

Original article

Polygalacturonase (pectinase), a new oilseed rape allergen

Background: Type I hypersensitivity to rapeseed pollen allergens was described as the result of a cross-sensitization with various pollens that could constitute an aggravating factor in birch or grass pollen allergies. Recently, a few rapeseed pollen allergens were described. The aim of the present work was to identify new rapeseed pollen allergens by using two-dimensional gel analysis, microsequencing, and mass spectrometry.

Methods: Water extractable proteins from oilseed rape pollen or stamen were separated by two-dimensional gel electrophoresis. The proteins were then electroblotted onto a nitrocellulose (NC) sheet. The NC sheets were successively incubated with (1) individual human sera pre-selected for their immunoglobulin E (IgE) reactivity to rapeseed pollen proteins, (2) alkaline phosphatase (AP)-conjugated goat anti-human IgE and (3) AP substrate. The allergens localized by this method were then identified by microsequencing and MALDI-TOF mass spectrometry analysis.

Results: Of the 18 sera studied, five recognized a wide multispot zone with a molecular mass around 43 kD and pIs between 6.5 and 8.5. The results obtained with two representative sera are shown. From this zone, two isoforms of the polygalacturonase enzyme were identified by microsequencing. Confirmation was obtained through MALDI-TOF mass spectrometry analysis.

Conclusion: The present results allow the identification of a new rapeseed allergen that can be the main allergen for some patients.

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Possible adverse effects caused by oilseed rape cultivation have been suggested since many years, especially in Britain, and several reports discuss the scientific basis of such effects (1–5). These adverse effects have been mainly attributed to volatile organic compounds that may be involved in the irritancy and/or in the allergenicity of rapeseed proteins (6). The evaluation of allergenicity includes clinical parameters (symptoms, reactivity to cutaneous tests) and biological tests (specific IgE levels), and McSharry (2) focused on the need for standardized extracts to improve the reliability of the various tests.

There is now a general agreement that oilseed rape pollen is allergenic, with oligo- or mono-sensitized patients and a high prevalence of sensitization in cases of occupational exposure (7, 8). Moreover, Suh et al. (9) also reported the case of a patient allergic to oilseed rape seeds and Alvarez et al. (10) recently reported three cases of occupational asthma caused by oilseed rape flour.

In 1997, Monsalve et al. (11) isolated and sequenced an allergen of oilseed rape seeds which obtained the nomenclature designation Bra n 1. In pollen, the characterization of oilseed rape allergens has been developed recently with the works of Hemmer et al. (8), Focke et al. (12) and Chardin et al. (13). Taken together, these reports suggested that oilseed rape pollen allergens could

be divided into three main groups: a 6–8 kD allergen, a 14 kD allergen and a cluster of molecules of molecular masses ≥ 27 kD. The 6–8 kD allergen was identified as a calcium-binding protein, the 14-kD allergen was related to profilins (12), and three proteins were identified in the ≥ 27 kD cluster: the cobalamin-independent methionine synthetase, an analogue of the berberin bridge protein from *Arabidopsis thaliana*, and putative protein kinase receptor-like proteins (13). Focke et al. also suggested that sugar residues are implicated in the recognition of the ≥ 27 kD cluster (12), a fact that could explain some cross-reactions between various species.

The aim of the present work was to pursue the characterization of the oilseed rape pollen allergens by the identification of an as yet undescribed 43-kD allergen, that was recognized solely and not as a part of a cluster.

Materials and methods

Human sera

Human sera from birch and/or orchard grass pollen allergic patients were screened for their immunoglobulin E (IgE) reactivity to oilseed rape pollen proteins separated by isoelectric focusing (IEF) (see ref. 13 for details). The specificity of 18 IEF selected sera was further

analyzed in two-dimensional (2-D) blot. Five of these 18 sera displayed an analogous IgE specificity and the results obtained with two of them, considered as representative sera, are presented in this study. These sera were used as immunochemical reagents to identify allergens recognized by IgE.

Protein extraction

Six hundred milligrams of oilseed rape (*Brassica napus*) pollen (Allergon, Angelholm, Sweden) was incubated for 1 h at 22°C in 1.4 ml of distilled water. After centrifugation (10 000 g, 10 min, 4°C) the supernatant was recovered.

Oilseed rape stamens (Drakkar wild type) were provided by Dr Angenon and Dr Dewaele (Vrije Universiteit, Brussels, Belgium). The proteins were extracted as described above with a ratio of 143 mg of stamen per 1.4 ml of distilled water. A filtration on glass wool was performed before the centrifugation.

Two-dimensional gel electrophoresis

The oilseed rape pollen extract was submitted to an IEF separation performed in a polyacrylamide gel (4%T, 3%C) containing 5% v/v Servalyt® pH 2–11 (Serva, Heidelberg, Germany) on a flat bed electrophoretic chamber (Multiphor II, Amersham-Biosciences, Uppsala, Sweden) cooled at 15°C. Two 4 mm wide strips of the focused gel were cut, incubated in the equilibration buffer and submitted to SDS-PAGE separation on an 8–18% gradient gel (ExcelGel, AB). One 2-D separation was silver-stained according to Blum et al. (14) and the other used for a Western blot.

Western blot

After the electrophoretic separation the proteins were electroblotted onto a nitrocellulose (NC) sheet with a semi-dry Novablot apparatus (AB) following the manufacturer's instructions (1 h, 1 mA/cm²). Just after blotting, the NC sheet was dried and blocked with phosphate-buffered saline (PBS) containing 1% Bovine serum albumin and 0.1% Tween 20 (PBS-BSA-Tw) for 1 h at 22°C. Then, the NC sheet was incubated with individual serum (1 : 10 in PBS-BSA-Tw, overnight, 22°C), followed by alkaline phosphatase (AP)-conjugated goat anti-human IgE (1 : 700 in PBS-BSA-Tw, 2 h, 22°C, Sigma Biochemicals, St Louis, MO). The AP activity was detected using 5-Bromo-4-Chloro-3-Indolyl Phosphate and Nitro Blue Tetrazolium (Sigma Biochemicals) in 0.1 M Tris buffer pH 9.5.

To evaluate the implication of sugar residues in IgE binding, the NC sheet was incubated with the individual serum in the presence of 300 mM methyl- α 2 glucopyranoside or after a periodate oxidation of the blotted proteins according to Yasuda et al. (15). Briefly, an NC sheet was treated with 26 mM sodium metaperiodate in acetate buffer pH 4.5 (20 h, 4°C in darkness) before incubation with the individual serum.

Detection of sugar residues by Concanavalin A (ConA)

After blotting, the NC sheet was dried and saturated with PBS containing 0.3% Tween (PBS-Tw 0.3). Then, the sheet was incubated with biotinylated Con A (1 : 80.000 in PBS-Tw 0.3, 1 h, 22°C), followed by AP-conjugated streptavidine (1 : 5000 in PBS-Tw 0.3, 1 h, Amersham, UK), and NBT-BCIP as described above.

The Con A binding was also performed after a periodate oxidation of the blotted proteins or in the presence of 300 mM methyl- α 2 glucopyranoside.

Amino acid sequence analysis and mass spectrometry

Two spots of proteins were selected based on their ability to bind to IgE (see Fig. 1, arrows) and the sequences of their N-terminal amino acids were determined.

After an IEF separation, the protein bands at pIs of 7.2 and 7.7 were cut out and submitted to SDS-PAGE in an 8–18% gradient gel [double one dimension electrophoresis according to Altland (16)]. The proteins were electroblotted overnight onto a ProBlott membrane (Applied Biosystems, Weiterstadt, Germany) in 50 mmol/l borate, 50 mmol/l Tris (pH 8.3). After Coomassie staining, the bands of interest (apparent molecular mass of 43 kD) were cut out and submitted to microsequencing. Microsequencing was performed by Edman degradation with an Applied Biosystems sequencer (model 473A). Amino acid sequence similarity with already known proteins was searched by using the Basic Local Alignment Search Tool (BLAST) algorithm (17) (PIR, Swiss-Prot and Genpept databases).

The spots of interest were also analyzed by peptide mass fingerprinting according to Henzel et al. (18) with slight modifications using an Applied Biosystem Voyager DE-RP instrument. Databases were queried with Protein Prospector (<http://prospector.ucsf.edu/>) and Profund (<http://prowl.rockefeller.edu/>) programs.

Results

The comparative 2-D gel analysis of oilseed rape pollen and stamen extracts is shown in Fig. 1. The result of the silver staining (Fig. 1a,b) shows similar profiles: numerous proteins were present in both extracts. A careful visual comparison of the two stained gels shows that most protein spots were present in the two extracts, but some of them in variable amounts from one extract to the other. The glycosylation pattern revealed by ConA binding (Fig. 1c) shows the presence of numerous glycoproteins with molecular masses $\geq$ 30 kD.

The 2-D blot analysis of the specificity of two human serum IgEs to oilseed rape pollen and/or stamen proteins is shown in Fig. 2a,b. These two sera strongly recognized a series of spots of proteins with an apparent molecular mass of 43 kD and pIs varying from 6.5 to 8.5. Serum no. 17 (Fig. 2a) also recognized various spots in the same pI range but with higher and lower molecular masses, which could represent polymers and degradation products of the previous molecules respectively. Moreover, serum no. 72 (Fig. 2b) faintly recognized numerous spots with various pIs and molecular masses $\geq$ 30 kD.

The N-terminal amino acid sequences of two spots at 43 kD (see Fig. 1b, arrows for identification) were identical. The amino acid sequence found was EVFTAGGP. This sequence is 100% identical to amino acids 23–30 of *Brassica napus* polygalacturonase (precursor). This identification was confirmed through MALDI-TOF mass spectrometry analysis where 43% of the tryptic peptides matched with the polygalacturonase and covered 38% of the protein. Thus, these two spots are isoforms of the *B. napus* polygalacturonase.

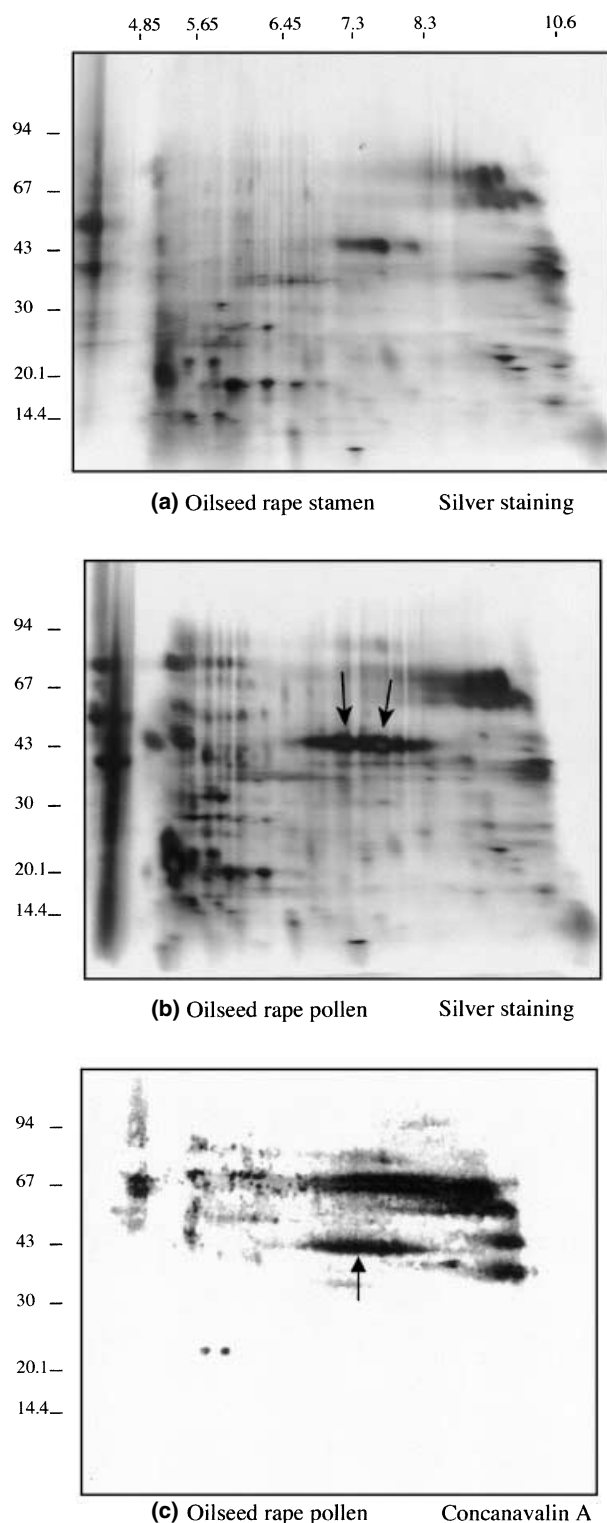


Figure 1. Comparison of stamen and pollen oilseed rape extracts by 2-D gel electrophoresis. The proteins are evidenced by silver staining (1a,b) and the glycoproteins by Con A binding after blotting (1c). Molecular masses (kD) are indicated on the left and pIs on the top. (b) Arrows indicate the proteins submitted to N-terminal amino acid sequence analysis. (c) Arrow indicates that polygalacturonase binds Con A.

The results of the IgE binding capacity of serum no. 17 in the presence of a large excess of methyl, α 2 glucopyranoside or after protein deglycosylation are shown in Fig. 2c,d. The results show that, even if the polygalacturonase bound ConA (Fig. 1c, arrow), the binding capacity of the IgE was maintained when the sugar residue was added into the medium (Fig. 2c) or after deglycosylation of the blotted proteins (Fig. 2d). ConA binding was completely abolished in the same conditions. After periodate oxidation, the IgE binding capacity of serum no. 17 was reduced (Fig. 2d), as well as the silver nitrate staining of the NC sheet (data not shown).

Discussion

The present study allows the characterization of a not yet described oilseed rape (*Brassica napus*) allergen: the polygalacturonase (or pectinase, accession number P35337). This allergen was evidenced in pollen and flower extracts. The pollen extract was always found to be the richest source in plant aero-allergens as compared with leaf or flower extracts. Classical palynological studies have shown that some of the pollen proteins migrate from the male flower and concentrate into the pollen (19). This is true for pollen allergen as well. In this study, we compared pollen and flower extracts from rapeseed and found the same allergens in both extracts, sometimes in different concentrations. Thus, flower extracts may be a good alternative source to study pollen allergens.

Using SDS-PAGE separation of the proteins followed by immunoblotting, Hemmer et al. (8) studied the individual response of patients known to be allergic to oilseed rape pollen, and found that of six patients, one was strongly and mono-sensitized to an allergen of a molecular mass around 45 kD. More recently, using the same method, Focke et al. (12) found two patients mono-sensitized to an allergen of 42 kD, when a total of 56% of their patients recognized an allergen in the same molecular mass range. As this mono-sensitization was observed only in a few patients, this allergen has not been further characterized. Our own results, based on a 2-D blot analysis, showed that five of the 18 sera tested displayed IgE to the polygalacturonase (28%). Thus, polygalacturonase seems to be recognized in 28–56% of the cases of oilseed rape allergy with detectable immunoblot IgE binding and as a single allergen in approximately 4% of the cases.

As Focke et al. (12) suggested that the IgE binding to the proteins of the ≥ 27 kD cluster was mostly due to sugar residues, we tested the implication of these residues in the IgE binding of serum 17 to polygalacturonase. We found that binding was maintained after treatment with sodium metaperiodate or when methyl, α 2 glucopyranoside was added. The decrease of the IgE binding after metaperiodate treatment is most likely due to the drastic condition of the periodate oxidation that can alter the blotted proteins, and

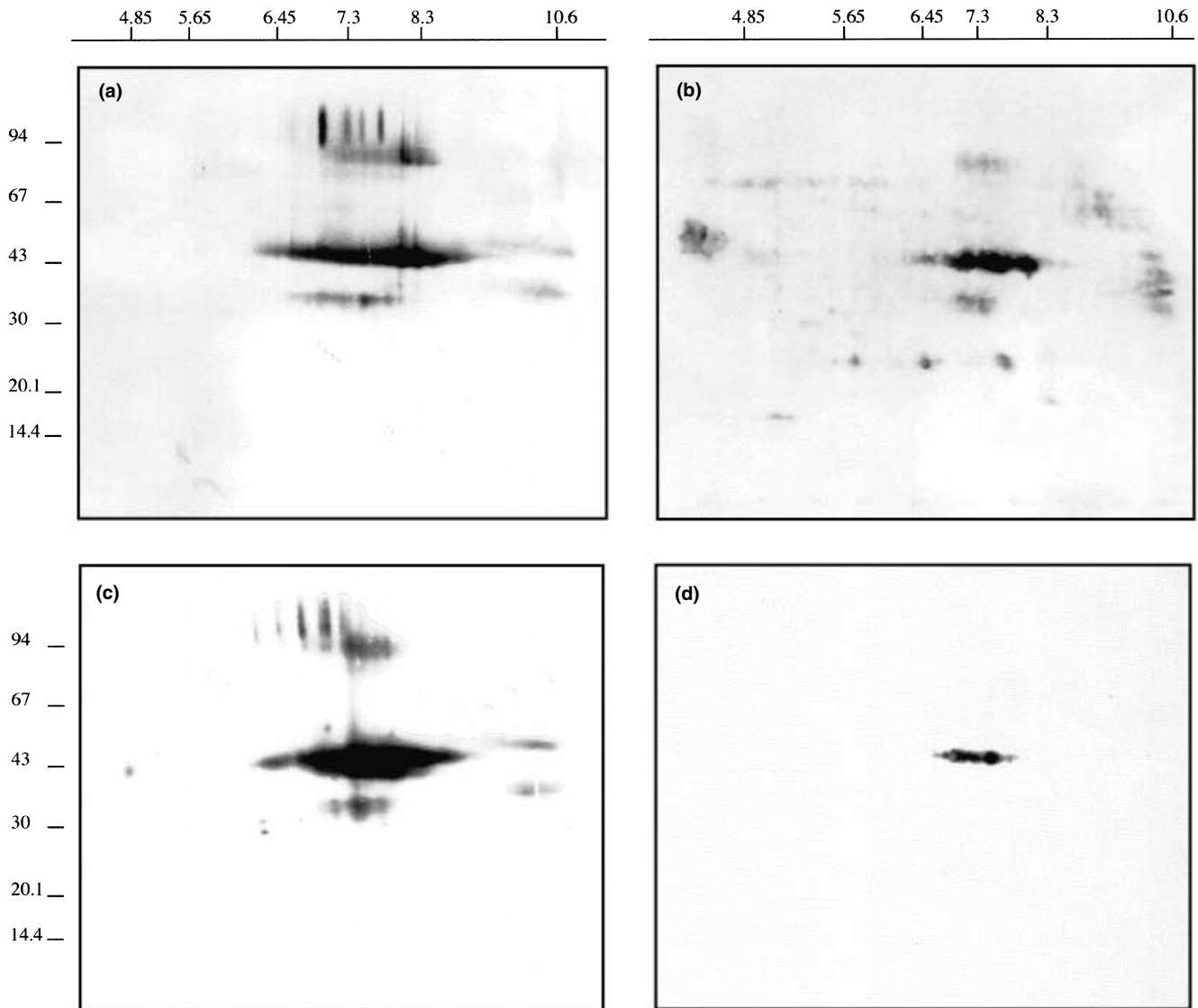


Figure 2. 2-D blot analysis of the IgE binding capacity of two human sera selected after IEF screening. First dimension: IEF in 4% polyacrylamide gel, pH 2–11. Second dimension: 8–18% SDS-PAGE. Molecular masses (kD) are indicated on the left and pIs on the top. (a) IgE binding of serum no. 17 on oilseed rape pollen proteins, (b) IgE binding of serum no. 72 on oilseed rape stamen proteins, (c) IgE binding of serum no. 17 in the presence of methyl, α 2 glucopyranoside (oilseed rape pollen proteins), (d) IgE binding of serum no. 17 after deglycosylation by metaperiodate treatment of the NC sheet (oilseed rape pollen proteins). Note that the IgE binding capacity was not modified by the addition of methyl, α 2 glucopyranoside (2c), but decreased after metaperiodate oxidation (2d).

that also reduces the silver nitrate staining. Otherwise, as the IgE binding was mainly (or solely) limited to polygalacturonase, and the glycosylation revealed by ConA was widely distributed among high molecular mass proteins, it is very unlikely that the IgE recognize a sugar epitope only expressed by this enzyme. Thus, sugar residues do not seem to be mainly implicated when only one (or very few) protein is recognized, as is the case for polygalacturonase in this study.

Polygalacturonase (or pectinase) is a pollen protein that belongs to family 28 of glycosyl hydrolases. These

enzymes digest the 1,4- α -D-galactosiduronic linkages in pectate and other galacturonans and may function during pollen development, germination or tube growth by pectin depolymerization. Another polygalacturonase enzyme, already described as an allergen in timothy grass, obtained the official nomenclature Phl p 13 (<http://www.allergen.org>; accession number Q9XG86) (20). The capacity of oilseed rape proteins to induce allergic response has been a matter of debate because several authors consider oilseed rape mainly as a species that cross-reacts with various grass allergens (7). Cross-reac-

tivity between oilseed rape and timothy grass calcium-binding allergens has been previously demonstrated (12), and the profilin is also a common allergen that cross-reacts between various species (21). However, it was recently indicated that sensitization to oilseed rape is not due to cross-sensitization to grass pollen because there is no cross-inhibition of the IgE binding, even if proteins of similar molecular masses are recognized (22). In the present case, oilseed rape and timothy grass polygalacturonases display various sequence similarities, especially around the polygalacturonase active site, where 100% identity is observed between the aminoacids 229–243 and 234–248 of the oilseed rape and timothy grass proteins

respectively. The level of cross-reactivity between these two allergens should be evaluated in further studies.

In conclusion, this study identifies a not yet described oilseed rape pollen allergen, polygalacturonase (or pectinase), and we estimate that 28–56% of the oilseed rape pollen allergic patients are sensitized to this allergen, with a mono-sensitization in approximately 4% of the cases.

Acknowledgments

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