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Peptide Mapping Analysis of Group I Allergens of Grass Pollens

Key Words

Group I allergens
Peptide mapping
Grass pollen allergens
Proteolysis
Cyanogen bromide digestion
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Protein purification

Abstract

Group I allergens, from eight different clinically important grass pollens of the Pooideae (rye-grass, canary, Kentucky bluegrass, orchard and timothy), Chloridoideae (Bermuda grass) and Panicoideae (Johnson grass, maize) were isolated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and subjected to limited partial proteolysis and cyanogen bromide cleavage. The generated digests were visualised on gels by silver staining. Replicate gels were blotted and screened with *Lol p* I-specific mAb FMC A1 and IgE binding to an allergic human serum pool, to determine the degree of antigenic and IgE-binding similarities, respectively. The highest antigenic and IgE-binding similarities were between orchard and rye-grass pollens, both in the same tribe Poaceae, and among the closely related grass genera. No peptide mapping of major grass pollen allergens has previously been undertaken. This study proves peptide mapping is a powerful method for the preliminary identification and internal amino acid microsequencing of common antigenic and IgE-binding determinants of allergens, providing information that is useful in the development of reagents for the treatment of grass-pollen-associated allergies.

Introduction

The examination of patients with type I allergy usually reveals human IgE-binding proteins or glycoproteins as the causative agents, for example, from grass pollen [1]. The presence of grass pollens in the atmosphere is the major outdoor cause of hayfever and seasonal asthma from early spring to late summer, which affects about 20% of humans mainly in temperate climatic zones [2]. The allergic (IgE-mediated) response, associated with grass pollen allergy, is first induced by the release of the allergenic (or IgE-binding) proteins when the grass pollen grains contact the moist surface of the human upper respiratory tract which then leads to a cascade of events resulting with the typical symptoms associated with grass pollen allergy. The most clinically significant elicitor of allergy is the ma-

ajor IgE-binding component of grass pollen, which has been classified as group I allergen by the International Union of Immunological Societies (IUIS) [3].

Pollen extracts are commonly used for diagnosis and prognosis of grass pollen allergy. However, the crude pollen extracts are heterogeneous mixtures of allergenic and non-allergenic components which can induce, in some patients, sensitivity to some pollen constituents to which they were not originally sensitive [4]. Therefore, the use of reagents with only the purified and well-characterised allergenic proteins, as well as the identification of antigenic and IgE-binding epitopes that are relevant to these allergies, would be essential for immunotherapy [5, 6]. Thus, identification of antigenic and IgE-binding determinants within the polypeptide chains of group I, and other grass pollen allergens, is a preliminary and essential step

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for subsequent investigations of immunological characteristics.

There is considerable evidence indicating that pollen allergens of botanically related grasses are cross-reactive [7]. This has come from a variety of in vitro and in vivo techniques, most notably radioallergen sorbent test inhibition assays, crossed radioimmuno-electrophoresis and skin prick testing [8]. It is also possible to detect cross-reacting allergens with distinct electrophoretic mobilities by combination of polyacrylamide gel electrophoresis (PAGE), electrophoretic transfer to nitrocellulose and detection with grass-pollen-allergic human sera [4]. Furthermore, hybridoma technology has been employed to produce monoclonal antibodies (mAb) directed against individual antigenic epitopes on a variety of allergens. Such antibodies raised against *Lolium perenne* (rye-grass) pollen extract have demonstrated the presence of cross-reacting epitopes on other pollen species [9].

Immunoblotting analysis of crude extracts is a powerful tool for detecting cross-reactivity between different allergens. However, individual allergens can be subjected to further biochemical dissection to assess their antigenic and IgE-binding similarities. Peptide mapping [10-13] is a useful tool which is well suited for this kind of study. Peptide mapping involves partial enzymatic proteolysis of proteins, that have been isolated from gels containing sodium dodecyl sulphate (SDS), and subsequent analysis of the cleavage products by PAGE. Furthermore, peptide mapping analysis of group I grass pollen allergens have not been previously undertaken.

Knowledge of immunological cross-reactivities among protein components of different pollen allergens (i.e. group I) is considered to be of clinical importance. This may lead to identification of components that would be useful in development of reagents for immunotherapy [14].

The aim of the present study is to answer the following question: are group I allergens from different grass pollens identical or closely related? We have set out to use peptide mapping for the preliminary assessment of the degree of antigenic and IgE-binding similarities between group I pollen allergens isolated from clinically important grasses of the Pooideae (rye-grass, canary, Kentucky bluegrass, orchard and timothy), Chloridoideae (Bermuda grass) and Panicoideae (Johnson grass, maize) using group-I-specific mAb and a grass-pollen-allergic human serum pool. Amino acid sequence information from common antigenic and IgE-binding epitopes of these allergens can then be obtained using microsequencing.

Materials and Methods

Pollen Extract

Pollen was collected from canary grass (*Phalaris aquatica*) flowering heads in the field, on hot and still mornings during the flowering season in spring, and stored under liquid nitrogen until use. Dry *L. perenne* (rye-grass), *Cynodon dactylon* (Bermuda grass), *Poa pratensis* (Kentucky bluegrass), *Dactylis glomerata* (orchard grass), *Phleum pratense* (timothy grass), *Sorghum halepense* (Johnson grass) and *Zea mays* (maize) pollen was purchased from Greer Laboratories, Inc., Lenoir, N.C., USA.

The pollen (100 mg) from all grasses was separately shaken in phosphate-buffered saline (PBS; 1 ml) at 4°C for 3 h with constant shaking, as previously described by Singh and Knox [9]. After a brief vortex, the pollen solutions were centrifuged (13,600 rpm × 5 min) in a microcentrifuge to remove the pollen debris. The protein concentration of the extracts was determined with a Bio-Rad dye-binding protein microassay [15] using BSA as the standard.

Sera

Sera were obtained from 10 grass-pollen-allergic subjects selected on the basis of a previous clinical record of spring hay fever symptoms, who had not earlier undergone hyposensitization treatment, including a positive skin test, RAST and immunoblotting analysis with crude rye-grass pollen. Sera were pooled and stored at -20°C in small aliquots. All skin tests were performed by the puncture technique with use of commercial rye-grass pollen extracts (Hollister-Stear, Spokane, Wash., USA). As positive and negative controls, we tested histamine (10 mg/ml) and diluent, respectively. The formation of a wheal more than 3 mm in diameter was considered to indicate a positive response. RAST was performed according to standard methods (Phadezyme RAST, Pharmacia-LKB, Uppsala, Sweden).

SDS-PAGE Protein Isolation

Crude extracts of the different grass pollens (20 µg protein for 'mini-gel' (Bio-Rad) per well) were mixed with equal volumes (2 µl) of sample buffer (125 mM Tris buffer (pH 6.8), 0.4% SDS, 0.008% di-thiothreitol (DTT), 2% glycerol), boiled for 5 min, allowed to cool to room temperature and separated by 'mini' (10 × 7.5 cm) 10% homogeneous polyacrylamide gels (SDS-PAGE), using the Laemmli buffer system [16]. The gels were electrophoresed in tank buffer (25 mM Tris (pH 8.3), 190 mM glycine, 0.1% SDS) at 200 V (for 'mini-gels') until the dye-front just ran out. Low molecular weight (MW) standards (Pharmacia) used to determine the molecular weight of the pollen components were phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD) and α-lactalbumin (14.4 kD). When electrophoresis was completed, the gels were stained for 10 min at 60°C with Coomassie Brilliant Blue-R250 (CBB-R250, Bio-Rad; 0.25% w/v in 50% v/v methanol and 10% v/v acetic acid) and destained for up to 45 min at 60°C (5% v/v methanol and 7.5% v/v acetic acid). Major protein bands identified as group I allergens were excised and equilibrated in tank buffer for 1 h at 18°C. The gel slices containing the protein bands were loaded into the wells of a second gel and re-isolated, stained/destained, excised and re-equilibrated.

Proteolytic Digestion

Equilibrated gel slices containing the isolated allergens were loaded into the stacking gel wells of a 'mini' 15% homogeneous polyacrylamide gel, covered each slice with 5 µl/well of gel slice overlay so-

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lution (20% v/v glycerol, 0.125 M Tris, 0.1% w/v SDS, 3% v/v mercaptoethanol, 0.005% w/v bromophenol blue, pH 6.8) and one of the endoproteases (Glu-C or *S. aureus* V8, Lys-C, Arg-C; Promega) added on top of the gel slices at the correct working dilution (5 µl of 40 µg/µl protease stock) in equal volume of diluent solution (50% v/v glycerol, 0.625 M Tris, 0.5% w/v SDS, 0.025% w/v bromophenol blue, pH 6.8). The gel was run at 100 V until the dye-front reached the interface between stacking and resolving gel and interrupted for 35 min to allow the proteolytic digestion to occur. The gel was then allowed to resolve at 200 V until the dye-front just ran out. The partial digests were visualised by silver staining [17].

Cyanogen Bromide Digestion

Allergens, isolated by 10% homogeneous 'mini' PAGE as described above, were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore Corp., Bedford, Mass., USA) pre-wet in absolute methanol and soaked in distilled water for 1-2 min, at 200 mA for 2 h [18] in a transblot buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol). Cyanogen bromide (CNBr) digestion of the allergens immobilised on PVDF membrane was the same as described by Simpson et al. [19]. Briefly, the PVDF membrane was washed in distilled water (d.H₂O) for 5 min, stained with CBB R250 (0.1% w/v in 50% v/v methanol) for 5 min and destained (50% v/v methanol, 10% v/v acetic acid) until the background stain cleared and washed in d.H₂O for 10 min. The target bands were excised and incubated in 200 µl of CNBr solution (1% w/v in 70% v/v formic acid) for 6 h in an enclosed and light insulated tube in a fume hood. The tubes were vented for 15 min, the solution transferred into a fresh tube and added 200 µl of peptide elution solvent (PES: 0.2% v/v trifluoroacetic acid, 1.1% w/v thioglycolic acid, 1.8% w/v lysine in 70% v/v isopropanol) into each tube containing the membranes with CNBr digests and incubated for 2 h with occasional vortexing. The PES solution was then transferred into the tube containing the initial CNBr solution and the membranes were incubated with a further 200 µl of PES solution for 2 h. The PES solution was pooled with the other solutions and evaporated by placing tubes under slow argon stream while sitting tubes in a 37°C waterbath. When the samples were close to complete drying, 20 µl deionised d.H₂O was added and dried in speed vacuum. Samples were resuspended in 20 µl deionised d.H₂O and re-dried in speed vacuum. The samples were resuspended in deionised d.H₂O, equal volume of sample buffer added, separated by 15% homogeneous SDS-PAGE and digests visualised with silver staining, as described previously.

Immunoblotting Analysis

Protease and CNBr digests from replicate gels were transferred onto nitrocellulose (NC) membranes as described earlier. The NC membranes were screened with a serum pool of 10 highly grass-pollen-allergic subjects and *Lol p 1*-specific (FMC A1) and *Lol p 1X*-specific (FMC A7) mAbs [20, 21] as described elsewhere [22]. Briefly, NC membranes obtained after SDS-PAGE were soaked in blotto (10% skim milk powder in PBS) for 1-2 h and washed twice in PBS with 0.1% Tween 20 (TPBS) and twice in PBS only, 5 min each with vigorous shaking. The NC membranes were then incubated with mAb FMC A1 or A7 (diluted 1:1,000 in PBS with 0.5% BSA) or the serum pool (diluted 1:4 in PBS with 0.5% BSA) overnight, as previously described by Sutton et al. [4]. After washing with TPBS and PBS (as described above), the serum NC membranes were incubated in rabbit immunoglobulins to human IgE (DAKO; diluted 1:200 in PBS with 0.5% BSA) for 1 h and mAb NC membranes incubated in horse-

radish-peroxidase-conjugated sheep anti-mouse immunoglobulins (Silenus; diluted 1:1,000 in PBS with 0.5% BSA) for 2 h. The serum NC membranes were washed once again in TPBS and PBS, as previously described, and incubated in horseradish-peroxidase-conjugated anti-rabbit IgG (Promega; diluted 1:2,500 in PBS with 0.5% BSA) for 1 h. Both serum and mAb NC membranes, after washing in TPBS and PBS for the final time, were developed in peroxidase substrate (0.06% 4-chloro-1-naphthol, 0.006% hydrogen peroxide and 20% methanol in PBS) at 37°C for 5-10 min and development was stopped by washing in distilled water.

Digest Pattern Comparison and Assessment

All the digest patterns observed on the immunoblots underwent densitometric analysis (fig. 3) with UltraScan XL (Pharmacia-LKB, Sweden) for accurate, precise comparisons between different digest profiles. For each protease and CNBr digest profile each sample was compared with each other, the number of common fragments (sharing the same mobility) between two samples determined and expressed as a percentage of similarity over the maximum number of fragments generated by each protease or CNBr in any sample. All the similarity percentage values for all protease and CNBr digests were averaged and expressed as a single value separately for serum immunoblots (for IgE-binding similarity) and mAb immunoblots (for antigenic similarity). These two sets of data then separately underwent conventional cladistic clustering analysis [23] to identify the reciprocal nearest neighbours and finally presented as tree diagrams (fig. 4).

Results

Identification of Group I Allergens

Crude grass pollen extracts were resolved by 10% SDS-PAGE and the protein profiles visualised by CBB staining (fig. 1A), and by Western analysis using the allergic serum pool and a *Lol p 1*-specific mAb FMC-A1 (data not shown). Both IgE antibodies and the specific mAb A1 confirmed that the group I allergens are the most strongly stained bands within the group I MW range as determined by Marsh et al. [3]. After identification, the bands were excised and re-run to check purity (fig. 1B). The purity of these allergens, for the purpose of this study, is satisfactory, since the allergens formed single bands and the absence of group IX/V allergens was confirmed by lack of binding of *Lol p 1X*-specific mAb FMC A7 (data not shown).

Peptide Mapping of Group I Allergens

When the isolated group I allergens were partially digested with the proteases, Glu-C, Lys-C and Arg-C, and cyanogen bromide, distinct patterns or finger-prints for each allergen were generated on the gel upon silver staining (fig. 2). Maximum numbers of peptide bands were generated by Glu-C (fig. 2IA) while endoproteases Lys-C and Arg-C (fig. 2IIA, IIIB, respectively) gave fewer pep-

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ride fragments. However, when blotted and screened for human IgE and mAb FMC-A1 binding, many more fragments were identified (fig. 2IIB, C, IIIB, C).

Cyanogen bromide gave the best cleavage of all the allergens, resulting in generation of many peptide fragments (fig. 2IVA). Unusually, Bermuda grass did not show any IgE or mAb FMC-A1 binding (fig. 2IVB, C) when digested with CNBr. Extensive similarities were evident between canary, orchard, Kentucky blue and rye-grass pollens when qualitative comparisons between different grass digests were made with each digestion agent.

For accurate quantitative assessment of degree of similarity, the peptide fingerprints were scanned with a densitometer in order to detect minor bands (fig. 3). Every peptide fragment was detected. This method allowed quick and accurate identification of different grass pollen allergen digests sharing common mobilities.

The number of fragments common to the different grasses was estimated for each of the 4 different digestion agents, and the degree of similarity was expressed as tree diagrams (fig. 4). The IgE-binding similarity was assessed from serum immunoblot finger-prints (fig. 4B) and antigenic similarity from mAb FMC-A1 immunoblot finger-prints (fig. 4C).

▶ The highest IgE-binding similarity was observed between orchard and rye-grass (53%) and between rye-grass and canary grass (43%). No IgE-binding similarity was observed between maize and other grasses (fig. 4B).

The highest antigenic similarity (fig. 4C) was also observed between rye-grass and orchard grass (76%) and the lowest similarity between maize (23%), and Bermuda (10%) with other grasses.

Taken together, the antigenic and IgE-binding similarities between these grass pollen allergens were in agreement with the botanical classification of grasses into sub-families and tribes (fig. 4A). The highest level of homology is between rye-grass and orchard grass (both genera of tribe Poeae). As might be expected, Bermuda grass and maize show very low levels of homology with the other grasses, consistent with their different botanical classification.

Discussion

In this paper, we have used a biochemical dissection technique, peptide mapping, to demonstrate the major antigenic and IgE-binding similarities between group I allergens from eight clinically important grass pollens.

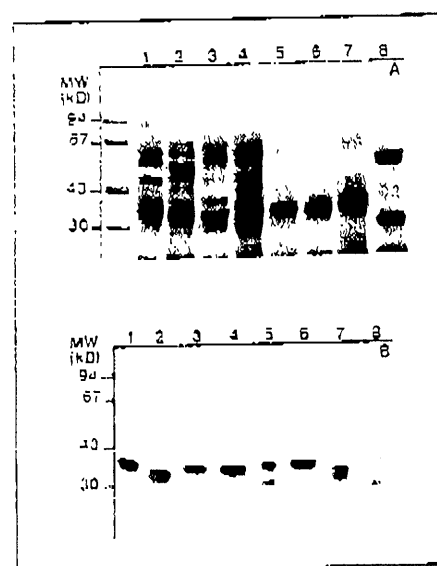


Fig. 1. SDS-PAGE isolation of group I allergens from eight different clinically important grass pollens. Crude extracts were resolved and stained with CBB (A). Group I allergens identified, excised and re-run on a second gel to check purity after silver staining (B) before peptide mapping. First lane of A represents MW markers. 1-8 represent perennial rye, Bermuda, canary, Johnson, Kentucky blue, orchard, timothy grass and maize, respectively.

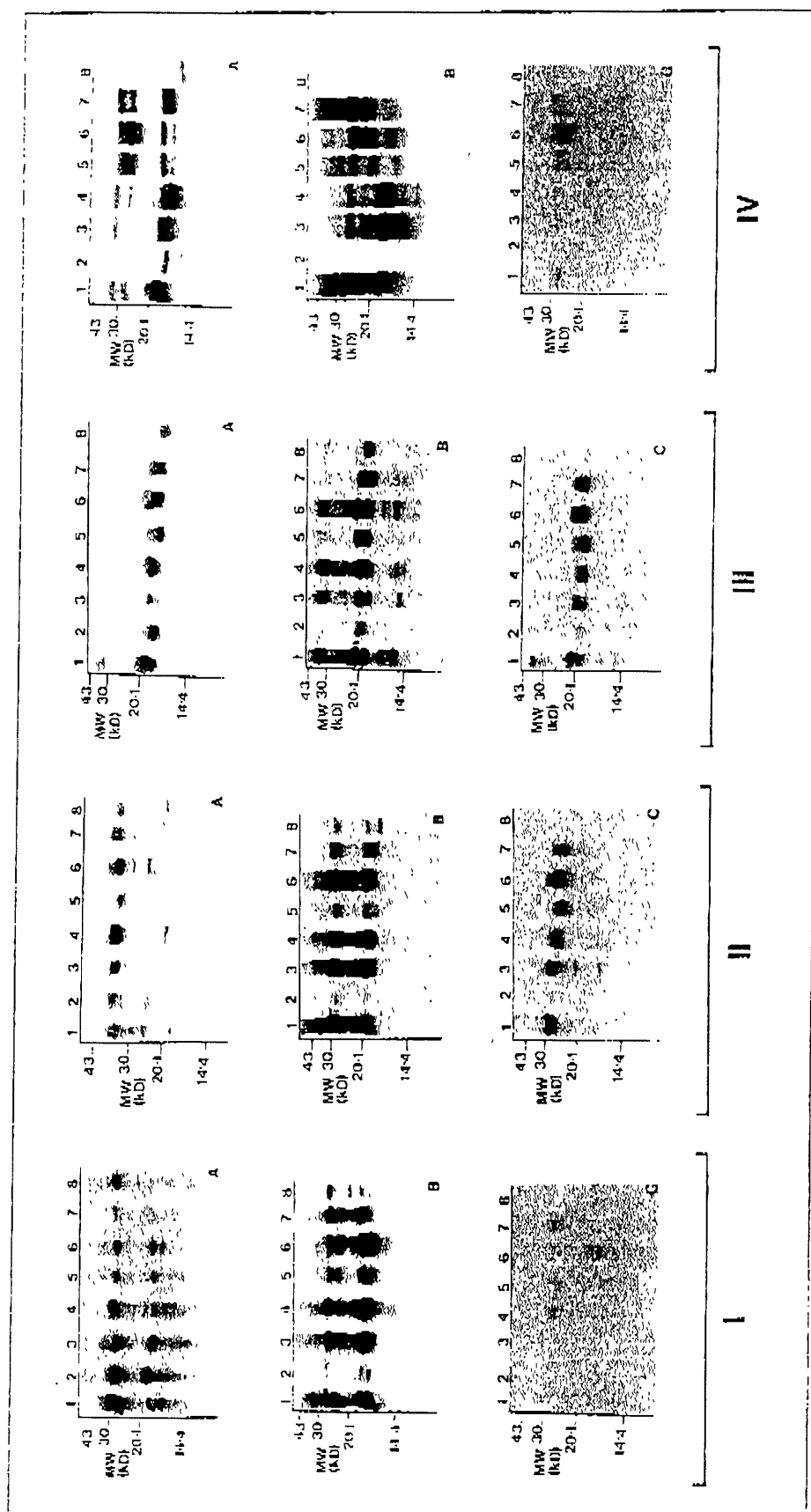
The major protein digests were visualised on gels by silver staining. Since this study only deals with samples that are isolated by single dimension only, there may be other contaminating proteins occupying the same MW, thus interfering with the finger-print patterns. Thus, the Coomassie brilliant blue stained gel finger-prints were considered unsuitable for the assessment of structural similarities between the different major allergens. However, since similar or highly cross-reactive allergens are known to contain common antigenic and IgE-binding determinants, these gel digests were blotted and screened for their degree of antigenic and IgE-binding similarities.

IgE-Binding Similarity

We have decided to use the immunoblotting technique for the assessment of similar antigenic and IgE-binding capacity of the different allergens as there is considerable evidence from the literature [24-27] that most grass pollen allergens are not affected by SDS, that is the epitopes are continuous or non-conformational, although it is known that partial re-naturation of proteins may take place during SDS removal and transfer of proteins onto membranes [28]. From this study, the highest IgE-binding similarity was observed between orchard and rye-grass, which belong to the same tribe Poeae. Next highest were canary,

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Fig. 2. Peptide maps of group I grass pollen allergens. **A** Silver-stained gels of peptide fragments and blots (from replicates of the same gel) screened with grass-pollen-allergic patients' serum pool (**B**) and (**C**) mAb FMC A1 (specific to *Lol p I* of rye-grass pollen). I-IV Partial digests with *S. aureus* V8 endoproteinase (Glu-C), Lys-C and Arg-C endoproteinases and chemical agent cyanogen bromide, respectively. 1-8 represent different grasses as in figure 1.



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timothy and Kentucky bluegrass which, together with the tribe Poaceae, belong in the same supertribe Pooideae. Johnson and Bermuda grass showed a low degree of similarity with other grasses as they both separately belong to different subfamilies. Maize, on the other hand, did not show any IgE-binding, suggesting that its IgE-binding epitope structurally differs somewhat from the others or may be composed of continuous and discontinuous determinants. Furthermore, it is interesting that Bermuda grass did not show any IgE-binding when digested with CNBr, suggesting that its IgE-binding determinant may be made up of C and N-terminal sequences which the CNBr may have cleaved, interrupting its IgE-binding capacity.

Antigenic Similarity

When screened for antigenic similarity with mAb FMC A1, the highest similarity was observed, once again, between orchard and rye-grass, both from the same tribe of Poaceae. The remainder of the grasses slotted into their respective places, resembling the serum tree. Once again, maize and Bermuda grass shared the lowest antigenic similarity with the other grasses. Bermuda grass showed, as with the IgE-binding study, absence of mAb FMC A1-binding with the CNBr digestion. This may indicate that both the antigenic and IgE-binding determinants may be shared/overlapped, and upon CNBr cleavage, the binding is interrupted.

Conclusions

From this study, there are correlations between antigenic and IgE-binding similarities, or conserved determinants between group I allergens of botanically related grass pollens. Furthermore, the antigenic and IgE-binding similarities are both in agreement with the botanical classification. There is evidence that botanically closely related grasses share cross-reactive antigenic and IgE-binding determinants of their group I allergens.

In conclusion, peptide mapping of group I allergens of grass pollens, by limited partial proteolysis and cyanogen bromide cleavage, successfully demonstrated the degree of antigenic and IgE-binding similarities between different allergens. Furthermore, this technique can be used to access internal amino acid sequences of allergens for the determination of their antigenic and IgE-binding determinants: especially when N-terminal sequencing is not possible, for example due to blocking by hydroxyproline residues. Efforts can now be directed towards obtaining amino acid sequence information from common antigenic

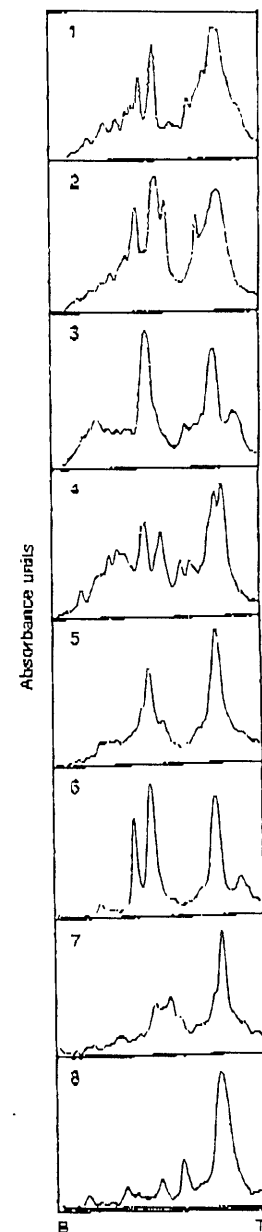


Fig. 3. Densitometric analysis of Glu-C partial digest profile of group I allergens from silver-stained gel, same as in figure 2IA. Absorbance was measured in arbitrary densitometric units by UltraScan XL (Pharmacia-LKB, Sweden). Scanning began from the bottom (B) of the gel towards the top (T). 1-8 represent different grasses as in figure 1.

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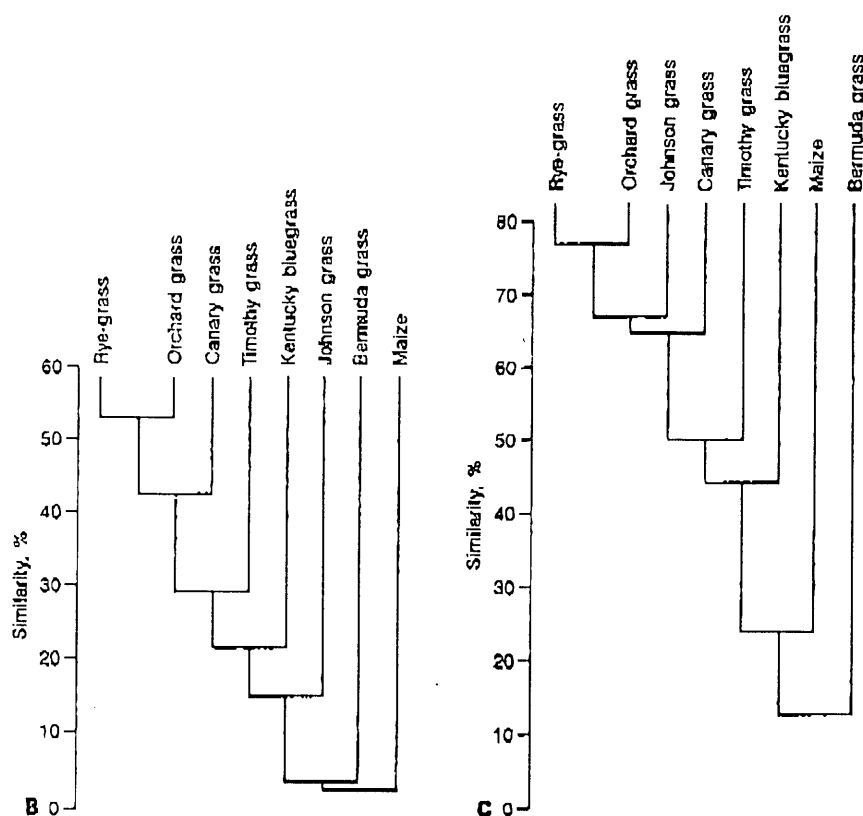
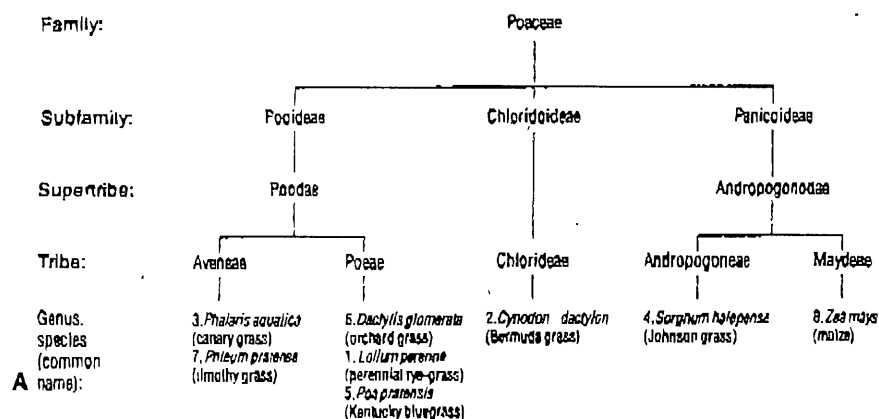


Fig. 4. Comparison of botanical classification (**A**) with the degree of IgE-binding (**B**) and antigenic (**C**) similarities between different group I grass pollen allergens. Antigenic and IgE-binding similarities were assessed by comparing the allergen partial proteolytic and CNBr digests with each other from mAb and allergic serum pool immunoblots, respectively, and subjected to cladistic clustering analysis [23]. 1-8 represent different grasses as in figure 1.

and IgE-binding fragments using microsequencing. The information presented here can be useful in the development of reagents for the treatment of allergies caused by these major allergens.

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