allergen of birch, Bet v I [3]. However, aqueous extracts (fig. 1) have some disadvantages. First, extracting for 24 h at neutral or slightly alkaline pH yields, in addition to proteins, high amounts of non protein material (up to 30% on a weight basis) that interferes with methods of protein quantitation and, more inconveniently, with most of the chromatographic procedures applied for separation and purification of antigens and allergens from pollen [Scheiner, unpubl. observ.]. Secondly, there might be a substantial loss of relevant minor allergens during a 24-hour extraction period. Allergens of constant quality obtained by recombinant DNA techniques would, therefore, be of great help for diagnosis of pollen-induced allergies as well as for hypo-sensitization treatment.

We used in vitro translation techniques in order to characterize BV pollen allergens at a molecular level. Immunoblotting techniques were used to identify some of the in vitro synthesized proteins as allergens, thus characterizing the mRNA isolated as being biologically active and capable of being translated into IgE-binding proteins, i.e. allergens.

When comparing the immunoblots of proteins isolated from pollen with those of proteins translated in vitro from mRNA of male inflorescences, a few interesting facts come to mind.

First, it is remarkable that patients' IgE antibodies

can be used for the detection of allergens in immunoblots, and most certainly this technique will be used for the characterization of both allergens of different origin and the diagnosis of an individual patient's IgE response in the future (fig. 1, 6).

Second, the characteristic pattern of allergenic proteins observed in pollen extracts is reflected by the proteins translated in vitro (fig. 3, 5, 6). This immediately shows that glycosylation of the allergenic proteins (which does not occur in the wheat germ in vitro translation system) does not play a major role for the allergic response. However, this statement needs two qualifications. (1) The minor allergens in the MW range of 30 kD are not detected after in vitro translation. A possible explanation for this fact could be that carbohydrate residues of these proteins may be responsible for provoking an IgE response in allergic patients. (2) The 17-kD allergen is seen only as a weak band in immunoblots of in vitro translated proteins. Possible explanations are: the steady-state concentration of this mRNA might be low; this mRNA might be selectively degraded during isolation, and it might be less efficiently translated in vitro. The series of experiments (fig. 4, 5) also shows that there was virtually no change in mRNA of male inflorescences between June (very immature inflorescences) and January (after meiosis, but before maturity of the pollen). This is surprising, because it could be anticipated that the pollen proteins are synthesized during pollen maturation only. This suggests that these mRNAs are very long-lived.

Third, the results obtained with the three serum pools A, B and C (fig. 6, 7) pose a problem. These three pools chosen represent three types most often found among a large number of patients (58 tested [6]). It can be observed (fig. 6) that individual patients show marked differences in their response to the allergens present in birch pollen (only untreated patients were included in these studies). The minor allergens of 12.5 kD (serum type C) and 40 kD (serum type B) were well recognized by these sera among the in vitro translated proteins. However, the allergen of 17 kD, which shows strong IgE binding in pollen extracts, was recognized in the first series of experiments among in vitro translated proteins, but not by the three serum types in the following experiments (fig. 7). Several explanations are possible, as already mentioned above. We think that the mRNA isolated from pollen or male inflorescences coding for Bet v I is less stable than, e.g., the mRNA coding for the

12.5-kD protein, or, alternatively, the 17-kD protein might be less efficiently translated by the wheat germ system.

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