

Allergenic Fragments of Ryegrass (*Lolium perenne*) Pollen Allergen *Lol p IV*¹

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Abstract. To facilitate studies on establishing the nature of structure/function relationships of allergens, ryegrass pollen allergen, *Lol p IV*, was cleaved into smaller fragments by cyanogen bromide (CNBr) and the resulting peptides were further digested with trypsin. The resulting peptides were then fractionated by high performance liquid chromatography (HPLC) on a C-18 reverse phase column. The allergenic activity of the HPLC fractions was evaluated in terms of their ability to inhibit the binding of ¹²⁵I-*Lol p IV* to serum IgE antibodies of a grass-allergic patient. Many of these fractions inhibited the binding between the native allergen and IgE antibodies in a dose-dependent manner. The inhibitions were specific, i.e., the fractions did not inhibit the binding between ¹²⁵I-*Lol p I* (a group-I ryegrass pollen allergen) and the IgE antibodies present in the allergic human serum. The possibility that the allergenic peptide fractions were contaminated by the native undegraded allergen, which might have accounted for the observed inhibition, was ruled out by the fact that the native allergen could not be detected by SDS-PAGE and the elution profiles of allergenically active peptides did not coincide with that of native allergen. One of the allergenic sites recognized by monoclonal antibody (Mab) 90, i.e., site A, was located in HPLC fractions 90-100 while another allergenic site B (recognized by Mab 12) appeared to be lost following the sequential digestion of *Lol p IV* with CNBr and trypsin.

Introduction

In recent years, many studies have reported on the isolation and characterization of allergens. However, only a few attempts have been made to identify the regions on an allergen that bind human IgE antibodies [1-4]. Following elucidation of the amino acid sequence of ragweed allergen Ra3, the determinants capable of binding human IgE antibodies were identified in a novel approach using synthetic overlapping peptides representing the entire protein [3, 5]. Thus, the identification and characterization of allergenic determinants are important primary steps which provide information essential for the synthesis of allergenic peptides. Moreover, allergenic peptides of known amino acid sequence could also serve as a basis for the construction of synthetic probes for use in cloning allergens by molecular recombinant tech-

niques [6-8]. Application of similar strategies to an allergen such as *Lol p IV* (molecular weight about 57 kD) would first require controlled degradation of the allergen in order to elucidate the amino acid sequence of the various peptide fragments possessing the relevant allergenic determinant(s).

We have recently reported on the identification of allergenic sites on *Lol p IV*, which is one of the major allergens of ryegrass pollen [9]. With the aid of murine anti-*Lol p IV* monoclonal antibodies (Mabs), at least two different allergenic sites recognized by human IgE antibodies have been detected and designated as sites A and B [10]. In another study, we found that cleavage of *Lol p IV* with cyanogen bromide (CNBr) produced peptides, of which two, CB-1 (17.4 kD) and CB-2 (22 kD) retained allergenic activity [11]. In the present study, an attempt was made to produce even smaller fragments of the allergen by cleaving *Lol p IV* first with CNBr and then further digesting the resulting peptides with trypsin. The resulting peptides were fractionated by high performance liquid chromatog-

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raphy (HPLC) and the peptide fractions eluted from HPLC were analyzed for their ability to bind to human serum IgE antibodies by a sensitive radioimmunoassay (RIA) inhibition procedure.

Materials and Methods

Allergic Human Serum

The serum of a patient (B.W.) sensitive to grass pollens was selected on the basis of positive skin prick test and RAST results. At the time that blood was drawn, the patient had not received immunotherapy with grass pollen extracts. The RAST was performed on polyvinyl microtiter plates which had been coated with *Lol p IV* (2 µg/ml in 0.1 M carbonate-bicarbonate buffer, pH 9.0), blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and followed by overnight incubation with 100 µl of the allergic serum. After washing the wells with PBS, ¹²⁵I-labelled mouse monoclonal anti-human IgE (clone No. CIA/E/4.15, kindly supplied by Dr. A. Saxon, UCLA, Los Angeles, Calif.) was added to the wells (about 100,000 cpm/well) and incubated overnight at room temperature. The wells were washed 5 times with PBS containing 1% Tween 20 and cut out of the plates. The levels of radioactivity were measured in a Beckman gamma counter. The *Lol p IV*-specific IgE antibodies could be detected by RAST up to a serum dilution of 1:80.

Isolation of *Lol p IV*

Lol p IV was isolated from the retentate fraction of ryegrass (*Lolium perenne*) pollen according to the procedure described earlier [9]. The homogeneity of *Lol p IV* was evaluated by SDS-PAGE, in 10% gel, using the electrophoresis Phast system (Pharmacia Fine Chemicals, Montreal, Que., Canada).

Cleavage of *Lol p IV* with CNBr and Trypsin

Lol p IV was cleaved with a 100-fold molar excess of CNBr according to the procedure described earlier [11]. Briefly, 4 mg of *Lol p IV* was dissolved in 3.92 ml of 70% formic acid; 80 µl of a solution of CNBr (100 mg/ml of 70% formic acid) was added and the reaction was allowed to proceed in the dark in a glass-stoppered round-bottom flask for 18 h. The digest mixture was then diluted with 50 ml of distilled water and lyophilized in the same flask. The lyophilized material was reconstituted in 1.92 ml of 0.05 M NH₄HCO₃ (pH 7.75) and 0.08 ml of trypsin tosylphenylethyl chlormethyl ketone (Worthington Biochemical Co., N.J.; 1 mg/ml in NH₄HCO₃ buffer) was added. The mixture was kept on a horizontal shaker for 18 h and the digestion was stopped by the addition of 6 drops of 2 N acetic acid.

Fractionation of Digest Mixture by HPLC

The *Lol p IV* peptides (approximately 2 mg) generated by CNBr and trypsin digestion were fractionated by HPLC (Waters Associates Inc., Milford, Calif.) using a Vydac C-18 reverse phase column (4.6 mm × 25 cm; 218 TP 54.6 C-18, Vydac, Hesperia, Calif.). An aqueous solution of 0.1% trifluoroacetic acid (TFA) was used as solvent A and 0.1% TFA in 70% propanol as solvent B. A linear gradient was applied for 2 h and the absorbance of the eluent was measured at 214 nm. The eluted fractions of 0.5 ml each, were collected, lyophilized and reconstituted in 0.35 ml of 1% BSA in PBS

prior to testing for their ability to bind IgE antibodies by an RIA inhibition procedure described below.

Identification of Allergically Active Peptide Fractions by a RIA Inhibition Procedure

The ability of each peptide fraction to bind to human IgE antibodies was analyzed by an RIA inhibition procedure as described elsewhere [12]. Briefly, polyvinyl microtiter plates were coated with mouse monoclonal anti-human IgE (cell line 193.23C4.3/56.09; 0.15 µg/well), blocked with 2% BSA in PBS, followed by overnight incubation with the serum of a grass allergic patient (B.W.). After washing the wells with PBS, the HPLC peptide fractions were each premixed with ¹²⁵I-labelled *Lol p IV* (about 100,000 cpm) for 5 h at room temperature before adding to the wells. For control, ¹²⁵I-*Lol p IV* was mixed with 1% BSA in PBS. The extent to which each peptide fraction inhibited the binding between ¹²⁵I-*Lol p IV* and the human IgE antibodies on the solid phase was calculated using the formula: Inhibition (%) = [(A-B)/A] × 100 where A = cpm bound by the wells in the absence of a peptide fraction and B = cpm bound by the wells in presence of the peptide fraction. The test was performed in triplicate.

Detection of Sites A and B in the Peptide Fractions

The murine anti-*Lol p IV* Mab 90 (anti-site A) and Mab 12 (anti-site B) [10] were used to identify, respectively, the presence of sites A and B in the peptide fractions separated by HPLC. An amount of approximately 1 mg of the *Lol p IV* peptides generated by CNBr and trypsin digestion were fractionated by HPLC as described above. The eluted fractions of 0.5 ml each were lyophilized and reconstituted in 0.5 ml of coating buffer (0.05 M NH₄HCO₃, pH 7.5), and volumes of 100 µl of each fraction were used to prepare the solid phase by coating wells of the flexible polyvinyl microtiter plate overnight. The wells were washed with PBS-Tween solution and blocked with 2% BSA in PBS. This was followed by overnight incubation with ¹²⁵I-labelled preparations of Mab 90 or Mab 12 (approximately 100,000 cpm/well). The ability of these labelled Mab preparations to bind to the solid phase peptide fractions was expressed in terms of the amount (cpm) of Mab which could bind to a given peptide fraction relative to that amount of Mab which bound the intact *Lol p IV*: i.e., binding capacity (%) = [A-B/A] × 100, where A = cpm bound by the *Lol p IV* and B = cpm bound by the peptide fraction. The test was performed in triplicate.

SDS-PAGE

SDS-PAGE was performed on 8-25% gradient Phast gels obtained from Pharmacia Fine Chemicals, Sweden, using the Phast system. The gels were stained with Coomassie brilliant blue and destained on the Phast system according to the manufacturer's instructions.

ELISA

The ELISA was performed on flat-bottom microtiter plates (Costar Serocluster, Cambridge, Mass.) coated with 0.2 µg of *Lol p IV* in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.0) at 4°C, overnight and then blocked with an aqueous solution of gelatin (3%) at room temperature for 3 h. Following washings with PBS-Tween 20 (1%) solution, the wells were incubated overnight with purified Mab 12 (2 µg/ml). The wells were again washed with PBS-Tween 20 solution and incubated with rabbit anti-mouse IgG alkaline phosphatase (Sigma Chemicals, St. Louis, Mo.; 1:1,000

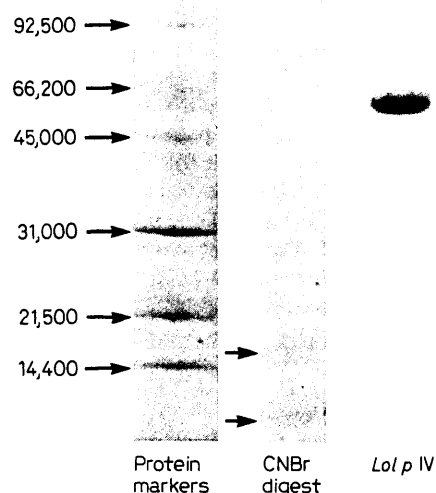


Fig. 1. SDS-PAGE on 8–25% gradient polyacrylamide gel. Proteins separated in the gel were visualized with Coomassie blue stain.

v/v; diluted in PBS containing 1% BSA) for 1 h. The wells were again washed with PBS-Tween, 100 μ l of substrate solution was added and 4 min later, the absorbance at 410 nm was measured using an automated ELISA reader (Dynatech Laboratories, Inc., Alexandria, Va.).

Amino Acid Sequence Analysis

The amino acid sequences of the peptide fractions were determined by Dr. D.J. McKay at the Protein Sequencing Facility, University of Calgary, Ala., Canada.

Results

Cleavage of Lol p IV with CNBr and Trypsin

Peptides with molecular weights lower than 20,000 daltons were obtained by cleaving Lol p IV with a 100-fold molar excess of CNBr (fig. 1). Intact Lol p IV was not detectable in the CNBr digest by SDS-PAGE (fig. 1) or on Western immunoblot analysis with Mab 12 (not shown). Further cleavage of the CNBr digest with trypsin produced peptides which could not be detected by SDS-PAGE in 8–25% gradient gel (not shown). These results clearly demonstrated that the CNBr digest did not contain intact Lol p IV.

Allergenic Activity of the Peptide Fractions of Lol p IV Separated by HPLC

Fractionation of the CNBr and tryptic-derived peptides by HPLC gradient elution on a Vydac C-18 column resulted in about 180 fractions (fig. 2). The allergenic activity of these peptide fractions was evaluated in terms of their ability to inhibit the binding between ^{125} I-Lol p IV and human IgE antibodies. In this assay, many peptide fractions were found to possess the inhibitory capacity, some as high as 80% (fig. 3). The peptide fractions (68, 73, 81, 105, 132) with the highest degree of inhibition were all found to inhibit the binding of ^{125} I-Lol p IV to human IgE antibodies in a dose-dependent manner (fig. 4). Fraction 81, which exhibited a high degree of inhibition between Lol p IV and human IgE antibodies did not inhibit the binding between ^{125} I-Lol p I (a ryegrass group-I allergen) and human IgE antibodies, suggesting that

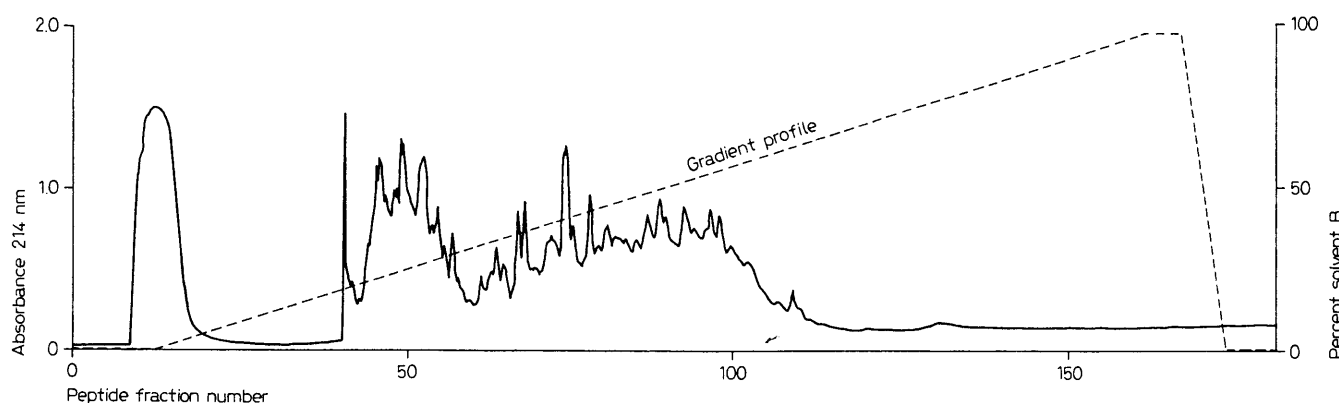


Fig. 2. Separation of CNBr and tryptic digest of Lol p IV (2 mg) by HPLC on a reversed phase C-18 column equilibrated with solvent A (0.1% TFA); a linear gradient of solvent B (70% propanol, 0.1% TFA) was applied over a period of 2 h.

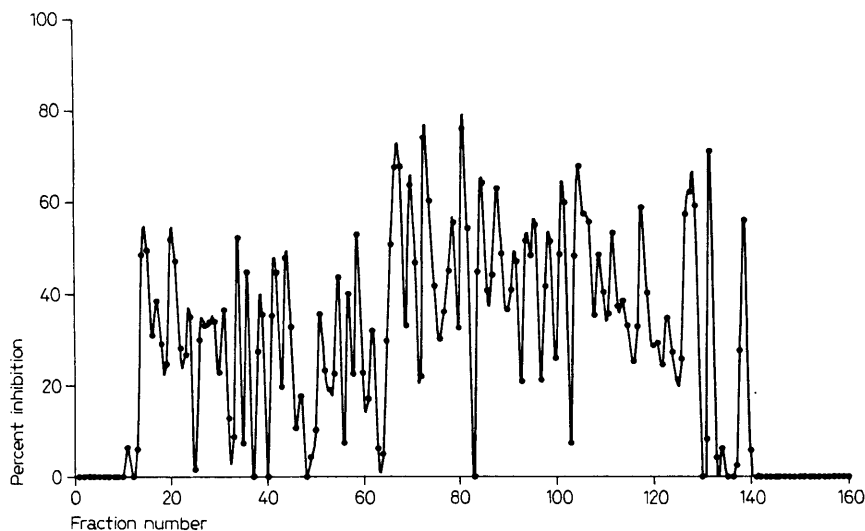


Fig. 3. Allergenic activity of CNBr and tryptic derived peptides of *Lol p* IV separated by HPLC. The polyvinyl microtiter plates were coated with murine monoclonal anti-human IgE (0.15 μ g/well). Volumes of 100 μ l of allergenic serum (B.W.) was then added and incubated overnight. Following washings with PBS-Tween solution ^{125} I-*Lol p* IV was pre-mixed with fractionated peptides and added to the wells. The percent inhibition was calculated using the formula: Inhibition = $[(A-B)/A] \times 100$, where A = cpm bound to the well in absence of peptide fraction and B = cpm bound to the well in presence of peptide fraction.

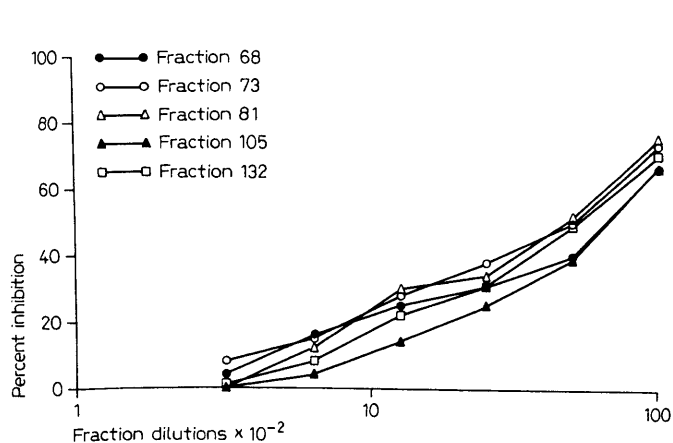


Fig. 4. Dose-related inhibition of allergenic active peptides of *Lol p* IV. The test was performed as described in figure 3 using 2-fold serial dilutions of peptide fractions.

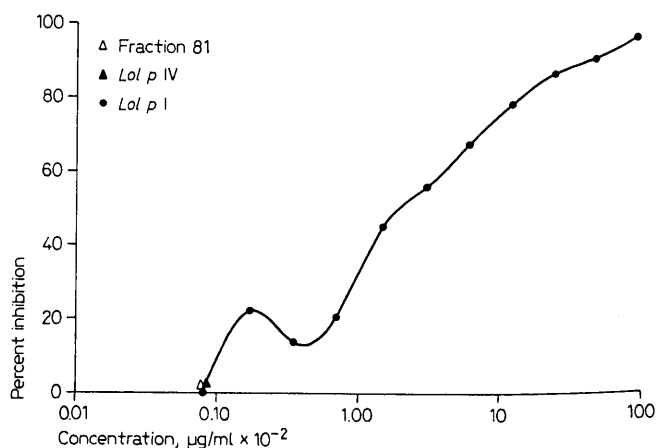


Fig. 5. Specificity of inhibition demonstrated by the allergenic peptide fraction analyzed by RIA inhibition, using an unrelated system of ^{125} I-*Lol p* I (a rye grass group-I allergen) and the IgE antibodies present in B.W. serum. The test was performed as described in figure 3.

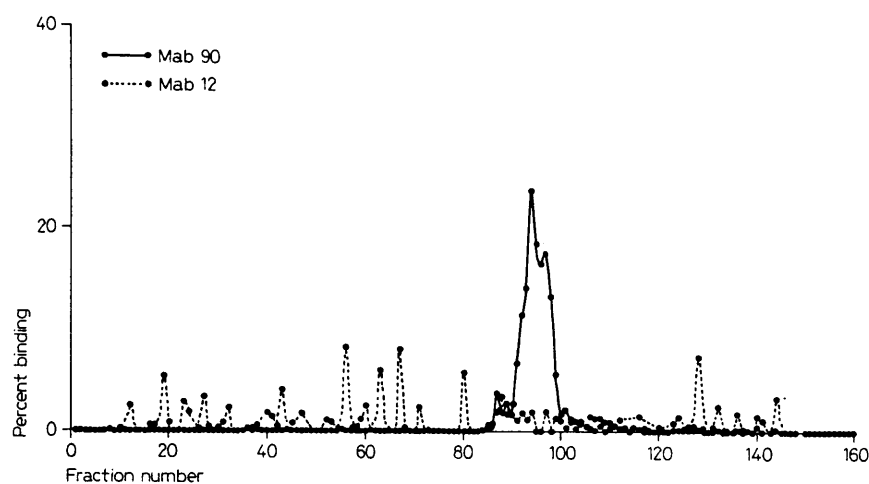


Fig. 6. Identification of allergenic sites A and B in CNBr and tryptic-derived peptide fractions of *Lol p IV* using anti-*Lol p IV* murine Mab 90 (anti-site A) and Mab 12 (anti-site B). The ability of ^{125}I -Mab 90 and Mab 12 to bind to peptide fractions coated onto microtiter plate was expressed as percent Mab bound by a given peptide fraction relative to intact *Lol p IV*.

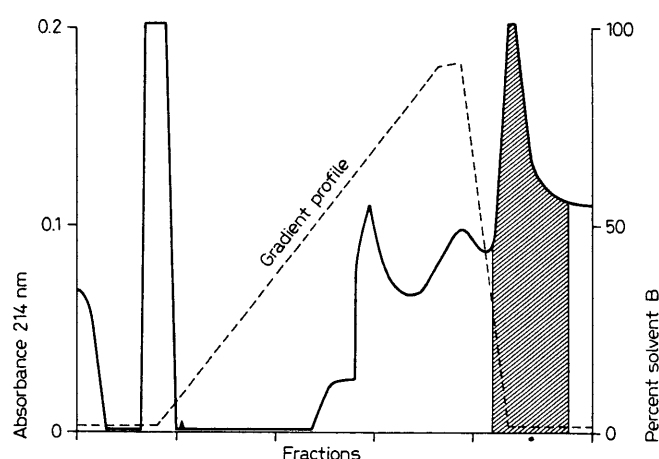


Fig. 7. Elution profile of intact *Lol p IV* by HPLC on a reversed phase C-18 column equilibrated with solvent A (0.1% TFA); a linear gradient of solvent B (70% acetonitrile, 0.1% TFA) was applied for 40 min. The shaded area indicates fractions that bound Mab 12 in a direct ELISA procedure.

the inhibitions obtained with the *Lol p IV* system were indeed specific (fig. 5). On the basis of the collective results of these experiments, it was concluded that many peptide fractions retained their allergenic activity.

Identification of sites A and B in the Peptide Fractions of Lol p IV

An evaluation of the capacity of ^{125}I -labelled preparations of Mab 90 (anti-site A) and Mab 12 (anti-site B) to bind to peptide fractions indicated that only HPLC fractions 92–98 bound Mab 90 while none of the peptide fractions bound Mab 12 to an extent greater than 10% (fig. 6). These results suggest that site A was associated with HPLC fractions 92–98 while site B appeared to be destroyed by

the sequential cleavage of *Lol p IV* with CNBr and trypsin.

Amino Acid Sequence Analysis

Most of the allergenic peptide fractions, including HPLC fractions 92–98 containing peptides with site A were found to be a mixture of several peptides which proved difficult to obtain meaningful sequence data. However, HPLC fraction 55, which gave a significant degree of inhibition in the RIA (fig. 3), was sequenced as Phe-Leu-Glu-Pro-Val-Leu. A less reliable sequence of Gly-Leu-Ile-Phe-Pro-Ala-Gly-Val was obtained for an octapeptide which constituted fraction 77. Both the sequences did not match with any sequences presently available in protein sequence data banks.

Discussion

Lol P IV contains 11 methionine, 31 lysine and 24 arginine residues [9]. Cleavage of *Lol p IV* at methionine residues with CNBr alone fragmented the allergen into smaller subunits (fig. 1). Sequential cleavage of *Lol p IV* with CNBr, followed by digestion with trypsin at arginine/lysine residues, produced peptides which were not detected following SDS-PAGE (results not shown). Since, the gel system employed could only resolve peptides with a molecular weight of 5 kD or higher, it was evident that the peptides produced by the sequential cleavage and digestion were lower than 5 kD. Separation of the digest mixture by HPLC and analysis of the resulting fractions by an RIA inhibition procedure, revealed that many of these fractions had the ability to inhibit the binding between *Lol p IV* and human serum IgE antibodies of a grass allergic individual. Some of these peptide fractions were inhibitory to the extent of 80% (fig. 3) in a specific and dose-dependent manner (Fig. 4). A similar observation has been reported for the tryptic peptides TM1 and TM2 of cod allergen M. In the RAST assays, these peptides inhibited the binding of allergen M to human serum IgE antibodies of fish allergic individuals to the extent of approximately 85% [1, 2]. On the other hand, the results of the present study are in contrast to recent reports [13, 14] where the authors observed a major reduction in the capacity of a crude ryegrass pollen extract to bind human IgE antibodies after cleavage of the crude extract with CNBr and nitromiocyano benzoic acid. However, a direct comparison between these studies is not possible since our experiments were performed with a highly purified allergen and not the crude pollen extract.

It is unlikely that the high levels of allergenic activity of many of the peptide fractions of *Lol p IV* were simply due to contamination with intact allergen because: (i) intact *Lol p IV* could not be detected in the digest mixture (fig. 1); (ii) a comparison of the HPLC elution profiles revealed that intact *Lol p IV* was eluted much later (fig. 7) than any of the CNBr and tryptic derived peptide fractions (fig. 2), and (iii) examination of the HPLC fractions taken from the region where intact *Lol p IV* would have eluted revealed that the fractions of the digest were devoid of any capacity for binding with human IgE antibodies or murine anti-*Lol p IV* Mabs. The following reasons may explain why so many peptide fractions are able to in-

hibit the RIA: (i) the human serum IgE antibodies are of polyclonal origin and, therefore, the RIA inhibitions (fig. 3) represent a repertoire of specificities capable of recognizing *Lol p IV*, and (ii) evidence was available which suggested that some fractions contained more than one peptide and it was not surprising that many fractions could readily compete with the intact allergen for binding to the IgE antibodies.

The possibility existed that trace amounts of the trypsin enzyme used in digesting *Lol p IV* may have coeluted from HPLC with at least some of the peptide fractions and be responsible for digesting the human IgE antibodies bound to the solid phase. In this case, the results would erroneously be interpreted as an increase in the degree of RIA inhibition. Such a possibility was ruled out by the fact that none of these fractions was able to inhibit the interaction between *Lol p I* (another Ryegrass pollen allergen) and the human IgE antibodies present in the sera of the grass allergic individual (fig. 5). In summary, the activity demonstrated by these peptide fractions was considered to be specific.

We have previously reported on the identification of two allergenic sites A and B on *Lol p IV* using murine anti-*Lol p IV* Mabs [10]. In the present study, site A was detected by Mab 90 only in HPLC peptide fractions 92–98. On the other hand, site B was probably destroyed following the sequential digestion of *Lol p IV* by CNBr and trypsin (fig. 6). This finding confirms our earlier observation that anti-site B, Mab 12 failed to bind to the tryptic digest of the CB-2 peptide fraction derived from cleavage of *Lol p IV* by CNBr [10]. It can therefore be speculated that lysine/arginine residues either constitute a part of site B or contribute to its allergenic/antigenic integrity.

Given that many peptide fractions exhibit the allergenic activity, it may be reasonably concluded that there are other allergenic sites on *Lol p IV* in addition to sites A and B described to date. Since, the IgE antibody response to *Lol p IV* can differ from one individual to another [10], it is more than likely that testing of peptide fragments with other grass allergic patients' sera would reveal an allergenic activity profile different from the one obtained in the present study (fig. 3). It is clear that identifying the repertoire of specificities of allergenic epitopes which may be recognized by grass allergic individuals is a major undertaking. Such studies are currently in progress.

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References

- 1 Elsayed S, Apold J, Aas K, et al: The allergenic structure of allergen M from *Cod* I. Tryptic peptides of fragment TM 1. *Int Arch Allergy Appl Immunol* 1976;52:59-63.
- 2 Elsayed S, Apold J: Allergenic structure of allergen M from *Cod*. Allergenicity of the limited tryptic hydrolysis peptides of fragment TM 2. *Int Arch Allergy Appl Immunol* 1977;54:171-175.
- 3 Atassi H, Atassi MZ: Localization of the continuous allergenic sites of ragweed allergen Ra3 by a comprehensive synthetic strategy. *FEBS Lett* 1985;188:96-100.
- 4 Ekramoddoullah AKM, Kisil FT, Bundesen DG, et al: Determinants of ryegrass cytochrome c recognized by human IgE and murine monoclonal antibodies. *Molec Immunol* 1984;21:375-382.
- 5 Atassi H, Atassi MZ: Antibody recognition of ragweed allergen Ra3. Localization of full profile of the continuous antigenic sites by synthetic overlapping peptides representing the entire protein chain. *Eur J Immunol* 1986;16:229-235.
- 6 Chua KY, Stewart GA, Thomas WR, et al: Sequence analysis of cDNA coding for a major house dust mite allergen, *Der p* I. Homology with cysteine proteases. *J Exp Med* 1988;167:175-182.
- 7 Hatton TW, Hill RD, Ekramoddoullah AKM, et al: Molecular cloning of Kentucky bluegrass (KBG) pollen allergens (abstract). *J Allergy Clin Immunol* 1988;81:183.
- 8 Thomas WR, Stewart GA, Simpson RJ, et al: Cloning and expression of DNA coding for the major house dust mite allergen *Der p* I in *Escherichia coli*. *Int Arch Allergy Appl Immunol* 1988;85:127-129.
- 9 Ekramoddoullah AKM, Kisil FT, Schon AH: Immunochemical characterization of a high molecular weight basic allergen (HMBA) of rye-grass (*Lolium perenne*) pollen. *Molec Immunol* 1983;20:465-473.
- 10 Jaggi KS, Ekramoddoullah AKM, Kisil FT, et al: Identification of two distinct allergenic sites of a rye grass pollen allergen, *Lol p* IV. *J Allergy Clin Immunol* 1989;83:845-852.
- 11 Ekramoddoullah AKM, Kisil FT, Schon AH: Partial characterization of an antigenic site of high molecular weight basic antigen, a rye-grass pollen allergen, using a monoclonal antibody. *Molec Immunol* 1986;23:111-117.
- 12 Bose R, Rector ES, Fischer J, et al: Production and characterization of mouse monoclonal antibodies to allergenic epitopes on *Lol p* I (Rye I). *Immunology* 1986;59:309-315.
- 13 Cottam GP, Wheeler AW, Cook RM, et al: Immunological properties of chemically produced fragments of rye grass pollen extract. *Immunol Lett* 1988;17:345-350.
- 14 Wheeler AW, Spackman VM, Cottam GP, et al: Retained T-cell reactivity of rye grass pollen extract following cleavage with cyanogen bromide and Nitrothiocyanobenzoic acid. *Int Arch Allergy Appl Immunol* 1988;86:1-8.

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