

Cloning, Expression, and Immunological Characterization of Recombinant *Lolium perenne* Allergen *Lol p II**

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The molecular cloning of the cDNA encoding for an isoallergenic form of *Lol p II*, a major rye grass (*Lolium perenne*) pollen allergen, was performed by polymerase chain reaction amplification on mRNA extracted from pollen. The amino acid sequence derived from the cDNA was truncated by 4 and 5 residues at the NH₂- and COOH-terminal ends, respectively, and differed only in one position from that previously reported. This cDNA was expressed in *Escherichia coli* by fusion to the carboxyl terminus of the human ferritin H-chain. The molecule was produced in high yields as a soluble protein and was easily purified. The protein retains the multimeric quaternary structure of ferritin, and it exposes on the surface the allergenic moiety, which can be recognized in Western blotting and in enzyme-linked immunosorbent assay experiments by specific IgE from allergic patients. The recombinant allergen was used to analyze the sera of 26 patients allergic to *L. perenne* compared with control sera. The results were in good agreement with the values obtained with the radioallergosorbent test assay. In addition, histamine release experiments in whole blood from an allergic patient and skin prick tests showed that the recombinant allergen retains some of the biological properties of the natural compound. These findings indicate that the availability of homogeneous recombinant allergens may be useful for the development of more specific diagnostic and therapeutic procedures. Moreover, this expression system may be of more general interest for producing large amounts of soluble protein domains in *E. coli*.

About 20% of the population in the United States suffers from IgE-mediated atopic diseases such as allergic rhinitis, rhinoconjunctivitis, and bronchial asthma (1). The wind-pollinated species of grasses are among the more important

etiological agents of seasonal atopic allergy (2). The allergenic products contained in the pollen of grasses are complex mixtures of various proteins which are difficult to isolate and characterize; however, this problem can now be approached by cloning and expression of the genes for the more common allergens, because the recombinant products are expected to represent pure, well defined, and reproducible compounds (3).

Rye grass (*Lolium perenne*) is a widely distributed species responsible for a major portion of grass pollen allergies worldwide. The pollen of this species contains at least three major classes of allergenic proteins, each composed of multiple, immunologically indistinguishable isoforms. At least one protein from each of these classes has been isolated and characterized in some detail (4–9).

The major allergen *Lol p I* is a glycoprotein of about 27 kDa to which 85–90% of rye grass allergic patients are sensitive (4). The cDNAs of two isoforms of *Lol p I* have been sequenced, and they show numerous nucleotide differences, encoding proteins of the same size (240 residues) with four amino acid substitutions (5).

Lol p II and *Lol p III* are the other two major allergens of rye grass. *Lol p II* is the second major allergen, to which 45% of rye grass allergic patients are reactive (4), and it is comprised of a group of acidic proteins with at least two isoforms (A and B). *Lol p II B* may contain two or more isoelectric forms. The variant, *Lol p II A*, has been purified and sequenced; it consists of 97 residues with no cysteine and glutamine and no evidence of glycosylation (6).

Lol p III is a group of basic nonglycosylated proteins, with two major isoforms, A and B. The complete primary structure of the most abundant one (variant B) has been reported (7). *Lol p III B* and *Lol p II A* have the same size (97 amino acids) and 59% identity. *Lol p II* and *Lol p III* proteins are immunologically cross-reactive, but they do not show apparent cross-reactivity with *Lol p I* allergen (7).

The presence of an allergen-specific T cell response has been demonstrated at the clonal level in atopic subjects as well as in normal individuals. It has been found that human IgE and IgG responsiveness to *Lol p I–III* is significantly associated with HLA DR3, and in the case of *Lol p III*, this association extends to DR5 also (4–7).

The availability of purified soluble allergen molecules may allow the elucidation of the role of the individual isoallergens in the allergic response to rye grass pollen. In this work we describe the molecular cloning, expression, and immunological characterization of the *L. perenne* allergen *Lol p II*. The cDNA obtained by PCR¹ amplification of pollen encoded a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X73363.

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¹ The abbreviations used are: PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; RAST, radioallergosorbent test.

form of *Lol p II* A, which was truncated by 4 and 5 residues at the NH₂ and COOH termini, respectively, and differed by one amino acid substitution from the previously reported sequence obtained by protein sequencing (6). The coding sequence of *Lol p II* was fused to the COOH terminus of human ferritin H-chain, a thermostable protein that is expressed with high yields in *Escherichia coli* (10, 11). The chimeric recombinant protein was expressed by *E. coli* as a stable and soluble protein with the multimeric structure (24 subunit) typical of ferritin and with the allergenic moiety exposed on the protein surface. It could be produced and purified in milligram amounts. The *Lol p II*-ferritin construct was recognized by the IgE of allergic patients in Western blotting experiments and in an ELISA assay. *In vitro* histamine release tests and *in vivo* skin prick analysis confirmed that the recombinant allergen has biological properties similar to the pollen extract.

MATERIALS AND METHODS

Reagents—All reagents were purchased from BDH Biochemical Reagents unless otherwise stated. Purified recombinant human H-chain ferritin was obtained as described in Ref. 10. The crude extract of *L. perenne* pollen (Allergon AB, Engelholm, Sweden) was prepared as described in Ref. 6.

Synthetic Oligonucleotides—All oligonucleotides were synthesized by Primm s.r.l. (Milano, Italy). Primers for PCR amplification were LII/SP, sense primer 5'-AAT-CTA-GA(A/G)-TT(T/C)-AC(T/G)-GT(T/G)-GA(A/G)-AA-3' and LII/AP, antisense primer 5'-TTG-GTA-CC(A/G/C/T)-AC(T/C)-TT(A/G)-AA(A/G)-TC-3'. The primer for site-directed mutagenesis performed on pEMBLexHFT/222 was SH10, 5'-GAC-AAG-CAC-ACT-CTA-GAA-GAC-AGT-GGT-ACC-GAA-AGC-TAG-3'.

Cloning and Sequencing of *Lol p II* cDNA—Total RNA was extracted from pollen of *L. perenne* as described in Ref. 5. cDNA was synthesized from total RNA with oligo(dT) priming following the manufacturer's instructions (Amersham cDNA Synthesis System Plus). This cDNA was used as a template for amplification. PCR was performed in 50 µl of a solution containing 2 µl of first-strand cDNA, 1 × reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 1.5 mM MgCl₂), dNTP mix (1 mM), and Taq DNA polymerase from Perkin-Elmer Cetus Instruments (50 milliunits/ml) (12, 13). Each primer was then added to a final concentration of 8 µM, and the mixture was subjected to 40 cycles of PCR amplification at the following temperatures: melt 93 °C, 45 s; primer anneal 48 °C, 45 s; and primer extension 72 °C, 90 s. PCR products purified by electrophoresis on 1.2% agarose gel were digested with *Xba*I/*Kpn*I restriction enzymes (Boehringer Mannheim). Fragments were ligated into M13mp18 and -mp19 sequencing vectors (14, 15). Restriction enzyme reactions and ligations were performed following standard protocols (16). Dideoxynucleotide chain termination sequencing was carried out using an Applied Biosystems 370A DNA sequencer (Foster City, CA) following the manufacturer's conditions.

Construction and Expression of the Chimeric Recombinant *Lol p II*—The plasmid for the overexpression of the *Lol p II*-ferritin fusion protein was constructed by inserting the coding sequence of *Lol p II* into the modified plasmid pEMBLex2HFT. This vector encodes for a mutant of human ferritin H-chain named 222, which carries the substitutions K86Q, to enable protein crystallization, and E62K plus H65G, which abolishes the ferroxidase activity and increases ferritin stability (17, 18). Restriction sites for *Xba*I and *Kpn*I were obtained by site-directed mutagenesis on this plasmid following standard procedures (11). The insertion of the two restriction sites in the vector region induced the substitutions G176E, D179G, and N180T at the COOH terminus of the ferritin H-chain. This vector was named pVH. The DNA fragment coding for *Lol p II* was cloned in these restriction sites obtaining a construct (pVH-*Lol p II*) coding for a chimeric ferritin-*Lol p II* protein.

The expression of this protein is under the control of a λ P_L promoter, and it was induced by raising the temperature from 30 to 42 °C and incubating for another 3 h as described by Levi *et al.* (11, 19). The bacterial strain used for the expression was *E. coli* GC76 (*ara str^r Δ lac-pro F' lac I^a lacZ Δ M15 pro⁺*). Fermentation of the recombinant strain was performed in a 7-liter fermentor, following

standard conditions. The cells were collected by centrifugation, and from 4 liters of culture we obtained about 20 g of cellular paste (wet weight).

Purification and Characterization of the Recombinant Protein—The recombinant protein was purified with the procedure previously developed for recombinant ferritins (10, 11, 19, 20). The cell paste was disrupted by sonication, and the soluble cellular homogenate was heated at 65 °C for 10 min, clarified, and treated with DNase and RNase. The sample was then precipitated with ammonium sulfate (520 g/liter), resuspended, and dialyzed in 20 mM Tris-HCl, pH 7.4. Then it was loaded on a Sepharose 6B column; the peak was collected, concentrated, and stored at 4 °C with the addition of 1 mM EDTA, 0.1% sodium azide, 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin A, and 10 mM leupeptin. The recombinant protein showed a purity above 80%, as judged by gel electrophoresis. About 5 mg of purified protein were obtained from 1 liter of cell culture.

Molecular size was estimated by gel filtration on a Superose 6 column (fast protein liquid chromatography, Pharmacia LKB Biotechnology Inc.) calibrated with gel filtration markers (Pharmacia), equilibrated in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl. The recombinant fusion proteins, run on native PAGE or SDS-PAGE, were transferred to nitrocellulose membranes (21, 22). After blotting, un-specific protein binding sites were blocked with 5% defatted milk in Tris-buffered saline (50 mM Tris-HCl, pH 7.5, 0.9% NaCl). The nitrocellulose membranes were incubated for 2 h at room temperature with different human sera previously tested by RAST (radioallergosorbent test, Pharmacia). After washing, the membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated rabbit immunoglobulins to human IgE (1:250) (Sigma). Color development was obtained by adding 4-chloro-1-naphthol (Bio-Rad), following the supplier's instructions.

Dotting experiments were performed by spotting 1 µg of purified recombinant proteins or 5 µg of crude extract from *L. perenne* pollen and by incubating the filter with sera of normal and allergic patients followed by incubation with a 250-fold dilution of peroxidase-labeled rabbit anti-human IgE.

ELISA assay was performed as follows. Microtiter plate wells (Greiner) were saturated overnight at 4 °C in 50 mM sodium carbonate, pH 9.6, with 1 µg of purified recombinant protein per well. Plates were washed in phosphate-buffered saline, pH 7.4, and 0.05% Tween 20. The plates were then incubated for 2 h at 37 °C with serial dilutions of patient sera in 2% standard defatted milk in phosphate-buffered saline and 0.05% Tween 20. Final incubation was performed for 2 h at 37 °C with horseradish-peroxidase-anti-IgE (Sigma) diluted 1:500 in 2% standard defatted milk in phosphate-buffered saline (0.05 M potassium phosphate, 0.15 M NaCl), pH 7.4, and 0.05% Tween 20. Colorimetric reaction was performed by adding *o*-phenylenediamine (Sigma); the absorbance at 492 nm was measured using an ELISA reader.

In Vitro Histamine Release—The test for histamine release in whole blood was performed as indicated in a commercial radioimmunoassay kit (Immunotech International, France). The test is based on the competition between the histamine in the sample and the iodinated histamine tracer for the binding to the antibody coated on tubes (23). Blood was collected in heparinized tubes, and the tests were performed within a few hours from collection. The cells were challenged with various dilutions of the extract from *L. perenne* pollen and with the purified recombinant allergen or wild type H-ferritin. After incubation, the amount of the released histamine was determined.

Skin Testing—Immediate type I skin reactions were performed on two volunteers (E. T. and A. S.) with informed consent. One subject was allergic to *L. perenne* (RAST class 3), and the other one was a healthy control. The proteins were spotted at various dilutions onto the forearm and pricked. The reaction was read after 15 min, measuring the maximal longitudinal (*D*₁) and transversal (*D*₂) diameter of the wheal and vascular flare reaction. The surface area of the wheal skin was calculated using the formula $((D_1 + D_2)/2)^2$ (24). Patients were considered to be sensitive to a particular allergen if the wheal had a surface area greater than 9 mm². Histamine (10 mg/ml) was used as positive control. Recombinant human H-ferritin, recombinant ferritin-*Lol p II*, and extract from the pollen of *L. perenne* were used at various concentrations.

Human Sera—Human sera for ELISA and dot blot tests were obtained from a general hospital. Sera samples were classified on the

basis of the RAST analysis (1–6 and negative) (Pharmacia). The sera were stored at 4 °C and used within 24 h.

RESULTS

Molecular Cloning of *Lol p II* Allergen—The sequence coding for *Lol p II* protein was amplified by PCR from first strand cDNA prepared from pollen RNA, as described under "Materials and Methods." Primers corresponding to the 5' and 3' ends contained *XbaI* and *KpnI* restriction sites for cloning in suitable vectors. These oligonucleotides have only two degeneracies for the amino acids glycine, threonine, and valine, considering the high bond energy between guanine and thymine bases.

A single DNA fragment was produced after amplification, approximately 300 base pairs long, in good agreement with the size expected from the protein sequence of *Lol p II* (6). This fragment was cloned in M13mp18 and -mp19 vectors for sequencing. The DNA and derived amino acid sequences are shown in Fig. 1A. The *Lol p II* cDNA sequence encoded for a protein truncated by 4 amino- and 5 carboxyl-terminal amino acids with an amino acid sequence in complete agreement with the one published by Ansari *et al.* (6), except for the substitution of Trp in place of Arg in position 77 (Fig. 1B).

Expression and Immunological Analysis of Recombinant *Lol p II*—In order to obtain large amounts of *Lol p II* protein, its coding sequence was fused to the COOH terminus of the human ferritin H-chain, which is a protein expressed in high yields by *E. coli* that is easy to purify (10, 11). The chimeric construct (named pVH-*Lol pII*) encodes for a sequence of 266 amino acids, 176 residues of which in the NH₂-terminal portion derive from the ferritin sequence and are followed by the 88 residues of *Lol p II* shown in Fig. 1, and 2 final amino acids from ferritin.

Fig. 2A shows that *E. coli* transformed with the plasmid pVH-*Lol pII* expresses specifically, and in high yield, a peptide of the expected molecular size of about 30 kDa (lanes 4–6). Nondenaturing gel electrophoresis of the soluble *E. coli* extracts (Fig. 2B) showed that the transformed cells specifically expressed a protein (lanes 4–6) with a mobility slightly slower than the assembled 24-mer H-ferritin wild type (lane 1). The recovery of soluble recombinant proteins appeared comparable in the wild type H-ferritin and in the chimeric protein.

The chimeric protein was purified with the procedure used for recombinant ferritins involving a heat treatment at 65 °C and gel filtration on Sepharose 6B. The preparations thus obtained were considered sufficiently pure (more than 80% of recombinant protein) for further characterization. The expression yields were of about 10 mg/liter culture, giving about 5 mg/liter purified protein.

The chimeric protein was subjected to preliminary biochemical characterization. Gel filtration analysis showed that it has an apparent molecular mass of about 700 kDa; it was resistant to incubation in 0.1% SDS at room temperature and to 65 °C (data not shown). These properties are characteristic of natural and recombinant ferritins and demonstrate that the chimeric protein assembles into a 24-mer protein. Incubation with proteolytic enzymes induced a reduction of the molecular weight of the fusion protein while it did not affect the size of the H wild type peptide (not shown). Analogous results were reported with a similar construct in which the COOH terminus of ferritin was fused to a 10-kDa peptide and indicated that the COOH terminal extension with the foreign peptide is exposed to the solvent (38).

The chimeric protein was recognized by anti-H-ferritin monoclonal antibodies in double diffusion (not shown) and in

blotting experiments (Fig. 3B, lane 5). The wild type H-ferritin and the chimeric protein were subjected to blotting experiments from denaturing (Fig. 3A) and nondenaturing PAGE (Fig. 3B), incubated with sera of atopic allergic patients and with negative control sera, followed by incubation with anti-IgE-labeled antibody. Wild type ferritin was not recognized by the IgE of patients (Fig. 3, A and B, lane 4) and control negative subjects (Fig. 3, A and B, lane 8), whereas the chimeric protein was recognized by the IgE contained in the sera of allergic patients (Fig. 3, A and B, lane 3) but not of control subjects (Fig. 3, A and B, lane 7).

To perform an ELISA assay, microtiter plates were coated with the purified fusion protein and then incubated with various dilutions of sera from allergic patients or control sera, followed by incubation with horseradish peroxidase-labeled anti-human IgE rabbit antisera. Fig. 4A shows that two RAST-positive sera gave dilution-dependent responses significantly higher than normal control sera, and a positive serum absorbed with the recombinant *Lol p II* protein. The specific signal disappeared at dilutions of 1:16 and higher, and the dilution 1:4 was chosen for analysis of some clinical samples. Inter- and intrareproducibility tests showed a coefficient of variation below 15%, which was considered acceptable for preliminary clinical analysis. The results of the ELISA assay on sera of 26 atopic individuals classified on the basis of RAST and on 9 nonallergic control subjects are shown in Fig. 4B. All control sera gave responses below the cutoff value. Using this value as the limit of normality, only three RAST-positive patients (12%) were negative on the ELISA assay. The significance of the assay was confirmed by the finding that all sera which were positive in the less sensitive dot blot assay showed ELISA values above normality, and an additional 10 RAST-positive sera, which were dot blot negative, were also above normality. The highest mean values were observed in class 5 and 6 patients, *i.e.* in the patients with the highest titer of specific IgE.

Further studies to analyze the allergenic properties of the recombinant protein involved histamine release tests from basophils of one allergic subject (Fig. 5) and skin prick tests on nonallergic healthy and allergic volunteers (Table I). The recombinant *Lol p II* induced a significant allergic response in both tests, whereas the wild type recombinant ferritin produced no detectable response. The extract of *L. perenne* pollen was used as a positive control in the tests, and, interestingly, the magnitude of the allergic responses induced by the recombinant allergen and the natural homogenate was comparable. The large difference in the concentration at which the allergic response was obtained was expected and is explained by the presence of various types of allergens in the pollen and by the structure of the recombinant allergen.

DISCUSSION

In the last few years several groups have been able to characterize at the molecular level the mRNA coding for various allergens (25–37), but very few have reported, to the best of our knowledge, the production of recombinant allergens in high yields either for diagnostic use or specific immunotherapy (39).

The cloning of the cDNA of *Lol p II* allergen was approached by PCR amplification with degenerated primers designed from the amino acid sequence of the protein (6). A single amplified fragment was obtained, and the derived amino acid sequence was, as expected, a truncated form of *Lol p II*. It matches entirely the sequence of *Lol p II* A (6), except for the presence of Trp instead of Arg at position 77 (Fig. 1B). This

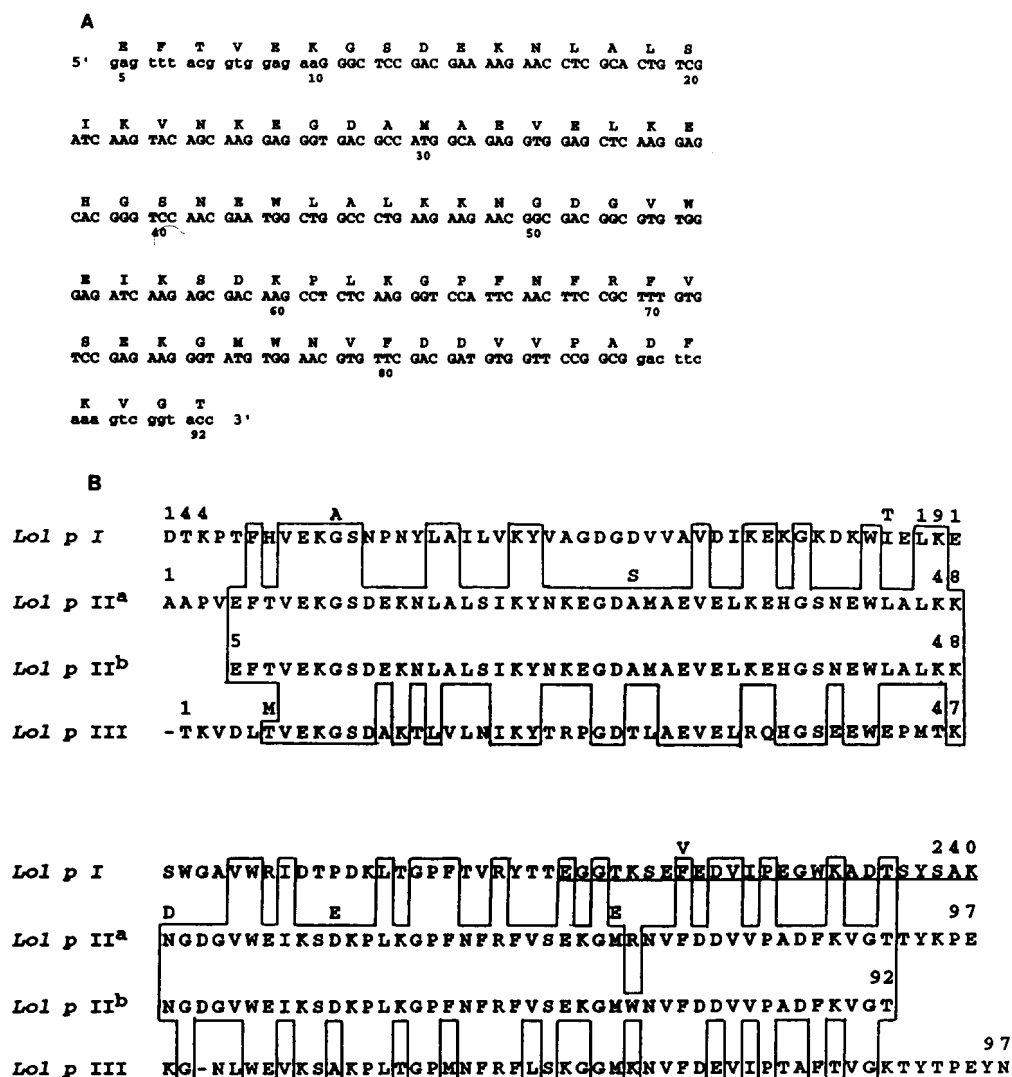


FIG. 1. A, nucleotide sequence and the deduced amino acid sequence of the *Lol p II* allergen. This DNA sequence was obtained as described in the text. The deduced amino acid sequence is numbered as reported in Ref. 6. The DNA sequences corresponding to the oligonucleotide primers used for PCR amplification are in *small letters*. B, alignment of amino acid sequences of *Lol p I*, *II*, and *III*. Identical residues are boxed. The *Lol p II* sequence (a) is from Ansari *et al.* (6), and the sequence derived from cDNA (b) is from this study. The *Lol p I* sequence is from Perez *et al.* (5) and the *Lol p III* sequence is from Ansari *et al.* (7). The residue in position 77 of the *Lol p II* sequence derived from cDNA is Trp instead of Arg. The antibody binding fragment of *Lol p I*, as defined in Refs. 5 and 7, is underlined. Variant residues found in sequences derived from protein analysis are indicated in the upper part of the sequence. Numbering refers to the sequences published in Refs. 5–7.

substitution may be related to the heterogeneity of *Lol p II* or to errors introduced by the PCR technique. The variation can be introduced by the transition of A to T in the first nucleotide of codon 77, but T was found in all the different clones we sequenced. The cloned *Lol p II* shares 38% identity (33 residues) with the carboxyl-terminal domain of *Lol p II* and 58% identity (51 residues) with the *Lol p III* amino acid sequence.

Our preliminary attempts to directly express in *E. coli* the cloned *Lol p II* cDNA were unsuccessful (data not shown); thus we tried to express it by fusion to a protein carrier. We used ferritin as a carrier because of its peculiar advantages; it is expressed in *E. coli* in high yields as a soluble, 24-mer protein (10, 11), it is thermostable, and it has a large molecular size (about 500 kDa), all properties that facilitate its purification. In addition, the multimeric structure of this protein offers the possibility to expose 24 copies of the fused peptide on a single molecule, a characteristic that should increase

immunoreactivity and may be exploited for the construction of multifunctional molecules. We fused *Lol p II* to the COOH terminus of a human ferritin H-chain previously modified to allow crystallization and to increase physical stability (17, 18). The chimeric protein was efficiently expressed in *E. coli* as an assembled 24-mer complex of about 700 kDa, which is soluble, stable, and easy to purify.

Ferritin is made of a bundle of four long α -helices, a long loop connecting helices B and C, a fifth short E helix at an acute angle to the bundle, and short NH_2 - and COOH-terminal extensions (17). In native ferritin, the COOH-terminal extensions are directed toward the cavity of the molecule and are not accessible to macromolecules. However, it was shown that the fusion of a 10-kDa peptide to the COOH terminus of ferritin changes the conformation of the COOH terminus such that it becomes exposed to solvent and easily accessible to proteolytic digestion (38). This also appears to occur in the

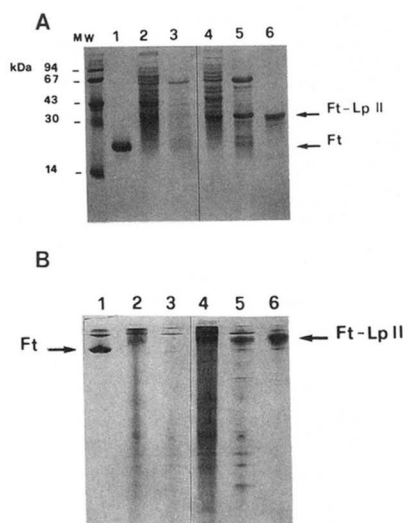


FIG. 2. Expression analysis of the recombinant protein. Denaturing SDS-PAGE in 15% polyacrylamide (panel A) and native PAGE in 7.5% polyacrylamide (panel B) stained with Coomassie Blue G. Lane MW, molecular mass markers; lane 1, purified recombinant human H-ferritin; lane 2, soluble extract of *E. coli* strain GC76 wild type; lane 3, same as 2 but heated at 65 °C; lane 4, soluble extract of *E. coli* strain GC76 transformed with pVH-Lol p II; lane 5, same as 4 but heated at 65 °C; lane 6, purified fusion protein. The mobility of recombinant human H-ferritin (*Ft*) and fusion protein (*Ft-Lp II*) is indicated by an arrow.

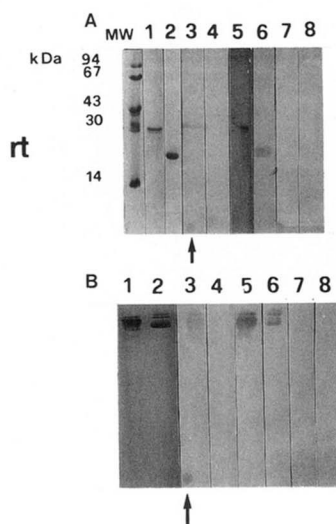


FIG. 3. Western blotting of the recombinant protein. Denaturing SDS-PAGE in 15% polyacrylamide (panel A) and native PAGE in 7.5% polyacrylamide (panel B) of the purified fusion protein (lanes 1, 3, 5, and 7) and of recombinant human H-ferritin (lanes 2, 4, 6, and 8). The gels were transferred to nitrocellulose membrane and treated as follows: Coomassie Blue staining (lanes 1 and 2), incubation with a pool of sera from *L. perenne* allergic patients (lanes 3 and 4), incubation with the monoclonal antibody specific for human H-ferritin RH02 (lanes 5 and 6), and incubated with a control negative serum (lanes 7 and 8). Lane MW contains molecular mass markers. An aliquot of a protein extract from *L. perenne* pollen was spotted before incubation onto the lower part of the filter in lanes 3, and it is indicated by an arrow.

present chimera as shown by the accessibility of the *Lol p II* sequence to proteolytic enzymes and to the specific IgE of allergic patients. Thus, the chimeric product is expected to have the structure of a large shallow sphere carrying 24 allergenic peptides on its surface. This construct seems to solve two major problems for the production of recombinant proteins, expression in high yields and solubility. The other

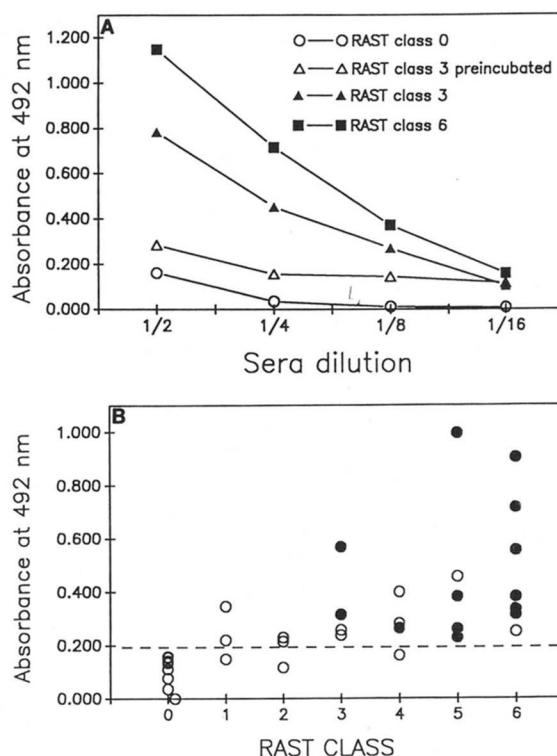


FIG. 4. ELISA and dot blot immunoassays for the determination of *Lol p II*-specific IgE in serum. A, serial dilutions of sera from healthy control and allergic subjects. A positive serum (RAST class 3) was preincubated for 2 h at 37 °C with 20 µg of the recombinant fusion protein. Means are of duplicate determinations. The coefficient of variation of intra- and interassay determinations was below 15%. B, ELISA assay of control ($n = 9$) and allergic sera ($n = 26$) classified on the basis of RAST analysis. The samples were diluted 1:4, and the mean values obtained in duplicate determinations are reported. The normality cutoff value of the ELISA assay, determined as the average of the negative samples plus two standard deviations, corresponds to 0.197 A, and it is indicated by the dashed line. The sera were also analyzed in dot blot immunoassays; solid and empty symbols indicate the samples that gave positive and negative responses, respectively.

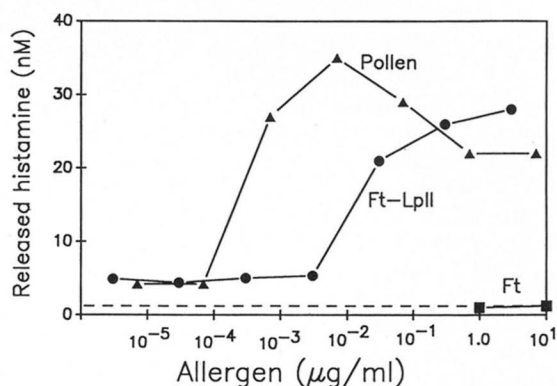


FIG. 5. Histamine release assay on basophils from an allergic subject. Whole blood was collected from a RAST class 3 patient and challenged with various dilutions of crude extract from *L. perenne* (Pollen, 7 µg/ml starting solution), recombinant *Lol p II* (*Ft-LpII*, 3 µg/ml) and recombinant H-ferritin (*Ft*, 10 µg/ml). Spontaneous histamine release is indicated by a dashed line. Data are means of duplicate experiments.

important question is whether the fused peptide retains its functionality. The functional properties of *Lol p II* are unknown, and probably not relevant to the present study; how-

TABLE I
Wheat and flare reaction from skin tests

Prick tests were performed as described under "Materials and Methods." The surface area of the reaction is given in mm². The results with proteins at concentrations of 1600, 16, and 1.6 µg/ml are reported.

Patient	NaCl	Histamine ^a	Crude extract ^b	Recombinant ferritin	Ferritin <i>Lol p II</i>
A. S.	0	64	0/0/0	0/0/0	0/0/0
E. T.	0	81	144/25/16	0/0/0	49/9/0

^a Histamine concentration was 10 mg/ml.

^b Crude pollen extract from *L. perenne* was prepared as described under "Materials and Methods."

ever, the major interest in this product is its allergenicity, which can be tested by its capacity to bind the IgE of allergic patient sera and to induce allergic responses in allergic patients.

The present study shows that the recombinant *Lol p II* fusion protein has the following properties. It specifically binds to the IgE of patients allergic to *L. perenne* (as shown in Figs. 3 and 4), it induces histamine release from basophils of allergic subjects (Fig. 5), and it provokes a flare reaction in skin prick tests on an allergic subject (Table I). None of these properties are shared by the ferritin carrier protein, and it was concluded that the cloned cDNA codes for the expected allergen and behaves as such in the chimeric construct.

How much of the allergenicity of natural *Lol p II* is retained by the recombinant fusion protein? The answer to this important question is difficult, mainly due to the lack of purified natural *Lol p II*. Comparison with the extract of *L. perenne* pollen is somewhat misleading because this is a heterogeneous composition of various allergens, which may have different allergenic effects and possibly may also act synergistically. The large difference in the potency of the recombinant protein and of pollen extract found in the histamine release and skin prick tests (Fig. 5 and Table I) may be due also to the particular structure of the chimeric product. The allergen accounts for only 30% of its mass, and a single molecule exposes 24 allergens. These structural constraints may allow only one or a few of the 24 allergens to interact with cells to trigger the allergic response and, therefore, in these assays, only a minor proportion of the total mass (calculated to be 1–3%) has biological activity. This does not apply to *in vitro* IgE binding tests, in which many more antibody molecules can bind to a single chimeric complex. The suggestion that the recombinant allergen retains much of the IgE binding property of the natural product is supported also by the finding that it is active in histamine release tests at concentrations of ng/ml, which is the concentration range in which other purified allergens are active (23, 35).

The production of large amounts of a purified allergen also has a number of potential applications for diagnosis and therapy. Recombinant *Lol p II* was used to set up dot blot and ELISA immunoassays in order to recognize who among the patients with IgE specific for the pollen of *L. perenne* (i.e. the RAST-positive subjects) has IgE for *Lol p II*. The results, summarized in Fig. 4B, show that 13 out of 26 allergic sera contained levels of IgE for the recombinant *Lol p II* detectable in dot blot tests, and most of these patients belong to RAST class 5 and 6.

This proportion of positivity is in good agreement with the one previously obtained using an analogous dot blot assay based on natural *Lol p II* (4, 40). The setting-up of an ELISA test was complicated by the absence of a suitable reference

standard, but a normality limit (cutoff) could be roughly determined at 0.2 A based on the results of the nine sera from nonallergic healthy controls (Fig. 4B). With this reference value, 88% of the allergic sera were found to contain IgE binding *Lol p II*. To our knowledge, this is the first attempt to evaluate anti-*Lol p II* IgE with ELISA, and the results suggest that it is more sensitive than dot blot assays and that the population of individuals sensitive to *Lol p II* is possibly larger than previously indicated. These results encourage further clinical evaluation of the ELISA assay.

In conclusion, the present study indicates that the ferritin-*Lol p II* artificial structure retains many of the properties of the two molecules, the high levels of expression, the stability and complex structure of the ferritin, and the allergenic properties of *Lol p II*. This new reagent and its derivatives may be useful to map IgE binding epitopes, to identify T cell determinants, and to study its immunogenicity. The possibility of producing large amounts of homogeneous allergens may help to improve diagnosis and therapy of atopic allergy. Ferritin may be exploited as a carrier of other recombinant allergens. Its multimeric structure also offers the possibility of constructing molecules exposing various selected peptide domains from different allergens, and preparing mono- and polyspecific compounds for specific immunotherapy.

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