

Lol p XI, a new major grass pollen allergen, is a member of a family of soybean trypsin inhibitor-related proteins

Ronald van Ree, PhD,^a Donald R. Hoffman, PhD,^b Willem van Dijk, PhD,^c
Virginie Brodard, MSc,^d Karin Mahieu, MSc,^a
Carolien A. M. Koeleman, BSc,^c Marjolein Grande, MSc,^a
W. Astrid van Leeuwen, BSc,^a and Rob C. Aalberse, PhD^a *Amsterdam,*
The Netherlands, Greenville, N.C., and Paris, France

Background: Monoclonal antibodies were obtained against an unknown allergen from *Lolium perenne* grass pollen. The allergen had an apparent molecular mass of 18 kd on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Earlier immunoblotting studies had shown that carbohydrate-specific IgG antibodies recognize an antigen of similar size.

Objective: We sought to characterize the allergen biochemically and immunologically.

Methods: The amino acid sequence of the allergen was determined by automated Edman degradation, and its monosaccharide composition was determined by gas chromatographic analysis. A panel of 270 grass pollen–positive sera was assessed in a RAST with the purified allergen. Protease digestion (proteinase K) and chemical deglycosylation (trifluoromethane sulfonic acid) were used to distinguish between carbohydrate and peptide epitopes for IgE antibodies.

Results: The allergen was shown to be a glycoprotein with a molecular mass of 16 kd, of which 8% is carbohydrate. Its amino acid sequence shares 32% homology with soybean trypsin inhibitor (Kunitz) but lacks its active site. No homology was found with known grass pollen allergens, hence it was designated Lol p XI. A high degree of homology (44%) was found with a tree pollen allergen, Ole e I, the major allergen of olive pollen. More than 65% of grass pollen–positive sera had IgE against Lol p XI. IgE reactivity was demonstrated both with the carbohydrate moiety and the peptide backbone.

Conclusions: Lol p XI is a new major grass pollen allergen carrying an IgE-binding carbohydrate determinant. Lol p XI is structurally related to the major allergen from olive pollen. (*J ALLERGY CLIN IMMUNOL* 1995;95:970-8.)

Key words: Lol p XI, monosaccharide composition, amino acid sequence, anti-carbohydrate

Grass pollen is a major cause of seasonal allergies. During the past decades several allergens from grass pollen were identified and characterized, mainly from *Lolium perenne* (Lol p): Lol p I,¹⁻³ Lol p II and III,³⁻⁵ Lol p IV,⁶ Lol p V⁷ (sometimes referred to as Lol p IX,⁸ cytochrome c (Lol p X),⁹ and more recently profilin.^{10, 11} Lol p I and Lol p V were shown to be the major allergens

Abbreviations used

BSA:	Bovine serum albumin
Lol p:	<i>Lolium perenne</i>
PBS:	Phosphate-buffered saline solution
SBTI:	Soybean trypsin inhibitor
SDS-PAGE:	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TFMS:	Trifluoromethanesulfonic acid

From ^aCentral Laboratory of The Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam; ^bDepartment of Pathology, East Carolina University School of Medicine, Greenville; ^cDepartment of Medical Chemistry, Faculty of Medicine, Free University, Amsterdam; and ^dUnité d'Immuno-Allergie, Institut Pasteur, Paris.

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Reprint requests: R. C. Aalberse, PhD, p/o Publication Secretariat, C.L.B., Plesmanlaan, 1066 CX Amsterdam, The Netherlands.

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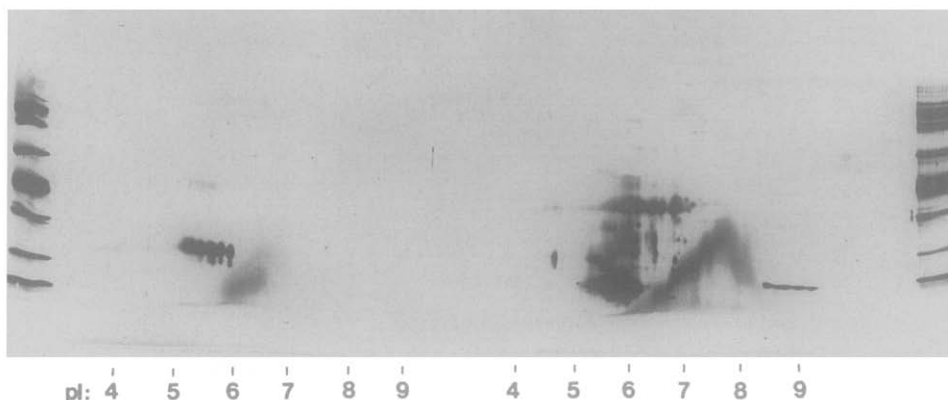


FIG. 1. Two-dimensional analysis of *Lol p XI* (silver stained). *Left*, Two-dimensional separation of 10 μ g of *Lol p XI*; *right*, two-dimensional separation of 36 μ g of total *Lol p* extract. Lanes at *far left* and *far right* contain molecular weight markers (14.4, 20.1, 30.0, 43.0, 67.0, and 94.0 kD).

against which 75% to 95% of all patients with grass pollen allergy have IgE antibodies.

Some patients with grass pollen allergy were shown to have IgE antibodies directed to carbohydrate determinants, resulting in extensive cross-reactivity of IgE toward pollen, vegetable foods, animal foods of invertebrate origin, and insect venoms.^{12, 13} On phospholipase A₂, a major allergen from bee venom, an IgE-binding N-linked carbohydrate structure was demonstrated.^{14, 15} For grass pollen individual allergens with IgE-binding sugar groups have not been reported so far. In a previous investigation rabbit IgG directed to carbohydrate determinants was shown to bind preferentially to a band with an apparent molecular mass of 18 kD on grass pollen immunoblot.¹³ We recently produced monoclonal antibodies (mAbs) directed to an allergen of similar size.¹⁶ The aim of this study was (1) to determine the importance of this allergen and its relation to known grass pollen allergens and (2) to investigate whether this allergen is a glycoprotein with an IgE-binding carbohydrate group.

METHODS

Grass pollen extract

Grass pollen (*Lol p*) was purchased from Diephuis Laboratories, Groningen, The Netherlands. Pollen extract was made as described elsewhere.⁷ For application in a RAST 5 mg of lyophilized extract was coupled to 1 gm of cyanogen bromide-activated Sepharose medium (Pharmacia, Uppsala, Sweden).

Purification of *Lol p XI*

Sepharose-coupled mAb 2H4 (10 mg/gm) was used for affinity purification of *Lol p XI*. Lyophilized *Lol p* pollen extract (370 mg) was dissolved in phosphate-

buffered saline solution (PBS) (10 mg/ml) and incubated end over end at room temperature for 4 hours with 5 gm of 2H4-Sepharose. After washing, bound allergen was eluted with 0.1 mol/L glycine HCl (pH 2.5), followed by immediate neutralization with 1 mol/L tris(hydroxymethyl) aminomethane. The eluate was dialyzed for 24 hours against demineralized water, followed by concentration by ultrafiltration with an YM10 filter (Amicon Corp., Danvers, Mass.). The concentrate was gel filtered on a Sephacryl HR S-100 column (60 $\times$ 1.4 cm) equilibrated in demineralized water. The peak fractions containing *Lol p XI* were pooled and freeze dried. In this way 330 μ g was purified as measured in a Pierce protein determination.

Protein determination

Protein was measured with Pierce BCA protein assay reagent (Pierce, Rockford, Ill.) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as a protein standard.

Proteinase K digestion

Lol p pollen extract (5 mg in 1 ml PBS) was digested with 20 μ g of proteinase K (from *Tritirachium album*; Boehringer Mannheim, Mannheim, Germany) at 50° C for 8 hours. Proteinase K was subsequently inactivated for 5 minutes at 100° C. For application in a RAST an equal amount of digested pollen extract was coupled as in the case of undigested extract (5 mg/gm).

Treatment with trifluoromethanesulfonic acid

Deglycosylation with trifluoromethanesulfonic acid (TFMS) was essentially performed as described by Edge et al.¹ TFMS (Merck, Darmstadt, Germany) and anisole (Aldrich Chemical Corp., Milwaukee, Wis.) were mixed in a 2:1 ratio. To 1 ml of this reaction mixture 5 mg of lyophilized pollen extract was added. Deglycosylation was performed for 10 minutes under nitrogen. Protein

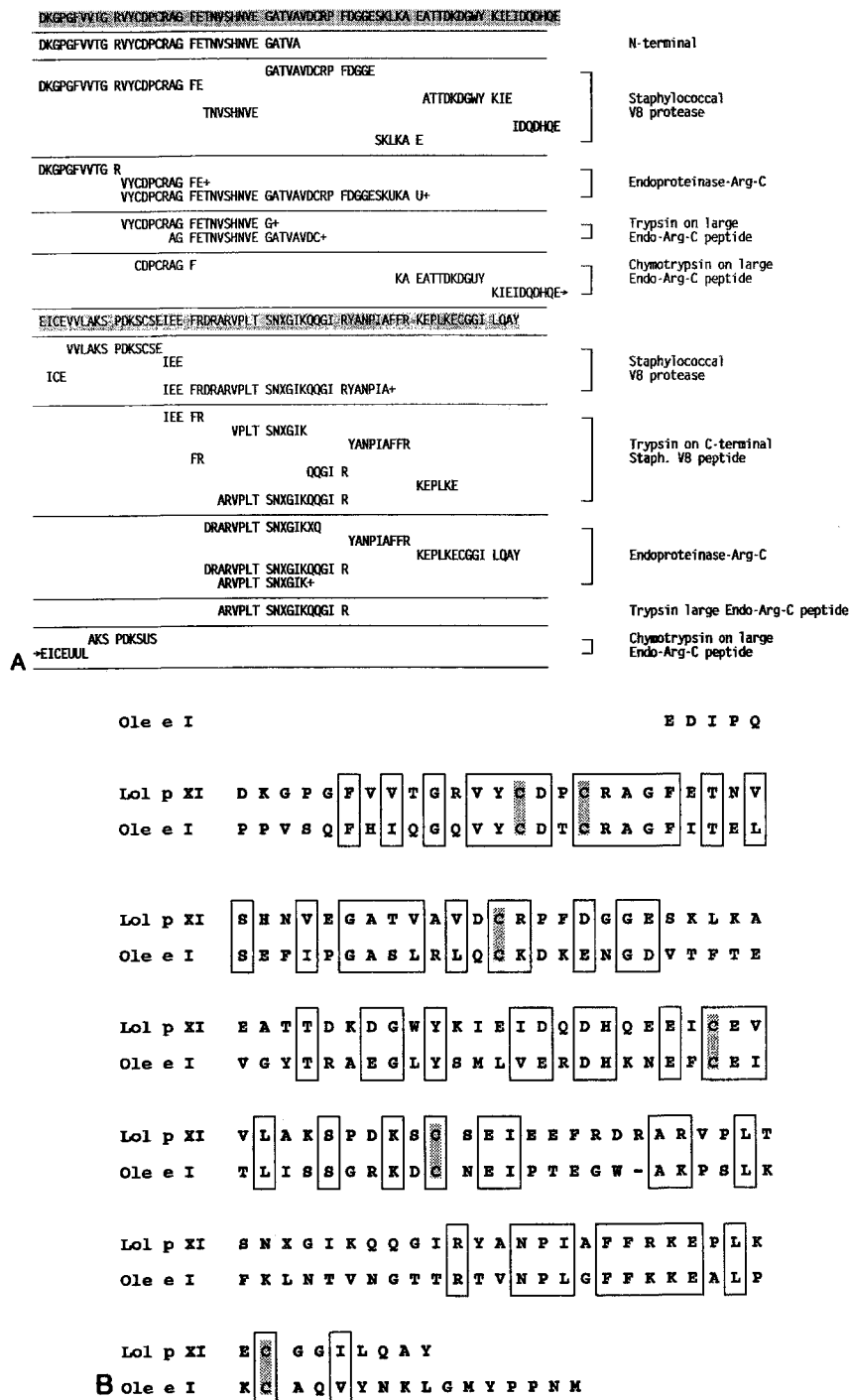


FIG. 2. A and B. Amino acid sequence analysis of *Lol p XI*. **A**, Complete amino acid sequence of *Lol p XI* (shaded area) contains 134 amino acids. Position 24 is N-linked glycosylation site, position 103 (X) represents atypical residue. Amino acid at this position is not a hydroxyproline, phosphoserine, phosphotyrosine, or a carbohydrate-modified asparagine. Under the complete sequence all separate peptides obtained with four different enzymes are shown. Some peptides were longer than could be sequenced (indicated by +). Some residues could not be identified in some peptides (indicated by U). Peptide starting with amino acid 61 obtained after chymotrypsin digestion continues in second half of figure (arrows). **B**, Complete sequence of *Lol p XI* was aligned for maximum homology with *Ole e I*.²⁴ In the latter one insertion (-) was made to obtain maximum homology. Boxes were drawn around identical and similar residues. The following groups of amino acids were regarded similar: (V,I,L), (T,S), (D,E), (K,R), (N,Q), and (F,Y). Conserved positions of six cysteines are in shaded regions.

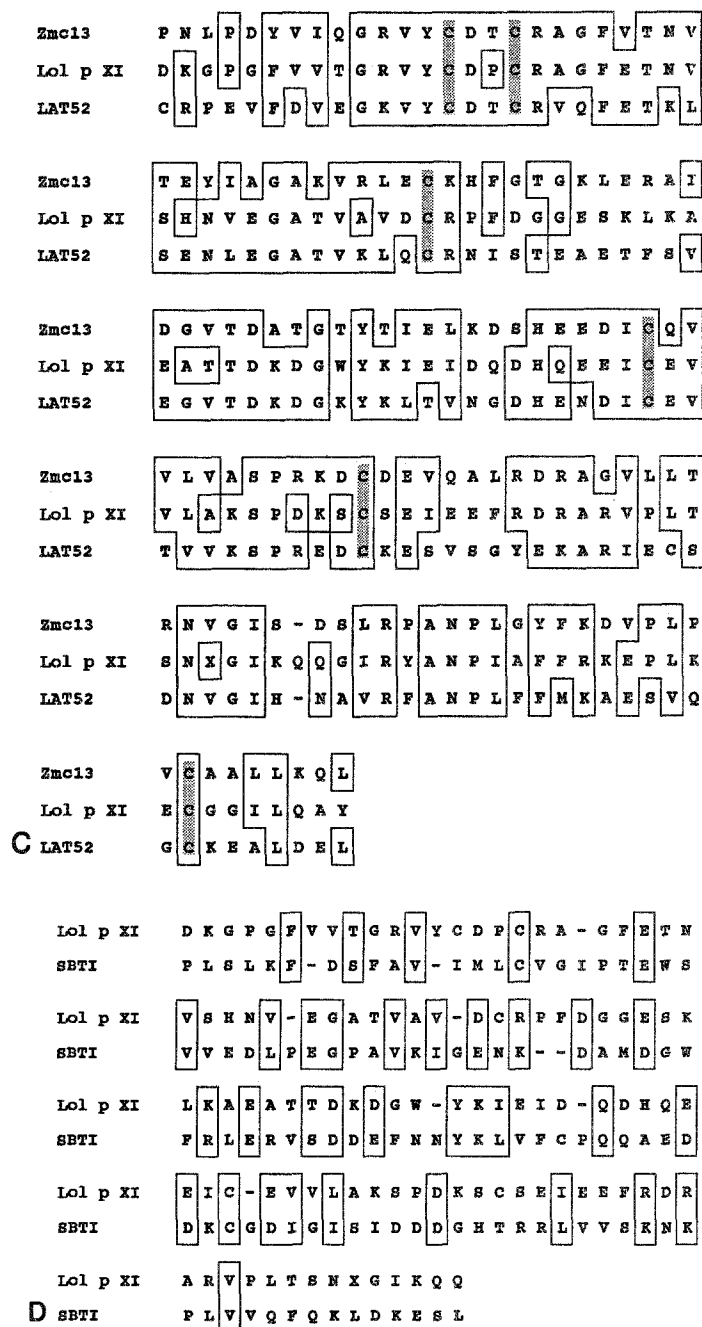


FIG. 2. C, Alignment for maximum homology of *Lol p XI* and amino acids 30 to 162 of maize pollen protein Zmc13²⁵ and 22 to 154 of tomato pollen protein LAT52.²⁶ One insertion (—) was made in sequences of Zmc13 and LAT52. **D,** Figure shows 108-amino-acid overlap of *Lol p XI* (1 to 108) with SBTI (72 to 181). For maximum homology, insertions were made in both sequences. Active site of SBTI is not included in overlap (63 and 64) and is not present in *Lol p XI*. (Sequence was submitted to Protein Identification Resource, National Biomedical Research Foundation, Georgetown University Medical Center, 3900 Reservoir Rd., N.W., Washington, DC 20007; accession No. A54002.)

was allowed to precipitate at -30°C for 1 hour in a 50-fold excess of diethyl ether plus 10% *n*-hexane (vol/vol). After centrifugation the precipitate was subjected to this procedure once more. The precipitated protein was dissolved in 1 ml PBS.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed as described earlier.¹⁸ For the first dimension IPG Dry Strips (pH 3 to 10.5; Pharmacia-LKB) were used; for the second dimension ExcelGel SDS 8% to 18% gels (Phar-

TABLE I. Monosaccharide composition

Monosaccharide	Molar monosaccharide/sialic acid ratio
GlcNAc*	4
Mannose	7
Fucose	3
Xylose	9
Galactose	2
Arabinose	3
Sialic acid	1
Glucose	4

*GalNAc was not detected.

macia-LKB) were used. Gels were subsequently silver stained according to Blum et al.¹⁹

Amino acid sequence determination

Amino acid sequencing was performed by automated Edman degradation on a Milligen-Bioscience model 6625 Prosequencer (Millipore Corp., Marlborough, Mass.). Purified *Lol p* XI was reduced with dithiothreitol in 6 mol/L guanidine HCl and carboxymethylated with iodoacetic acid. Digests were prepared with sequence grade staphylococcal strain V8 protease, trypsin, endoproteinase Arg C, and chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) and individual peptides isolated by reversed-phase chromatography on a Vydac 218TP54 column (The Separations Group, Hesperia, Calif.) with a gradient of acetonitrile in water with 0.1% trifluoroacetic acid.^{20, 21} Sequences were determined with amounts exceeding 100 pmol per peptide, which is comfortably greater than the detection limit of the analytical system. The complete structure was established by alignment of the sets of overlapping peptides from the digestions of whole molecule and the larger fragments.

Monosaccharide determination

For sugar analysis *Lol p* XI was subjected to methanolysis in 1 mol/L HCl in methanol for 14 hours at 80° C in sealed tubes under nitrogen. The sugar composition was determined after derivatization to methylsilicone ethers by means of gas chromatography on a Hewlett-Packard model 5993 apparatus equipped with a crosslinked methylsilicone capillary column as described by Savage et al.²²

Monoclonal antibody

Monoclonal antibody 2H4 directed to *Lol p* XI was obtained after immunization of Balb/c mice with partially purified *Lol p* II, which proved to be contaminated with *Lol p* XI.¹⁶ Production of hybridomas was performed according to standard procedures.²³ The mAb was of the IgG1 subclass and was purified from culture supernatants with protein A-Sepharose (Pharmacia) according to the manufacturer's instructions.

In a similar way mAb Dp6 was obtained after immunization of Balb/c mice with *Dermatophagoides pteronyssinus* (house dust mite) extract. In this case ascites rather than culture supernatant was used. The mAb was of the IgG1 subclass. This mAb was directed to a carbohydrate determinant as was judged by its broad spectrum of cross reactivity with pollen and vegetable foods and by the sensitivity of this binding to periodate oxidation.

Sera

All sera ($n = 270$) used in this study were sera submitted for routine diagnostic allergy testing. They were selected on the ground of a positive RAST result for grass pollen; 43 demonstrated weak binding to grass pollen-Sepharose (RAST class 1+/2+; 2% to 10% anti-IgE binding), and 227 had strong binding (RAST class 3+/4+/5+; >10% binding).

RAST and RAST inhibition

RAST was performed as described earlier.¹² Briefly, Sepharose-coupled *Lol p* XI (0.25 mg/test) or Sepharose-coupled *Lol p* extract, untreated or proteinase K-treated (0.5 mg/test), was incubated overnight with 50 μ l of serum (undiluted) in a final volume of 300 μ l of PBS with 0.3% BSA and 0.1% polysorbate 20 (PBS-AT). After washing, bound antibodies were detected with iodine 125-labeled polyclonal sheep anti-human IgE. Results were expressed as the percentage of binding of total counts added. A result of less than 2% was regarded as negative. In case of RAST inhibition, serum was preincubated for 2 hours with 50 μ l of inhibitor (treated or untreated *Lol p* extract). Results were expressed as the percentage of the uninhibited value (50 μ l of PBS-AT instead of inhibitor added). In RAST inhibition mAbs 2H4 and Dp6 were used in a 1:10,000 dilution. Bound antibodies were detected with ¹²⁵I-labeled monoclonal rat anti-mouse κ light chain.

RESULTS

Purification of *Lol p* XI

By means of affinity purification with Sepharose-coupled mAb 2H4, followed by gel filtration on a Sephacryl HR S-100 column, an allergen was isolated from *Lol p* grass pollen extract. It had an apparent molecular mass of 18 kd on SDS-PAGE (not shown). On a two-dimensional gel several isoforms were detected, with an isoelectric point ranging from 5.0 to 6.0 (Fig. 1). The apparent molecular mass on this gel was higher (between 20 and 24 kd), which might be due to the iodoacetamide treatment.

Amino acid sequence analysis

The complete amino acid sequence was found to be: a 134-amino-acid protein with a calculated molecular mass of 14,875 d (Fig. 2, A). The

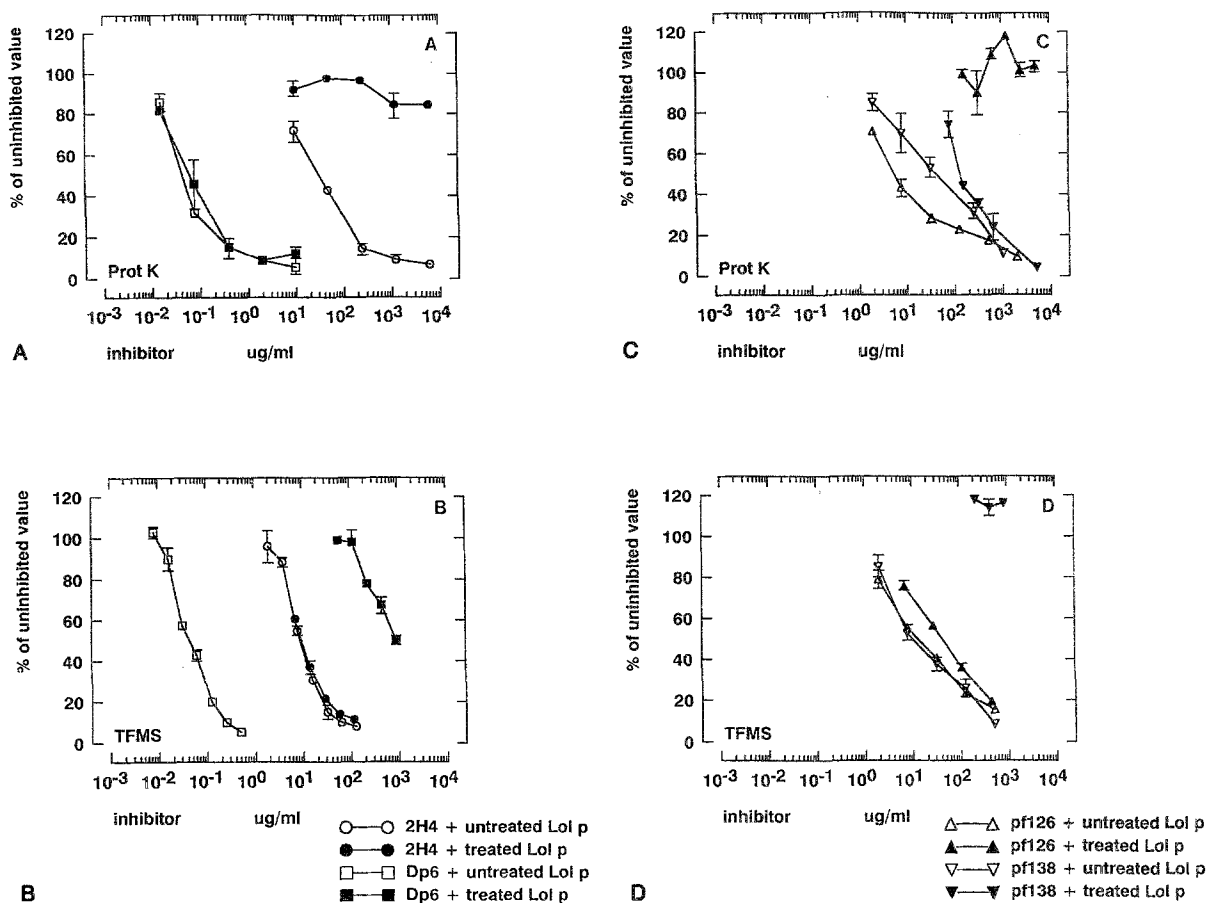


FIG. 3. Proteinase K and TFMS treatment. **A**, Influence of proteinase K treatment on binding of mAbs 2H4 (anti-peptide) and Dp6 (anticarbohydrate) to *Lol p* XI. Results are expressed as percentage of uninhibited value. **B**, Influence of TFMS treatment on binding of mAbs 2H4 and Dp6 to *Lol p* XI. **C** and **D**, Influence of proteinase K (**C**) and of TFMS (**D**) treatment on binding of IgE from serum with predominantly anticarbohydrate IgE activity (pf138) and from serum with predominantly antipeptide activity (pf126).

sequence contained one putative N-glycosylation site at position 24. No significant sequence homology was found with known grass pollen allergens; hence, the allergen was designated *Lol p* XI. Homology (44.0%) was found with the major allergen of olive pollen, *Ole e* I (Fig. 2, B).²⁴ An even higher degree of homology was found with a maize pollen protein, Zmc13,²⁵ and with a tomato pollen protein, LAT52;²⁶ 55.2% and 53.0%, respectively (Fig. 2, C). The position of six cysteines is conserved in these four proteins. All four demonstrated resemblance to soybean trypsin inhibitor (SBTI).^{27, 28} *Lol p* XI showed 32.4% homology with SBTI in a 108-amino-acid overlap (Fig. 2, D). None of the four proteins contained the active site of SBTI, located at position 63/64. In agreement with this, no inhib-

itory activity of *Lol p* XI toward trypsin could be demonstrated (data not shown).

Monosaccharide composition

By gas chromatographic analysis it was shown that the allergen contained 8% carbohydrate, resulting in a molecular mass of *Lol p* XI of approximately 16 kd. The following monosaccharides were detected: N-acetyl glucosamine, mannose, fucose, xylose, galactose, sialic acid, arabinose, and glucose (Table I).

IgE reactivity

In a RAST with Sepharose-coupled *Lol p* XI 179 of 270 (>65%) grass pollen-positive sera demonstrated greater than 2% binding. When the sera were divided into RAST classes 1+/2+ and 3+/-

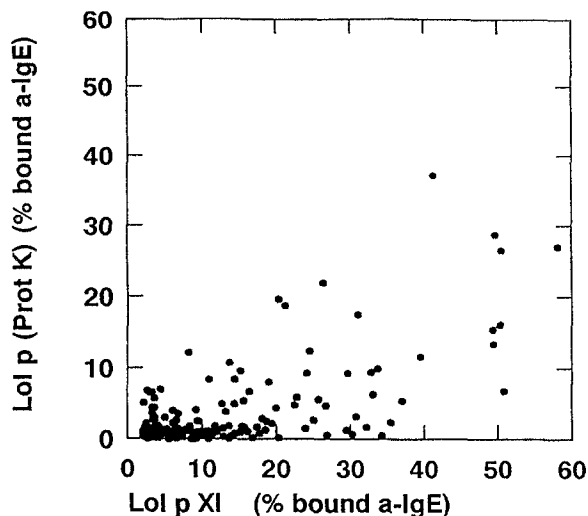


FIG. 4. Anticarbhydrate IgE in *Lol p* XI-positive sera. RAST results are expressed as percentage binding of total [^{125} I] anti-IgE added.

4+/5+, the proportions of positive sera were 11 of 43 (>25%) and 168 of 227 (74%), respectively. The mean ($\pm$ SE) percentage binding of the 179 positive sera was $12.7\% \pm 0.9\%$, with 36 sera showing binding of greater than 20%.

Grass pollen extract (*Lol p*) was protease digested (with proteinase K) to destroy its peptide epitopes or chemically deglycosylated to remove its carbohydrate epitopes. The efficiency of these treatments was monitored in a RAST inhibition with Sepharose-coupled *Lol p* XI, and with mAb Dp6 (anticarbhydrate) or mAb 2H4 (antipeptide). After proteinase K digestion less than 0.1% of the 2H4-binding capacity remained, leaving more than 95% of the binding of Dp6 intact (Fig. 3, A). The TFMS treatment resulted in a loss of more than 99.9% of the Dp6-binding capacity, whereas the binding of 2H4 was retained for 95% (Fig. 3, B). Sepharose-coupled proteinase K-digested pollen extract was used in a RAST to test sera for anticarbhydrate IgE. In a panel of 165 sera with IgE against *Lol p* XI, 64 (>35%) demonstrated anticarbhydrate IgE (Fig. 4). Approximately 25% of the carbohydrate-reactive sera showed a comparable percentage binding to *Lol p* XI and the proteinase K-treated pollen extract, suggesting predominance of anticarbhydrate reactivity in their anti-*Lol p* XI IgE response. The existence of sera with either predominantly anticarbhydrate IgE or antipeptide IgE was confirmed in *Lol p* XI-RAST inhibition assays with protein-

ase K-treated and TFMS-treated pollen extract as inhibitor (Fig. 3, C and D).

DISCUSSION

A new major allergen from *Lol p* grass pollen was isolated and characterized. No sequence homology with known grass pollen allergens was found. Therefore we designated it *Lol p* XI. The allergen probably has its homologues in pollen of other grass species, because the mAb 2H4 bound to several other grass pollen extracts, including *Dactylis glomerata*, *Festuca rubra*, and *Phleum pratense* (not shown). The existence of a similar protein in other grass pollen (to which mAb 2H4 did not bind) was confirmed by the homology of *Lol p* XI with a protein from maize pollen, Zmc13.²⁵ Homology was further found with proteins from non-grass pollen origin, LAT52 from tomato pollen,²⁶ and *Ole e* I, the major allergen from olive pollen.²⁴ After profilin,^{10, 11} this is the second allergen shown to be present in both grass and tree pollen.

Lol p XI, together with Zmc13, LAT52, and *Ole e* I, belongs to a group of proteins that share homology with Kunitz SBTI, but they all lack the active site (Arg⁶³-Ile⁶⁴) of the inhibitor.^{27, 28} It has been suggested that such "inactive" members of this inhibitor family might have a role as storage proteins.²⁹

In more than 65% of the grass pollen-positive sera tested, IgE antibodies against *Lol p* XI were detected. How is it possible that such an important allergen escaped attention so far? A possible explanation is the poor recognition of the allergen on immunoblot. The conformation of the allergen is probably changed drastically under reducing conditions during SDS-PAGE, which is not unexpected, with three potential disulfide bridges (six cysteines) present. Sera with IgE predominantly directed either to the peptide structure of *Lol p* XI, or to mAb 2H4 failed to detect the allergen on immunoblot, whereas sera with antibodies directed to the carbohydrate moiety clearly bound to an 18 kd band (not shown), indicating that this epitope is not affected by the reducing conditions used in SDS-PAGE.

Lol p XI was shown to be a glycoprotein with one N-linked glycosylation site. Monosaccharide analysis revealed the presence of sugar residues, also found in N-linked polysaccharides of the complex mannose type on glycoproteins from plants and invertebrate animals, such as ascorbate oxidase (from zucchini) and bee venom

phospholipase A₂.^{15,30} Binding of IgE to *Lol p* XI was inhibited by more than 90% by both phospholipase A₂ and ascorbate oxidase, suggesting that the *N*-glycan on *Lol p* XI might indeed be related to the carbohydrate structures on these glycoproteins (not shown).

The *N*-glycan structure discussed previously does not account for all monosaccharides found for *Lol p* XI. Besides glucose, arabinose and extra xylose were demonstrated. Glucose is usually present only at intermediate stages in the glycosylation process. The glucose therefore probably is an artifact introduced by the dextran-based gel-filtration medium (Sephacryl) used in the purification of *Lol p* XI. The remaining xyloses and arabinoses could be part of an arabinoxylan attached to a serine residue, as was suggested for *Lol p* IV.³¹

The identification of *Lol p* XI as a major grass pollen allergen is of importance for improvement of the standardization of grass pollen extracts used in diagnostics and immunotherapy. In addition, the presence on *Lol p* XI of an IgE-binding carbohydrate group offers the possibility to study the role of anticarbohydrate IgE in allergy.

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