Identification and Cloning of Prs a 1, a 32-kDa Endochitinase and Major Allergen of Avocado, and Its Expression in the Yeast Pichia pastoris*

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Slawomir Sowka, Li-Shan Hsieh‡, Monika Krebitz, Akira Akasawa§, Brian M. Martin†, David Starrett‡, Clemens K. Peterbauer, Otto Scheiner, and Heimo Breiteneder**

From the Department of General and Experimental Pathology, University of Vienna, AKH-EBO-3Q, Waehringer Guertel 18-20, A-1090 Vienna, Austria, the ‡Division of Oncology Drug Products, DNDC 1, CDER HFD-150, Rockville, Maryland 20892, the §Department of Allergy, National Children’s Hospital, 3-35-31, Taishido, Setagaya-Ku, Tokyo, 154 Japan, the ¶Unit of Molecular Structures, Clinical Neuroscience Branch, National Institute of Mental Health, Bethesda, Maryland 20892, and †Biology Department, Southeast Missouri State University, Cape Girardeau, Missouri 63701

Avocado, the fruit of the tropical tree Persea americana, is a source of allergens that can elicit diverse IgE-mediated reactions including anaphylaxis in sensitized individuals. We characterized a 32-kDa major avocado allergen, Prs a 1, which is recognized by 15 out of 20 avocado- and/or latex-allergic patients. Natural Prs a 1 was purified, and its N-terminal and two tryptic peptide sequences were determined. We isolated the Prs a 1 encoding cDNA by PCR using degenerate primers and 5'-rapid amplification of cDNA ends. The Prs a 1 cDNA coded for an endochitinase of 326 amino acids with a leader peptide of 25 amino acids. We expressed Prs a 1 in the yeast Pichia pastoris at 50 mg/liter of culture medium. The recombinant Prs a 1 showed endochitinase activity, inhibited growth and branching of Fusarium oxysporum hyphae, and possessed IgE binding capacity. IgE cross-reactivity with latex proteins including a 20-kDa allergen, most likely prohevein, was demonstrated, providing an explanation for the commonly observed cross-sensitization between avocado and latex proteins. Sequence comparison showed that Prs a 1 and prohevein had 70% similarity in their chitin-binding domains. Characterization of chitinases as allergens has implications for engineering transgenic crops with increased levels of chitinases.

Food allergy is a well known condition, afflicting a portion of the adult population that is hard to define. If one relies on self-perception, a prevalence of 15–20% of food allergic patients could be assumed (1, 2). However, on the basis of in vitro and in vivo (skin prick test) diagnosis, the percentage might be as low as 1.4–1.8% (1, 2). Self-perception has the drawback of being based on imponderable psychological effects. On the other hand, extracts used for diagnostic procedures are, in particular in the case of food allergens, often of questionable quality. This is probably due to the varying composition and stability of the food extracts. For this reason, recombinant DNA techniques have significantly contributed to a reliable characterization of the responsible allergens from food extracts (3–6).

Allergy to avocado is of increasing importance, especially in Mexico and the United States, where consumption of avocado-based dishes is common. To judge from the few data available, the prevalence of avocado allergy in the general population could be estimated to be around 1% (8% in atopic individuals; Refs. 1 and 7). Avocado allergy is of particular relevance in the “latex-fruit syndrome” observed in at least 40% of latex-allergic individuals (8–10). Since 5–10% of health care workers are sensitized to latex, which is much more than the risk of latex allergy in the general population (11, 12), the percentage of health care workers affected with the latex-fruit syndrome may be as high as 2–4%. In this context, a precise characterization of the respective allergens is of special interest.

Avocado can induce IgE-mediated reactions with different clinical manifestations including a high percentage of anaphylaxis (8, 13). For avocado pear extracts, immunoblotting studies revealed several antigenic constituents between 10 and 120 kDa (14, 15), none of which have been characterized on a molecular level. Cross-reactivity of avocado and latex proteins has been reported (14, 15). The predominant allergen in avocado, shown to be cross-reactive among avocado, latex, and banana, is about 30 kDa (14). The cross-reactivity suggests that this avocado allergen might share antigenic determinants with some latex allergens, although Persea americana and Hevea brasiliensis are botanically unrelated.

Here we report the cloning and expression of Prs a 1, a 32-kDa major allergen of avocado, cross-reactive with latex allergens. This cross-reactivity of the recombinant protein provides the first molecular basis of the association of type I allergic reactions to latex and avocado. rPrs a 1 displayed endochitinase activity and inhibited the growth of Fusarium oxysporum in vitro.

EXPERIMENTAL PROCEDURES

Patients—A total of 20 individual serum samples from patients with positive case histories, positive skin prick tests, positive RASTs (RAST classes higher than 4), and characteristic type I allergic reactions to latex was used in this study. Seven out of 20 patients (35%) reported symptoms after the ingestion of avocado. A serum pool from 22 healthy individuals with no histories of any type I allergy, negative skin prick tests, and negative RAST results to avocado and/or latex allergens was used as negative control.

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** To whom correspondence should be addressed. Tel.: 43-1-40400-5130; Fax: 43-1-40400-5130; E-mail: Heimo.Breiteneder@akh-wien.ac.at.

† The abbreviations used are: rPrs a 1, recombinant Prs a 1; nPrs a 1, natural Prs a 1; RAST, radioallergosorbent test; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction.
Major Avocado Allergen

Protein Extracts—Ten grams of avocado pear mesocarp tissue (P. americana Miller cv. Haas) were homogenized in a Waring blender and mixed with 20 ml of extraction buffer consisting of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM diethylthiocarbamate, and 10 mM sodium sulfate. The mixture was then centrifuged at 40,000 × g for 1 h. The supernatant and pellets were collected and lyophilized. To complete the cDNA, the fragment was extended by 5′-rapid amplification of cDNA ends using the AmpliFinder™ kit (CLONTECH, Palo Alto, CA). The full-length Psa 1 cDNA sequence was analyzed for possible cleavage sites using the program SIGSEQ (18).

DNA Sequencing—Sequence analysis was performed using the Thermoscript RT-PCR System (Ambion, Austin, TX) and labeled Primer Cycle Sequencing kit (Amersham Life Science) and the LI-COR DNA sequencer model 4000L (LI-COR, Lincoln, NE). Both strands of six different clones were analyzed to yield the final sequence of the amplified fragment.

Computer Search for Sequence Homology—The FASTA program provided with the Wisconsin Package (Genetics Computer Group, Madison, WI) was used to search for protein sequence homologies of the 32-kDa avocado allergen to proteins in the SWISSPROT data base.

Expression of the Recombinant Psa 1 in the Yeast P. pastoris—The cDNA corresponding to the mature Psa 1 protein was amplified by PCR using the phosphorothioate-modified primers: sense, 5′-TATCTCC-GAGAAAAAGAACATGTTGAGAAGCT-3′; antisense, 5′-TATTGCCGCGCGCTCTTTAGGATGACAGCAAGGA-3′ (priming regions underlined) (New England Biolabs, Beverly, MA). The sequence CTC GAG AAA AGA GAA, corresponding to the amino acid sequence LEKRE, was necessary to recreate the signal peptide cleavage site of the Saccharomyces cerevisiae a factor leader peptide present in the P. pastoris expression vector pPIC9 (Invitrogen). The signal peptide cleavage in the above sequence lies between arginine and glutamic acid residues.

Fortunately, glutamic acid is also the first amino acid of the mature Psa 1, so no cloning artifacts were produced. The XhoI/NorI-digested PCR product was ligated to the respective sites of P. pastoris vector pPIC9 and sequenced to confirm the identity of the insert. The transformation of the P. pastoris strain GS115 (Invitrogen), screening for recombinant Psa 1-producing clones, and extracellular expression were performed according to the instruction manual.

Enzymatic Assays—The endochitinase assay with the purified Psa 1 was performed in 50 mM potassium phosphate, pH 8.0, according to Wirth et al. (19) using carboxymethyl-substituted soluble chitin labeled with remazol brilliant violet 5R (Loewe Biochemica, Otterfing, Germany). The exochitinase activity was measured using 4-nitrophenyl-chitobioside (simulates a trimer; Sigma) according to Ref. 21. Lysozyme activity was determined by a modification of the method reported by Shugar et al. (22). Briefly, 0.2 mg of Micrococcus lysodeikticus cell walls (Sigma) in 900 μl of 100 mM potassium phosphate, pH 8.0, were mixed with 100 μl of enzyme solution, incubated at 37 °C, and the absorbance of the reaction mixture at 570 nm was measured every 10 min to determine the decrease in turbidity. Hen egg white lysozyme (Merck, Darmstadt, Germany) was assayed at 55 °C in 100 mM Tris-HCl, 100 mM NaCl, pH 9.0, as a positive control.

Fungal Growth Inhibition Assay—For the growth inhibition assay, F. oxysporum spores were collected from 8-day-old cultures grown on potato dextrose agar plates (Difco). Assay mixtures contained 12 μl of the 5× potato dextrose broth (Difco), 3000 spores of the test fungus in 10 μl of water, and 38 μl of the Psa 1 solution. The effect of rPsa 1 was tested at concentrations of 1, 5, 10, 15, 20, 25, 30, 40, 50, and 100 μg/ml. In the controls, heat-denatured rPsa 1 and sterile water were used instead of the solution containing the enzyme. After 40 h of incubation at 25 °C, portions of the samples were placed on microscope slides, and the lengths of the first 20 germ tubes were measured and averaged.

RESULTS

IgE Binding Analysis of Avocado Extracts—Nineteen out of 20 serum samples from patients allergic to avocado and/or latex reacted with proteins from the avocado extract. Fifteen of them reacted with a 32-kDa protein, nine reacted with a 46-kDa protein, four reacted with a 28-kDa protein, and two reacted with a 14-kDa protein (Fig. 1).

Purification and Sequence Analysis of the Prs a 1 Protein—Both natural and recombinant Psa 1 eluted at 0.26 M NaCl in 20 mM citric acid buffer, pH 3.8, from the HiTrap 153™ SP
nases (EC 3.2.1.14), enzymes that catalyze the hydrolysis of
mismatches. Chitinases from family 19 belong to endochito-
nallergic individuals (Controls included the serum pool from 22
nonallergic individuals (N) and buffer instead of serum (B)). The position of Prs a 1
at 32 kDa is indicated.

Carbohydrate Analysis—The de-acetylated monosaccharides
found by high pH anion exchange chromatography with pulsed
amperometric detection were GalNH2 and Gal. The lack of Man
and GlcNH2 is consistent with the absence of potential
N-glycosylation sites in the Prs a 1 sequence. The molar ratio of
the O-linked carbohydrates GalNH2 and Gal to each other was
3:5:1.

cDNA Coding for Prs a 1—Fig. 3 depicts the Prs a 1 sequence
with the identified motifs as analyzed by DNA sequencing of
six independent cDNA clones. The cDNA codes for a polypep-
tide of 326 amino acids including a leader peptide of 25 residues
as determined by N-terminal amino acid microsequencing and
software analysis. The cleavage of the leader sequence results
in the mature protein with a calculated molecular mass of 32.0
kDa. No potential N-glycosylation sites were detected. A chitin
recognition and/or binding motif corresponding to the consen-
sus pattern CX2-CX3GXXGXX(F/Y/W)C (Fig. 3) was de-
tected by the program MOTIFS (Wisconsin Package). Two addi-
tional consensus patterns, CX2-FY(S/T)X(F/Y)/L/I/V/M/
FXAX2/F/X2X2/G/S/A) and (L/I/V/M/G/S/A)FX(S/T/A/G)X(L/I/
V/M/F/Y)W(W/F/Y/W/L/I/V/M) (Fig. 3), which are characteristic
for chitinases from family 19 of glycosyl hydrolases according to
the classification by Henrisaat et al. (23), were identified with-
out mismatches. Chitinases from family 19 belong to endochito-
nases (EC 3.2.1.14), enzymes that catalyze the hydrolysis of
β-1,4-β-D-N-acetylglucosamine linkages in chitin polymers. En-
zymes from family 19 are also known as class I A (another
notation for class I A is 1) and class I B (II) endochitinases.
Class I A and I B endochitinases differ in the presence (I A) or
absence (I B) of an N-terminal chitin-binding domain. The catalytic
domain of these enzymes consists of about 220–240
amino acid residues. In this nomenclature, Prs a 1 is a class I
A (class I) chitinase. In the Prs a 1 sequence, a glycine-
and serine-rich hinge region of about 20 amino acids connects the
chitin-binding domain (amino acid residues 1–39 in Fig. 3) with
the C-terminal catalytic domain of about 240 amino acid residues.

Sequence Homology Analysis—Prs a 1 shares substantial
sequence similarities with endochitinases from plants. Only
similarities to chitinases present in plant-derived foods were
taken into account, since they may play important roles in the
context of plant food allergies. The Prs a 1 endochitinase from
avocado shares 77.0% identity with a chitinase from Triticum
aestivum (in 300-amino acid overlap), 73.8% with Oryza
sativa (in 301 amino acids), 73.5% with Solanum tuberosum (in
294 amino acids), 72.9% with Brassica napus (in 292 amino
acids), and 71.0% with Cucumis sativa (in 300 amino acids)
endochitinases. Another similarity to a known major latex al-
lergen was revealed by the sequence comparison of Prs a 1 with
prohevein (Fig. 4)). The similarity between the two proteins is
confined to their chitin-binding domains. The degree of identity
over this region of 43 amino acid residues is 70%. Two other
similarities of interest in the context of the latex-fruit syn-
drome, to a 33-kDa banana allergen (24) and a latex 29-kDa
allergen (25) were found (Fig. 4 and 5).

Expression of Prs a 1 in P. pastoris—The extracellular ex-
pression using the pPIC9 vector yielded a prominent band of 32
kDa (Fig. 5, lane 1). The yield, estimated by the method of
Bradford was approximately 50 mg/liter culture supernatant.

The rPrs a 1 protein could be separated from low molecular

FIG. 1. IgE binding of sera from av-
ocado- and/or latex-allergic patients
tested on avocado extracts. Nitrocel-
llose-blotted avocado extracts were probed
with individual sera from patients alleric
to avocado and/or latex (lanes 1–20).
Controls included the serum pool from 22
nonallergic individuals (N) and buffer in-
stead of serum (B). The position of Prs a 1
at 32 kDa is indicated.

FIG. 2. Coomassie-stained SDS-PAGE of purified natural and recombinant Prs a 1. Lane 1, 400 ng natural Prs a 1 purified from
avocado fruit; lane 2, 600 ng of recombinant Prs a 1 purified from the
culture supernatant of P. pastoris.
weight degradation products by one-step purification over a HiTrap™ SP column (Fig. 2, lane 2). Under standard SDS-PAGE conditions, the protein migrated exactly the same as natural Prs a 1 (Fig. 2). The experimental pI of the rPrsa1 was determined to be 8.8 (data not shown).

Enzymatic Assays—rPrsa1 exhibited endochitinase activity but no exochitinase activity (Table I). In addition, as some plant chitinases also display the activity defined in EC 3.2.1.14 (lysozyme), we tested rPrsa1 for lysozyme activity with hen egg white lysozyme as a control (Table I). Interestingly, hen egg white lysozyme showed a weak endochitinase activity, but Prs a 1 showed no lysozyme activity.

Inhibition of Fungal Growth by Natural and Recombinant Prsa1—Growth of F. oxysporum was inhibited by 95% at a concentration of 35 g/ml purified rPrsa1 and 33 g/ml purified nPrsa1. The inhibition curve of the purified nPrsa1 was equivalent to the recombinant product within the S.D. values. As a control, heat-denatured rPrsa1 did not inhibit the growth of the test fungus. In addition, an altered morphology was observed in samples treated with rPrsa1. Only 15% of the germ tubes incubated with 35 mg/ml of rPrsa1 were branched, all of them having only a single branch. In contrast, most of the control mycelia were highly branched.

Immunological Reactivity of rPrsa1—The IgE binding capacity of rPrsa1 was assessed by direct binding of IgE from a serum pool of avocado- and/or latex-allergic patients to solid phase bound rPrsa1. The 32-kDa rPrsa1 was the only component of P. pastoris culture supernatants, which bound serum IgE (Fig. 5, lane 3). No IgE binding was detected with untransformed P. pastoris strain GS115, which was used as negative control (Fig. 5, lane 4).

Immunoblot Inhibition Experiments with the rPrsa1—Using rPrsa1, we could achieve an 80–90% inhibition of IgE binding to the 32-kDa band in avocado extracts (Fig. 6, lanes 3 and 4).
latex was preincubated with 30°C avocado extracts. Lane 1, molecular mass standards; lane 2, 60-μg protein extracts from avocado fruit; lane 3, IgE binding of the serum pool to avocado proteins; lane 4, inhibition of IgE binding to avocado proteins by the addition of 30 μg of purified rPrs a 1. Lanes 1 and 2 are Coomassie-stained SDS-PAGE gels. The position of Prs a 1 is indicated. The same amount of protein was loaded in lanes 2–4.

and 4). In immunoblot inhibition experiments using solid phase bound latex B-fraction proteins, a weakening of the IgE binding to the 20-, 28–30-, and 36-kDa allergens was observed, whereas the reactivity to the 18-kDa allergen remained unaffected (Fig. 7, lanes 3 and 4). In contrast, the pattern of IgE binding to latex C serum proteins did not change after incubation with rPrs a 1 (data not shown).

DISCUSSION

We purified a 32-kDa protein from avocado pear and characterized it as a major avocado allergen, Prs a 1 by protein sequencing and cDNA cloning. The open reading frame of the Prs a 1 cDNA encodes a polypeptide of 326 amino acid residues with no consensus N-glycosylation sites. The first 25 amino acids are absent in the mature peptide as determined by protein microsequencing and constitute a leader peptide (Fig. 3). The presence of a leader indicates that Prsa1i is not a cytoplasmic protein. At the N terminus of the mature Prs a 1 resides a chitin-binding and/or recognition domain (Fig. 3), a conserved domain of 43 amino acid residues found in several plant and fungal proteins that has a common binding specificity for oligosaccharides of N-acetylglucosamine (26). This domain is found in endochitinases (EC 3.2.1.14) from class I A, in a number of nonleguminous plant lectins, and in prohevein, a major allergen and wound-induced protein from natural rubber latex (27, 28). Based on the amino acid motifs found in the sequence, Prs a 1 belongs to endochitinases from family 19 of glycosyl hydrolases or, alternatively, to class I endochitinases (Fig. 3; Ref. 23). Sequence analysis revealed that Prs a 1 shares 60–70% amino acid identity with the majority of class I endochitinases.

We expressed Prs a 1 in the yeast P. pastoris to take full advantage of the eucaryotic folding machinery. Expression levels of approximately 50 mg of Prs a 1/liter of medium were achieved. We performed an enzymatic analysis demonstrating that rPrs a 1 had endochitinase activity. The specific activity of rPrs a 1 was similar to the activity of Chi-I (29), a class I endochitinase from tobacco (1.4 and 1.0 OD units/mg of protein, respectively). Prs a 1 lacks exochitinase activity as we showed using p-nitrophenyl substrates simulating dimers and trimers (Table I). Since some plant chitinases also display lysozyme activity, we tested for it using hen egg white lysozyme as a positive control, but none was found (Table I). Taken together, Prs a 1 is a class I endochitinase with enzyme activities well within the range of other class I endochitinases. Comparison of the inhibition curves of F. oxysporum mycelial growth showed comparable biological in vitro activities for rPrs a 1 and nPrs a 1. rPrs a 1 and nPrs a 1 exhibited 95% inhibition of growth of hypheae of F. oxysporum at concentrations of 35 ± 3 μg/ml and 33 ± 3 μg/ml, respectively.

In addition to endochitinase activity, the purified rPrs a 1 displayed IgE binding capacity (Fig. 5, lane 3). This indicates that the recombinant protein was correctly folded and equivalent to its natural counterpart. rPrs a 1 could inhibit IgE binding to patients' serum pool. This suggests that the recombinant protein can be used as a diagnostic tool for allergy screening.

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>EC number</th>
<th>Substrate</th>
<th>rPrs a 1</th>
<th>HEWL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endochitinase</td>
<td>EC 3.2.1.14</td>
<td>CM-chitin-RBV</td>
<td>1411.7 ± 0.05 OD units/mg</td>
<td>1.6 ± 0.05 OD units/mg</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>EC 3.2.1.17</td>
<td>M. lysodeikticus cell walls</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Exochitinase (formerly)</td>
<td>EC 3.2.1.14</td>
<td>4-Nitrophenyl-N,N-diacyetyl-β-D-chitobioside</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Exochitinase (formerly)</td>
<td>EC 3.2.1.14</td>
<td>4-Nitrophenyl-N-acetyl-β-D-glucosaminide</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* HEWL, hen egg white lysozyme.

**FIG. 6. Inhibition of patients' IgE binding to proteins in avocado extracts.** A serum pool from patients allergic to avocado and/or latex was preincubated with 30 μg of recombinant Prs a 1 and then incubated with allergens blotted onto nitrocellulose strips. Lane 1, molecular mass standards; lane 2, 60-μg protein extracts from avocado fruit; lane 3, IgE binding of the serum pool to avocado proteins; lane 4, inhibition of IgE binding to avocado proteins by the addition of 30 μg of purified rPrs a 1. Lanes 1 and 2 are Coomassie-stained SDS-PAGE gels. The position of Prs a 1 is indicated. The same amount of protein was loaded in lanes 2–4.

**FIG. 7. Inhibition of patients' IgE binding to proteins in latex B fraction with purified rPrs a 1.** A serum pool from patients allergic to avocado and/or latex was preincubated with 30 μg of purified Prs a 1 and then incubated with nitrocellulose-blotted latex B fraction proteins. Lane 1, molecular weight standards; lane 2, 60 μg of latex B fraction proteins; lane 3, IgE binding of the serum pool to proteins from B fraction; lane 4, inhibition of IgE binding to B fraction proteins by 30 μg of purified rPrs a 1. The same amount of protein was loaded in lanes 2–4. The position of the 20-kDa allergen is indicated.
binding to the 32-kDa protein band in avocado extracts although the inhibition was not complete (Fig. 6, lanes 3 and 4). The remaining IgE binding could be due to the presence of epitopes from other endochitinases, since it is well known that most plants contain several different chitinases. In tobacco (Nicotiana tabacum), for example, five different chitinases with similar molecular weights have been described, and in peanut (Arachis hypogaea) and in potato (Solanum tuberosum) four different ones each have been described (SWISSPROT data base). Furthermore, the presence of other allergens of the same or similar molecular weight in avocado extracts cannot be excluded.

We tested the cross-reactivity between the avocado and latex allergens by inhibition of IgE binding from sera of avocado and/or latex allergic patients to proteins from the B fraction and C serum from latex with rPrs a 1. IgE binding to solid phase-bound 20-, 28-, 30-, and 36-kDa allergens was partially inhibited by rPrs a 1, whereas the reactivity to the 18-kDa allergen remained unaffected (Fig. 7, lanes 3 and 4). The 20-kDa allergen very likely represents prohevein because of its molecular weight and presence in latex B-fraction (27). The inhibition could be explained by structural similarity between the chitin-binding domains of the two proteins (Fig. 4A). As expected, the inhibition is only partial, because the 14-kDa C-terminal domain of prohevein still binds IgE from 30% of latex-allergic patients' sera (30). Additional evidence for the cross-reactivity between Prs a 1 and the 20-kDa allergen from latex is provided by the observation of two patients whose IgE recognized exclusively prohevein in natural latex B fraction; IgE from sera of these patients bound exclusively to the 32-kDa Prs a 1 in avocado extracts.2 The amino acid sequence similarity between the major latex allergen hevein and the N-terminal portion of Prs a 1 suggests that the chitin-binding motif of Prs a 1 may form an important cross-reactive IgE epitope. The partial inhibition of the 28–30-kDa allergens could be due to the presence of endochitinases in natural rubber latex, since latex contains high levels of chitinase activities (31) and a 29-kDa chitinase has been identified as an allergen in latex (25, Fig. 4C). However, the decrease in IgE binding to a 36-kDa allergenic component of latex cannot be explained with sequences of allergens characterized so far.

There is evidence that other plant chitinases could also be allergens. A polyclonal antibody raised against hevein recognized a 33-kDa banana protein (24), which may also be an endochitinase (Fig. 4B). In immunoblot inhibition experiments, purified hevein, at the low concentration of 10 ng/ml, completely inhibited IgE binding to the 33-kDa banana protein (24).

Endochitinases belong to group 3 of pathogenesis-related proteins in the classification of Stintzi et al. (32). Chitinases are a part of the plant's basic defense system against fungal pathogen attack. Many endochitinases present in food, including chitinases from chestnut (71.0%), rice (73.8%), potato (73.5%), and wheat (77%), display more than 65% amino acid identity with Prs a 1. Thus, one would expect IgE cross-reactivity with other plant-derived endochitinases. Consequently, chitinases from many different sources of plant origin, including pollen and vegetables, may form another group of so-called "pan-allergens," as was first described for profilins (33). This hypothesis is currently under investigation.

In hyphal extension-inhibition assays, endochitinases have been shown to be very effective in preventing the invasion of fungal mycelia into plant tissues (34–36). A hybrid endochiti-