A Novel Acidic Allergen, Hev b 5, in Latex
PURIFICATION, CLONING AND CHARACTERIZATION*

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Akira Akasawa‡§, Li-Shan Hsieh‡§, Brian M. Martin†, Teresa Liu, and Yuan Lin†

From the Division of Allergenic Products and Parasitology, Center for Biologies Evaluation and Research, Food and Drug Administration, Rockville, Maryland 20852 and the Unit on Molecular Structures, Clinical Neuroscience Branch, National Institute of Mental Health, Bethesda, Maryland 20892

Latex allergy is recognized as a serious health problem among health care workers and children with spina bifida. A number of IgE-reactive proteins have been identified in natural and processed latex products. One of the most acidic proteins in the cytoplasm of laticifer cells of rubber trees (Hevea brasiliensis) is demonstrated to be a potent allergen in eliciting allergic reactions in humans. This protein, with pI = 3.5, has a molecular mass of 16 kDa with a blocked N terminus and an unusual amino acid composition. This acidic protein was found in extracts prepared from latex gloves, which were shown to be allergenic. The purified protein elicits histamine release from human basophils passively sensitized with serum from latex-allergic individuals in a dose-dependent manner.

From a latex cDNA library, the cDNA coding for this protein was isolated and sequenced. The deduced amino acid sequence shows a high degree of homology to another acidic protein identified in kiwifruit (Actinidia deliciosa var. deliciosa). The sequence homology (47% sequence identity) between these two acidic proteins suggests a molecular explanation for the high frequency of fruit hypersensitivity in latex-allergic patients.

Allergy to natural rubber products has been recognized as a serious medical problem especially among health care workers and children with spina bifida (1, 2). Five to 10% of surgeons and operating room nurses were reported to be allergic to latex gloves (3, 4), and as high as 34% of children with spina bifida were allergic to latex gloves or other rubber products (5). Agents responsible for eliciting the type I hypersensitivity have been demonstrated in native latex proteins with molecular sizes ranging from 5 to 200 kDa (6–11).

Latex of the rubber tree (Hevea brasiliensis) is produced by a group of specialized cells, laticifers. The cytosol of these cells contain rubber particles and a large number of proteins. Upon wounding, the cytoplasmic content of these cells is expelled in the form of latex. For the manufacturing of rubber goods, the fresh milky latex is collected in strong ammonia solution (20% buffer solution as described previously (10). The mixture was centrifuged at 50,000 × g at 4°C for 2 h to separate the rubber particles from the C-serum. The floating layer, consisting mostly of rubber particles, was desalted in a 20 mM citrate buffer, pH 3.0, by passing through a PD-10 column (Sephadex G-25M, Pharmacia Biotech Inc.). The fraction collected in the void volume was subjected to ion-exchange chromatography on a Pharmacia cation exchange column (HiTrap™ SP) using a FPLC system: buffer A, 20 mM sodium citrate. When proteins from the glove extracts were analyzed on gel electrophoresis, a smeared pattern, indicative of degraded protein, was observed (10). Nevertheless, strong allergic reactions can be elicited by the proteins/epitopes remaining on the surface of the latex product.

When latex is subjected to centrifugation, it can be separated into a floating yellow rubber particle layer and a clear pale yellow C-serum fraction. We have previously identified an acidic protein (pI = 3.5) in the C-serum fraction of the latex. The protein has an apparent molecular mass of 25 kDa on SDS-PAGE1 and was found by IgE immunoblotting to be reactive in 52% of latex-allergic patient sera (10). It has been shown that allergen recognition patterns differ between adults and children; this acidic protein, however, is recognized by both (10). Due to its acidic nature and possible association with isoprene residues, it migrates through the nitrocellulose membrane during the electroblotting process and thus escaped previous detection.

Patients with latex allergy are often reported to be allergic to fruits (13), particularly avocado, chestnut, banana, and kiwi-fruit (14). The cross-reactivity suggests that constituents of these fruits might share common antigenic determinants with some latex allergens, even though these fruits are botanically unrelated to latex. The purpose of this study was to determine the amino acid sequence of the acidic protein and to demonstrate its allergenicity based on its ability to induce histamine release. The sequence of this protein was compared to proteins in the Swiss Protein data bank for possible elucidation of the physiological roles of this protein in latex.

MATERIALS AND METHODS

Purification of the pI 3.5 Protein from Latex—Latex was collected from a H. brasiliensis (clone RRIM 600) from Malaysia in a glycercinated buffer solution as described previously (10). The mixture was centrifuged at 50,000 × g at 4°C for 2 h to separate the rubber particles from the C-serum. The floating layer, consisting mostly of rubber particles, was carefully removed. Glycerol was added to the C-serum to a final concentration of 50% (w/v) to prevent degradation and the growth of microorganisms. The mixture was stored at 4 °C until use.

The C-serum was desalted in a 20 mM citrate buffer, pH 3.0, by passing through a PD-10 column (Sephadex G-25M, Pharmacia Biotech Inc.). The fraction collected in the void volume was subjected to ion-exchange chromatography on a Pharmacia cation exchange column (HiTrap™ SP) using a FPLC system: buffer A, 20 mM sodium citrate

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; HPLC, high performance liquid chromatography; LB, Luria-Bertani medium; PIPES, piperazine-N, N'-bis(2-ethanesulfonic acid); PCR, polymerase chain reaction; MALDI, matrix-assisted laser desorption ionization.
buffer, pH 3.8; buffer B, 20 mM sodium citrate buffer, pH 3.8, and 1 M NaCl; and buffer C, a 20 mM Tris-HCl buffer, pH 8.0, and heated for 15 min at 60°C. 5% buffer B was added to approximately 5% buffer C, creating the pH 3.5 protein. This fraction was further purified by a C-4 reverse phase column (Vydac) with a gradient of 0.12% trifluoroacetic acid in water, 0.1% trifluoroacetic acid in acetonitrile.

Trypsin and Endoproteinase Asp-N Digestions and Separation of Digested Fragments—For tryptic digestion, 50 μg of purified pH 3.5 protein was dissolved in 10 ml of 4 M urea in 0.1 M Tris-HCl, 2.0 mM CaCl2, pH 8.0, and heated for 15 min at 60°C. Tryptic digestion was performed at room temperature overnight (15). For endoproteinase Asp-N digestion, 50 μg of purified protein was dissolved in 0.1 M Tris-HCl buffer, pH 8.0, and digestion was performed at 37°C overnight with a substrate/enzyme ratio of 50/1. The resulting peptides were separated by reverse-phase HPLC using a C-4 or a C-18 column and eluted with a gradient of 0.12% trifluoroacetic acid, 0.1% trifluoroacetic acid in acetonitrile (15).

Amino Acid Analysis and Amino Acid Sequence Analysis—Amino acid analysis was carried out after hydrolysis in 6 N HCl for 90 min at 150°C by using a Beckman model 6300 amino acid analyzer (16, 17). The amino acid sequences of several peaks from the tryptic digestion were sequenced by automated Edman degradation and analyzed on a model 890A protein microsequencer (Applied Biosystems) connected to an on-line model 120A phenylthiohydantoin analyzer. The endoproteinase Asp-N peptides were sequenced on a model LP 3000 Beckman protein sequencer with on-line phenylthiohydantoin analyzer.

Latex Glove Extract, Ammoniated Latex Extract, and Heat-treated Latex Extract—Glove extracts were generous gifts from Dr. Robert Hamilton (The Johns Hopkins University, Baltimore, MD) and Dr. John Yunginger (Mayo Clinic, Rochester, MN). The extracts were prepared and concentrated as described previously (10, 18, 19). Ammoniated latex extract was from Dr. Robert Hamilton. To test the stability of the pH 3.5 protein at high temperature, a condition used in the manufacture of latex gloves, a preparation of the latex extract was heated in an autoclave (250°F, 18 psi) for 20 min (19).

SDS-PAGE, IEF Gel Analysis, and Immunoblotting—SDS-PAGE was performed with a 16% gel under non-reducing conditions and proteins were visualized with silver stain as described previously (10). IEF gel electrophoresis was performed with Ampholine® PAG plates, pH 3.5–9.5 (Pharmacia), at 500 V for 90 min. After completion of the electrophoresis, immunoblotting was carried out with passive capillary blotting by pressing a nitrocellulose membrane directly against the gel for 45 min. The membrane was reacted with a serum (31 years old, female) that reacts strongly to the pH 3.5 protein followed by peroxidase-labeled anti-human IgE antibody. The IgE-reactive bands were detected by chemiluminescence horseradish peroxidase system (Amersham Life Science) according to the manufacturer’s instructions.

Histamine Release from Human Basophils—Passive sensitization of human basophils (20), venous blood was collected from a volunteer who is not allergic to latex. Twenty ml of heparinized blood was mixed with 25 ml of 154 mM NaCl solution containing 6% dextran for 1 h. After sedimentation of red cells, the polynuclear leukocytes were collected. The basophil-containing supernatant was centrifuged at 150 × g for 8 min at 4°C. The cell pellet was washed twice with PAG solution (25 mM Pipes, pH 7.4, 110 mM NaCl, 5 mM KCl containing 0.1% dextran and 0.003% human serum albumin) and once with saline. To strip IgE antibody from basophils, 5 ml of 10 mM lactic acid, pH 3.9, 140 mM NaCl, 5 mM KCl was added and incubated for 3.5 min at room temperature. Cells were washed once with PAG solution by centrifugation. To sensitize the cells, the cell pellet was incubated with 100 μl of patient’s serum, 300 ml of PAG solution, and 100 ml of 20 mM EDTA containing 0.05% heparin for 60 min at 37°C. Following this incubation, the cells were washed twice with PAG solution. The cell pellet was resuspended in 5 ml of PAG-DM solution (PAG solution with 1 mM MgCl2 and 1 mM CaCl2 added and diluted into 14 tubes) in duplicate. Tubs were prewarmed at 37°C for 2 min, and serially diluted pH 3.5 protein (1000, 100, 10, and 1 ng/ml) and 1% perchloric acid (to determine total histamine in cells) were added and incubated for 45 min at 37°C. After incubation, tubes were centrifuged at 150 × g for 8 min at 4°C and supernatants were subjected to histamine measurement by an automatic histamine analyzer (Tosoh Manufacturing Co. Ltd., Tokyo, Japan) with sensitivity of 0.1 ng/ml. Spontaneous histamine release was measured with PBSA+B cells on a C-4 reverse phase column (Vydac) with a gradient of 0.12% trifluoroacetic acid in water, 0.1% trifluoroacetic acid in acetonitrile.

Purification of the PI 3.5 Protein—For purification of the PI 3.5 protein, a preparation of the latex extract was heated in an autoclave (250°F, 18 psi) for 20 min (19).

RESULTS

Purification of the PI 3.5 Protein—Fractions collected from the cation-exchange column were analyzed by SDS-PAGE and IEF immunoblotting. The fraction containing the pH 3.5 protein was purified to homogeneity by HPLC reverse-phase chromatography (Fig. 1). This acidic protein could not be stained by Coomassie Blue and had poor staining using alkaline phosphatase-labeled antibody, but it could easily be detected by silver stain and by chemiluminescence using peroxidase-labeled antibody.

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A 0.5 ml of 5.0 M MgSO4 to an Amn of 5.0. In a 50-ml conical tube, 1 × 10^6 XLI-Blue MRF cells, 10^6 Assay helper phage, and 10^2 plaque-forming units of amplified latex cDNA library were combined and incubated at 37°C for 15 min. To this mixture, 25 ml of LB was added and incubated for another 2–2.5 h at 37°C with shaking and cells were spun down for 15 min at 2000 × g. The supernatant was transferred to a fresh tube and heated at 70°C for 15 min, then spun for 15 min at 4000 × g. The supernatant that contained the excised phagemid pBluescript, packaged as filamentous phage particles, was decanted into a sterile tube. To this mixture MgSO4 was added to a final concentration of 7% and stored at –80°C. For DNA preparation, the heat-treated ph哲more and ammoniated latex was dissolved in acetic acid in acetonitrile.

Isolation of cDNA Encoding the pH 3.5 DNA by Polymerase Chain Reaction (PCR)—Based on the amino acid sequences of a tryptic fragment, a degenerate 23-base oligonucleotide was synthesized. The degenerate oligonucleotide, T7 primer, and the phagemid DNA as a template, a 500-base pair DNA fragment was generated by PCR. The fragment was gel-purified and cloned into a Bluescript plasmid for sequence analysis. This was found to be the 3’ portion the cDNA coding for the latex pH 3.5 protein.

The cDNA that contains the entire coding sequence of the latex pH 3.5 protein was generated by PCR using a T3 primer and a specific 22-base oligomer (GAG ATG AGT TAC TGT GAG GTC T) from the 3’ end of the cDNA sequence. A 800-base pair DNA fragment identified by Southern blot was isolated and cloned into a Bluescript plasmid. Five DNA clones containing coding sequences for pH 3.5 protein were isolated and sequenced.

DNA Sequencing—DNA sequencing was performed using an Amer sham/U. S. Biochemical Corp Sequenase™ version 2.0 DNA sequencing kit. The initial sequencing was carried out using M13 (+) and T3 primers for the C-terminal portion of the cDNA. The complete coding sequence of latex pH 3.5 cDNA was achieved by using specific internal primers 5–1 (GAG ATG AGT TAC TGT GAG GTC T), 5–1R (CCA CAG CAT TGC CAA AG), 5–2R (GCA CAA GCG GAA GCT CCA G) and 5–2R (AGG TGC ACG TTC GTC TGC).

Computer Search for Sequence Homology—The FASTA program provided with PCGene was used to search for protein sequence homology of the pH 3.5 protein with other proteins of known sequence in the Swiss Protein sequence bank.

Acidic Latex Allergen

For purification of the PI 3.5 protein—Fractions collected from the cation-exchange column were analyzed by SDS-PAGE and IEF immunoblotting. The fraction containing the pH 3.5 protein was purified to homogeneity by HPLC reverse-phase chromatography (Fig. 1). This acidic protein could not be stained by Coomassie Blue and had poor staining using alkaline phosphatase-labeled antibody, but it could easily be detected by silver stain and by chemiluminescence using peroxidase-labeled antibody.

Acidic Latex Allergen

The pH 3.5 protein can be readily detected by passive transfer of an IEF gel to a nitrocellulose membrane, and by treating the membrane with latex-allergic patient serum. The IEF immu

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Most of the glove extracts contained this protein in varying concentrations. The pl 3.5 protein appeared to be unaffected by autoclaving, while most of the other proteins formed aggregates that remained near the origin of the sample loading zone (Fig. 2, lane 3). To demonstrate that the acidic proteins from the glove extracts were the pl 3.5 protein, an inhibition immunoblot experiment was performed. The serum was treated with the purified pl 3.5 protein prior to immunoblotting. Once the IgE specific for this acidic protein was removed, the band at pl 3.5 was no longer detectable (data not shown).

Amino Acid Analysis and Amino Acid Sequence—The amino acid composition derived from the amino acid analysis of the purified protein is comparable with that from the sequence deduced from the cDNA. The protein contains unusually high amounts of glutamic acid (46 residues), threonine (21 residues), alanine (29 residues), and proline (22 residues) and no aromatic amino acids, methionine or cysteine/cystine.

Amino acid sequence analysis indicated that the N terminus of the pl 3.5 protein was blocked. Several amino acid sequences were obtained from HPLC-purified, trypsin-generated, and endopeptidase Asp-N-generated peptides of the purified protein (Fig. 3). The deduced amino acid sequence from the cDNA was nearly identical to that obtained by amino acid sequencing, with the exception of two amino acids at positions 67 and 103 (Fig. 3). At position 81, both Ser and Pro were found in different

cDNA clones while amino acid sequence indicated a Pro at this position. The poly(A) tails are 12–24 bases in length among different clones sequenced. Amino acid sequences obtained from trypsin-digested peptides were indicated with a dashed line, and those from Asp N-digested peptides were indicated with a solid line.

Molecular Mass Determination by Mass Spectroscopy—The molecular mass of the purified pl 3.5 protein as determined by mass spectroscopy was 16001.2 Da. The molecular mass calculated from the deduced amino acid sequence was 15957.5 Da. The discrepancy of 43 Da is mostly likely from an acetyl group (43 Da) attached to the N-terminal alanine residue.

Complementary cDNA Coding for the pl 3.5 Protein—Fig. 3 is the result of DNA sequence analysis of five independent clones of cDNAs coding for the pl 3.5 protein. The DNA sequences for the coding region of all clones were identical with the exception of position 312, which is either a C or a T. This change gave rise to a different amino acid, either proline or serine. The noncoding regions at the 3'-end adjacent to the poly(A) tail were quite variable. Fig. 3 depicts the sequence of a cDNA clone with the shortest sequence at the 3'-end.

Sequence Homology Analysis—The computer search identified an acidic protein (pl 3.5) from kiwifruit that has a substantial sequence homology to the latex acidic protein (22). Fig. 4 is the sequence comparison of the latex pl 3.5 protein with a kiwifruit acidic protein, which has a molecular mass of 18.9 kDa with a pl of 3.5. The latex acidic protein has a truncated middle region when the sequences of these proteins are aligned with maximal sequence homology. The homology is most striking in the segments around the N and C termini.

Histamine Release Induced by Purified Latex pl 3.5 Protein—To analyze the allergic properties of the pl 3.5 protein, histamine release was performed using passively sensitized human basophils. Fig. 5 demonstrates that the purified pl 3.5 protein induced a dose-dependent histamine release when cells were first incubated with sera from allergic patients. The amount of histamine release also correlated with the amount of pl 3.5-specific IgE present in the individual serum. (The higher the level of pl 3.5-specific IgE, the better the responses to dosage of pl 3.5 proteins.) Patient 6 was allergic to latex but with a very low level of pl 3.5-specific IgE in the serum. When this serum was used for sensitization of basophils, no detecta-
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Latex ASVEVEAALASHFEW--EYTVKEHTYSEIV--AAPP--SEQTYAQ--ATYKV--KEE 51
Kiwi ATYVVMTAWLSEETSAYTVKQRPQAAPAARPHTEFAAHPVKEF 61

Latex TAAAFK----------------------AEYPDEKSFSEIVTKEFPAIAQDTQTKHPK 93
Kiwi AAIKESKARQEEVEAEHSEVIYRFAPFPSKAEVTSRASETKSYTAFK 120

Latex ALRPEYVAMKHESH---------------------KTSPPAPASPKEKPDHFHSTATRTVPSK 140
Kiwi APYSPYEEKDAAPPKEADYMEVAPAPPKEFAVAYKEEYVKEF VAYAQAVSYVPSK 179

Latex KE 150
Kiwi KE 181

Fig. 4. Sequence comparison of pl 3.5 protein from latex (upper sequence) and an acidic protein from kiwifruit (lower sequence). Colon (:) indicates identical sequence; period (.) indicates similar sequence.

Fig. 5. Human basophil histamine release stimulated by pl 3.5 protein from latex. Serum from 6 latex-allergic patients were used for sensitization of cells. The level of IgE specific to pl 3.5 protein is expressed as percent to the patient who has the highest level of specific IgE. Patient 1 (C), 100%; patient 2 (B), 87.7%; patient 3 (A), 85.7%; patient 4 (D), 90.1%; patient 5 (L), 20.4%; patient 6 (O), 2.6%.

Histamine was released after incubation with the pl 3.5 protein.

DISCUSSION

Many laboratories have identified IgE-binding latex proteins with different immunoreactivity profiles (6–11). The variability may be in part due to differences in the methods of extraction, the diversity of latex sources, and the specificity of allergic patient sera used for immunoblot analysis. We have evaluated the IgE reactivities of extracts of nonammoniated latex, ammoniated latex, and extracts of commercial latex gloves by SDS-PAGE and IEF (19). Despite the fact that some of the antigens may have been denatured during “milking” of latex into ammoniated solutions, later compounding with chemicals, and vulcanization, a number of proteins or protein fragments remained IgE-reactive. One of the proteins that survived these harsh conditions was an acidic protein with a pl of 3.5. In the IEF immunoblot (Fig. 2), almost all gloves showed to produce an allergenic response contained this acidic protein. In some gloves, this is the only band detected by the allergic patient serum. However, the IEF blotting conditions used in this study favor the detection of this acidic protein. Other proteins in the glove extracts reactive to IgE in the serum might not have been detected due to the short transfer time (30–45 min) used with passive contact transfer of proteins to the nitrocellulose. Proteins with different molecular masses were visible as smears when they were assayed by an immunoblot of SDS-PAGE using electroblot (200 V and 1 h) (19). The stability of this protein and its presence in most of the glove extracts tested demonstrate the potential importance of this protein in allergy to latex products. The fact that the purified pl 3.5 protein induced histamine release in IgE sensitized-basophils confirms that it is an allergen, Hev b 5. Histamine release could be detected at an allergen concentration as low as 10 ng when cells were sensitized with serum from patients who had a high IgE titer to this specific protein.

In a previous report (23), rubber elongation factor (molecular mass, 14.5 kDa), Hev b 1, was shown to react with sera from all 13 latex-allergic patients in the study. In our experience with 50 latex-allergic patient sera, only 22% reacted to the rubber elongation factor, while 52% of latex-allergic sera reacted with the pl 3.5 protein (10). Due to the large number of allergens in latex, it does not seem feasible to use a single allergen for immunotherapy or for diagnostic purposes.

The pl 3.5 protein in latex is composed of only 14 of the 21 naturally occurring amino acids, with unusually high numbers of glutamic acid and proline residues in the repeated motif of XEEX or XEEEX (X can be any amino acid, but most frequently Lys or Ala residues). The molecular mass determined by MALDI mass spectrometry (16001.2 Da) agrees well with that calculated from the amino acid composition deduced from the cDNA (15957.5 Da). The difference can be explained by the presence of an acetyl group, which blocked the N terminus. There were sequence heterogeneities among the five cDNA clones, suggesting the possible existence of a family of genes coding for the proteins.

Recently a high percentage of latex allergy patients have been reported to also have food allergy (13). Fruits frequently have been reported to have cross-reactivities with latex including chestnut, banana, papaya, avocado, and kiwifruit (14). A health care worker who developed allergy to latex gloves after working in the hospital later also developed allergy to avocado and kiwifruit. The most likely explanation for the cross-reactivities is the existence of constituents with common antigenic determinants in latex and various fruits. In our study, the acidic latex protein shares a 47% sequence identity with the kiwifruit pl 3.5 protein. The homology is most striking in the N- and C-terminal segments. The pl 3.5 protein in kiwifruit is one of the proteins that appears in the early stage of fruit development (21), but its biological function is not known. The highest level of mRNA coding for the acidic protein in kiwifruit appears on day 6 after anthesis. The biological role of the pl 3.5 protein in latex is also unknown and needs further investigation. The molecular bases of allergenic cross-reactivity between latex and other fruits are currently under investigation.

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