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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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 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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1 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 diethyldithiocarbamate, and 10 mM sodium sulfate. The mixture was then centrifuged at 40,000 × g for 1 h. The supernatant was decanted and filtered through Miracloth (Calbiochem, La Jolla, CA). Freshly harvested field latex was fractionated by ultra centrifugation (40,000 × g at 4 °C for 1 h) into (i) the rubber particles in the upper fraction, (ii) a translucent aqueous layer known as C-serum, and (iii) a pellet containing organelles collectively called “lutoïds” (16). The bottom fraction was resuspended in 50 mM Tris-HCl buffer, pH 8.0, containing 0.05 M Triton X-100. After this was centrifuged at 100,000 × g for 1 h, the supernatant was discarded and the pellet washed once with 50 mM sodium chloride. The pellet was then treated with 0.1 M NaCl. In a further step, the protein was purified by a Vydac C-4 column (Hercules, CA). The column was equilibrated with 0.1 M NaCl in 20 mM citric acid buffer, pH 3.8, from the HiTrap 153TM SP column after being called the B fraction. C serum was again centrifuged for 1 h (40,000 × g, 4 °C) to remove residual latex particles. The pH of the extract was adjusted with acetic acid to pH 7.0, dialyzed against water, lyophilized, and stored at −80 °C until use. For the experiments, the lyophilized protein extracts were resuspended in 200 mM NaCl.

**SDS-PAGE, Isoelectric Focusing Gel Analysis, and Immunoblotting**—rPrs 1, nPrs 1, latex B fraction, C serum, and avocado extracts were analyzed by SDS-PAGE in 12% polyacrylamide gels under reducing conditions. For immunodetection, the separated proteins were transferred to ProBlott membranes (Applied Biosystems, Foster City, CA). Membrane strips were incubated with sera from allergic patients, and bound IgE was detected using phosphatase-labeled anti-human IgE (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and the Chemiluminescence Horseradish Peroxidase system from Amersham Life Science (Little Chalfont, United Kingdom) according to the manufacturer’s instructions (Fig. 1). Alternatively, bound IgE was detected using 125I-labeled rabbit anti-human IgE (RAST RIA, Pharmacia, Upsala, Sweden) diluted 1:10 (Figs. 5–7). Isoelectric focusing gel electrophoresis was performed with Ampholine® PAGplates, 3.5–9.5 (Pharmacia) at 500 V for 90 min.

**Immunoblot Inhibition with the Recombinant Prs 1**—For the immunoblot inhibition experiments, a serum pool from 19 avocado and/or latex allergic patients (sera 2–20 in Fig. 1) was preincubated overnight at 4 °C with 30 μg of purified rPrs 1. Preincubated sera were used to probe the ProBlott membrane strips containing total avocado extracts, latex B fraction, or latex C serum as described above.

**Purification and Sequence Analysis of Prs 1**—Twelve milliliters of avocado extracts were dialyzed against 20 mM citric acid buffer, pH 3.8, and subjected to a HiTrap™ SP cation exchange column (Pharmacia) at a flow rate of 0.5 ml/min using a fast protein liquid chromatography system: buffer A, 20 mM citric acid buffer, pH 3.8; buffer B, buffer A and 1 M NaCl. In a further step, the protein was purified by a Vydac C-4 reverse phase column (Western Analytical). The elution was done with a linear gradient of 0.12% trifluoroacetic acid in water (buffer C) and 0.12% trifluoroacetic acid in acetonitrile (buffer D). The rPrs 1 was purified by one-step purification over the HiTrap 153TM SP column as described above. The protein concentration was assessed with the Protein Assay kit from Bio-Rad with bovine serum albumin as a standard. The amino acid sequences of the N terminus and two tryptic peptides of rPrs 1 were determined by automated Edman degradation and analysis on a 477A gas phase microsequencer (Applied Biosystems) connected on-line to the phenylisothiocyanate analyzer, model 120A.

**Carbohydrate Analysis of rPrs 1**—A sample of the purified rPrs 1 was desalted by extensive dialysis, dried down under a stream of nitrogen, and then dissolved in 50 μl of HPLC grade water. Neutral sugars were released by hydrolysis with trifluoroacetic acid added to a final concentration of 2 M in a sealed polypropylene microcentrifuge tube. After 6 h of hydrolysis at 100 °C, the sample was evaporated to dryness. The sample was redissolved in 55 μl of water and analyzed directly by high pH anion exchange chromatography with pulsed amperometric detection. The monosaccharides were separated on a Dionex Bio-LC system (Dionex, Sunnyvale, CA) equipped with a PAD 2 detector. The elution was performed at 15 m M NaOH isocratic with a flow of 1 ml/min, 200 mM NaOH as eluant A and water as eluant B.

**Isolation of RNA and cDNA Synthesis**—RNA was prepared from avocado pear mesocarp tissue as described previously (17). Samples corresponding to different stages of maturation were pooled for reverse transcription. The reverse transcription was performed with 1 μg of total RNA using an oligo(dT) 16 primer and the GeneAmp RNA PCR kit (Perkin-Elmer).

**Isolation of cDNA Encoding Prs 1**—From the obtained N-terminal peptide sequence, two different degenerate sense oligonucleotides were designed for use in PCR: AV01, 5'-TG/TCT/GTG/T/AG/GT/TCA/AG/TT/C/TGGTG/T/CG/GG-3', and AV02, 5'-TG/TCT/GTG/T/AG/GT/TCA/AG/TT/C/TGGTG/T/CG/GG-3', both corresponding to CCSQFGWG. The antiensine oligonucleotide was the lock-docking oligo(DT) primer 5'-T(CT)_9/G(A/C)/G(A/T)/C/T'-3'. The PCR products were cloned into pCRII (TA Cloning kit, Invitrogen, San Diego, WI) and sequenced. 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 Prs a 1 cDNA sequence was analyzed for possible cleavage sites using the program SIGSEQ2 (18).

**DNA Sequencing**—DNA sequencing was performed using the Thermoscript™Reverse Transcriptase and Fluorescent Labeled Primer Cycle Sequencing kit (American Type Culture Collection, Rockville, MD) 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 Prs 1 in the Yeast P. pastoris**—The cDNA corresponding to the mature Prs a 1 protein was amplified by PCR using the phophorosioate-modified primers: sense, 5'-TATCTCC- GAGAAAGGACAAATGTTAGCAAGCT-3'; antisense, 5'-TATTCGCGCCGCCTCTATTAGGATGAGACAGCGAGG-3' (priming regions of the introns were omitted) (21). The 549-bp fragment was cloned into the expression vector pPIC9 (Invitrogen). The signal peptidase cleavage in the above sequence lies between arginine and glutamic acid residues. For immunodetection, glutenic acid is also the first amino acid of the mature Prs 1, so no cloning artifacts were produced. The XhoI/NcoI-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 Prs 1-1 producing clones, and extracellular expression were performed according to the manufacturer's instructions.

**Enzymatic Assays**—The endochitinase assay with the purified rPrs 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-N-acetyl-D-glucosaminide (simulates a dimer; Serva, Heidelberg, Germany). The exochitinase activity was measured using 4-nitrophenyl-N-acetyl-D-glucosaminide (simulates a dimer; 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 5× potato dextrose broth (Difco), 3000 spores of the test fungus in 10 μl of water, and 38 μl of the rPrs 1 solution. The effect of rPrs 1 was tested at concentrations of 1, 5, 10, 15, 20, 25, 30, 35, 40, 50, and 100 μg/ml. In the controls, heat-denatured rPrs 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 Prs a 1 eluted at 0.26 M NaCl in 20 mM citric acid buffer, pH 3.8, from the HiTrap 153TM SP...
Fig. 1. IgE binding of sera from avocado- and/or latex-allergic patients tested on avocado extracts. Nitrocellulose-blotted avocado extracts were probed with individual sera from patients allergic to avocado and/or latex (lanes 1–20). 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.

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.

column. Fractions containing natural Prs a 1 were collected and then further purified to homogeneity by HPLC reverse-phase chromatography (Fig. 2, lane 1), eluting at 38% buffer D. For the rPrs a 1, the first purification step was sufficient (Fig. 2, lane 2). The first 26 N-terminal amino acid residues determined by protein microsequencing were EQCCGRQAGGALCPGGLCCSQPFWCGGS. To confirm that this sequence was the N terminus of the mature peptide, an additional cleavage site analysis was performed using the SIGSEQ2 software. The cleavage site indicated by this program matched exactly the experimental data. The amino acid sequences of the two tryptic peptides derived from nPrs a 1 were GPIQISYNYNYPAGA and TALWFWMTPQSPK. All three peptide sequences matched exactly the deduced amino acid sequence of Prs a 1 (Fig. 3).

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 polypeptide 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 consensus pattern CX3GCX5GXCGX(F/Y/W)(F/Y/W/C (Fig. 3) was detected by the program MOTIFS (Wisconsin Package). Two additional consensus patterns, CX2FX(F/Y)/(S/T)(F/Y)/(L/I/V/M/F)AX4/FX2FX(F/G/S/A) and (L/I/V/M/G/S/A)FX(S/T/A/G)(L/I/V)/M/(F/Y)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 Henrissat et al. (23), were identified without mismatches. Chitinases from family 19 belong to endochitinases (EC 3.2.1.14), enzymes that catalyze the hydrolysis of β-1,4-linkages in chitin polymers. Enzymes from family 19 are also known as class I A (another notation for class I A is I) 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 a 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 allergen was revealed by the sequence comparison of Prs a 1 with prohevein (Fig. 4A). 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 syndrome, to a 33-kDa banana allergen (24) and a latex 29-kDa allergen (25) were found (Fig. 4B and C).

Expression of Prs a 1 in P. pastoris—The extracellular expression 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
weight degradation products by one-step purification over a HiTrapTM 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—rPrs a 1 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 rPrs a 1 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 Prs a 1—Growth of F. oxysporum was inhibited by 95% at a concentration of 35 ± 6 mg/ml purified rPrs a 1 and 33 ± 6 mg/ml purified nPrs a 1. The inhibition curve of the purified nPrs a 1 was equivalent to the recombinant product within the S.D. values. As a control, heat-denatured rPrsa 1 did not inhibit the growth of the test fungus. In addition, an altered morphology was observed in samples treated with rPrsa 1. Only 15% of the germ tubes incubated with 35 mg/ml of rPrsa 1/ml were branched, all of them having only a single branch. In contrast, most of the control mycelia were highly branched.

Immunological Reactivity of rPrs a 1—The IgE binding capacity of rPrs a 1 was assessed by direct binding of IgE from a serum pool of avocado- and/or latex-allergic patients to solid phase bound rPrs a 1. The 32-kDa rPrs a 1 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 rPrs a 1—Using rPrs a 1, we could achieve a 80–90% inhibition of IgE binding to the 32-kDa band in avocado extracts (Fig. 6, lanes 3).
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.

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 Prs a 1 is a secreted 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, 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 *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
Major Avocado Allergen

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 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 endochitinase gene under a constitutive promoter introduced into rapeseed oil (Brassica napus var. oleifera) inbred line rendered the transgenic plants more resistant to three different fungal pathogens (37). Transgenic rice plants constitutively overexpressing chitinases were produced to enhance resistance to fungal pathogens (38), and considerable commercial interests are involved. Concerning the ongoing discussion about the allergenicity of transgenic crops (39, 40), we emphasize that endochitinases are widely used to enhance the resistance of crops against fungal attack. Since one of the chitinases is now characterized as an allergen, the risk of creating food with a higher content of proteins with known allergenic potential should be taken into consideration when producing transgenic crops.

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REFERENCES
1. European Commission (1997) Study of Nutritional Factors in Food Allergies and Food Intolerances (Ortolani, C., and Pastorello, E. A., eds) pp. 93–94, European Commission, Office for Official Publications of the European Communities, Luxembourg.

2. Young, E., Stoneham, M. D., Petruckevitch, A., Barton, J., and Bona, R. (1994) *Lancet* 343, 1127–1130.

3. Vanek-Krebitz, M., Hoffmann-Sommergruber, K., Laimer-da-Camara-Machado, M., Susani, M., Ebner, C., Kraft, D., Scheiner, O., and Breiteneder, T. (1995) *Biochem. Biophys. Res. Commun.* 214, 538–551.

4. Breiteneder, H., Hoffmann-Sommergruber, K., O’Riordain, G., Susani, M., Ahorn, H., Ebner, C., Kraft, D., and Scheiner, O. (1995) *Eur. J. Biochem.* 233, 484–489.

5. Burks, A. W., Cockrell, G., Stanley, J. S., Helm, R. M., and Bannon, G. A. (1995) *J. Clin. Invest.* 96, 1715–1721.

6. Leung, P. S., Chu, K. H., Chow, W. K., Ansari, A., Bandea, C. I., Kwan, H. S., Nagy, S. M., and Gershwin, M. E. (1994) *J. Allergy Clin. Immunol.* 94, 882–890.

7. Teale-Diaz, G., Ellis, M. H., Morales-Russo, F., and Hein, D. C. (1995) *Allergy* 40, 241–243.

8. Blanco, C., Carrillo, T., Castillo, R., Quiralte, J., and Cuervas, M. (1994) *Ann. Allergy* 73, 309–314.

9. Brehler, R., Theissen, U., Mohr, C., and Luger, T. (1997) *Allergy* 52, 404–410.

10. Beezhold, D. H., Sussman, G. L., Liss, G. M., and Chang, N.-S. (1996) *Clin. Exp. Allergy* 26, 416–422.

11. Slater, J. E. (1997) in *Allergy and Allergic Diseases* (Kay, A. B., ed) Vol. 2, p. 981–993, Blackwell Science, Oxford.

12. Turjanmaa, K., Alenius, H., Makinen-Kiljunen, S., Reunala, T., and Palosuo, T. (1996) *Allergy* 51, 593–602.

13. Alenius, H., Kalkkinen, N., Lukka, M., Reunala, T., and Palosuo, T. (1994) *Allergy* 49, 454–459.

14. Leung, P. S., Chu, K. H., Chow, W. K., Ansari, A., Bandea, C. I., Kwan, H. S., Nagy, S. M., and Gershwin, M. E. (1994) *J. Allergy Clin. Immunol.* 94, 882–890.

15. Fitz, R. J., and Gordon, J. I. (1987) *Biochem. Biophys. Res. Commun.* 146, 870–877.

16. Wirth, S. J., and Wolf, G. A. (1990) *J. Microbiol. Methods* 12, 197–205.

17. Berghem, L. E. R., and Pettersson, L. G. (1974) *Biochim. Biophys. Acta* 385, 227–234.

18. Shugar, D. (1952) *Biochim. Biophys. Acta* 12, 214 (abstr).

19. Wirth, S. J., and Wolf, G. A. (1990) *J. Microbiol. Methods* 12, 197–205.

20. Berghem, L. E. R., and Pettersson, L. G. (1974) *Biochim. Biophys. Acta* 385, 227–234.

21.Starrett, D. A., and Laties, G. G. (1993) *Plant Physiol.* 103, 227–234.

22. Shugar, D. (1952) *Biochim. Biophys. Acta* 12, 214 (abstr).

23. Posch, A., Chen, Z., Wheeler, C., Dunn, M., Rauf-Heimsoth, M., and Baur, X. (1997) *J. Allergy Clin. Immunol.* 99, 385–395.

24. Flach, J., Pilet, P. E., and Jolles, P. (1992) *Experientia* 48, 701–716.

25. Alenius, H., Kalkkinen, N., Lukka, M., Reunala, T., Turjanmaa, K., Makinen-Kiljunen, S., Yips., E., and Palosuo, T. (1995) *Clin. Exp. Allergy* 25, 659–665.

26. Borkert, I., Lee, H.-J., Kusah, A., Chua, N.-H., and Raikhel, N. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 7633–7637.

27. Melchers, E., Apotheker-de-Groot, M., van der Knaap, J. A., Ponteijn, A. S., Sela-Buurlage, M. B., Bol, J. F., Cornelissen, B. C. J., van den Elzen, P. J. M., and Linthorst, H. J. M. (1994) *Plant J.* 5, 469–480.

28. Alenius, H., Kalkkinen, N., Reunala, T., Turjanmaa, K., and Palosuo, T. (1996) *J. Allergy Clin. Immunol.* 109, 1618–1625.

29. Martin, M. N. (1991) *Plant Physiol.* 95, 469–476.

30. Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdingluo, S., Kauffmann, S., Geoffroy, P., LeGrandr, M., and Fritig, B. (1993) *Biochimie* 75, 657–706.

31. Valenta, R., Duchene, M., Ebner, C., Valenti, P., Sillaber, C., Devillier, P., Ferre, F., Tejl, M., Edelmann, H., and Kraft, D. (1996) *J. Exp. Med.* 179, 377–385.

32. Roberts, W. K., and Selitrennikoff, C. P. (1986) *Biochim. Biophys. Acta* 880, 161–170.

---

2 S. Sowka, L.-S. Hsieh, M. Krebitz, A. Akasawa, B. M. Martin, D. Starrett, C. K. Peterbauer, O. Scheiner, and H. Breiteneder, unpublished results.
35. Roberts, W. K., and Selitrennikoff, C. P. (1988) *J. Gen. Microbiol.* **134**, 169–176
36. Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. (1991) *J. Biol. Chem.* **266**, 1564–1573
37. Grison, R., Grezes-Besset, B., Schneider, M., Lucante, N., Olsen, L., Leguay, J.-J., and Toppan, A. (1996) *Nature Biotechnol.* **14**, 643–646
38. Lin, W., Anuratha, C. S., Datta, K., Petrykus, I., Muthukrishnan, S., and Datta, S. K. (1994) *Bio/Technology* **12**, 686–691
39. Nordlee, J. A., Taylor, S. L., Townsend, J. A., Thomas, L. A., and Bush, R. K. (1996) *N. Engl. J. Med.* **334**, 688–692
40. Metcalfe, D. D., Astwood, J. D., Townsend, R., Sampson, H. A., Taylor, S. L., and Fuchs, R. L. (1996) *Crit. Rev. Food Sci. Nutr.* **36**, 165–186