

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 1¹ 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.

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.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Z78202.

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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 collected and filtered through a Millex-HV filter (Millipore Corp., Bedford, MA). Freshly harvested field latex was fractionated by ultracentrifugation (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 “lutoids” (16). The bottom fraction was resuspended in 50 mM Tris-HCl buffer, pH 8.0, containing 0.05% Triton X-100 and will hereafter be 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 extracts 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 a 1, nPrs a 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 goat serum (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, Uppsala, 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 a 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 a 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 a 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 Products, Temecula, CA) with a linear gradient of 0.12% trifluoroacetic acid in water (buffer C) and 0.12% trifluoroacetic acid in acetonitrile (buffer D). The rPrs a 1 was purified by one-step purification over the HiTrap 153™ 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 purified nPrs a 1 were determined by automated Edman degradation and analysis on a 477A gas phase microsequencer (Applied Biosystems) connected on-line to the phenylthiohydantoin analyzer, model 120A.

Carbohydrate Analysis of nPrs a 1—A sample of the purified nPrs a 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 the strong anion exchange column CarboPac PA1 (4 × 250 mm), a PA 1 guard column, and a PAD 2 detector. The elution was performed at 15 mM NaOH isocratic with a flow of 1 ml/min, 200 mM NaOH as eluent A and water as eluent 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 and used 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 a 1—From the obtained N-terminal peptide sequence, two different degenerate sense oligonucleotides were designed for use in PCR: AVO1, 5'-TG(T/C)TG(T/C)AG(C/T)CA(A/G)T-

T(T/C)GG(A/G/T/C)TGTTG(T/C)GG-3', and AVO2, 5'-TG(T/C)TG(T/C)TC(A/G/T/C)CA(A/G)TT(T/C)GG(A/G/T/C)TGTTG(T/C)GG-3', both corresponding to CCSQFGWCG. The antisense oligonucleotide was the lock-docking oligo(dT) primer 5'-(T)₃₀(G/A/C)(G/A/C/T)-3'. The PCR products were cloned into pCRII (TA Cloning kit, Invitrogen, San Diego, CA) 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—Sequence analysis was performed using the Thermosequenase Fluorescent 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 Prs a 1 in the Yeast *P. pastoris*—The cDNA corresponding to the mature Prs a 1 protein was amplified by PCR using the phosphorothioate-modified primers: sense, 5'-TATCTC-GAGAAAAGAGAAACAATGTGGTAGACAAGCT-3'; antisense, 5'-TTA-GCGGCCGCTCATTAGGATGAAGCAGCAAGG-3' (priming regions underlined, *Xho*I and *Not*I sites in italics) and the Vent Polymerase (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* α factor leader peptide present in the *P. pastoris* expression vector pPIC9 (Invitrogen). The signal peptidase cleavage in the above sequence lies between arginine and glutamic acid residues. Fortunately, glutamic acid is also the first amino acid of the mature Prs a 1, so no cloning artifacts were produced. The *Xho*I/*Not*I-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 a 1-producing clones, and extracellular expression were performed according to the instruction manual.

Enzymatic Assays—The endochitinase assay with the purified rPrs a 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-β-D-glucosaminide (simulates a dimer; Serva, Heidelberg, Germany) as described previously (20) and 4-nitrophenyl-*N,N'*-diacetyl 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.

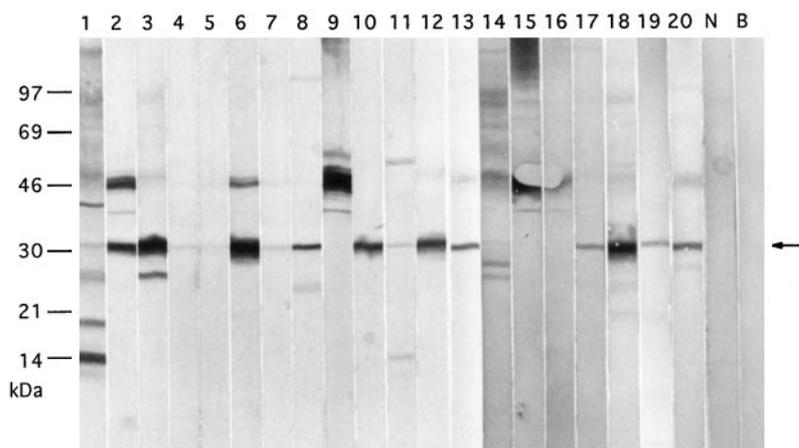
Fungal Growth Inhibition Assay—For the growth inhibition assay, *F. oxysporum* spores were collected from 8-day-old cultures grown on potato dextrose agar plates (Difco). Assay mixtures contained 12 µl of 5× potato dextrose broth (Difco), 3000 spores of the test fungus in 10 µl of water, and 38 µl of the rPrs a 1 solution. The effect of rPrs a 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 a 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 153™ 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.



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 EQCGRQAGGALCPGGLCCSQFGWCGS. 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 GPIQISYNYGPPAGA and TALWFWMTSPK. 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 CX_{4,5}CCSX₂GXCGX₄(F/Y/W)C (Fig. 3) was detected by the program MOTIFS (*Wisconsin Package*). Two additional consensus patterns, CX_{4,5}FY(S/T)X₃(F/Y)(L/I/V/M/F)XAX₃(Y/F)X₂F(G/S/A) and (L/I/V/M)(G/S/A)FX(S/T/A/G)₂(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-N-acetyl-D-glucosamine 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

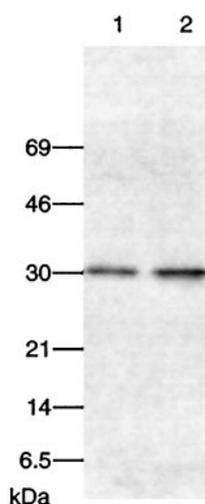


FIG. 2. Coomassie-stained SDS-PAGE of purified natural and recombinant Prs a 1. Lane 1, 400 ng natural Prs a 1 purified from avocado fruit; lane 2, 600 ng of recombinant Prs a 1 purified from the culture supernatant of *P. pastoris*.

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. 4, B 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

TABLE I
Chitinase and lysozyme activities of rPrs a 1 and hen egg white lysozyme

Enzyme activity	EC number	Substrate	rPrs a 1	HEWL ^a
Endochitinase	EC 3.2.1.14	CM-chitin-RBV ^b	1411.7 ± 0.05 OD units/mg	1.6 ± 0.05 OD units/mg
Lysozyme	EC 3.2.1.17	<i>M. lysodeikticus</i> cell walls	NM ^c	1.413 ± 0.08 × 10 ⁵ units/mg
Exochitinase	EC 3.2.1.14 (formerly 3.2.1.29)	4-Nitrophenyl- <i>N,N</i> -diacetyl-β-D-chitobioside	NM ^c	ND ^d
Exochitinase	EC 3.2.1.14 (formerly 3.2.1.30)	4-Nitrophenyl- <i>N</i> -acetyl-β-D-glucosaminide	NM ^c	ND ^d

^a HEWL, hen egg white lysozyme.

^b CM-chitin-RBV, carboxymethyl chitin labeled with remazol brilliant violet 5R.

^c NM, not measurable.

^d ND, not determined.

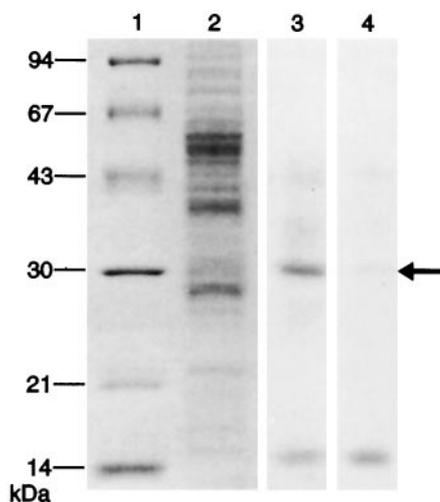


FIG. 6. Inhibition of patients' IgE binding to proteins in avocado extracts. A serum pool from patients allergic to avocado and/or latex was preincubated with 30 μg of recombinant Prs a 1 and then incubated with allergens blotted onto nitrocellulose strips. Lane 1, molecular mass standards; lane 2, 60-μg 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.

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 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

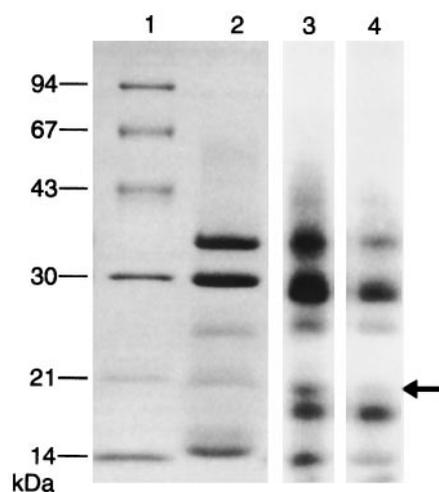


FIG. 7. Inhibition of patients' IgE binding to proteins in latex B fraction with purified rPrs a 1. A serum pool from patients allergic to avocado and/or latex was preincubated with 30 μg of purified Prs a 1 and then incubated with nitrocellulose-blotted latex B fraction proteins. Lane 1, molecular weight 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.

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/μg 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 hyphae 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 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 hypogea*) 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-

nase gene under a constitutive promoter introduced into rapeseed oil (*B. 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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