

IgE and IgG cross-reactivity among *Lol p I* and *Lol p II/III*

Identification of the C-termini of *Lol p I*, *II*, and *III* as cross-reactive structures

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In this study, the homologous C-termini of *Lol p I*, *Lol p II*, and *Lol p III* were shown to contain cross-reactive B-cell epitopes. This was demonstrated by inhibition studies with purified *Lol p I*, *II*, and *III* and synthetic peptides of their C-termini. It was ruled out that the observed cross-reactivity was caused by cross-contamination of the purified allergens. Both human IgE and IgG bound to the C-terminus of *Lol p I*. These antibodies were cross-reactive with *Lol p II* and, more specifically, with its C-terminus. Within a small panel of allergic patients, no cross-reactivity with *Lol p III* was found. A hyperimmune polyclonal rabbit antiserum against *Lol p I* also recognized the *Lol p I* C-terminus. As for human antibodies, cross-reactivity with *Lol p II* and its C-terminus was demonstrated. Cross-reactivity with *Lol p III* was demonstrated with C-terminal peptides, but not with native *Lol p III*. A polyclonal rabbit antiserum against *Lol p II* bound to the C-terminal peptides of both *Lol p II* and *III*. This binding was inhibited with *Lol p I*, confirming that cross-reactive structures exist not only on the C-termini of *Lol p II* and *Lol p I*, but also of *Lol p III* and *Lol p I*. The existence of cross-reactivity between *Lol p I* and *Lol p II* and *III* possibly contributes to the frequently observed cosensitization for these allergens in grass-pollen-allergic patients.

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After the identification of *Lol p I*, *II*, and *III* as important grass pollen allergens by Marsh and co-workers (15–18), it was demonstrated that IgE against *Lol p II* and *III* is hardly ever seen without IgE against *Lol p I* (6, 11). Ansari et al. investigated whether this concordance is caused by cross-reactive antibodies. From inhibition studies with human IgG (from patients receiving grass pollen immunotherapy) and rabbit IgG, it was concluded that cross-reactivity between *Lol p I* and *Lol p II/III* does not exist (2). Although cross-inhibition was demonstrated between *Lol p I* and *Lol p II/III*, it was concluded that this was caused by cross-contamination instead of cross-reactivity. Therefore, researchers proposed similar HLA-restriction (HLA-DR3) of the antibody responses to *Lol p I* and to *Lol p II* and *III* as explanation (2–5, 12).

Recent progress in allergen research has resulted

in the determination of the complete amino acid sequences of several allergens, among them the three grass pollen allergens (5, 6, 13, 19). The C-termini of *Lol p I*, *II*, and *III* proved to be highly homologous (6). It was further demonstrated that the 28 C-terminal amino acids of *Lol p I* contain an important IgE-binding site (9). Computer analysis of the sequences of *Lol p II* and *III* predicted the same for their C-termini (6). These new insights prompted us to investigate whether cross-reactivity could be demonstrated by focusing on antibodies reactive with the C-terminus of *Lol p I*. So that cross-contamination was not mistaken for cross-reactivity, the purified allergens from *Lolium perenne* were carefully checked for maximum impurities. Here we report the identification of the C-termini of *Lol p I*, *II*, and *III* as cross-reactive structures for both human IgE and IgG and for rabbit IgG.

Material and methods

Allergen extracts and allergens

Ryegrass (*L. perenne*) pollen was obtained from Diephuis Laboratories (Groningen, The Netherlands). The pollen was extracted as described previously (24). In short, it was extracted with distilled water (5% w/v) for 2 h. The pH was maintained at 8.5 by readjustment with NaOH (0.1 mol/l) every 15 min. After centrifugation, the supernatant was defatted, dialyzed against distilled water, and freeze-dried.

Purified allergens from *L. perenne*, *Lol p I, II, and III*, were obtained from the National Institute of Allergy and Infectious Diseases (Bethesda, MD, USA): NIH research reagents A611-901-185, A612-901-185, and A613-901-185, respectively. For radiolabeling of *Lol p I*, a different *Lol p I* preparation was used. In this case, *Lol p I* was purified physicochemically, as described elsewhere (24).

Peptides

Synthetic peptides were made by the T-bag method (14), adopted for Fmoc-chemistry (21). The peptides synthesized are listed in Fig. 1. Peptides were analyzed by reversed-phase HPLC (Delta Pak C-18, gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile, UV-detection at 214 and 280 nm). Nine showed one main peak and only minor contaminations. For four peptides (*Lol p II* (61–75), *Lol p II* (71–85), *Lol p III* (61–75), and *Lol p III* (66–80)), a second large peak was detected (67, 95, 95, and 80% of the highest peaks, respectively). The main peak of three peptides was therefore analyzed (*Lol p I* (216–240), *Lol p II* (73–97), and *Lol p II* (61–75)). After hydrolysis with 6 N HCl for 48 h at 110°C, and derivatization with PITC, HPLC analysis of the PITC derivatives revealed that the main peak contained the correct amino acids. Because *Lol p I* (216–240) is crucial for this study, the second peak (about 20% of the major peak) was purified and analyzed as well. It appeared that the amino acid composition of this peak was identical to that of the major peak. The observed difference in retention time was possibly caused by inefficient deprotection of side-chains for part of the *t*-peptides. Immune reactivity of crude and purified peptides was compared in a RAST and a binding assay with radiolabeled peptide. No differences were found (data not shown).

Radiolabeling

Synthetic peptides *Lol p I* (216–240), *Lol p II* (73–97), and *Lol p III* (71–97), and purified *Lol p I* were radiolabeled by the chloramine-T method with

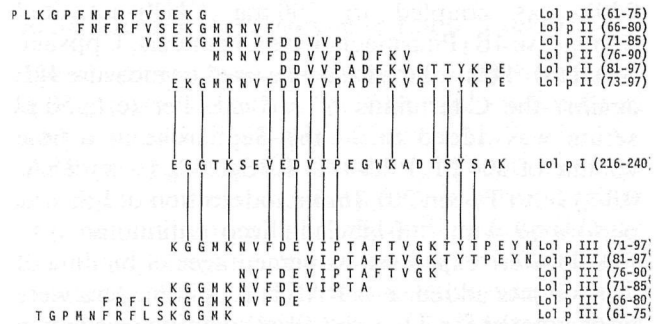


Fig. 1. Peptides synthesized of C-termini of *Lol p I, II, and III*, aligned for maximum homology. Vertical lines indicate identical or similar residues.

carrier-free ^{125}I (Amersham, Amersham, UK); 20 μg of peptide or allergen was labeled with 1 mCi of ^{125}I . Unbound ^{125}I was separated from the labeled peptide on a Sephadex G-25 column equilibrated in PBS with BSA (3 mg/ml). For purified allergens, a Sephadex G-75 column was used.

Sera

Sera used in the RAST were selected from sera submitted for serologic allergy testing (RAST). For 46 sera, a positive RAST for grass pollen (> 10% binding) was used as selection criterion. Twenty-three sera with a negative RAST for grass pollen (< 2% binding), but with IgE against other inhalant allergens, were used as negative control. Three of the RAST-positive sera that showed the highest binding to Sepharose-coupled *Lol p I* (216–240) were used for RAST-inhibition studies.

Sera ($n = 47$) used to measure IgG against the *Lol p I* C-terminus were selected from sera of grass-pollen-allergic patients that received immunotherapy. The presence of specific IgG4 against *Lol p I* was used as selection criterion. Two of these sera that showed the highest binding of radiolabeled *Lol p I* (216–240) were used in inhibition assays with ^{125}I -labeled-*Lol p I* (216–240).

Rabbit antisera

Rabbit polyclonal anti-*Lol p I* was prepared by immunization with purified *Lol p I*, as previously described (24). Rabbit polyclonal anti-*Lol p I* (LMW) is an antiserum raised by immunization with a partially purified *Lol p II* preparation (20). Besides *Lol p II*, it also recognizes *Lol p III*, an 11-kDa degradation product of *Lol p V*, and an unidentified 17-kDa allergen.

RAST

RAST was performed as described previously (1). An amount of 100 μg crude peptide *Lol p I* (216–

240) was coupled to 100 mg CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). This solid phase was used to measure IgE against the C-terminus of *Lol p I*. Per test, 50 μ l serum was added to 0.5 mg Sepharose in a final volume of 300 μ l (PBS-AT: PBS/0.3% (w/v) BSA/0.1% (v/v) Tween 20). Immunodetection of IgE was performed with ^{125} I-labeled-sheep antihuman IgE. Results were expressed as percentages of binding of total counts added. For RAST-inhibition, sera were preincubated for 2 h with (serial) dilution of allergen or synthetic peptide before addition of Sepharose. Results were expressed as percentages of the uninhibited value (without addition of inhibitor).

Competitive RIA

For detection of human IgG or rabbit IgG against the C-terminus of *Lol p I*, II, or III, a binding assay was applied with protein A-Sepharose (Pharmacia) as IgG antibody-capturing agent and radiolabeled *Lol p I* (216–240), *Lol p II* (73–97), or *Lol p III* (71–97). Human sera were tested in a 1:10 dilution (50 μ l) with 0.5 mg Sepharose and 12 000 cpm ^{125} I-labeled-peptide in a final volume of 350 μ l (PBS-AT). Rabbit anti-*Lol p I* and rabbit anti-*Lol p*_(LMW) were tested in dilutions of 1:50 and 1:10, respectively. Results were expressed as cpm bound to the solid phase. This assay was also performed as a competitive RIA, in which antiserum was preincubated for 2 h with serial dilutions of allergen or peptide before addition of Sepharose and radiolabeled peptide. Results were expressed as percentage of the uninhibited value.

Lol p I assay

A crucial control for this study was the measurement of contamination of *Lol p II* and III with *Lol p I*. For quantification of *Lol p I*, a competitive RIA was used with radiolabeled *Lol p I* and a group I specific monoclonal antibody (mAb) SV 24.64, kindly provided by Dr R. E. Esch (Chapel Hill, NC, USA). This antibody binds at a site distant from the *Lol p I* C-terminus (10). Sepharose-coupled goat antibodies against mouse IgG were used as antibody-capturing agent instead of protein A-Sepharose. It cannot be excluded that the mAb SV 24.64 is, to some degree, cross-reactive with *Lol p II* and *Lol p III*. The results obtained in this assay should therefore be regarded as the upper limit of *Lol p I* contamination.

Results

Lol p I content of purified *Lol p II* and III

Purified *Lol p II* and *Lol p III* were tested for contamination with *Lol p I* by a competitive RIA with

a group I specific moab (SV 24.64) and ^{125} I-labeled-*Lol p I*. Both *Lol p II* and *Lol p III* contained 0.1% of *Lol p I* activity on a molar basis. These values are upper limits of contamination, since the antigroup I antibody could be cross-reactive with group II and/or group III allergen.

Reactivity with the C-terminus of *Lol p I*

A panel of 46 sera with IgE against grass pollen was tested for IgE-reactivity with the C-terminus of *Lol p I*. For this purpose, a Sepharose-coupled synthetic peptide of the 25 C-terminal amino acids of *Lol p I* was used as solid phase in a RAST. More than 40% showed IgE-reactivity (>2% binding). None of the 23 grass-pollen-negative sera with a positive RAST for other inhalant allergens showed reactivity with the C-terminal peptide (Fig. 2A). For three sera, the

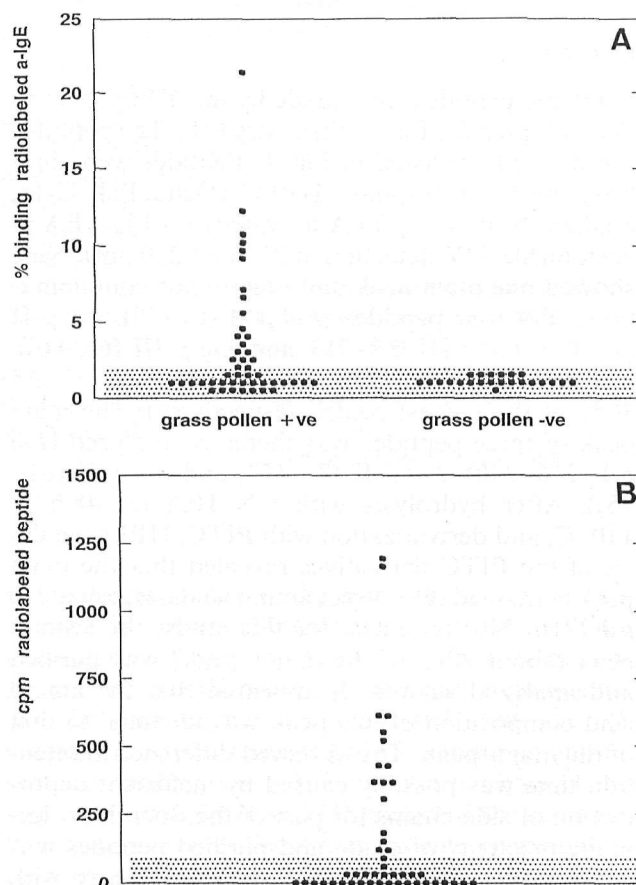


Fig. 2. Reactivity of human antibodies with C-terminus of *Lol p I*. A) IgE reactivity with *Lol p I* (216–240). Sera were tested in RAST with Sepharose-coupled C-terminal peptide of *Lol p I*. All sera that scored >2% binding were regarded as positive. In grass-pollen-positive group, >40% was positive. In control group, no positive sera were found. B) Binding of ^{125} I-labeled-*Lol p I* (216–240) to human IgG. Binding of radiolabeled *Lol p I* C-terminus to human IgG was shown for 16 of 47 sera tested. All sera were from patients that received grass pollen immunotherapy.

binding of IgE to *Lol p* I (216–240) was inhibited with grass pollen extract. At 0.5 mg per test, one serum was inhibited for 75%, whereas the binding of IgE from both of the other sera was hardly affected (20% and <1% inhibition). IgG reactivity of patients receiving immunotherapy for grass pollen was tested with radiolabeled C-terminal peptide. Of 47 sera with specific IgG against *Lol p* I, 16 had IgG that bound to the *Lol p* I C-terminus (Fig. 2B). The specificity of this binding was shown by inhibition with purified *Lol p* I (Fig. 3A). The C-terminus was 10 times more potent as inhibitor on a molar basis than the native allergen. Polyclonal rabbit antiserum raised with purified *Lol p* I also recognized the radiolabeled *Lol p* I C-terminus. A rabbit antiserum against cat dander used as a negative control was not reactive. The binding to anti-*Lol p* I was inhibited with grass pollen extract, but not with extract of house-dust mite (data not shown). As for human IgG, the synthetic C-terminus was more potent as inhibitor than native *Lol p* I (Fig. 3B).

Reactivity with the C-termini of *Lol p* II and III

The 47 sera of patients receiving grass pollen immunotherapy were tested in an RIA with radiolabeled peptides *Lol p* II (73–97) and *Lol p* III (71–97). No significant binding was found (<100 cpm), as was the case for rabbit anti-*Lol p* I. A polyclonal rabbit antiserum raised with partially purified *Lol p* II (anti-*Lol p*_(LMW)) was reactive with *Lol p* II C-terminal peptide (3240 cpm) and cross-reactive with *Lol p* III C-terminal peptide (840 cpm). The binding of both radiolabeled peptides was inhibited with *Lol p* II and III and its C-termini (Fig. 4A). Except for inhibition of the binding of ¹²⁵I-labeled-*Lol p* III (71–97) with *Lol p* II and its C-terminus, it was observed that the C-terminal peptides were stronger inhibitors than the native allergen.

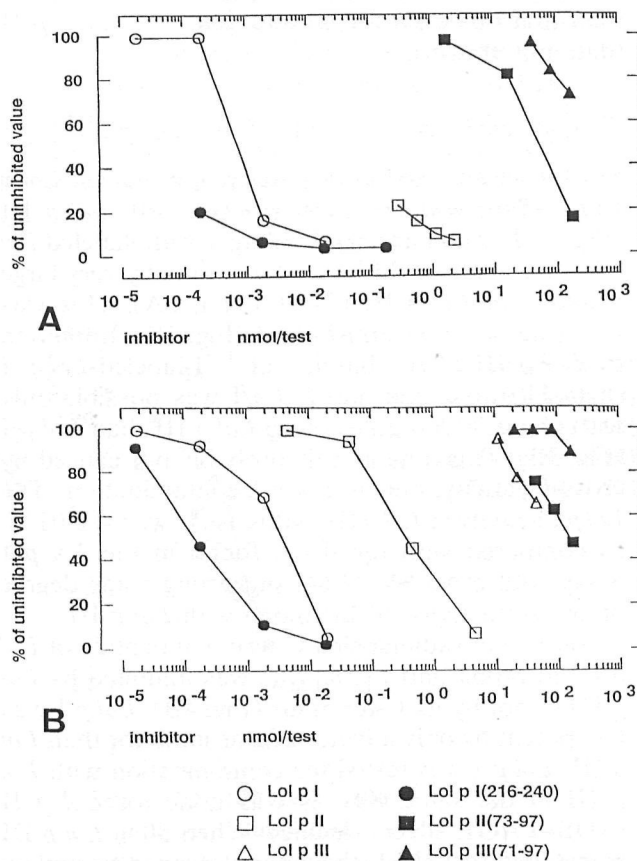


Fig. 3. Inhibition of binding ¹²⁵I-labeled-*Lol p* I (216–240) to IgG. A) Binding of radiolabeled *Lol p* I C-terminus to human IgG was inhibited by *Lol p* I and II and C-termini of *Lol p* I, II, and III. B) Inhibition of binding of radiolabeled *Lol p* I C-terminal peptide to rabbit anti-*Lol p* I by *Lol p* I, II, and III and their C-termini.

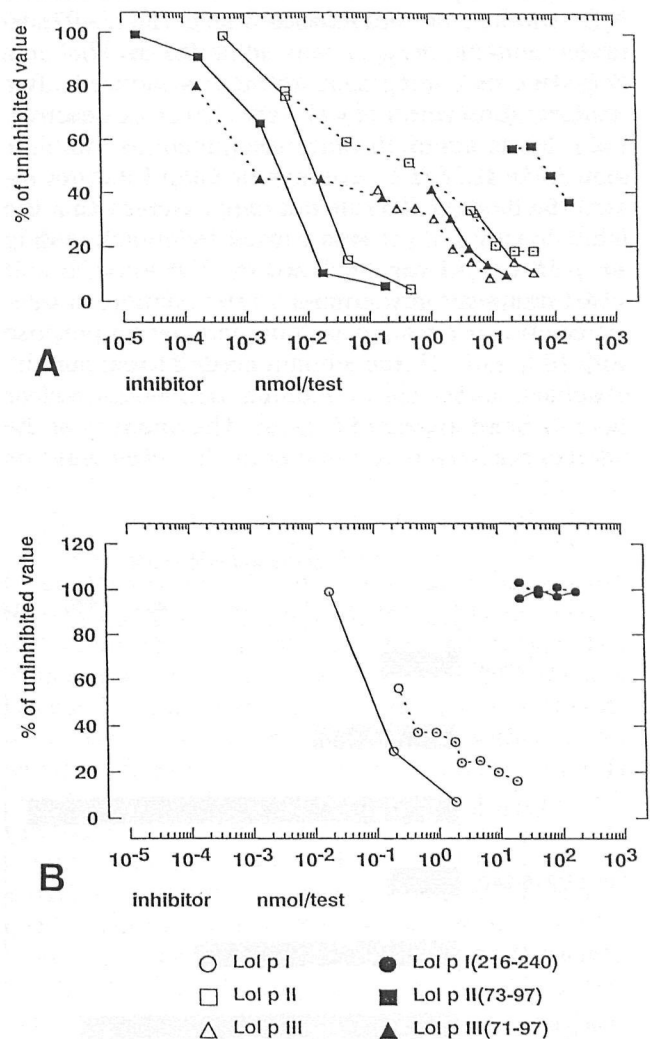


Fig. 4. Inhibition of binding of radiolabeled C-termini of *Lol p* II and III to rabbit anti-*Lol p*_(LMW). Solid line: ¹²⁵I-labeled-*Lol p* II (73–97), dotted line: ¹²⁵I-labeled-*Lol p* III (71–97). A) Inhibition with *Lol p* II and III and its C-termini. B) Inhibition with *Lol p* I and its C-terminus.

Cross-reactivity between Lol p I and Lol p II

Binding of IgE to Lol p I (216–240) was inhibited by Lol p I and II and their C-termini (Fig. 5). The concentrations used were 1 and 5 mg/ml, respectively. The binding of human IgG (from two sera) to radiolabeled Lol p I (216–240) was also inhibited by Lol p II and its C-terminus (Fig. 3A). On a molar basis, Lol p I was 200 times more efficient as inhibitor than Lol p II. This means that the inhibitory potency of Lol p II was about five times higher than would be expected from the upper limit of Lol p I contamination (0.1%). The inhibitory potency of the C-terminus of Lol p II was 10^6 times lower than that of Lol p I (216–240).

Binding of radiolabeled Lol p I (216–240) to rabbit anti-Lol p I was also inhibited by Lol p II and its C-terminus (Fig. 3B). The relative inhibitory potencies were comparable to those observed with human IgG. Binding of radiolabeled Lol p II (73–97) to rabbit anti-Lol p_(LMW) was inhibited by Lol p I (Fig. 4B), indicating that antibodies induced after immunization with Lol p II were also cross-reactive. Lol p I was about 10 times less potent as inhibitor than Lol p II. The C-terminus of Lol p I did not inhibit the binding. To rule out the possibility that the inhibition by Lol p I was caused by contaminating Lol p II, Lol p I was analyzed by SDS-PAGE and silver staining. Only a minor contamination was detected. When Lol p I was contaminated on purpose with 10% Lol p II (the amount needed to explain the observed inhibition by contamination alone), a clear Lol p II band appeared (Fig. 6). The intensity of the band of the Lol p II contamination in Lol p I was less

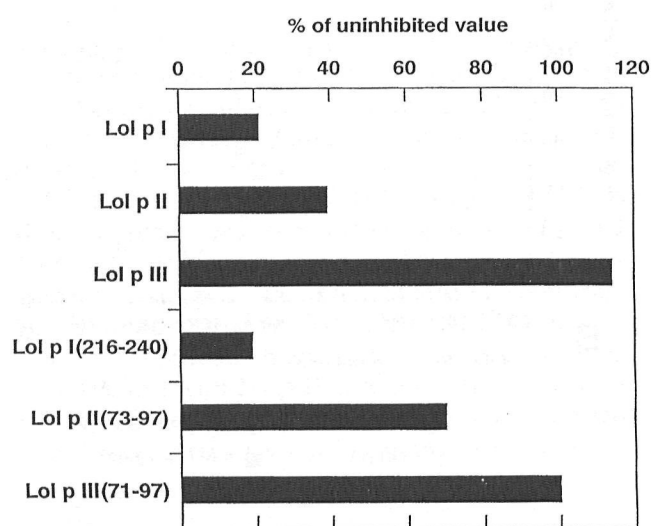


Fig. 5. Inhibition of Lol p I (216–240) RAST. Inhibition is shown for one serum with Lol p I, II, and III (1 mg/ml) and its C-termini (5 mg/ml). Uninhibited % binding was 12.3%; blank without serum, 0.7%.

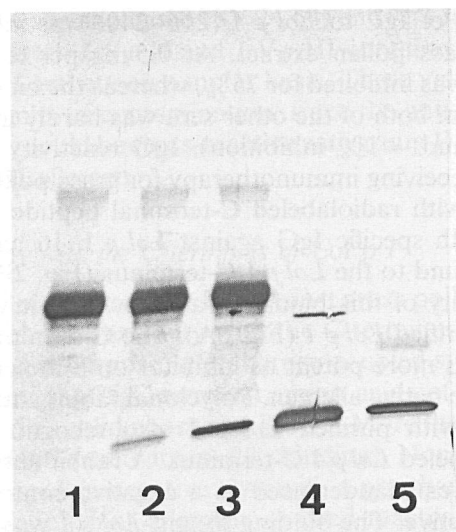


Fig. 6. SDS-PAGE analysis of purified allergens from *Lolium perenne*. Allergens were analyzed on 8–18% gradient gel (Excel-gel, Pharmacia). Lanes 1, 2, and 3: 1 µg of Lol p I. In lane 2, 40 ng of Lol p II was added (about 10% on molar basis); in lane 3, 200 ng of Lol p III (about 50% on molar basis); in lanes 4 and 5, 1 µg of Lol p II and 1 µg of Lol p III, respectively.

than that observed for an addition of 1% Lol p II (data not shown).

Cross-reactivity between Lol p I and Lol p III

For the serum used in this study, IgE against Lol p I (216–240) was not cross-reactive with Lol p III (Fig. 5). For human IgG, binding of radiolabeled Lol p I C-terminus could be inhibited only by very large amounts of Lol p III (71–97) (Fig. 3A). This was also true of rabbit anti-Lol p I (Fig. 3B). Inhibition by Lol p III of the binding of ^{125} I-labeled-Lol p I (216–240) to rabbit anti-Lol p I was possible only with about 30 000 times more Lol p III than Lol p I (Fig. 3B). This inhibition is probably not caused by cross-reactivity, but by Lol p I contamination. The Lol p I activity in Lol p III in this assay was $<0.01\%$, as compared with the 0.1% found in the Lol p I assay with mAb SV 24.64, suggesting some degree of cross-reactivity of this moAb with Lol p III.

Binding of radiolabeled C-terminal peptide of Lol p III to rabbit anti-Lol p_(LMW) was inhibited by Lol p I, but not by its C-terminus (Fig. 4B). Lol p I was less potent by only a factor of 2 as inhibitor than Lol p III. Lol p I was tested for contamination with Lol p III in the same way as was done for Lol p II (SDS-PAGE/silver staining). When 50% Lol p III was added to Lol p I (the amount needed to explain the inhibition by contamination alone), a clear band was demonstrated, and this was virtually absent in purified Lol p I (Fig. 6). As for Lol p II, the Lol p III contamination was $<1\%$.

Which amino acids form the cross-reactive site for rabbit anti-*Lol p I*?

For rabbit anti-*Lol p I*, the amino acids in *Lol p II* and *III* responsible for the observed cross-reactivity were more precisely determined. The binding of *Lol p I* (216–240) was inhibited by shorter overlapping peptides of the C-termini of *Lol p II* and *III*. For *Lol p II*, peptide 66–80 was the best inhibitor (Fig. 7). For *Lol p III*, a similar pattern was observed (data not shown). In addition, *Lol p III* (81–97) inhibited the binding of *Lol p I* (216–240) as strongly as *Lol p III* (66–80).

Discussion

In earlier studies, the 28 C-terminal amino acids of *Lol p I*, obtained after CNBr-cleavage and tryptic digestion, were shown to contain an important IgE-binding site (9). We now confirm this observation with a synthetic peptide comprising the 25 C-terminal amino acids. In addition, it was shown that the C-terminus of *Lol p I* is recognized by human and rabbit IgG antibodies that were induced after immunization with grass pollen extract or purified *Lol p I*, respectively. Binding of radiolabeled *Lol p I* C-terminus to these antibodies was inhibited by *Lol p I* and its C-terminus. In contrast to studies with peptides derived from *Der p II* (21) and *Fel d I* (22, 23), synthetic peptide was 10 times more potent as inhibitor on a molar basis than the native allergen. This phenomenon was also observed for anti-

bodies induced by immunization with *Lol p II*. *Der p II* and *Fel d I* derived peptides were about 10^6 times less potent than *Der p II* and *Fel d I*, respectively. The exceptionally high inhibitory potency of the C-terminal peptides in this study indicates that the preferential conformation of the synthetic peptides closely resembles that of the C-termini in the native allergens. In addition, it could indicate direct stimulation of antibody production by peptides formed by degradation of native allergen.

In contrast to earlier reports that cross-reactivity between *Lol p I* and *Lol p II/III* does not exist (2, 6, 7), we have clearly shown that a cross-reactive structure is present on the C-termini of these allergens. Ansari et al. had shown that binding of IgG to *Lol p I* was inhibited by *Lol p II* and *Lol p III*, and vice versa. They concluded that this was caused by cross-contamination, since the degree of inhibition did not exceed the inhibition that could be expected from the contaminations (0.01%–0.1%). In our system, we found that the degree of inhibition was several times higher than what could be accounted for by contamination. We cannot completely exclude the possibility that part of the inhibition of the binding of the *Lol p I* C-terminus to *Lol p II* and *III* was caused by contamination with proteolytic fragments of *Lol p I*. Inhibition by synthetic C-termini of *Lol p II* and *III* did, however, confirm the existence of cross-reactivity. The difference between our and Ansari and coworkers' studies is probably caused by the selection of the antibodies used to demonstrate cross-reactivity: in the latter studies antinative allergen as opposed to anti-C-terminal peptide in the present study. Among antinative antibodies, the share of cross-reactive antibodies was probably too small to be detected. Human IgE against the *Lol p I* C-terminus was cross-reactive with *Lol p II* and its C-terminus. Cross-reactivity with *Lol p III* was not found for the serum tested. RAST-inhibition assays with more sera are needed to determine whether IgE against *Lol p I* can be cross-reactive with *Lol p III*. Human IgG antibodies against the *Lol p I* C-terminus were also shown to be cross-reactive with *Lol p II* and its C-terminus. At high concentrations, *Lol p III* (71–97) inhibited the binding of *Lol p I* (216–240). Inhibition of the binding of the *Lol p I* C-terminal peptide to rabbit anti-*Lol p I* by native *Lol p III* was possible, although 30 000 times more *Lol p III* was needed than *Lol p I*. It is most likely that this inhibition was caused by *Lol p I* contamination of *Lol p III*. By the *Lol p I* assay with mAb SV 24.64, this contamination was estimated to be no higher than 0.1%. The inhibition of the binding of radiolabeled *Lol p I* (216–240) indicates that the contamination is <0.01%, and that SV 24.64 is, to some extent, cross-reactive with *Lol p III*. To prove unequivocally the existence of cross-reactivity between *Lol p I* and

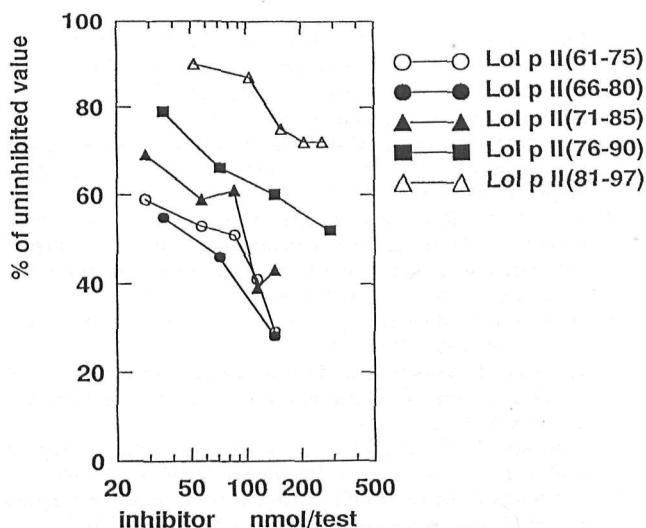


Fig. 7. Inhibition of binding of ^{125}I -labeled-*Lol p I* (216–240) to rabbit anti-*Lol p I* with overlapping peptides of *Lol p II* C-terminus. For more accurate identification of cross-reactive structure on C-termini of *Lol p I* and *II*, binding of ^{125}I -labeled-*Lol p I* (216–240) was inhibited by short overlapping peptides from *Lol p II* C-terminus.

Lol p III, another antiserum was used: rabbit anti-*Lol p*_(LMW). Binding of the C-terminus of *Lol p* III was inhibited by *Lol p* I. This inhibition could not be accounted for by contamination of *Lol p* I with *Lol p* III, since *Lol p* I was weaker by only a factor of 2 as inhibitor than *Lol p* III. It is unlikely that purified *Lol p* I contains 50% of *Lol p* III on a molar basis.

Ansari et al. (6) performed computer analysis on the amino acid sequences of *Lol p* II and *Lol p* III, according to Fraga and Hopp & Woods. For *Lol p* II and III, a B-cell epitope was predicted in the C-terminal part of the molecule, amino acids 75–85 and 73–83, respectively. In addition, we performed the analysis according to Chou & Fasman (8). B-cell epitopes were predicted for *Lol p* II around amino acids 71–79, and for *Lol p* III around 68–75 and 90–97. Although the value of prediction programs is debatable, the results basically agreed with the data presented in this paper. This was especially true of the analysis according to Chou & Fasman. The inhibition of the binding of ¹²⁵I-labeled-*Lol p* I (216–240) to rabbit anti-*Lol p* I with short overlapping peptides from the C-termini of *Lol p* II and III was most efficient by *Lol p* II (66–80), *Lol p* III (66–80), and *Lol p* III (81–97). The epitopes predicted by the method of Chou & Fasman all fall within the sequences of these synthetic peptides.

IgE against *Lol p* II/III is almost always accompanied by IgE against *Lol p* I. Ansari et al. proposed similar HLA-restriction to explain this phenomenon (2). The existence of cross-reactive structures on the C-termini of *Lol p* I, II, and III is an additional factor that might contribute to the observed cosensitization for these allergens. Recently, the synthetic C-terminus of *Lol p* I was used to immunize rabbits. Resulting antisera were shown to react with native *Lol p* I, and were possibly cross-reactive with *Lol p* II (paper in preparation). A synthetic peptide that induces antibodies with a broad spectrum of (cross-) reactivity is of potential interest for application in immunotherapy. The C-terminal peptide used in this study, *Lol p* I (216–240), does not have maximum overlap with the cross-reactive structures identified on the C-termini of *Lol p* II and III. For this reason, a synthetic peptide was made that spans amino acids 204–228 of *Lol p* I. It will be investigated whether this peptide mimics the cross-reactive site with *Lol p* II and III more closely. This would considerably increase its potential for application in immunotherapy.

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