

Short communication

Comparison of allergenic potentials of timothy (*Phleum pratense*) pollens from different pollen seasons collected in the Belgrade area

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We investigated extracts of timothy grass pollen from four seasons (1989, 1990, 1991, and 1994) by protein content, SDS–PAGE, immunoblot, RAST, RAST inhibition, and crossed immunoelectrophoresis. Extract of the pollen from 1991 showed the lowest yield in quantitative assays. SDS–PAGE, crossed immunoelectrophoresis, RAST, and RAST inhibition expressed approximately comparable patterns for all extracts except that from 1991. Obviously, the quality of grass pollens, as shown for some ragweed (*Ambrosia elatior*) pollens depend on year of collection. Our findings are partially in agreement with some earlier examinations of the quality of timothy pollen from different pollen seasons.

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Up to 20% of the population of developed countries suffer from seasonal or perennial morbidity due to allergic diseases, which are largely the results of aberrant immune responses to environmental allergens (1).

Among aeroallergens, pollen represents the biggest and the most important group eliciting type 1 allergy. In this group, grass pollen is the main cause of IgE-mediated hypersensitivity of the respiratory tract (2). Timothy grass pollen is the most common cause of allergic diseases in Yugoslavia.

Allergen extracts used for diagnostic purposes must support safe, reliable, and reproducible allergy testing. Moreover, to improve hyposensitization therapy, components of the administered extracts should be clearly defined. Therefore, it is important to have pollen extracts of good quality, with equilibrated potencies between different pollen batches (3).

The aim of our investigation was to examine the allergenic potency of timothy pollen, collected in Serbia, from four different pollen seasons, by employing various biochemical and immunochemical

methods. Pollen extracts were compared for protein content and composition by SDS–PAGE; total allergenic activity by RAST and RAST inhibition; and allergen and antigen composition by immunoblot and crossed immunoelectrophoresis (CIE), respectively.

Material and methods

Source of timothy grass pollen

Pollen samples, obtained from the Institute for Immunology and Virology, Torlak, Belgrade, Yugoslavia, were from four pollen seasons as follows: pollen lots 1989, 1990, 1991, and 1994 (codes A, B, C, and D, respectively). Pollens were stored at –20°C in metal containers until use.

Microscopic analysis

Microscopic analysis of the four lots (A, B, C, and D) of pollens was performed by examining pollen grains for identity and purity. All pollen samples contained less than 1% foreign pollen and/or mold spores.

Preparation of the extracts

All timothy pollen extracts were obtained under the same conditions. The extracts were prepared from pollen grains as 10% w/v suspension by stirring the raw material, at 4°C in distilled water and 0.015 M NaN_3 , for 16 h. After extraction, the samples were centrifuged and defatted with carbon tetrachloride 1:1 (v/v). After centrifugation, samples were stored at -20°C before analysis.

Solutions of the extracts were always freshly prepared in distilled water without NaN_3 for immunization of rabbits.

Sera

Antibodies against timothy pollen whole extracts were raised in rabbits essentially according to Harboe & Ingild (4). The animals were injected with 0.5 ml of a 1:1 emulsion of pollen extract (2 mg/ml) in complete Freund's adjuvant. The injection was repeated after 14, 28, and 42 days and every month with incomplete adjuvant. Bleeding was performed 50 days after first immunization and every 2 weeks. The immunoglobulins were isolated by ammonium sulfate fractionation (50% saturation).

Human sera were collected from 20 patients who had positive skin tests, as well as documented positive clinical histories for timothy pollens. All patient sera were screened with Phadebas RAST® and showed specific IgE binding (RAST classes 2-4).

Protein determination

Protein concentrations were determined by a modified Lowry procedure (5, 6). A calibrated solution of bovine serum albumin (BSA) (Torlak, Belgrade, Yugoslavia) served as standard.

Polyacrilamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a vertical slab gel 4/12%, according to Laemmli (7), in a Bio-Rad apparatus. About 20 µl of each protein extract was applied onto the gel. SDS-PAGE was performed in reducing conditions at 80 V for 1 h and 120 V for another 2 h. The gel was stained with 0.05% Coomassie brilliant blue R-250 in water:methanol:acetic acid (50:40:10).

Western immunoblot

Electrophoretically separated proteins were transferred from the gel to a nitrocellulose membrane (0.45 µm) by electrophoretic transfer in a Trans-

Blot cell (Bio-Rad). The gel was equilibrated for at least 15 min in blotting buffer that was composed of 25 mM Tris, 192 mM glycine, 20% methanol, and 1% SDS, pH 8.3. The blot was performed at 300 mA for 3 h. After the transfer was complete, the unoccupied protein binding sites on nitrocellulose membrane were blocked by equilibration for 60 min in 20 mM Tris buffer saline (TBS), pH 7.4, containing 0.3% BSA. After blocking, the nitrocellulose membrane was washed in 20 mM TBS, pH 7.4, 3×15 min, and incubated overnight at room temperature with the patient sera pool diluted five times with 20 mM TBS, pH 7.4, 1% BSA. After washing for 4×15 min, the membrane was incubated with alkaline phosphatase-labeled anti-IgE (Byk Gylden Pharmazeutica, Germany) in a 1:1000 dilution for 2 h. The membrane was washed 4×15 min in 20 mM TBS, and immersed in chromogenic substrate for 30 min at room temperature. The mixture of 0.15 mg/ml BCIP and 0.3 mg/ml NBT in 0.1 M Tris, pH 9.5, and 0.5 mM MgCl_2 was used as substrate. The color reaction was stopped by washing the membrane in distilled water.

Crossed immunoelectrophoresis (CIE)

CIE was performed as described by Løwenstein (8). Briefly, about 7 µl of each protein extract was applied to 1% agarose gel, on a 5×7 cm glass plate. Electrophoresis was carried out at 15°C in a buffer containing 5 mM barbital and 25 mM Na-barbital, pH 8.6, for 90 min at 8 V/cm. The second-dimension electrophoresis perpendicular to the first was carried out in 1% agarose gel containing 17 µl of rabbit antitimothy antibody per cm^2 gel, for 20 h at 2 V/cm.

Radioallergosorbent test (RAST)

Specific IgE antibodies for timothy pollens were determined by the RAST method. Paper disks (Schleicher & Schull, No. 2043, diameter 5 mm) were activated by CNBr, by the method of Ceska et al. (9). A pool of sera from 20 timothy-allergic patients, RAST classes 2-4, served as specific IgE source. Monoclonal anti-IgE (10) was performed in the laboratory for radioisotopes ("Vinca" Institute of Nuclear Sciences, Vinca, Belgrade, Yugoslavia). Affinity-purified anti-IgE monoclonal antibody (10 µg) was labeled with 37 MBq Na^{125}I (Polatom, Poland) by the chloramin-T method (11).

RAST inhibition

The allergenic activity of the extract was determined by RAST inhibition, with Phadebas allergen

paper disks prepared with a timothy pollen reference extract (Pharmacia, Uppsala, Sweden). RAST inhibitions were performed as described by Gleich et al. (12).

Results

Microscopic analysis

The micrographies of all four timothy pollens showed the same pollen grains of *Phleum pratense* species.

Protein content

Protein values, measured by a modified Lowry procedure, for different extracts were as follows: sample A, 3.10 mg/ml; sample B, 3.17 mg/ml; sample C, 1.04 mg/ml; and sample D, 3.30 mg/ml.

SDS-PAGE characterization of timothy pollen extracts

In SDS-PAGE, samples A, B, and D showed complex and mostly similar patterns (Fig. 1), different from sample C. Sample C lacked some protein bands of lower molecular weights which existed in other samples, but major bands of about 35 kDa and 55–65 kDa did appear, and they had lower intensity.

Allergenic patterns of timothy pollen extracts

The differences among allergen patterns of four timothy pollen batches in immunoblot (Fig. 2) were less than in SDS-PAGE. It is noteworthy that sample C, with weaker quality (shown in SDS-PAGE and CIE), retained the main allergenic bands of about 35 kDa and 55–65 kDa.

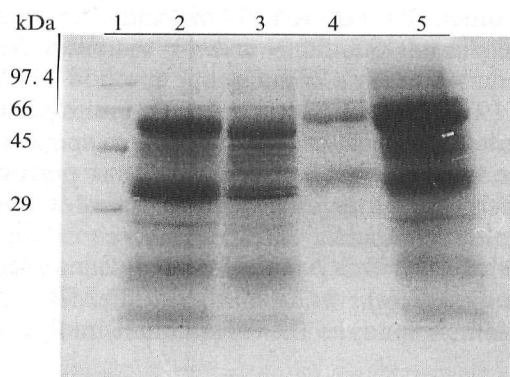


Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of timothy pollen extract. Molecular weight markers (lane 1), sample A (lane 2), sample B (lane 3), sample C (lane 4), and sample D (lane 5).

CIE characterization of timothy pollen extracts

The CIE patterns of samples A, B, and D were complex and similar, particularly samples A and B, while sample C showed the weakest pattern (Fig. 3). The antigenic profiles of pollen samples were as follows: sample A, 24; sample B, 26; sample C, 19; and sample D, 25 immunoprecipitates.

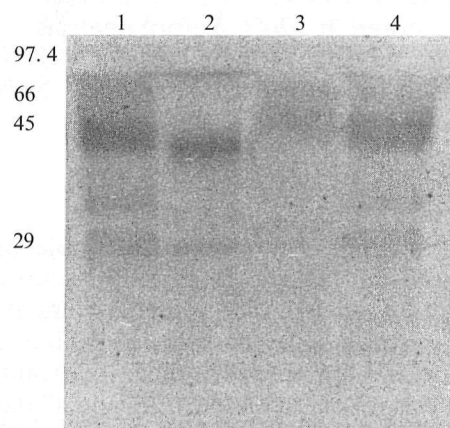


Fig. 2. Immunoblotting of timothy pollen extracts; sample A (lane 1), sample B (lane 2), sample C (lane 3), and sample D (lane 4).

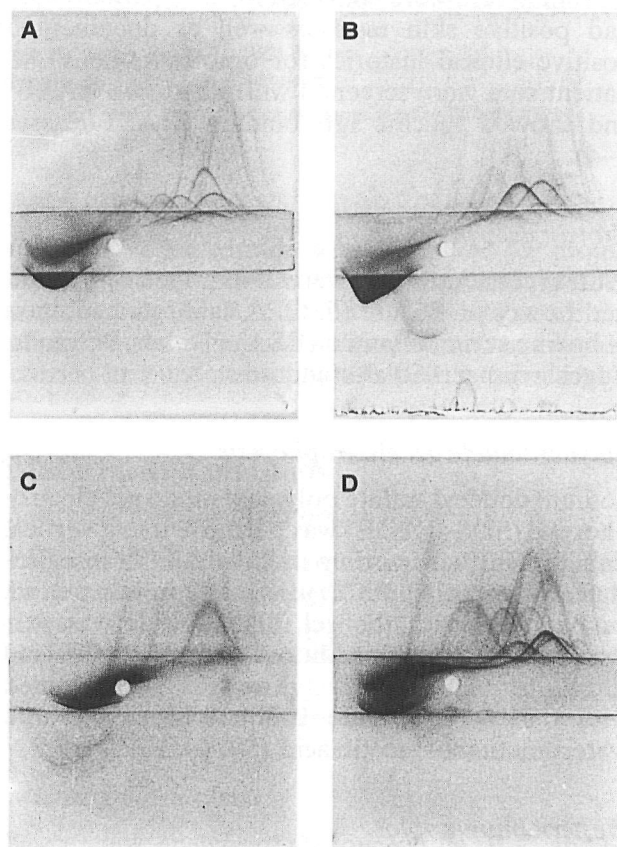


Fig. 3. Crossed immunoelectrophoresis of sample A (A), sample B (B), sample C (C), and sample D (D). → first dimension; ↑ second dimension.

Allergenic activity of timothy pollen extracts

In RAST (Fig. 4), sample D expressed the greatest allergenic potential (100%), and sample C the smallest (60%). The total allergenic activity, measured by RAST inhibition, was plotted as regression lines (Fig. 5). All extracts except extract C expressed nearly the same total allergenic potency.

Discussion

This paper reports year-to-year variations in the quality of timothy pollen, in partial agreement with some earlier investigations (13).

All pollen batches were collected in the Belgrade area by vacuuming plants in the field, and allowing the collected material to dry. The homogeneity of pollen grains was determined by examining raw material under $\times 400$ magnification. All four pollens contained less than 1% foreign pollen and/or mold spores. Only the color of sample C pollen was darker than the others, and this may have resulted from the meteorologic conditions for that year of collection (high humidity during the period of pollen maturation).

The protein values of the pollen extracts, measured by a modified Lowry procedure, were in the range of 1.04 (sample C) to 3.30 mg/ml (sample D).

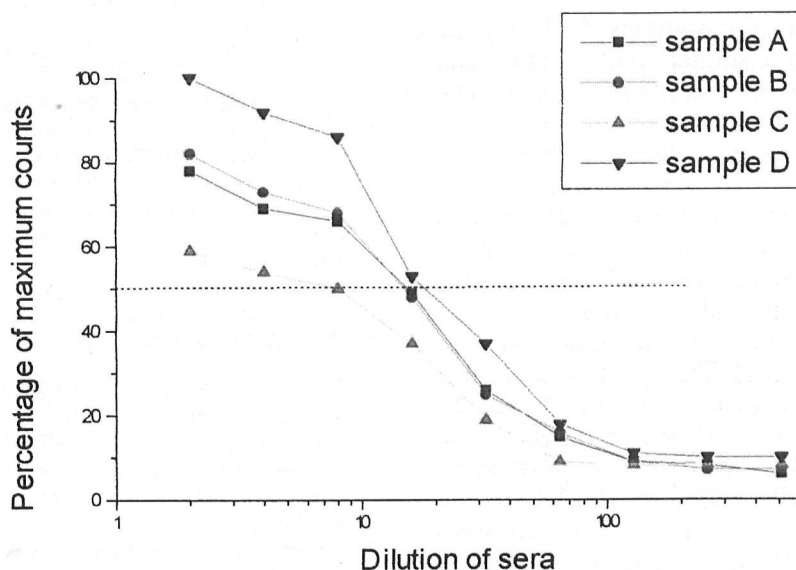


Fig. 4. Percentage of binding of antitimothy IgE to RAST disks of timothy pollen samples.

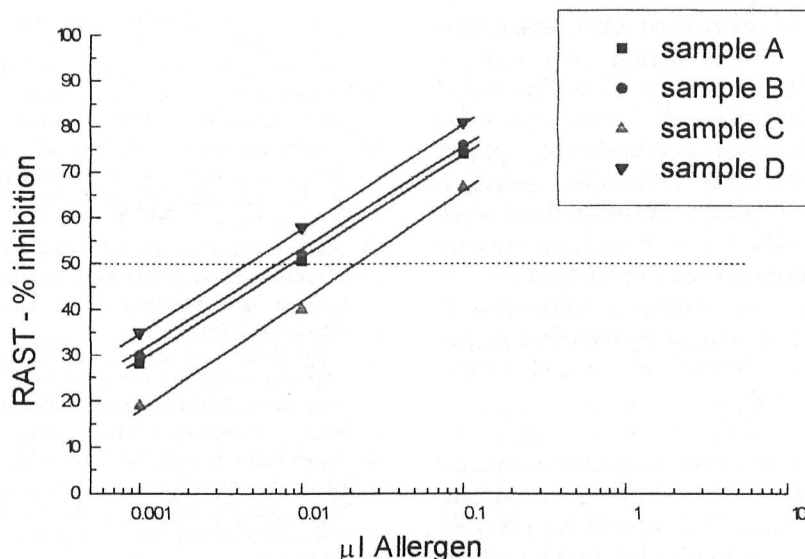


Fig. 5. Regression lines of RAST inhibition experiments with timothy pollen extracts.

These results showed a remarkably different content of extracted proteins for sample C compared with other samples, as confirmed by SDS-PAGE. The main protein bands, according to staining with Coomassie brilliant blue R-250, in all samples were about 30–35 kDa and 55–65 kDa. Sample C exhibited a very weak protein pattern in SDS-PAGE, particularly those of lower molecular weights of about 15–20 kDa.

In this study, we used immunoblotting to detect IgE specific for timothy pollen proteins. The most allergenic protein bands were the prominent bands in SDS-PAGE of about 30–35 kDa (allergens group 1/5) and 55–65 kDa (allergens group 4). In spite of weak quality, sample C also contained allergens of about 35 and 55 kDa, which were of lower intensity.

The CIE patterns of the samples A, B, and D were very complex and similar, particularly samples A and B, as opposed to sample C (Fig. 3). The most prominent band of all the samples was basic protein belonging to group 4 allergens. Moreover, this result is in agreement with the findings of Maasch et al. (13) that European timothy pollen samples show a marked band at about 60 kDa, corresponding to the basic allergen Phl p 4, unlike North American timothy pollen samples.

The allergenic potentials of four pollen batches were determined by RAST. The greatest allergenic potential (100%) was shown by sample D (Fig. 4). Pollen samples A and B exhibited about 80%, and sample C about 60% of allergenic potential. According to the RAST inhibition results, we can say that all pollen extracts were qualitatively similar in allergenicity, and only extract C differed in quantitative allergenicity. In other words, sample C showed four times less allergenic potential than the other samples in terms of average values, in contrast to the results of Maasch et al. (13), where the 50% inhibition values were within very narrow ranges (CV=25%). Furthermore, it should be noted that results from RAST (timothy pollen proteins from different batches being coupled to paper disks) and RAST inhibition (Phadebas allergen paper disks with reference to timothy pollen) were in good agreement, when the potencies among pollen batches were compared (Figs. 4 and 5).

The most similarities, according to SDS-PAGE and immunoblot, were expressed by samples A and D, but according to CIE, RAST, and RAST inhibition, by samples A and B.

Our investigations of extracts of four different timothy pollen batches showed comparable quality for samples A, B, and D, by protein content, SDS-PAGE, immunoblot, CIE, RAST, and RAST inhibition. All employed methods showed many

differences in results for sample C. From these results, we think that the three batches coded A, B, and D could serve as in-house reference preparations for timothy pollen extracts.

Because biologic systems are dynamic, it is reasonable to assume that allergen source materials vary (14, 15), and that meteorologic factors influence the allergen composition of an extract. These factors may explain the difference in quality, as well as in allergenic potential, of the timothy pollen sample C from the other pollen samples.

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