



PERGAMON

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Molecular basis of IgE-recognition of Lol p 5, a major allergen of rye-grass pollen

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Abstract

Grass pollen, especially of rye-grass (*Lolium perenne*), represents an important cause of type I allergy. Identification of IgE-binding (allergenic) epitopes of major grass pollen allergens is essential for understanding the molecular basis of interaction between allergens and human IgE antibodies and therefore facilitates the devising of safer and more effective diagnostic and immunotherapy reagents. The aim of this study was to identify the allergenic epitopes of Lol p 5, a major allergen of rye-grass pollen, immunodissect these epitopes further so that the amino acid residues critical for antibody binding can be determined and investigate the conservation and nature of these epitopes within the context of the natural grass pollen allergens. Peptides, 12–13 amino acid residues long and overlapping each other by 4 amino acid residues, based on the entire deduced amino acid sequence of the coding region of Lol p 5, were synthesised and assayed for IgE-binding. Two strong IgE-binding epitopes (Lol p 5 (49–60) and (265–276), referred to as peptides 7 and 34, respectively) were identified. These epitopes were further resolved by truncated peptides and amino acid replacement studies and the amino acid residues critical for IgE-binding determined (Lol p 5 (49–60) residue Lys₅₇ and (265–276) residue Lys₂₇₅). Sequences of these epitopes were conserved in related allergens and may form the conserved allergenic domains responsible for the cross-reactivity observed between pollen allergens of taxonomically related grasses. Furthermore, due to its strong IgE-reactivity, synthetic peptide Lol p 5 (265–276) was used to affinity-purify specific IgE antibodies which recognised proteins of other clinically important grass pollens, further indicating presence of allergenic cross-reactivity at the level of allergenic epitope. Moreover, Lol p 5 (265–276) demonstrated a strong capacity to inhibit IgE-binding to natural rye-grass pollen proteins highlighting the antibody accessibility to these sequences within the context of the natural allergens. Strong IgE-binding epitopes of Lol p 5 have been identified down to single critical amino acid residues and are shown to occur as linear or continuous domains in the natural conformation of natural Lol p 5 and other group 5 grass pollen allergens. The fact that such an allergenic synthetic epitope has the capacity to strongly inhibit IgE-binding to natural allergens highlight its potential for use as a candidate in future therapeutics to treat pollen-associated allergies. © 1998 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Grass pollen is the major outdoor seasonal source of airborne allergens in cool temperate climates such as

southern Australia and the allergenic role and importance of grass pollen in triggering hayfever and allergic asthma is well known and extensively documented (Knox and Suphioglu, 1996). In southern Australia and other cool

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Abbreviations: AR, activating reagent; AS, activating solution; BSA, bovine serum albumin; CBB, Coomassie brilliant blue; CRPE, crude rye-grass pollen extract; CS, cleaving solution; DCM, dichloromethane; DEE, diethylether; DMF, N,N-dimethylformamide; DP, deprotecting solution; ELISA, enzyme linked immunosorbent assay; FPLC, fast protein liquid chromatography; LS, lyophilising solution; mAb, monoclonal antibody; M_r, molecular mass; NC, nitrocellulose; OD, optical density; PBS, phosphate buffered saline; pI, isoelectric point; RAST, radioallergosorbent test; rec, recombinant; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TPBS, PBS with 0.1% Tween 20.

temperate climates, the most clinically significant grass is rye-grass (*Lolium perenne*). The major allergenic proteins of rye-grass pollen are Lol p 1 and Lol p 5, which together account for nearly all the IgE-binding reactivity of crude rye-grass pollen extract (Bond et al., 1993). These two allergens are very similar in molecular mass and both have been cloned and well characterised (Griffith et al., 1991; Singh et al., 1991; Ong et al., 1993). More than 90% of individuals allergic to grass pollen contain IgE antibodies in their sera that recognise Lol p 1 (Ford and Baldo, 1986). Although Lol p 5 is less abundant in pollen than Lol p 1, the number of individuals allergic to Lol p 5 is at least as great as the number of those allergic to Lol p 1 (Matthiesen and Løwenstein, 1991).

Detailed knowledge of the molecular organisation of the allergenic epitopes of pollen allergens would be invaluable in terms of allergen standardisation and diagnostics as well as designing mutants of these proteins with altered allergenicity. Such mutants would find use as therapeutic reagents in allergy treatment, and may provide a way of overcoming the serious side-effects associated with current therapy. However, although there has been a great deal of interest in the identification of grass pollen allergens, on the basis of their human IgE-binding capacity, the molecular details of the IgE-binding epitopes of these allergens are still lacking. This lack of molecular information is most likely a consequence of the nature of these epitopes in different allergens. That is, most epitope mapping techniques rely on continuous or linear epitopes and as a result the identification of conformational or discontinuous epitopes may not be possible. However, since such conformational epitopes may contain linear segments, the use of short synthetic peptides may also provide information about discontinuous epitopes (Gertzoff et al., 1988). Indeed, studies using peptide-based assays have identified the antigenically important portions of discontinuous epitopes (Redlich et al., 1991; Savoca et al., 1991), down to the resolution of single critical amino acids (Geysen et al., 1987; Appel et al., 1990).

The IgE-binding epitope of Lol p 1 has been previously localised to the last 28 C-terminal amino acid residues of the molecule (Esch and Klapper, 1989). On the other hand, the IgE-binding epitopes of Lol p 5 still remain to be elucidated. With this in mind, we report here, for the first time, the use of synthetic peptide strategy in mapping the strong IgE-binding epitopes of Lol p 5, which has been cloned and fully sequenced in our laboratory, as both rye-grass and the group 5 allergens are very important allergens and make a good model for immunoregulatory studies in humans. Moreover, we have identified the critical amino acid residues responsible for IgE-binding and have shown that an IgE-binding peptide of Lol p 5 has the capacity to strongly inhibit IgE-binding to natural rye-grass pollen allergens, highlighting the presence of such an epitope as a continuous or linear

segment within the context of natural Lol p 5 molecule. The identification and immunodissection of such allergenic epitopes are of considerable interest as these will be essential for devising safer and more effective immunotherapy strategies that can prevent allergens from binding and cross-linking mast cell/basophil bound IgE antibodies and therefore blocking the chain of events leading to the allergic disease. On the other hand, such B-cell information can be used in site-directed mutagenesis to abolish the molecule's IgE-reactivity, while retaining its T-cell activating capacity, for use in immunotherapy (Ferreira et al., 1996; Smith and Chapman, 1997).

2. Methods

2.1. Human sera

Sera were collected from subjects with a previous clinical record of spring hay fever symptoms, who had not earlier undergone hyposensitization treatment, positive skin-prick test and radioallergosorbent test (RAST) towards crude rye-grass pollen extract. The RAST scores for all sera was four (i.e., highly allergic). Sera were also obtained from two subjects, who were not atopic when assessed by RAST, and used as negative controls. All sera were stored at -20°C in 0.5–1 ml aliquots. Skin-prick and RAST tests were performed according to methods described elsewhere (Suphioglu et al., 1993).

2.2. Affinity purification of human IgE antibodies

Dot-blots (10 $\mu\text{g}/\text{dot}$) of either rye-grass pollen extract, SDS-PAGE and fast protein liquid chromatography (FPLC) purified Lol p 5 or Lol p 5 (265–276) (i.e., peptide number 34) (immobilised on nitrocellulose (NC) membrane as described in peptide dot-immunoassay section below) were blocked in 10% milk powder in phosphate buffered saline (PBS) and incubated in sera (diluted 1:4 in PBS containing 0.5% BSA and 0.1% sodium azide) overnight at room temp. (18°C). Blots were then washed once with PBS containing 0.1% Tween 20 (TPBS) and twice with PBS only and the bound IgE antibodies were eluted with 0.2 M glycine-HCl, pH 2.6, containing 0.5% BSA and 0.1% sodium azide with vigorous shaking for 10 min. After elution, the pH of the solutions were immediately neutralised with 2 M NaOH, and used to probe Western blots of different grass pollen proteins separated by SDS-PAGE.

2.3. Crude grass pollen extracts

2.3.1. Isolation of pollen proteins

Pollen was collected from *Phalaris aquatica* (canary grass) flowering heads in the field, on hot and still morn-

ings during the flowering season in spring, and stored under liquid nitrogen until use. Dry *L. perenne* (perennial rye-grass), *C. dactylon* (Bermuda grass), *D. glomerata* (orchard grass), *P. pratensis* (Kentucky bluegrass), *P. pratense* (timothy grass), *S. halepense* (Johnson grass), *F. elatior* (meadow fescue) and *T. aestivum* (wheat) pollen were purchased from Greer Laboratories, Inc., Lenoir, NC, U.S.A.

Soluble proteins from these pollen were obtained and their protein concentrations determined as described elsewhere (Suphioglu et al., 1993). The crude rye-grass pollen extract was used for SDS-PAGE, for affinity human IgE antibody purification and as positive controls in the peptide dot-blot and ELISA. Soluble proteins of the remainder grass pollens were only used for SDS-PAGE.

Lol p 5 from rye-grass pollen extract was purified by FPLC, as described elsewhere (Suphioglu et al., 1993). The purified Lol p 5 proteins were used as positive controls in the peptide dot-blot and ELISA immunoassays and for affinity human IgE antibody purification.

2.3.2. Gel electrophoresis

Protein samples were electrophoresed on 10% mini-gels (Bio-Rad Labs., California, U.S.A.) and protein bands visualised by Coomassie Brilliant Blue stain R250 (CBB) as described previously (Suphioglu et al., 1993).

Pollen proteins separated by SDS-PAGE were transferred to nitrocellulose (BA 0.45 μ m, Schleicher and Schuell, Dassel, Germany) as described by Suphioglu et al. (1993). Proteins from mini-gels were transferred at 200 mA for 2 h. After protein transfer, unreacted protein-binding sites were blocked by incubation of the NC filters in powdered milk (10% in PBS).

2.3.3. Screening with human sera or affinity-purified human IgE

Western blots were incubated in sera (diluted 1:4 in PBS containing 0.5% BSA and 0.1% sodium azide) or in affinity-purified human IgE (undiluted), overnight at room temp. (18°C). IgE-binding was visualised by incubation of the Western blots in ^{125} I-labelled anti-human IgE (Kallstad, TX, U.S.A.; diluted 1:7 in PBS containing 0.5% BSA and 0.1% sodium azide) overnight at room temp. (18°C). Binding of ^{125} I-labelled anti-human IgE was detected by exposure of Western blots to X-ray film (Kodak XAR) at -70°C with an intensifying screen, for periods of up to 48 h.

2.4. Synthetic peptides of Lol p 5

2.4.1. Peptide sequences

The amino acid (aa) sequence of Lol p 5 was deduced from the cDNA sequence of Lol p 5. The overlapping peptide aa sequences of Lol p 5 were constructed by using BT7400 manual peptide synthesizer (Biotech Instruments Ltd., Luton, U.K.).

2.4.2. Peptide synthesis

Thirty 12-mer and four 13-mer solid phase peptides were manually synthesized on a Biotech Instruments BT7400 manual multiple peptide synthesis block. The peptides were synthesized on C-terminal fluorenylmethoxycarbonyl (Fmoc)-protected aa resins (25 μ M; Auspep, Melbourne, Australia), held on a frit within a reaction well of the synthesis block.

2.4.3. Cleavage and deprotection of the peptides

After removal of the Fmoc protection group from the final aa of the completed peptide, the assembled peptide was cleaved with TFA, precipitated in DEE and lyophilised. Peptides were then subjected to analysis by FPLC and mass spectrometry to check peptide mass, sequence and purity. Peptides displayed $\geq 85\%$ purity with the correct aa mass and sequence ratios.

2.4.4. Peptide dot-blot immunoassays

Peptides were dissolved in sterile water to give a 1.6 mM (or 2 mg/ml) stock solution, where peptides were fully soluble. The peptides were then immobilised onto NC filter by using the method of Sithigorngul et al. (1991). The peptides were then screened for human IgE-binding with individual allergic sera and the bound antibodies detected with ^{125} I-labelled anti-human IgE, as described previously.

2.4.5. Densitometric analysis of peptide dot-blot immunoassays

All of the peptide dot-blot underwent densitometric analysis using UltraScan XL (Pharmacia-LKB, Sweden). The intensity of darkness of grey-scale of each dot was measured in comparison to the positive control (rye-grass pollen extract) and expressed as arbitrary densitometric units. The background reaction with negative control was subtracted from the final reading.

2.4.6. Peptide inhibition immunoassays

2.4.6.1. Inhibition dot-blot immunoassay. Peptide numbers 34, 6, 24 and 31 (last three used as negative controls) as well as FPLC purified Lol p 5 and rye-grass pollen extract (used as positive controls) were immobilised on NC and free protein-binding sites blocked with 10% milk powder in PBS as previously described. Two separate lots of 5 ml of sera (of serum F possessing peptide 34-specific IgE; diluted 1:4, as previously described) were pre-incubated overnight at room temp. (18°C) either with 50 μ g of rye-grass pollen extract (designated as inhibited serum) or without rye-grass pollen extract (designated as control serum). Peptide dot-blot were then separately incubated in the inhibited and control sera overnight. Human IgE antibody-binding was detected with ^{125} I-labelled anti-human IgE, as described previously.

2.4.6.2. Inhibition ELISA immunoassay. 10 µg/well of CRPE, in 50 µl/well of coating buffer (60 mM sodium bicarbonate/30 mM sodium carbonate, pH 9.3), was used to coat an ELISA microtiter plate. The proteins were allowed to coat the microtiter plate wells for 2 h at 37°C. After washing wells × 10 with distilled water, the wells were blocked with 1% BSA in PBS for 2 h at 37°C. After washing of all wells, the coated wells were incubated at 37°C for 1 h with 50 µl/well with either control serum pool or serum pool which was pre-incubated with different amounts of peptides (i.e., 6, 24, 31 used as negative control and 34 as the test) and CRPE for 1 h at room temp. (~18°C). After extensive washing, all wells were incubated with 50 µl/well of peroxidase-conjugated human IgE antibodies (Dakopatts, CA, U.S.A.: diluted 1 : 2000 in PBS/BSA) for 1 h each at 37°C. The plates were then incubated with 50 µl/well of peroxidase substrate (0.00024% w/v o-phenylenediamine, 0.0006% H₂O₂ in 0.1 M phosphate-citrate buffer, pH 5) for 10 min at 37°C and the reaction was stopped with 25 µl/well of 4 N HCl. The colour reaction was read at OD 492 nm. Percentage inhibition was calculated by the following formula:

$$\% \text{ Inhibition} = 100$$

$$- \left(\frac{\text{Absorbance value with inhibitor}}{\text{Absorbance value without inhibitor}} \times 100 \right)$$

2.4.7. Truncated peptides and alanine-replacement analysis

Truncated and alanine replacement peptides were synthesised, cleaved, immobilised on NC and immunologically screened as described previously for other peptides. However, truncated peptides varied in length from 4–11 amino acid residues, whereas alanine-replaced peptides were based on peptide 34 sequence and were all 12 amino acid residues in length. These peptides were screened for human IgE antibody-binding as described previously.

3. Results

3.1. IgE-binding characteristics of synthetic peptides based on Lol p 5 sequence

Thirty four overlapping peptides, covering the entire amino acid sequence of mature Lol p 5 protein were synthesised for mapping of potential IgE-binding epitopes of Lol p 5 (Table 1). In order to find a peptide that displayed high IgE-binding capacity, we screened all peptides with 41 sera from grass pollen-allergic individuals. Out of the 41 sera, 26 (63.4%) contained IgE which recognised at least one peptide. Although the majority of sera showed low to moderate IgE-reactivity, there were several displaying high reactivity (Fig. 1). For

example, sera A to G showed substantial reactivity with a selection of specific peptides (Fig. 1).

The peptides displaying the strongest IgE-reactivities were peptides 7, 16, 19, 21, 23, 30 and 34. Peptide numbers 7 and 34 were chosen for the later experiments, due to their very strong IgE-binding capacity (Fig. 1). The sequence of peptide number 34 corresponds to the last 12 C-terminal amino acid residues of the mature Lol p 5 protein.

All of the highly IgE-reactive synthetic peptides of Lol p 5 were found to be rich in the amino acid residue alanine. Another amino acid residue which was common to all these peptides was lysine. However, no strict amino acid composition pattern emerged. For example, while peptide number 34 contained 3 glycine residues, peptide number 7 had none.

Since most of the peptides displaying strong IgE-reactivity were alanine-rich, it could be suggested that they may bind human IgE non-specifically when compared with other peptides which contain fewer or no alanine residues. Accordingly, several immunoassays were performed with sera containing peptide 34-specific IgE antibodies. Binding studies were carried out with other non-IgE binding peptides that were either alanine-rich (i.e., peptides 6 and 31) or alanine-deficient (i.e., peptide 24). Dot-blot immunoassay of peptide 34 showed that IgE bound peptide 34 but not the other alanine-rich or alanine-deficient control peptides (Fig. 2). Thus, peptides containing high numbers of alanine residues do not appear to bind IgE non-specifically. When the same serum was pre-incubated with rye-grass pollen extract, the level of peptide 34-specific IgE-binding dropped significantly (Fig. 2), suggesting that the peptide 34 sequence may exist as a continuous (or linear) IgE-binding epitope in the natural Lol p 5 protein (or in other highly homologous or cross-reactive proteins) in the pollen extract. Moreover, in a preliminary inhibition experiment, we have shown that crude rye-grass pollen extract significantly inhibited IgE-binding to peptide 34 only (data not shown). However, this is not surprising and a more critical inhibition experiment would be the reciprocal of this where the extent to which peptide 34 inhibits IgE antibody binding to the natural protein counterparts in the crude extract should be investigated. Indeed we have conducted such an experiment and Fig. 3 shows that peptide 34 gave a high and significant degree of inhibition of IgE-binding to CRPE (more than 75%). Inhibition of IgE-binding to CRPE by peptide 34 is nearly as high as that by CRPE at the same inhibitor concentration (i.e., difference of only 15%). This slight difference may be due to the presence of many polymorphic forms of Lol p 5 and other proteins in the crude extract possessing the same epitope sequence. The two alanine-rich and one alanine-deficient peptides, used as negative controls, did not show any inhibition. In both of the immunoassays, the level of peptide 34-specific IgE-binding was as high

Table 1
Overlapping peptides synthesised according to the sequence of Lol p 5

Peptide Number	Position (aa)	Sequence	M _r * (Da)	pI*	IgE-binding
1	1–12	ADAGYTPAAAAT	1194	5.0	—
2	9–21	AAATPATPAATPA	1111	6.1	—
3	17–28	AATPAAAGGKAT	987	10.5	—
4	25–36	GKATTDEQKLLE	1333	5.4	—
5	33–44	KLLEDVNAGFKA	1306	8.0	5.0
6	41–52	GFKA AVAAAANA	1062	10.5	2.0
7	49–60	AANAPPADKFKI	1243	10.2	11.0
8	57–68	KFKIFEAAFSSES	1405	7.3	—
9	65–76	FSESSKGLLATS	1227	8.0	—
10	73–84	LATSAAKAPGLI	1113	10.5	—
11	81–92	PGLIPKLDTAYD	1303	5.2	—
12	89–100	TAYDVAYKAAEG	1259	5.3	—
13	97–108	AAEGATPEAKYD	1223	4.5	4.0
14	105–116	AKYDAFVTALTE	1329	4.2	—
15	113–124	ALTEALRVIAGA	1185	8.1	—
16	121–133	IAGALEVHAVKPA	1277	8.0	9.0
17	129–140	AVKPATEEVPA	1183	4.4	—
18	137–149	VPAAKIPTGELQI	1338	8.0	5.0
19	145–156	GELQIVDKIDAA	1272	4.4	11.0
20	153–164	IDAAFKAATAA	1163	7.0	—
21	161–172	ATAANAAPTNDK	1145	7.0	12.0
22	169–180	TNDKFTVFESAF	1407	4.2	2.0
23	177–188	ESAFNKALNECT	1327	4.4	10.0
24	185–196	NECTGGAYETYK	1336	6.1	5.0
25	193–204	ETYKFIPSLEAA	1370	4.4	3.0
26	201–212	LEAAVKQAYAAAT	1236	7.1	—
27	209–221	YAATVAAAPEVKY	1355	7.1	2.0
28	218–229	EVKYAVFEAALT	1342	4.4	—
29	226–238	AALTKAITAMTQA	1292	10.4	3.0
30	234–245	AMTQAQKAGKPA	1202	10.9	8.0
31	242–253	GKPAAAAATGAA	957	10.5	—
32	250–261	TGAATVATGAAT	992	8.2	—
33	258–269	GAATAAAGAATA	904	8.2	—
34	265–276	GAATAAAGGYKA	1009	10.3	13.0

The position of the peptides correspond to the amino acid sequence of the mature Lol p 5 protein. Molecular mass (M_r; kDa) and isoelectric point (pI) have been determined by Macro ProMass 2 software program. IgE-binding values are the highest arbitrary densitometric units, as assessed by the intensity of the grey scale of the dot-immunoassay results shown in Fig. 1.

as that of purified natural Lol p 5 and/or pollen extract and suggests that Lol p 5 is a clinically significant and dominant allergen of CRPE.

3.2. Conservation of allergenic epitopes of Lol p 5 in group 5 allergens from other grass pollens

IgE antibodies from serum F were affinity purified against peptide 34, SDS-PAGE and FPLC purified natural Lol p 5 and rye-grass pollen extract and then used to screen a panel of SDS-PAGE resolved crude extracts of nine clinically important grass pollens (Fig. 4A). The total IgE of serum F recognised major allergens from all of the nine grass pollens (Fig. 4B).

Affinity purified IgE from rye-grass pollen extract bound major allergens from seven of the pollens, includ-

ing canary, Bermuda, Orchard, timothy and Kentucky blue grasses, meadow fescue and wheat (Fig. 4C). Moreover, affinity purified IgE antibodies from both SDS-PAGE or FPLC purified Lol p 5 (Figs 4D and E, respectively) bound to major allergens of canary, orchard, Kentucky blue, timothy grasses and meadow fescue pollens suggesting that the IgE-binding epitopes of Lol p 5 protein must also exist in the pollen of these different grasses. More specifically, affinity purified IgE specific for peptide number 34 recognised the same proteins as those recognised by the Lol p 5-specific IgE (Fig. 4F). Further, although peptide 34-specific IgE antibodies bound to proteins occupying the correct molecular mass of Lol p 5 in rye-grass pollen, other grasses revealed an additional polypeptide, with an approximate molecular mass of 49 kDa, displaying strong IgE-binding. On the other hand,

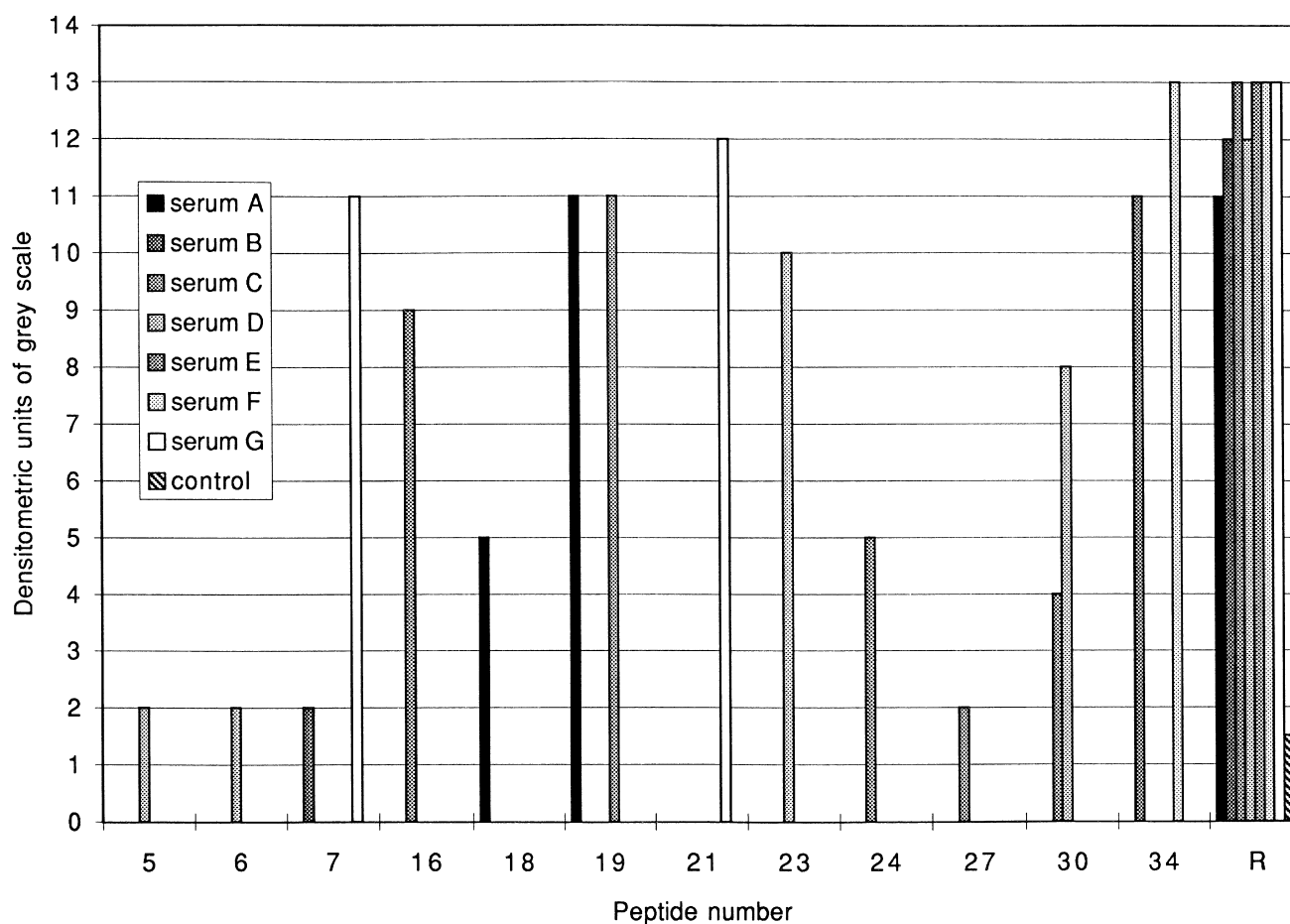


Fig. 1. Dot-blot immunoassay results for IgE-reactive synthetic peptides. Dot-blot immunoassays of top seven sera displaying the strongest IgE-binding with any one of the 34 peptides are shown in arbitrary densitometric units of grey scale (or intensity), measured by Pharmacia LKB UltraScan XL. Densitometric values ≥ 2 were considered positive in comparison to the background. Control serum refer to serum pool obtained from two individuals with no clinical record of grass pollen allergy, used as negative control. Human IgE-reactivity demonstrated by peptide numbers 7 and 34 were as much as that demonstrated by the crude natural rye-grass pollen extract, used as positive control (denoted by R).

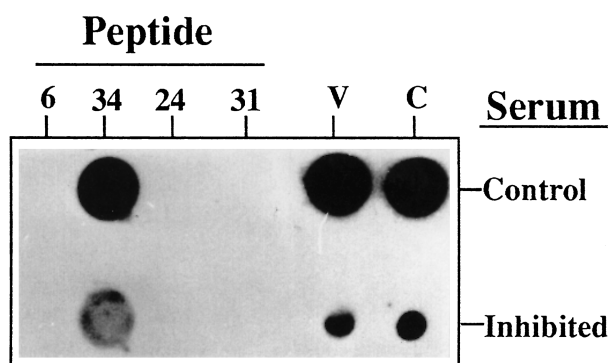


Fig. 2. Inhibition dot-blot immunoassays of IgE-reactive peptide 34 of Lol p 5. Crude rye-grass pollen extract inhibition of the level of IgE-antibody binding, from serum F, to peptide number 34. Peptide number 34, along with peptide numbers 6, 24 and 31 as negative controls and purified natural Lol p 5 [V] and rye-grass pollen extract [C] as positive controls, were immobilised onto NC and then incubated either with normal [control] serum or serum pre-incubated with 50 $\mu\text{g}/\text{ml}$ rye-grass pollen extract overnight [inhibited serum]. Antibody-binding was detected with ^{125}I -labelled anti-human IgE.

although recognised by total IgE from serum F, major allergens of Johnson and Bermuda grass pollens, which belong to different subfamilies, did not bind any of the affinity purified specific IgE antibodies from rye-grass pollen extract, Lol p 5 and peptide 34, suggesting absence of group 5 homologs in these grass pollens. No antibody binding to any of the pollen proteins was detected when incubated with affinity purified IgE from a milk powder blocked NC membrane (negative control).

3.3. Amino acid sequence identity with other group 5 grass pollen allergens

The degree of amino acid identity of the sequences of these peptides is compared with that of other group 5 allergens in Table 2, which lists the peptide sequences against the corresponding known sequences of other group 5 allergens. Table 2 shows that a high degree of sequence identity occurs between peptides 7 and 34 with

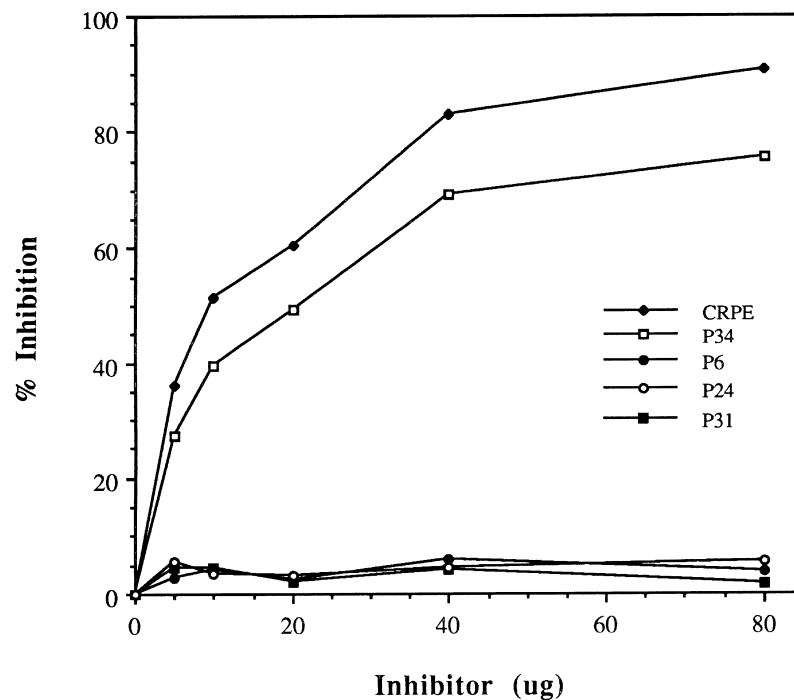


Fig. 3. Inhibition ELISA of IgE-reactive peptide 34 of Lol p 5. Synthetic peptides (P6, P24, P31, P34) and rye-grass pollen extract were used as the inhibitors and CRPE was immobilised onto the walls of ELISA microtiter plate wells. Peptide numbers 6, 24 and 31 were used as negative controls, while CRPE was used as positive control. Antibody-binding of a serum pool of 3 individuals allergic to rye-grass pollen and possessing peptide 34-specific IgE was detected colorimetrically at OD 492 nm.

all of the other group 5 allergens. Peptide 7 shares up to 58.3% amino acid identity with the corresponding sequences of other group 5 allergens. More importantly, amino acid residues Ala₄₉ and Lys₅₇ are strictly conserved in other allergens. Similarly, peptide 34 contains a 4 amino acid residue motif GGYK (amino acid residues 272–275) which is highly conserved in the sequences of the other group 5 allergens (Table 2).

3.4. Immunodissection of major IgE-binding epitopes of Lol p 5

To further resolve the molecular basis of the interactions between human IgE and strong IgE-reactive synthetic peptides of Lol p 5, sixteen truncated peptides, based on the sequences of peptide 7 and peptide 34, were synthesised (Table 3). The peptides were synthesised from both ends of the sequence so that each subsequent peptide truncated one amino acid residue at a time to a minimum peptide length of 4 amino acid residues. The results of dot-blot immunoassays of these truncated peptides, displaying human IgE antibody binding are shown in Table 3. Antibody binding is expressed only as a single + since the relative strength of binding was the same for all peptides when compared with that of the full length peptides (i.e., peptides 7 and 34).

For peptide 7, the dot-blot immunoassay results show that the key amino acid residue, within the context of the peptide, is Lys₅₇ for IgE-binding since KFKI peptide (i.e., #16) appears to be the minimal epitope and gives comparable binding to peptide 7 (Table 3). It is interesting to note that, while truncation 13 of peptide 7 is negative, further truncations are positive. This may be due to the proline residues' possible interference, within that particular length of the peptide, with accessibility of the human IgE. For peptide 34, the deduced key amino acid residues that may be critical for human IgE antibody-binding appear to be GGYK, occupying amino acid positions 272–275 (Table 3). It is interesting to note that amino acid residue Lys₅₇ of peptide 7 is strictly conserved at this position among other group 5 allergens (Table 2). Similarly, amino acid residues GGYK of peptide number 34 are strictly conserved among other group 5 allergens (Table 2). However, this does not mean that these linear epitopes will be accessible by IgE antibodies within the context of the different natural group 5 allergens.

In order to further resolve the IgE-binding capacity of GGYK, we have conducted an alanine replacement study (Table 3). We synthesised a series of peptides, corresponding to the sequence of peptide 34, in which every non-alanine amino acid residue was replaced with alanine, one at a time (Table 3). The peptides were then

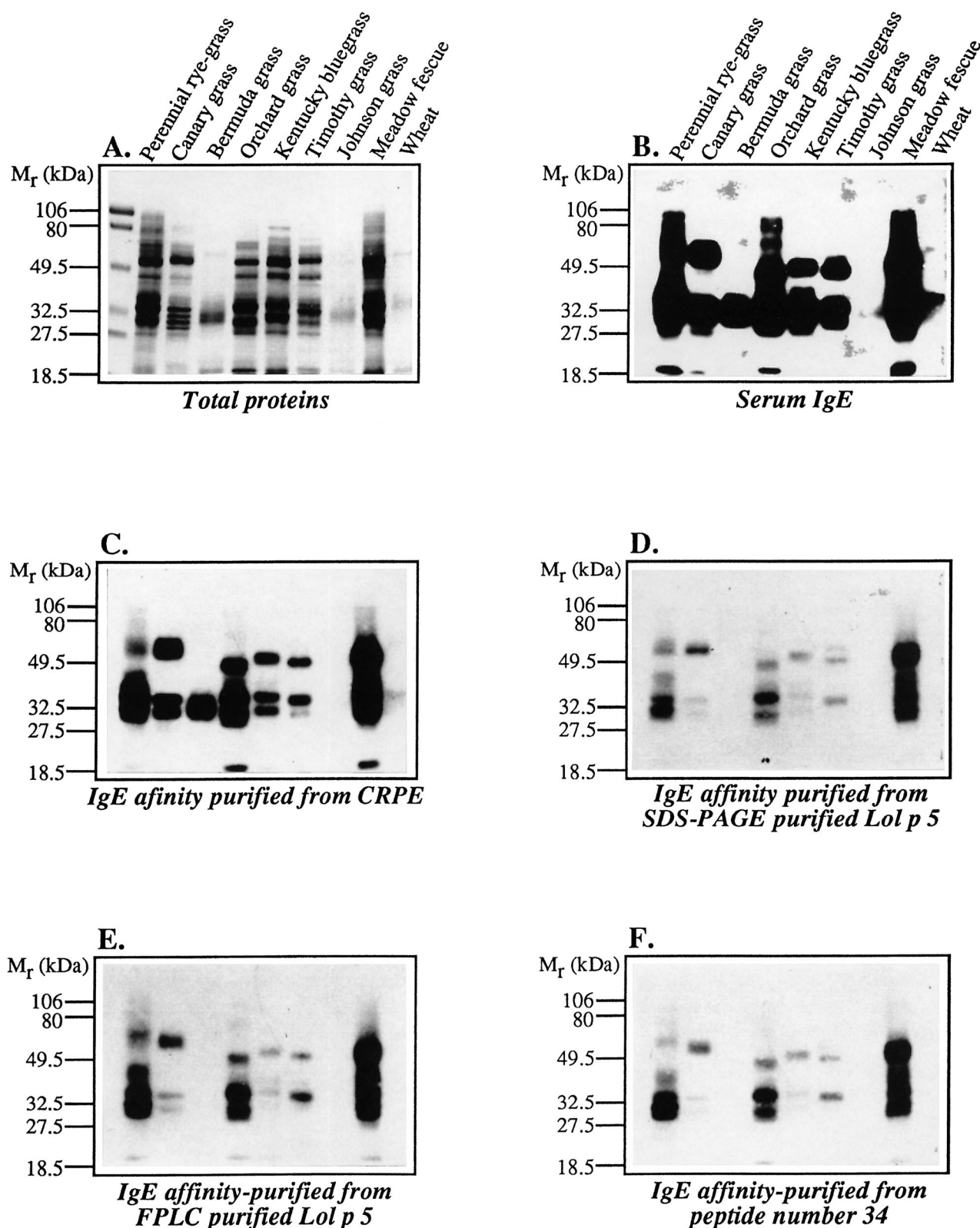


Fig. 4. Specificity of IgE affinity-purified from natural Lol p 5 rye-grass pollen extract and peptide number 34 for grass pollen proteins. Soluble pollen proteins from 9 grasses were separated by SDS-PAGE and either stained with coomassie brilliant blue R250 (A) or transferred to nitrocellulose (B, C, D, E, F). Western blots were screened either with total IgE from serum F (B) or IgE affinity-purified from rye-grass pollen extract (C), SDS-PAGE purified Lol p 5 (D), FPLC purified natural Lol p 5 (E) and peptide number 34 (F). IgE affinity-purified from a control piece of NC (which underwent the same steps as others) did not bind any of the proteins (not shown). Molecular mass (M_r) is denoted (kDa) at the left.

Table 2
Comparison of the immunoreactive peptide sequences of Lol p 5 with other group 5 grass pollen allergens

Grass	Allergen	IgE-binding peptides of Lol p 5									
		7	16	19	21	23	30	34			
		AANAPPADKFKI	IAGALEVHAVKPA	GELQIVDKIDAA	ATAANAAPTNDK	ESAFENKALINECT	AMTQAQKAGKPA	GAATAAAGGYKA			
<i>Lolium perenne</i>	Lol p 5B ¹	**VV**	*****	A*V*LI**V***	*****A**	*NT**N*IKVSL	**SE*E*EAT**	T*TP*****V			
<i>Phalaris aquatica</i>	Pha a 5 ²	W*SV*****T	*****	*****	*****S*****	*G*****IKS*	**SE***VA**P	*GSHR*R***V			
<i>Phleum pratense</i>	Phl p 5A ³	**GVQ*****YRT	*****	***VIE*V***	*****A***	*A***D*IKAS*	**SE***A***	*****T***V			
<i>Phleum pratense</i>	Phl p 5B ⁴	*RQR*A*****T	*****V	*****I*****	***AT**AD**	*A*****IKS*	**SEV**VSQ**	*****V*****V			
<i>Poa pratensis</i>	Poa p 5(31) ⁵	**GV*A*S*Y*T	*****G*****	***VI**V***	*****A***	*A***D*IKAS*	**S*****A***	*****V*****V			
<i>Poa pratensis</i>	Poa p 5(41) ⁵	**GV*AV**Y*T	*****	***VI**V***	*****A***	*A***D*IKAS*	**S*****A***	*****T*****T			
<i>Poa pratensis</i>	Poa p 5(60) ⁵	*GGV*A*N*Y*T	*****G*****	***VI**V***	*****A***	*A***D*IKAS*	**S*****A***	*****V*****V			

Similar amino acids in the corresponding sequences are indicated by an asterisk and different ones are listed. Underlined amino acids of the peptide sequences indicate their occurrence in all of the compared sequences. Lol p 5B is one of the isoforms of Lol p 5 that have been cloned and sequenced in our laboratory (Ong et al., 1993). References are represented by the bold numbers in superscript as follows: (1) Ong et al., 1993; (2) Suphioglu and Singh, 1995; (3) Bufe et al., 1994; (4) Bufe et al., 1995; (5) Silvanovich et al., 1991.

Table 3

Truncated and alanine-replacement peptides synthesised according to the sequences of peptides 7 and 34 of Lol p 5

Peptide Number	Position	Sequence	Human IgE-binding
(A) Peptide 7	49–60	AANAPPADKFKI	+
1	49–59	AANAPPADKFK	+
2	49–58	AANAPPADKFK	+
3	49–57	AANAPPADK	+
4	49–56	AANAPPAD	—
5	49–55	AANAPPA	—
6	49–54	AANAPP	—
7	49–53	AANAP	—
8	49–52	AANA	—
9	50–60	ANAPPADKFKI	+
10	51–60	NAPPADKFKI	+
11	52–60	APPADKFKI	+
12	53–60	PPADKFKI	—
13	54–60	PADKFKI	—
14	55–60	ADKFKI	+
15	56–60	DKFKI	+
16	57–60	KFKI	+
(B) Peptide 34	265–276	GAATAAAGGYKA	+
17	265–275	GAATAAAGGYK	+
18	265–274	GAATAAAGGY	—
19	265–273	GAATAAAGG	—
20	265–272	GAATAAAG	—
21	265–271	GAATAAA	—
22	265–270	GAATAA	—
23	265–269	GAATA	—
24	265–268	GAAT	—
25	266–276	AATAAAGGYKA	+
26	267–276	ATAAAGGYKA	+
27	268–276	TAAAGGYKA	+
28	269–276	AAAGGYKA	+
29	270–276	AAGGYKA	+
30	271–276	AGGYKA	+
31	272–276	GGYKA	+
32	273–276	GYKA	—
(C) Peptide 34	265–276	GAATAAAGGYKA	+
33	265–276	<u>AA</u> ATAAAGGYKA	+
34	265–276	GAAAAAAGGYKA	+
35	265–276	GAATAAAAGGYKA	+
36	265–276	GAATAAAGGYKA	+
37	265–276	GAATAAAGGAKA	+
38	265–276	GAATAAAGGYAA	—

(A) Truncated peptide numbers 1–16 are based on the sequence of peptide 7; as shown in Table 1. (B) Truncated peptide numbers 17–32 are based on the sequence of peptide 34; as shown in Table 1. (C) Peptide numbers 33–38 are based on the sequence of peptide 34 in which non-alanine residues have been replaced with alanine one at a time (shown in underlined A). Human IgE-binding is based on peptide 7 and peptide 34-specific IgE from serum G and F, respectively, shown in Fig. 1.

screened for human IgE antibody binding. The results clearly show that as soon as the amino acid residue Lys₂₇₅ was replaced with alanine, IgE antibody binding was abolished (Table 3). This highlights the importance of

this amino acid residue, within the context of peptide 34, for the molecular interaction with IgE antibodies. Moreover, the lysine residue (Lys₅₇) of peptide 7 was also found to be the critical aa residue for antibody binding and, along with Lys₂₇₅ of peptide 34, is strictly conserved among other group 5 allergens at these sequence positions, highlighting its importance within the context of the peptides for antibody binding and shared reactivity between different group 5 allergens.

4. Discussion

Here, we present our preliminary, but significant, data concerning the linear sequences of amino acids that constitute the strong IgE-binding epitopes of Lol p 5. Both rye-grass and group 5 allergens are very important allergens and make a good model for immunoregulatory studies in humans.

4.1. IgE-binding characteristics of synthetic peptides based on Lol p 5 sequence

In order to resolve the IgE epitopes of Lol p 5, we have used the synthetic peptide approach, with peptide lengths of 12–13 amino acids. Synthetic peptide approaches of epitope mapping have proved to be most useful for pollen allergens (Elsayed et al., 1991; Kawashima et al., 1992; Vik et al., 1993; van Ree et al., 1994, 1995). For example a synthetic peptide corresponding to the *N*-terminal segment of the major allergen of birch pollen (Bet v 1 23–38) was IgE-reactive when tested by specific IgE inhibition, skin-prick test, nasal provocation and Práusnitz–Küstner inhibition assays (Vik et al., 1993).

Synthetic peptides in our experiments were 12–13 amino acid residues in length, and overlapped each neighbouring peptide by at least 4 amino acids. The peptide and amino acid overlap lengths were chosen to maximise antibody accessibility and binding while minimizing continuous epitope disruptions (Scheidtmann, 1990).

As a result of screening our 34 overlapping synthetic peptides with serum IgE from grass pollen allergic individuals, we have identified two peptides, numbers 7 and 34, which display very strong IgE-reactivity when compared with other peptides and CRPE. More importantly, the IgE-reactive peptide, number 34, occupied the last 12 *C*-terminal amino acid residues of the molecule. It is possible that this amino acid segment of the allergen molecule is continuously exposed, perhaps at the surface of the folded natural protein, and thus may be readily accessible to the IgE antibodies. Indeed, in this study, we clearly demonstrate that IgE-reactivity of peptide 34 can be significantly inhibited by natural Lol p 5 and CRPE, and more importantly, peptide 34 showed significant inhibition of IgE-binding to CRPE highlighting that this

epitope occur continuously within the context of the natural allergen. Similarly, an IgE-reactive epitope of Lol p 1 has been located in the last 28 amino acid residues of the *C*-terminus (Esch and Klapper, 1989; van Ree et al., 1994). However, Lol p 1, unlike Lol p 5, contains many cysteine residues, which are required for the formation and stabilisation of secondary and tertiary structures. As a result, no further IgE-binding epitopes of Lol p 1 have been identified by synthetic peptide mapping, perhaps due to the conformational nature of the epitopes within the context of the natural protein as a result of its seven cysteine residues, which is involved in disulphide bonds and thus the stabilisation of tertiary structure of the molecule.

4.2. Conservation of allergenic epitopes of Lol p 5 in group 5 allergens from other grass pollens

Through the affinity purification of human IgE antibodies specific to natural and synthetic peptides of Lol p 5, we have demonstrated that there is considerable cross-reactivity between Lol p 5, and its strongly IgE-reactive peptide 34, and allergens from other clinically important grasses. Our results suggest that IgE-binding epitopes of rye-grass pollen allergens, and more specifically the epitope represented by peptide 34, are either cross-reactive with or are immunologically closely related to the major allergens of other clinically significant grass pollens. However, out of all the grass pollens assessed in this cross-reactivity study, only pollen from Johnson and Bermuda grass, which belong to different subfamilies, indicated lack of antibody-binding. It can be argued that these grass pollens may not possess Lol p 5-similar or -homologous proteins or alternatively, due to their natural protein conformation, the epitopes may not be exposed and accessible by the affinity-purified IgE antibodies used in this study. On the other hand, differences in intensity of IgE-binding between the different grass pollens may be due to the different amounts of group 5 allergens being expressed.

Since there is a considerable degree of allergenic cross-reactivity between allergens at the protein level, the question arises whether the sequences of the IgE-reactive synthetic peptides of Lol p 5 are conserved in group 5 allergens of other grass pollens. Indeed, the IgE-reactive synthetic peptides of Lol p 5 demonstrate strict motifs of amino acid sequence conservation with group 5 allergens from other grass pollens. These results highlight the importance of molecular analysis of cross-reactivity since common IgE-binding epitopes appear to be present in different allergens. Therefore, it is important to identify the critical amino acids involved in the recognition process of such cross-reactive epitopes as such information may greatly simplify the diagnosis and treatment of allergies towards these proteins.

4.3. Immunodissection of major IgE-binding epitopes of Lol p 5

In order to resolve these epitopes further and deduce the amino acid residues that may be critical for antibody-binding, we chose to perform truncated peptide studies of the strongest IgE-reactive peptides of Lol p 5, numbers 7 and 34, followed by alanine replacement studies. Each non-alanine amino acid residue of a given sequence was replaced, one at a time, with alanine in peptide 34 only, since truncated versions of this peptide did not resolve any single aa residue responsible for antibody binding. Since antibody-binding epitopes of proteins are known to be composed of continuous or discontinuous portions of the polypeptide chain, where the discontinuous epitopes are formed by two or more non-contiguous segments brought into proximity by the folding of the protein, it has been suggested that experimental approaches relying on the linear sequence of the protein to locate and define antigenic determinants, such as those employed in this study, would miss many biologically significant sites (van Regenmortel, 1989; Laver et al., 1990). On the other hand, other studies have shown that short synthetic peptides can also provide information about discontinuous epitopes, since all such discontinuous epitopes contain linear segments (Gertzoff et al., 1988). Indeed, recent studies using peptide-based assays have identified the antigenically important portions of discontinuous epitopes (Redlich et al., 1991; Savoca et al., 1991), down to the resolution of single critical amino acid residues (Geysen et al., 1987; Appel et al., 1990). For Lol p 5, we have successfully identified single amino acid residues of peptides 7 and 34 that are critical for the binding of human IgE antibodies. These were Lys₅₇ for peptide 7 and Lys₂₇₅ for peptide 34.

Since serum IgE from different individuals recognised the two peptides, it is suggested that different IgE antibodies may possess specificity towards a selection of different amino acid residues. However, if indeed the antibody binding to both of the peptides was significantly due to a single amino acid residue, in the context of the respective peptides, this residue is likely to be lysine, since it is the only amino acid residue that is present in all of the IgE-reactive truncated peptides and is the only residue of peptide 34 that abolished IgE-binding in alanine replacement studies. Moreover, these lysine residues of peptide numbers 7 and 34 are highly conserved in the corresponding sequences of other group 5 grass pollen allergens. On the other hand, since other non-IgE binding peptides presented in this study also contain the amino acid residue lysine, one can argue that a single amino acid residue is unlikely to be responsible for antibody binding. Indeed, Xing et al., 1992 demonstrated that for mucin mAbs, free peptides corresponding in sequence to the core amino acid residues did not necessarily inhibit the binding of the antibody to the antigen, indicating that

other amino acids were required for binding of the mAb. These 'flanking regions' may be important for providing the secondary or tertiary structure required for antibody binding (Cason et al., 1989). In another study, Geysen et al., 1984 identified leucine residues at positions 148 and 151 of the antigenic epitope of foot-and-mouth disease virus coat protein, corresponding to amino acid residues 146–152, as critical to antibody binding. Moreover, since we have demonstrated that the IgE-reactivity of peptide 34 can be significantly reduced with natural Lol p 5 and CRPE, Lys₂₇₅ may also play a critical role in the molecular recognition and binding process of human antibodies within the context of the natural protein. More importantly, peptide 34 was able to block IgE binding of sera to the whole pollen extract by 75%. This value is almost equal to the blocking by the extract itself (i.e., 75% of IgE antibodies, present in the sera tested, were directed to one peptide on one molecule). This is remarkable and highlights the clinical significance of Lol p 5 as the dominant allergen of crude rye-grass pollen extract and peptide 34 as an important B-cell epitope of Lol p 5, among the sera tested.

It will now be interesting to mutate the key B-cell epitope aa residues of recLol p 5 with site-directed mutagenesis and evaluate its IgE-reactivity, thereby not only providing hypoallergenic version of Lol p 5, which will be useful in immunotherapy, but confirming our synthetic peptide results and, more specifically, the true significance of these key aa residues in Lol p 5's IgE-reactivity. Indeed, such site-directed mutagenesis strategies have proved successful in reducing IgE-reactivity of house dust mite allergen Der p 2 (Smith and Chapman, 1997) and birch pollen allergen Bet v 1 (Ferreira et al., 1996). Availability of such hypoallergenic mutants, with reduced or abolished IgE-reactivity but maintaining T-cell activating capacity, will be useful candidates in immunotherapy. Since we have recently mapped the T-cell epitopes of Lol p 5 (Blaher et al., 1996) and present its B-cell epitopes here, we are now at an exciting stage where we can carry out site-directed mutagenesis of IgE-reactive recLol p 5 to abolish its IgE-reactivity and thereby providing material for use in immunomodulation experiments.

5. Conclusions

Taken together, the molecular basis of IgE-recognition of Lol p 5 clearly involves the recognition of key amino acid sequence motifs or single amino acid residues of the allergen. Our results suggest that these IgE-binding epitopes occur continuously in the context of the folded natural proteins of Lol p 5. Moreover, the conservation of the amino acid sequence of these IgE-binding epitopes in other clinically important grass pollen allergens highlight the importance of the cross-reactivity that is con-

stantly observed, both clinically and experimentally, between different grass pollen allergens.

Now that critical amino acid residues responsible for the IgE-recognition of Lol p 5 have been identified, the effect of these residues within the context of the entire recombinant protein can be investigated by mutating the gene encoding Lol p 5 at these critical sites of site-directed mutagenesis. Since we have already identified the T-cell epitopes of Lol p 5 (Blaher et al., 1996), expression of such modified allergens, without altering T-cell epitopes, are likely to be useful in immunotherapy without the side-effects of current crude pollen extracts.

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