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Isolation and partial characterization of Fes p 4 allergen

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SUMMARY

More than 75% of grass pollen-allergic patients produce specific IgE antibodies against group-4 allergens. Purification and characterization of different grass group-4 allergens should help to further understand their allergenicity. In this study, an attempt was made to isolate and characterize Fes p 4 allergen by several biochemical and immunochemical methods. Fes p 4 was purified by a combination of chromatographic techniques (gel permeation and ion exchange chromatography). Isolated protein revealed four main spots at a molecular weight of 60 kDa and a pI ranging from 8.7 to 9.1. Eight sera were selected from patients with positive result of skin prick test to the mixture of grass pollen extracts. ELISA inhibition technique was used to study Fes p 4-specific IgE in the patients' sera. ELISA to *Festuca pratensis* was inhibited up to 80% by *F. pratensis* pollen extract and up to 48% by Fes p 4. 2D-PAGE-immunoblot was used to identify allergenic and antigenic components of Fes p 4 with patients' IgE and monoclonal antibodies (MABs). Three components of purified protein expressed IgE binding ability. Two MABs which recognized unrelated regions on Phl p 4, bound three components of Fes p 4. The role of the carbohydrate moiety in allergenicity was examined with individual patient sera by using periodate-treated Fes p 4. Six out of eight patients reduced IgE binding to periodate-treated allergen. Isolated Fes p 4 glycoprotein consisted of four components, three of which were allergenic, and share common epitopes specific for grass group-4 homologs. The results of periodate oxidation of Fes p 4 suggest that the carbohydrate moiety is involved in IgE binding.

Key words: IgE reactivity - Fes p 4 - Glycoprotein - *Festuca pratensis* - Grass group-4 pollen allergen - Monoclonal antibody

INTRODUCTION

Within some communities, allergy to grass pollens is more prevalent than to other aeroallergens (1). The allergen extracts used in clinical work on allergy contain, beside allergens, a number of protein antigens without allergenic activity (2). The identification and characterization of grass pollen allergens is of substantial importance in diagnosis, treatment and basic research of grass pollen allergy (3).

Little is known about group-4 grass pollen allergens, although more than 75% of grass pollen-allergic patients produce specific IgE antibodies against group-4 allergens (4). These high molecular weight (50-65 kDa) allergens are basic (pI 9-10) and glycosylated (approx. 10-20% w/w) (5) and represented in various species (6).

Compared to natural allergen extracts, recombinant allergens have a clearly defined primary structure, which enables easy quantification (7). Beside being applicable in diagnostics and immunotherapy (8, 9), the expression of recombinant allergens in *Escherichia coli* lacks post-translational modifications e.g., glycosylation. According to van Ree *et al.* recombinant allergens produced in *E. coli* did not equal the IgE-binding capacity of their natural counterparts (10), while Petersen *et al.* found that rPhl p 1 from *Phicia pasteuris* is hyperglycosylated compared to nPhl p 1 (7). Therefore, the isolation and characterization of natural allergens, such as grass group-4 glycoproteins, is important for creating and comparing possible recombinant homologs with all the relevant IgE-binding epitopes. The existence of different isoforms of allergens must also be considered when recombinant proteins are used in immunothera-

py since all allergenic isoforms may need to be present in the evaluation and treatment of patients (11).

The aim of this study was to isolate and biochemically characterize Fes p 4 from *Festuca pratensis* pollen and determine its prevalence in eight patients with positive clinical history to grass pollen allergy. The role of the carbohydrate moiety in allergenicity was examined with individual allergic patients' sera by using periodate-treated Fes p 4.

MATERIALS AND METHODS

Pollen extract

F. pratensis pollen was obtained from the Institute for Immunology and Virology, Torlak, Yugoslavia. The extract was prepared from pollen grains as 10% (w/v) suspension by stirring raw material at 4 °C in distilled water and 0.015 M NaN₃ for 4 h. After extraction, the sample was centrifuged and defatted with carbon tetrachloride 1:1 (v/v). The sample was stored at -20 °C before analysis.

A solution of the extract was always freshly prepared in distilled water without NaN₃ for immunization of rabbits.

Antibodies

Antibodies against Fesp extract were raised in rabbits essentially according to Harboe and Ingild (12). An immunoglobulin fraction was prepared by ammonium sulfate fractionation (45% saturation).

Human sera were collected from eight adult patients with seasonal allergic rhinitis and positive results of skin prick test for grass pollens. These patients did not receive immunotherapy before the collection of their serum samples. For some experiments, a pool was made by mixing equal amount from each serum. A pool of three normal sera (nonatopic) was used as a negative control.

Murine monoclonal antibodies were produced after immunization with timothy grass pollen extract (13) or enriched Phl p 4 (14).

Isolation of Fes p 4 allergen

F. pratensis pollen extract was fractionated by ammonium sulfate to reach 50% saturation. The pellet was collected by centrifugation and dissolved in a minimal volume of 0.15 M NaCl. The protein solution was fractionated on a Sephadex G-100 Superfine (1.8 × 90 cm) column, preequilibrated with 0.15 M NaCl, 0.015 M NaN₃. Collected fractions were analyzed by fused rocket immunoelectrophoresis (15). The fractions containing cathodic antigen were pooled and concentrated by ul-

trafiltration. The protein solution was dialyzed against 30 mM Tris-HCl pH 8.6, and rechromatographed on a QAE-Sephadex A-50 (0.8 × 5.0 cm) column preequilibrated with 30 mM Tris-HCl pH 8.6.

Gel electrophoresis

SDS-PAGE

Protein analysis was performed under reducing conditions in a vertical slab gel (4/10%), according to Laemmli (16), in the Bio-Rad Mini-Protein II apparatus. A low molecular weight electrophoresis calibration kit (Pharmacia) was used for molecular weight determination. After electrophoresis proteins were either silver stained or transferred to a nitrocellulose membrane (Serva) for immunodetection according to Harlow and Lane (17).

Isoelectric focusing

Isoelectric focusing (IEF) was performed in the model 2117 Multiphor cell (LKB Pharmacia) according to the manufacturer's instructions. The samples applied on a polyacrylamide gel (80 × 83 × 7.5 mm³), containing 2% (pH 3-10) Pharmalyte[®] and 0.4% (pH 9-11) Servalyte[®] carrier ampholytes, were focused at 2000 Vh at 65 mA, 5 W at 10 °C. The Pharmacia broad pI calibration kit (pH 3.5-9.3) was used for pI determination.

Two-dimensional PAGE

In the first dimension, isoelectric focusing was performed as described above, then the gel was sliced in strips. After incubation for 10 min in a SDS-containing sample buffer, in the second dimension SDS-PAGE was carried out in discontinuous gel (4/10%) as mentioned above.

Western blot

After separation by SDS-PAGE or 2D-PAGE, the proteins were subsequently transferred on a nitrocellulose membrane by semi-dry blotting for an hour at 0.8 mA/cm². For further immunological examinations the blots were incubated with antibodies (human serum or murine monoclonal antibodies) and visualized with appropriate secondary antibodies, according to Harlow and Lane (17).

IEF-immunoelectrophoresis

IEF-immunoelectrophoresis was performed according to Smyth *et al.* (18). After IEF, the gel slice was

positioned by placing it on the antibody-free agarose gel. The gel was run 17 h at 2 V/cm.

Periodate oxidation of Fes p 4

Periodate oxidation of carbohydrate structures was performed after blotting Fes p 4 onto a nitrocellulose membrane by incubation in 10 mM NaIO₄ in 10 mM sodium acetate buffer pH 5.0. The reaction was performed in the dark at 4 °C for an hour. The control nitrocellulose was incubated in the same buffer without NaIO₄. The efficiency of oxidation was examined by incubation of control and periodate-treated nitrocellulose with concanavalin A (conA) (20 µg/ml) in 50 mM Tris-HCl pH 7.6, 0.15 M NaCl, 1 mM MnCl₂, 1 mM CaCl₂, 1 mM MgCl₂ for an hour. The binding of conA lectin to the nitrocellulose was detected by using horseradish peroxidase (25 µg/ml) and diaminobenzidine according to Harlow and Lane (17).

ELISA-inhibition

ELISAs were performed by the method of Voller *et al.* (19). Inhibition assays were performed in triplicate by preincubating 10 µl pooled patient sera with 40 µl inhibitor (Fesp extract and Fes p 4) in 10-fold diluted concentrations (ranging from 10 µg/ml to 0.001 µg/ml) for 30 min at 4 °C. The mixture was then added to *F. pratensis*-coated plate. Human serum from subjects with negative skin tests to grass pollen extracts was used as a negative control. After overnight incubation at 4 °C, specific IgE bound to the plate was detected with alkaline phosphatase labelled anti-IgE (Sigma) and with 4-NPP as a substrate. Absorbance measurements were done at 405 nm after adjusting the background with negative control. Percent of inhibition was calculated as %inhibition = OD of the test sample - (OD of the inhibited sample/OD of the test sample) × 100. IC₅₀ value was determined as a concentration of inhibitor at 50% of inhibition.

RESULTS

Purification of Fes p 4

Glycoprotein Fes p 4 was isolated from a pollen extract of *F. pratensis* by the combination of ammonium sulfate precipitation (50%), gel permeation chromatography and ion exchange chromatography. Purification steps were followed by reductive SDS-PAGE (Fig. 1), and a single broad protein band with a molecular weight about 60 kDa was obtained for Fes p 4. According to densitometric analysis of the silver stained gel, isolated Fes p 4 revealed more than 97% of purity. Fes p 4 also showed one band in Western immunoblot with anti-Phl

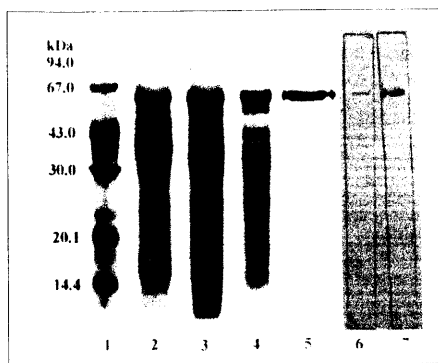


Fig. 1. SDS-PAGE analysis of Fes p 4 purification. Proteins were silver-stained. 1) molecular weight markers; 2) Fesp extract; 3) proteins fractionated by 50% ammonium sulfate; 4) enriched Fes p 4 after Sephadex G 100; 5) Fes p 4 after QAE-Sephadex; 6) Fes p 4 recognized by 3C.4 monoclonal antibody; and 7) Fes p 4 recognized by 2D8 MAb.

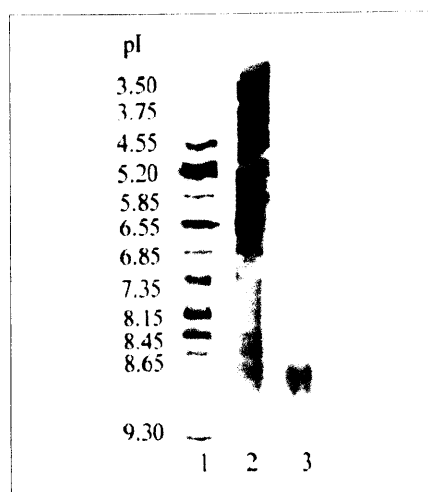


Fig. 2. Isoelectric focusing analysis of 1) pI markers; 2) Fesp extract; and 3) Fes p 4. Proteins were silver-stained.

p 4 MABs, which bound Phl p 4 homologs in various grass pollen extracts (4). In order to improve the resolution of Fes p 4 components, besides the broad range Pharmalyte® (pH 3-10), basic Servalyte® (pH 9-11) were added to the IEF gel. In IEF Fes p 4 revealed four isoforms with pI ranging from 8.7-9.1 (Fig. 2). Microheterogeneity of Fes p 4, concerning pI, can be seen as four spots in 2D-PAGE of Fesp extract (Fig. 3).

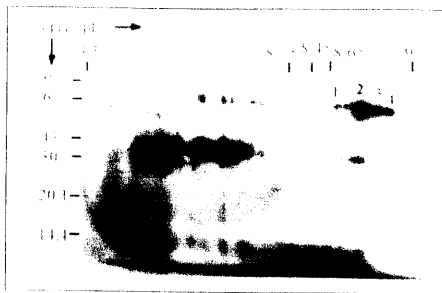


Fig. 3. 2D-PAGE analysis of Fesp extract. Proteins were silver-stained.

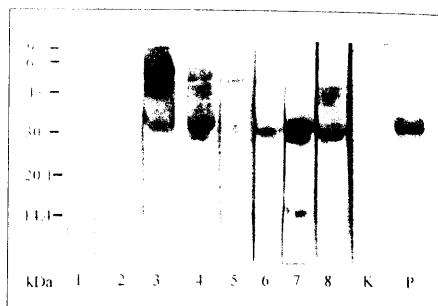


Fig. 5. Western immunoblot of Fesp extract with individual sera (1-8); K) control; P) nitrocellulose stained with Ponceau S.

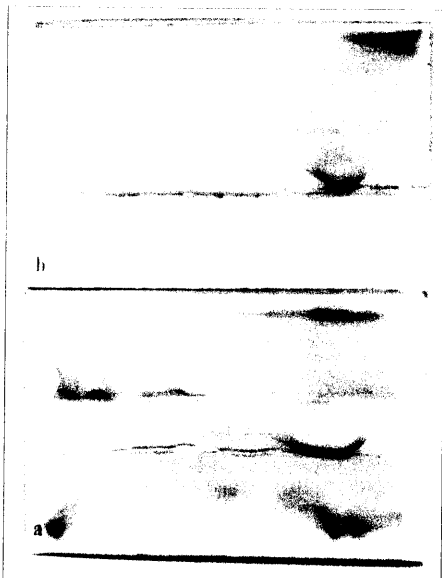


Fig. 4. IEF-immunoelectrophoresis of: a) Fesp extract; b) Fes p 4.

Antigenic identity as well as pI microheterogeneity of isolated Fes p 4 was shown by IEF-immunoelectrophoresis (Fig. 4).

Immunological detection

Human sera were collected from patients with positive skin prick test results for grass pollen extracts (SPT classes 3+ to 5+, with histamine used as a reference). The allergenic pattern of Fesp protein extract

was obtained for eight patients by Western immunoblot (Fig. 5). All patients were allergic mostly to the protein bands in the molecular weight range of 55-60 kDa (8/8), 30-40 kDa (8/8), and 15-18 kDa (6/8).

The immunological characterization of isolated Fes p 4 was carried out by 2D-PAGE followed by immunoblot. Figure 6 shows the binding patterns of 2D8 anti-

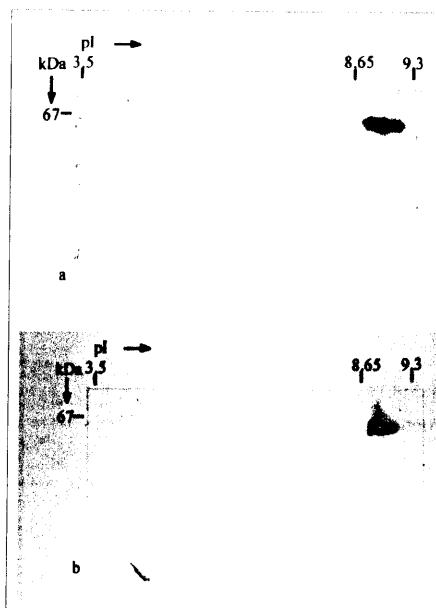


Fig. 6. 2D-PAGE-immunoblot of Fes p 4; a) nitrocellulose incubated with 2D8 monoclonal antibody; b) nitrocellulose incubated with pool of patients' sera.

Phi p 4 MAB (a) and pooled patients' sera (b). On both figures, three spots of Fes p 4 revealed antigenicity and allergenicity, respectively. The detection of the fourth component of Fes p 4 was more difficult, either as allergen or antigen, due to its small amount, as well as possible loss in the electrotransfer.

Periodate oxidation

The possibility of a carbohydrate moiety involvement in the IgE binding to Fes p 4 was investigated by periodate oxidation. The efficiency of periodate treatment of the carbohydrate moiety was confirmed by the disappearing of conA binding to the oxidized Fes p 4, compared to the control (Fig. 7). From eight patients with specific IgE for the allergen, only two retain almost the same ability to bind for periodate-treated Fes p 4. These results proved, in qualitative terms, the involvement of the carbohydrate moiety of Fes p 4 in allergenicity in six patients, and conA binding ability of the glycoprotein.

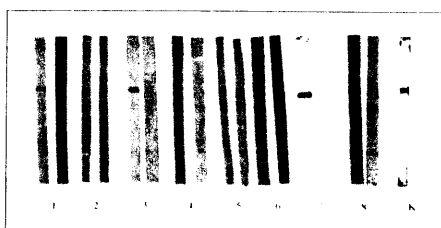


Fig. 7. Western blot of Fes p 4: (–) nitrocellulose incubated in 10 mM sodium acetate buffer pH 5.0; (+) nitrocellulose incubated in 10 mM NaIO₄/10 mM sodium acetate buffer pH 5.0; 1–8 nitrocellulose incubated with individual patient's sera; 9) nitrocellulose incubated with concanavalin A.

ELISA inhibition

The inhibition capacity of Fes p 4 was compared with inhibition capacity of the Fesp extract. Inhibition curve for Fesp extract achieved a plateau at about 80% of inhibition, and 50% of inhibition was attained at the concentration of 0.03 µg/ml. Inhibition curve for Fes p 4 as an inhibitor has a moderate slope, and at the concentration of 10 µg/ml there was a 48% of inhibition of IgE binding from a pool of allergic patient sera. By applying interpolation IC₅₀ value was determined for Fes p 4 to be 15 µg/ml.

DISCUSSION

Up to 75% of patients allergic to grass pollen were found to exhibit IgE reactivity to group-4 allergens, hence

they are classified as the major grass pollen allergens (20). Several grass group-4 allergen have been characterized thus far: Phi p 4 (20–26), Dac g 4 (6), Lol p 4 (27, 28), BG60 (29, 30).

The rapid progress made in the field of molecular allergen characterization appears likely to considerably improve the use of recombinant allergens in diagnosis and specific immunotherapy (31). It is well known that *E. coli*-derived recombinant allergens lack post-translational modifications (glycosylation) and sometimes are improperly folded (disulfide bonding), which could be expected for the expression of proteins with higher molecular weight. Although eukaryotic systems are preferred, Petersen *et al.* (7) gained hyperglycosylated rPhi p 1 compared to nPhi p 1 using *Pichia pasteuris* for expression. The same group of authors indicated that a single post-translational modification can alter the IgE reactivity of allergens or short peptides and should be taken into account when recombinant allergens are used for diagnostics and immunotherapy (32). Therefore, the isolation and characterization of natural allergens is indispensable to create their structural as well as functional recombinant counterparts.

Fes p 4 allergen, isolated by classical biochemical methods, consists of four components with pI ranging from 8.7–9.1, and the same molecular weight of about 60 kDa, according to SDS- and 2D-PAGE. Densitometric analysis of Fes p 4 showed that the components are not represented in equal amounts. Three allergenic components were identified by 2D-PAGE-immunoblot, with a pI from 8.8–9.1. This microheterogeneity was first observed by Su *et al.* (29, 30). Namely, they indicated that antigen BG60 contains four isoallergens, with an apparent molecular weight of 55–60 kDa as determined in SDS-PAGE. The lack of immunological detection of a Fes p 4 component with pI 8.7 may be due to its lower amount compared to the other three, or possible loss in the electrotransfer.

The development of monoclonal antibodies has had an important impact on characterization, purification and quantification of antigenic molecules. Allergen-specific MABs have been used in the development of sensitive and specific immunoassays for allergens and in the study of cross-reactivity and immune structure of allergens (4, 6). Two MABs, 2D8 and 3C4 (produced on unrelated regions of Phi p 4) which recognized grass group-4 homologs were used in characterization of purified Fes p 4. Western immunoblot showed that the cross-reactive determinants of Fes p 4 were preserved during the purification. According to 2D-PAGE-immunoblot all three components of Fes p 4 share the same or related epitopes specific for grass group-4 homologs.

In addition to genetic variants expressed in their polypeptide chains, almost all glycoproteins reveal another form of polymorphism associated with their carbohydrate residues (33). Expressed microheterogeneity of

Fes p 4 concerning pl could be either a consequence of a different degree of glycosylation of the protein core, or spotted mutations in amino-acid sequence or both. A further structural characterization should clarify the origin of pl microheterogeneity. We observed pl microheterogeneity for some other grass group-4 glycoproteins, such as Phl p 4, Dac g 4, Ant o 4, and Sec c 4 (unpublished results), while Haavik *et al.* reported microheterogeneity of Phl p 4 concerning the carbohydrate part of the glycoprotein (25).

The allergenic activity of the purified allergen was investigated in an ELISA inhibition experiment performed with the patient sera pool. Fes p 4 achieved 50% inhibition at an amount of 0.15 µg, while Fesp extract achieved 50% inhibition at 0.03 µg. This result is in agreement with findings that group-4 allergens seem to be frequently recognized in IgE-immunoblotting studies (8, 23), although the levels of specific-IgE are relatively low compared with group 1 and 5 (4, 34).

It was found that *F. pratensis* grass pollen is a very frequent cause of allergic reactions in Yugoslavia, almost as frequent as *Phleum pratense* (35, 36) and *Dactylis glomerata* (36). The major Fesp allergens belong to group 1/5, 4, 2/3 of grass pollen allergens. This result is in agreement with findings for major allergen groups in other grass pollen species, and thus supports the concept of defining a limited panel of recombinant allergens for each patient and using them in immunotherapy (8).

The importance of Fes p 4 was exhibited by the finding that all sera from grass pollen-allergic patients included in the study contained IgE antibodies to this allergen. The allergenicity of carbohydrate moiety was examined by periodate treatment of blotted Fes p 4. The lack of conA binding to periodate-treated nitrocellulose, the opposite to the control, confirm that the carbohydrate moieties were destroyed. Besides oxidation of carbohydrate structures, periodate treatment may also alter amino acids in the polypeptide chain (37). Although we chose milder conditions (10 mM NaIO₄/10 mM sodium acetate pH 5.0, 1 h, 4 °C) in comparison to optimized reaction conditions determined in comparable Western blotting studies on grasses (50 mM NaIO₄/10 mM sodium acetate pH 5.0, 2 h at room temperature) (38, 39), the periodate oxidation of Fes p 4 reduced IgE reactivity of six patients' sera. A similar result, but in quantitative values, was gained for the binding of allergic sera to oxidized BG60 which was reduced about 30-40% compared to the unoxidized BG60, suggesting that the carbohydrate of BG60 is involved in the human IgE binding domain (30). It should be mentioned that Haavik *et al.* (24) found that most of the binding sites for human IgE and IgG on the Phl p 4 allergen did not contain carbohydrate destroyed by periodate oxidation or removed by the glycosidases used in their study. Further structural characterization of the IgE-binding

epitopes should help in understanding allergenicity of group-4 allergens and creating possible recombinant homologs.

Our study clearly showed that isolated Fes p 4 allergen is not a homogenous glycoprotein. It consists of four components differing in pl with similar molecular weight, three of them bound IgE from pool of patients' sera. All three components share at least two common epitopes specific for grass group-4 homologs. It seems that carbohydrate structure takes part in allergenicity of Fes p 4 allergen.

REFERENCES

1. Becker, W.M. Grass pollen allergens and relevant substructures. XVI Eur Congr Allergol Clin Immunol 1995, 529-36.
2. Nielsen, L., Lowenstein, H., Weeke, B. Quantitative immunoelectrophoresis used in analysis of the antigens in timothy (*Phleum pratense*) pollen extract. Acta Allergologica 1974, 29: 385-401.
3. Fahlbusch, B., Müller, W.D., Diener, C.H., Jäger, L. Detection of crossreactive determinants in grass pollen extracts using monoclonal antibodies against group IV and group V allergens. Clin Exp Allergy 1993, 23: 51-60.
4. Fahlbusch, B., Müller, W.-D., Rudeschko, O., Jäger, L., Cromwell, O., Fiebig, H. Detection and quantification of group 4 allergens in grass pollen extracts using monoclonal antibodies. Clin Exp Allergy 1998, 28: 799-807.
5. Matthiesen, F., Klysner, S., Lowenstein, H. Characteristics of grass pollen allergens. Proceedings of Workshop held under the Aegis of the XIVth Congress of the European Academy of Allergy and Clinical Immunology. The UCB Institute of Allergy, Berlin 1989, 9-11.
6. Leduc-Brodard, V., Inaco, F., Jaquinod, M., Forest, E., David, B., Peltre, G. Characterization of Dac g 4, a major basic allergen from *Dactylis glomerata* pollen. J Allergy Clin Immunol 1996, 98: 1065-72.
7. Petersen, A., Grobe, K., Lindner, B., Schlaak, M., Becker, W.-M. Comparison of natural and recombinant isoforms of grass pollen allergens. Electrophoresis 1997, 18: 819-25.
8. Valenta, R., Vrtala, S., Ebner, C., Kraft, D., Scheiner, O. Diagnosis of grass pollen allergy with recombinant timothy grass (*Phleum pratense*) pollen allergens. Int Arch Allergy Immunol 1992, 97: 287-94.
9. Hakkaart, G.A.J., Harmsen, M.M., Chua, K.-Y., Thomas, W.R., Aalberse, R.C., van Ree, R. Expression of the house dust mite allergen Der p 2 in the baker's yeast *Saccharomyces cerevisiae*. Clin Exp Allergy 1998, 28: 45-52.
10. van Ree, R., van Leeuwen, W.A., Aalberse, R.C. How far can we simplify in vitro diagnostics for grass pollen allergy? A study with 17 whole pollen extracts and purified natural and recombinant major allergens. J Allergy Clin Immunol 1998, 102: 184-90.
11. Olsson, S., van Hage-Hamsten, M., Whitley, P., Johansson, E., Hoffman, D.R., Galvelin, G., Schmidt, M. Expression of two isoforms of Lep d 2, the major allergen of *Lepidoglyphus destructor*, in both prokaryotic and eukaryotic systems. Clin Exp Allergy 1998, 28: 984-91.
12. Harboe, N.M.G., Ingild, A. Immunization, isolation of immunoglobulins and antibody titre determination. Scand J Immunol 1983, 17 (Suppl. 10): 345-51.
13. Müller, W.-D., Diener, C., Jung, K., Jäger, L. Antigens of timothy and other grass pollen extracts identified by monoclonal antibodies. Allergol Immunopathol 1988, 16: 315-20.

14. Fahibusch, B., Muller, W.-D., Diener, C., Jager, L. Detection of crossreactive determinants in grass pollen extracts using monoclonal antibodies against group IV and group V allergens. *Clin Exp Allergy* 1993, 23: 51-60.
15. Harboe, N.M.G., Svendsen, P.J. Fused rocket immunoelectrophoresis. *Scand J Immunol* 1983, 17 (Suppl. 10): 107-12.
16. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 117: 680-5.
17. Harlow, E., Lane, D. Antibodies: A Laboratory Manual, 1st ed. Cold Spring Harbor Laboratory: New York 1988, 473-510.
18. Smyth, C.J., Soderholm, J., Wadstrom, T. Electrofocusing-immunoelectrophoresis. *Scand J Immunol* 1983, 17 (Suppl. 10): 233-41.
19. Voller, A., Bidwell, D. Enzyme-Linked immunosorbent assay. In: Manual of Clinical Immunology. Rose, N.R., Fridman, H., Fahey, J.L. (Eds.) American Society for Microbiology: Washington DC 1986, 99-109.
20. Fischer, S., Grote, M., Fahibusch, B., Muller, W.D., Kraft, D., Valenta, R. Characterization of *Phl p 4*, a major timothy grass (*Phleum pratense*) pollen allergen. *J Allergy Clin Immunol* 1996, 98: 189-98.
21. Lowenstein, H. Isolation and partial characterization of three allergens of timothy pollen. *Allergy* 1978, 33: 30-41.
22. Haavik, S., Paulsen, B.S., Wold, J.K. Glycoprotein allergens in pollen of timothy. I Investigation of carbohydrates extracted from pollen of timothy (*Phleum pratense*) and purification of a carbohydrate-containing allergen by affinity chromatography on concanavalin A-sepharose. *Int Arch Allergy Appl Immun* 1985, 78: 197-205.
23. Haavik, S., Paulsen, B.S., Wold, J.K. Glycoprotein allergens in pollen of timothy. II. Isolation and characterization of a basic glycoprotein allergen. *Int Arch Allergy Appl Immun* 1985, 78: 260-8.
24. Haavik, S., Frostad, A.B., Paulsen, B.S., Wold, J.K. Glycoprotein allergens in pollen of timothy. III. Immunochemical and biological properties of a basic glycoprotein. *Int Arch Allergy Appl Immun* 1987, 83: 182-9.
25. Haavik, S., Paulsen, B.S., Wold, J.K. Glycoprotein allergens in pollen of timothy. IV. Structural studies of a basic glycoprotein allergen. *Int Arch Allergy Appl Immun* 1987, 83: 225-30.
26. Haavik, S., Paulsen, B.S., Wold, J.K. Glycoprotein allergens in pollen of timothy. V. Significance of the carbohydrate moiety for the immunological activity of a basic glycoprotein allergen. *Int Arch Allergy Appl Immun* 1987, 83: 231-7.
27. Ekramoddoullah, A.K.M., Kisil, F.T., Sehon, A.H. Immunochemical characterization of a high molecular weight basic allergen (HMBA) of rye grass (*Lolium perenne*) pollen. *Molec Immun* 1983, 20 (4): 465-73.
28. Ekramoddoullah, A.K.M., Kisil, F.T., Sehon, A.H. Partial characterization of an antigenic site of high molecular weight basic antigen, a ryegrass pollen allergen, using a monoclonal antibody. *Molec Immun* 1986, 23 (2): 111-7.
29. Su, S.N., Lau, G.X., Tsai, J.J., Yang, S.-Y., Shen, H.D., Han, S.H. Isolation and partial characterization of Bermuda grass pollen allergen. BG-60a. *Clin Exp Allergy* 1991, 21: 449-55.
30. Su, S.N., Shu, P., Lau, G.-X., Yang, S.-Y., Huang, S.-W., Lee, Y.-C. Immunologic and physicochemical studies of Bermuda grass pollen antigen BG60. *J Allergy Clin Immunol* 1996, 98: 486-94.
31. Valenta, R., Vrtala, S., Laffer, S., Spitzauer, S., Kraft, D. Recombinant allergens. *Allergy* 1998, 53: 552-61.
32. Petersen, A., Schramm, G., Schlaak, M., Becker, W.-M. Post-translational modifications influence IgE reactivity to the major allergen *Phl p 1* of timothy grass pollen. *Clin Exp Allergy* 1998, 28: 315-21.
33. Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G., Strecker, G. Glycoproteins. In: Carbohydrate Analysis: A Practical Approach. Chaplin, M.F., Kennedy, J.F. (Eds.) IRL Press: Oxford-Washington DC 1992, 143-204.
34. Laffer, S., Vrtala, S., Duchene, M., van Ree, R., Kraft, D., Scheiner, O., Valenta, R. IgE-binding capacity of recombinant timothy grass (*Phleum pratense*) pollen allergens. *J Allergy Clin Immunol* 1994, 94: 88-94.
35. Gavrovic, M.D., Trtic, T., Vujcic, Z., Petrovic, S., Jankov, R. Comparison of allergenic potentials of timothy (*Phleum pratense*) pollens from different pollen seasons collected in the Belgrade area. *Allergy* 1997, 52: 210-4.
36. Lalic, L. Investigation of cross-sensitivity to grass pollens. Doctoral thesis, Belgrade 1992.
37. Clamp, J.R., Hough, L. The periodate oxidation of amino acids with reference to studies on glycoproteins. *Biochem J* 1965, 94: 17-24.
38. Petersen, A., Becker, W.-M., Moll, H., Blumke, M., Schlaak, M. Studies on the carbohydrate moieties of the timothy grass pollen allergen *Phl p 1*. *Electrophoresis* 1995, 16: 869-75.
39. Petersen, A., Vieths, S., Aulepp, H., Schlaak, M., Becker, W.-M. Ubiquitous structures responsible for IgE cross-reactivity between tomato fruit and grass pollen allergens. *J Allergy Clin Immunol* 1996, 98: 805-15.

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