

The Main IgE-Binding Epitope of a Major Latex Allergen, Prohevein, Is Present in Its N-Terminal 43-Amino Acid Fragment, Hevein¹

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Polypeptides of natural rubber latex (NRL) that elute from surgeon's gloves and other manufactured rubber products can sensitize exposed individuals and elicit severe hypersensitivity reactions. Previously, we showed that prohevein is a major allergen in NRL, the source material for rubber manufacturing. To analyze which region of the molecule carries the main IgE-binding epitope(s), we purified prohevein and its C-domain from NRL by gel filtration, reverse phase chromatography, and electroelution. In immunoblotting, prohevein bound IgE from 15 of 20 (75%), and the prohevein C-domain from 3 of 20 (15%) latex-allergic patient sera. In ELISA, 36 of 52 (69%) patient sera showed IgE binding to prohevein, whereas 11 of 52 (21%) sera had IgE to prohevein C-domain. We then purified from a brand of highly allergenic surgeon's gloves six hydrophilic peptides that revealed in amino-terminal sequencing 100% identity to the N-terminus of prohevein. In mass spectrometry, all purified peptides gave a molecular mass of 4719 ± 1.9 daltons, which corresponds to the molecular mass of hevein (4719.1 daltons), a 43-amino acid N-terminal fragment of prohevein. Purified hevein inhibited 72% of IgE binding from pooled sera of NRL-allergic patients to solid phase glove extract and 45% of IgE binding to solid phase NRL. Of the 43 NRL-allergic patient sera tested, 56% showed IgE Abs to purified hevein in ELISA. In skin prick testing, purified hevein elicited positive reactions in three-quarters of the latex-allergic patients tested. These results indicate that the majority of prohevein's IgE-binding ability resides in its N-terminal fragment, known as hevein. In one highly allergenic latex glove examined, the majority of IgE-binding ability was attributable to hevein molecules, suggesting that these peptides can be significant sensitizers in NRL allergy. *The Journal of Immunology*, 1996, 156: 1618–1625.

The importance of immediate hypersensitivity reactions to natural rubber latex (NRL)³ has emerged from increasingly reported cases during the last 15 yr. The frequency of NRL allergy among health care workers has ranged from 2 to 5% (1) and among instrumental nurses from 7 to 10% (2). Patients with histories of multiple operations show even higher frequencies of NRL allergy, and severe allergic reactions seem to be especially frequent in patients with spina bifida (3–5). In addition to medical NRL products, such as surgical gloves and catheters, people can become sensitized to nonmedical products, such as condoms, toy balloons, household gloves, and baby pacifiers (5–8). It is generally agreed that the symptoms of NRL allergy, ranging from contact urticaria and asthma to anaphylaxis and even death, are mediated by IgE Abs reacting with polypeptides eluting from various NRL products (4, 9, 10). Several IgE-binding proteins with a wide m.w. range (5–200 kDa) have previously been detected by immunoblotting in NRL, the source material for rubber products (11–

13). We recently reported that prohevein, a 20-kDa NRL protein comprising 187 amino acid residues, is a major NRL allergen (14). A few other NRL proteins, i.e., the rubber elongation factor, a 27-kDa protein, a 46-kDa protein, and a 36-kDa protein, evidently a rubber endoglucosidase, have been characterized at the primary structure level and suggested to be important latex allergens (14–17). However, virtually nothing is known about the IgE-binding epitopes of the NRL allergens or the molecular forms in which they are present in and elute from latex gloves and other manufactured NRL products.

To characterize IgE-binding regions in the prohevein molecule, we purified the 20-kDa protein and its 14-kDa C-terminal fragment and analyzed their immunoreactivity by immunoblotting and ELISA. We also analyzed hydrophilic peptides eluted from latex gloves for immunologic specificity, allergenicity, N-terminal amino acid sequence, and molecular mass.

Materials and Methods

Patient and control sera

Sera used for immunoblotting were obtained from 20 NRL-allergic patients. For ELISA studies, using purified prohevein and its C-domain as Ags, sera from 52 patients (40 females and 12 males, aged from 1–64 yr, including patients tested in immunoblotting) were available; of these sera, 43 were also analyzed against purified hevein. Twenty of the 52 patients were children, of whom 6 had spina bifida or other congenital anomalies and histories of multiple surgeries. Six control subjects without NRL allergy were used in immunoblotting, and 24 control subjects, of whom 12 were atopic, were used in ELISA studies. All patients had positive skin prick test (SPT) results, and controls had negative SPT results in response to NRL extract prepared and SPT performed as previously described (18). Two reference patient sera exhibiting strong IgE binding to the N-terminal region of prohevein were used in ELISA inhibition studies.

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³ Abbreviations used in this paper: NRL, natural rubber latex; SPT, skin prick test; ACN, acetonitrile; TFA, trifluoroacetic acid; HSA, human serum albumin; PBS-T, PBS containing 0.05% Tween-20; TBS, Tris-buffered saline; RAST, radioallergo sorbent test.

Extraction and purification of 20- and 14-kDa NRL proteins

Freshly collected NRL from the rubber tree (*Hevea brasiliensis*) was centrifuged at $43,000 \times g$ for 1 h, and the bottom fraction was freeze-thawed several cycles to rupture its membrane-bound organelles. After centrifugation, the supernatant was collected and stored at -70°C . Two hundred microliters of the supernatant was dried in a vacuum centrifuge, redissolved in 200 μl of equilibration buffer (50 mM sodium-phosphate and 0.6 M NaCl, pH 7.0), and filtered through a 0.45- μm Millex-HV membrane (Millipore, Molsheim, France). The sample was then subjected to gel filtration on a Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden) at a flow rate of 0.5 ml/min and monitoring at 280 nm. Fractions were collected and analyzed by SDS-PAGE as previously described (14). The fractions with the most intense bands at 20 and 14 kDa were collected and further purified by reverse phase chromatography on a ProRP 5/10 column (Pharmacia) using a linear gradient of acetonitrile (ACN; 20–35% in 40 min) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 ml/min. Elution was monitored at 280 nm, and fractions were collected and analyzed by SDS-PAGE. The fractions showing a single band at 20 or 14 kDa were collected and further analyzed by immunoblotting, ELISA inhibition, and N-terminal sequencing on an ABI 494 A Procise sequencer (Applied Biosystems, Foster City, CA).

To ensure that 20- and 14-kDa NRL proteins used in ELISA were highly purified, a further purification step by electroelution after gel electrophoresis was performed. The 20- and 14-kDa NRL proteins, purified as described above, were separated by SDS-PAGE according to the manufacturer's instructions (Mini Protean II Cell, Bio-Rad, Richmond, CA). Protein concentrations (measured by Lowry's method) were adjusted to approximately 1 mg/ml before gel electrophoresis. The samples were diluted 1/2 in electrophoresis buffer, boiled for 3 min, and applied to a 16% polyacrylamide gel (24 μl sample/6-mm wide well in 0.75-mm thick gel). Electrophoresis was conducted for 45 min at a constant voltage of 200 V, and then the gels were stained with Coomassie brilliant blue. The clear-cut bands with m.w. of 20 and 14 kDa were excised and subjected to electroelution. Electroelution was performed according to manufacturer's instructions using a commercial electroeluter (model 422 Electro-Eluter, Bio-Rad). Gel slices from 28 lanes were electroeluted at constant current of 9 mA for 3 h against 50 mM ammonium bicarbonate containing 0.1% SDS, using 12.5 kDa cut-off membrane caps. The removal of SDS was performed after electroelution by replacing the elution buffer with fresh 50 mM ammonium bicarbonate buffer without SDS and running gel slices containing tubes for 2 h more. Gel slices were removed from the tubes, and the remaining aqueous fractions were combined and concentrated by vacuum centrifugation and used as Ags for ELISA.

Extraction and purification of polypeptides from latex gloves

Five pairs (100 g) of surgical latex gloves (Triflex, lot 0892112 DPGN, Baxter, Valencia, CA) were cut into 0.5-cm² pieces and homogenized in a Waring blender in 450 ml of 20 mM ammonium-bicarbonate buffer, pH 8.4. Extraction was conducted for 2 days at 4°C with constant stirring. The eluate was then centrifuged at $40,000 \times g$ for 1 h, the supernatant was collected and filtered through 0.45- μm Millex-HV (Millipore) filter membrane and finally freeze-dried. The lyophilized preparation was stored at 4°C until used.

The extract was restored for use by solubilizing 10 mg of lyophilized glove eluate in 1 ml of phosphate buffer (50 mM sodium-phosphate, pH 7.0, containing 0.6 M NaCl and 0.1% TFA) and then filtered through 0.45- μm Millex-HV membrane. The sample was subjected to reverse phase chromatography on a ProRP 5/2 column (Pharmacia) using a linear gradient of ACN (constant 3% concentration for 20 min and 3–100% gradient in 10 min) in 0.1% TFA at a flow rate of 0.7 ml/min and monitoring at 280 nm. To obtain the most hydrophilic peptides, all fractions eluted at a constant ACN concentration of 3%, including the exclusion peak, were collected, pooled, and dried in a vacuum centrifuge. The resulting sample was then redissolved in 250 μl of equilibration buffer (50% ACN in 0.1% TFA), filtered through a 0.45- μm Millex-HV membrane, and then subjected to gel filtration on a Superdex Peptide HR 10/30 column (Pharmacia) at flow rate of 0.5 ml/min and monitoring at 214 nm. Fractions were collected and their IgE binding capacity was analyzed by an ELISA inhibition test as described below. The fractions showing strongest inhibition (>90%) of IgE binding to the 20-kDa NRL protein (purified as described above) in ELISA were pooled, dried in a vacuum centrifuge, and redissolved in 500 μl of 3% ACN in 0.1% TFA. The sample was then subjected to reverse phase chromatography on a PepRP 5/5 (Pharmacia) column using a linear gradient of ACN (3–40% in 160 min) in 0.1% TFA. Chromatography was performed at a flow rate of 0.7 ml/min, and elution was monitored at 214 nm. The fractions showing base line separation were collected and further analyzed by ELISA inhibition. The fractions showing

significant inhibition of IgE binding to the purified 20-kDa protein were subjected to N-terminal sequencing (ABI 494 A Procise Sequencer, Applied Biosystems) and mass spectrometric analysis.

Inhibition of IgE binding to purified 20-kDa NRL protein and to NRL and glove extracts by purified glove peptides in ELISA (ELISA inhibition)

Purified 20-kDa protein (1.5 $\mu\text{g}/\text{ml}$ in 50 mM carbonate buffer, pH 9.6) was applied to 96-well polystyrene microtiter plates (100 $\mu\text{l}/\text{well}$; Nunc, Roskilde, Denmark) and incubated for 3 h at room temperature and overnight at 4°C . Wells were then emptied and postcoated for 1 h with 100 μl of 50 mM carbonate buffer, pH 9.6, containing 1% human serum albumin (HSA). After rinsing three times with PBS containing 0.05% Tween 20 (PBS-T), 50 μl of reference serum (diluted 1/20 with PBS-T containing 0.2% HSA; PBS-T-HSA; two reference patient sera containing IgE Abs to prohevein were used as the source of IgE Abs) was incubated for 1 h at room temperature with an equal volume of inhibitor solution. Nine different concentrations of peptide 2 (Fig. 6A; protein concentrations of 70,000, 23,000, 7,000, 2,300, 700, 230, 70, 23, and 7 $\mu\text{g}/\text{ml}$ in PBS-T-HSA, determined by N-terminal amino acid sequencing) were used for inhibition. In addition, five fold dilutions of peptide 1 and four fivefold dilutions of peptides 3 to 6 were used as inhibitors. The inhibitor solution was replaced with an equal volume of PBS-T-HSA in control experiments. The mixture containing the serum and the inhibitor (or serum without inhibitor) was then added to the wells and incubated for 2 h at room temperature. Wells were washed three times with PBS-T, and biotinylated anti-human IgE (Vector, Burlingame, CA; diluted 1/1000 with PBS-T-HSA) was added and incubated for 1 h. Wells were again washed three times and streptavidin-conjugated alkaline phosphatase (Bio-Rad; diluted 1/3,000 with PBS-T-HSA) was incubated for 1 h. After three washes, the substrate development solution (Sigma Chemical Co., St. Louis, MO) was added, and the color that developed was read at 405 nm using an automated ELISA reader (Titertek Multiskan, Eflab, Espoo, Finland).

To control possible nonspecific inhibition, reference patient serum was inhibited with three unrelated polypeptides (OVA, gastrin, and substance P; Sigma Chemical Co.) using concentrations up to 1000 μg in 1 ml.

The ability of a purified glove peptide (peptide 2, Fig. 6A) to inhibit the binding of IgE to heterogeneous glove and NRL proteins in ELISA was examined to evaluate the peptide's significance as an NRL allergen. The serum pool obtained from 10 NRL-allergic patients, showing elevated levels of IgE Abs to solid phase NRL in ELISA and in immunoblotting, and a reference serum with apparently monospecific IgE Abs to prohevein N-terminus served as the source of the IgE Abs. Coating was performed with glove or NRL extract, with protein concentrations adjusted to 20 $\mu\text{g}/\text{ml}$ (optimized in preliminary checkerboard titrations). Seven different concentrations of peptide 2 (protein concentrations of 10,000, 1,000, 100, 10, 1, 0.1, and 0.01 $\mu\text{g}/\text{ml}$, determined by N-terminal amino acid sequencing) were used for inhibition. ELISA inhibition was performed as described above.

Mass spectrometric analysis of purified glove peptides

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was conducted using a Finnigan MAT Lasermat 2000 instrument. About 1 pmol (0.5 μl) of each purified peptide was mixed with 0.5 μl of matrix (33 mM α -cyano-4-hydroxycinnamic acid in 70% ACN) and air-dried onto the sample plate. The laser energy was manually optimized for each sample, and the spectrum was recorded from 10 repeated shots.

Skin prick testing using purified 20-kDa protein and a purified glove peptide

SPT with purified 20-kDa NRL protein (using concentrations of 0.1, 1, 10, and 50 $\mu\text{g}/\text{ml}$ in PBS, pH 7.2) was performed in four latex allergic patients (patients 1–4 in Table I) as described previously (18). Purified glove peptide (number 2 in Fig. 6A, using protein concentrations of 0.7 and 7 $\mu\text{g}/\text{ml}$) was used in a similar manner in SPT in the same four patients. Ten atopic subjects without latex allergy (with negative clinical history and negative SPT to NRL) were used as controls for both Ags.

Immunoblotting

To allow simultaneous demonstration of IgE Abs to both 20- and 14-kDa Ags, a mixture of the 20- and 14-kDa NRL proteins (containing ~1 part of the 20-kDa protein and 3 parts of the 14-kDa protein, with the total protein concentration adjusted to 165 $\mu\text{g}/\text{ml}$) was diluted 1/2 into electrophoresis buffer containing 2% SDS and 5% 2-ME and kept in boiling water for 2 min. The dissolved samples (350 μl sample/74-mm wide well and 0.75-mm thick gel) were electrophoresed for 45 min at a constant voltage

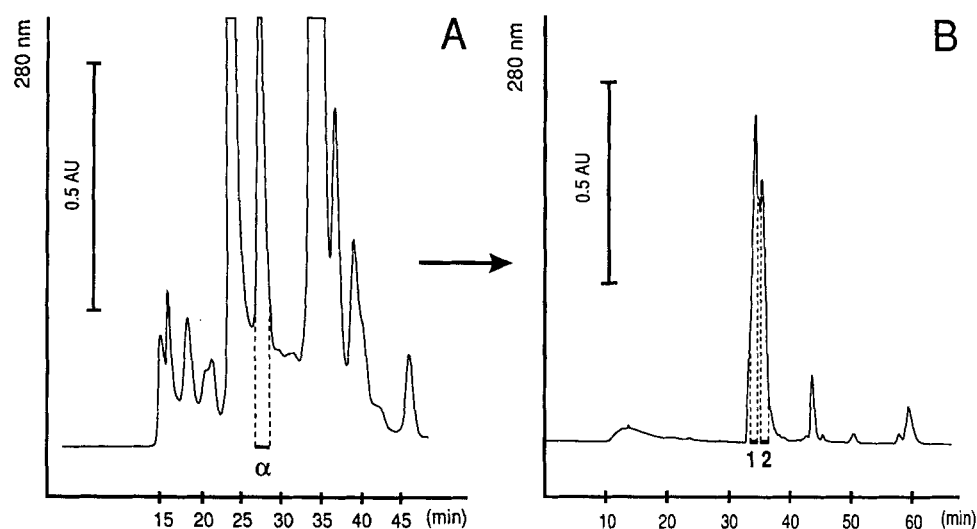


FIGURE 1. HPLC purification steps of 20- and 14-kDa NRL proteins. *A*, Gel filtration on a Superdex 75 HR 10/30 column. The fraction containing the 20- and 14-kDa proteins is denoted by α . *B*, Reverse phase chromatography of fraction α on a ProRP 5/10 column. Fractions 1 and 2 contained the 20- and 14-kDa proteins, respectively (see Fig. 2).

of 200 V (Mini Protean II Cell, Bio-Rad). After electrophoresis, the proteins were transferred from the gel to a 0.2- μ m pore size nitrocellulose filter (Trans-Blot, Bio-Rad) by electroblotting at 100 V for 1 h (Mini Trans-blott cell, Bio-Rad) and stained with Ponceau-S red to demonstrate successful transfer of proteins. The membrane was then cut into 3-mm strips, washed with Tris-buffered saline (TBS) containing 0.1% Tween 20, and incubated for 1 h in a blocking buffer containing 5% nonfat milk powder in TBS. The strips were incubated overnight at 4°C with patient or control sera (diluted 1/5). The strips were then washed and incubated with biotinylated goat anti-human IgE Ab (Vector) diluted 1/1,000. An additional blocking was conducted for 2 min after washing, and the membranes were then washed again. Streptavidin-conjugated alkaline phosphatase (Zymed, Camarillo, CA), diluted 1/24,000 in TBS, was added, and the strips were incubated for 30 min at room temperature. The strips were placed in a color development solution (Bio-Rad) after washing, and the reaction was stopped after 8 min by rinsing in tap water.

ELISA for IgE Abs to 20-kDa (prohevein) and 14-kDa (prohevein C-domain) NRL proteins and to purified glove peptide (hevein)

Coating with electroeluted 20- or 14-kDa NRL proteins (protein concentrations adjusted to 1.2 μ g/ml) and with purified glove peptide (peptide 2, Fig. 6A; 0.5 μ g/ml) and postcoating were performed as described above for ELISA inhibition. One hundred microliters of serum to be tested (diluted 1/20 with PBS-T-HSA) was added to the wells and incubated for 2 h at room temperature. Wells were then washed three times with PBS-T, biotinylated anti-human IgE was added, and the assay was continued as described above for ELISA inhibition. The mean OD + 3 SD of the controls was chosen as cut-off limit for positivity.

Modified trypsin and endoproteinase Glu-C digestion of purified 20-kDa protein and peptide separation

Approximately 200 μ g of the purified 20-kDa NRL protein was dissolved in 50 μ l of 50 mM ammonium-bicarbonate, 6 M urea, and 3 mM 2-ME, pH 7.8, for Glu-C digestion and in 50 mM Tris-HCl, 6 M urea, and 3 mM 2-ME, pH 8, for trypsin digestion, and heated at 90°C for 15 min. Samples were diluted by adding 275 μ l of 50 mM ammonium-bicarbonate, pH 7.8, for endoproteinase Glu-C digestion and 275 μ l of 50 mM Tris-HCl and 1 mM CaCl_2 , pH 7.6, for trypsin digestion, followed by the addition of 10 μ g of the enzyme (endoproteinase Glu-C, Promega V 5191 or Modified Trypsin, Promega V 5111, Promega Corp., Madison, WI). The samples were digested overnight at 37°C, and the resulting peptides were separated on a PepRP 5/5 column (Pharmacia) using a linear gradient of ACN (3–40% in 160 min) in 0.1% TFA. Chromatography was performed at a flow rate of 0.7 ml/min, and peptide elution was monitored at 214 nm. The eluted peptides were collected and further analyzed by ELISA inhibition, as described above.

Results

Purification and molecular characterization of prohevein and its C-domain

Gel filtration of NRL extract revealed several peaks, which were collected and analyzed by SDS-PAGE. The fraction containing the majority of the 20- and 14-kDa proteins (fraction α , Fig. 1A) was further purified by reverse phase chromatography (fractions 1 and 2, Fig. 1B). The purity and the electrophoretic mobility of the fractions were analyzed by SDS-PAGE (Fig. 2). Amino-terminal sequencing of the 20-kDa protein (15 residues analyzed) revealed 100% identity to the N-terminus of prohevein, and the 14-kDa protein (15 residues analyzed) gave 100% identity to the C-domain of prohevein (starting at amino acid position 67 of prohevein).

Enzymatic digestion of prohevein and immunoreactivity of the obtained peptides

In an attempt to identify IgE-binding epitopes in more detail, we cleaved the 20-kDa protein with trypsin and endoproteinase Glu-C. However, none of the collected 38 peptides separating in reverse phase chromatography after trypsin digestion of prohevein and none of the 15 peptides obtained by endoproteinase Glu-C digestion showed significant inhibition of IgE binding to purified prohevein by ELISA inhibition (data not shown).

Binding of IgE from latex-allergic patient sera to prohevein, to its C-domain, and to purified hevein

In immunoblotting, the purified 20-kDa prohevein bound IgE from 15 of 20 (75%), and the 14-kDa prohevein C-domain bound IgE from 3 of 20 (15%) latex-allergic patient sera. All six control sera gave negative results. Figure 3 shows typical IgE immunoblot reactions of selected sera to the purified prohevein and to its C-domain. In ELISA analysis, 36 of the 52 NRL-allergic patients (69%) had IgE Abs to purified prohevein, whereas only 11 patients (21%) exhibited IgE to the purified C-domain of prohevein. Of the 43 patients studied, 24 (56%) showed IgE Abs to purified hevein in the ELISA. The levels of IgE Abs to prohevein and hevein among the 43 NRL-allergic patients correlated significantly ($r = 0.63$, $p < 0.0001$, Pearson's linear correlation). All 24 control subjects were negative against all three allergens at the chosen

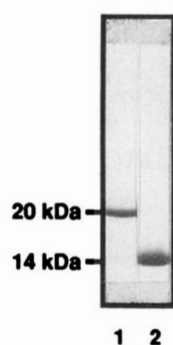


FIGURE 2. Electrophoretic mobility and purity of fractions 1 and 2 (Fig. 1B), as analyzed by SDS-PAGE. Lane 1, 20-kDa protein; lane 2, 14-kDa protein.

cut-off level for positivity. Figure 4 shows distribution IgE Abs to purified prohevein, to its C-domain, and to hevein in patient and control sera.

Purification of hydrophilic peptides eluting from latex gloves

When skin or mucous membranes are in contact with latex gloves, it is anticipated that the peptides eluting within the first minutes are the most hydrophilic ones. To obtain information on such hydrophilic peptides present in latex gloves, an eluate of a brand of highly allergenic gloves was subjected to reverse phase chromatography. The material eluting with 3% ACN was collected (indicated by the bar in Fig. 5A) and further subjected to gel filtration (Fig. 5B). The material showing the strongest inhibition (>90%) of IgE binding to purified prohevein in ELISA (indicated by the bar in Fig. 5B) was then subjected to peptide separation by reverse phase chromatography. Two aliquots of this material gave different peptide profiles in two reverse phase chromatography experiments, as shown, for example, in Figure 6 (A and B).

Immunologic specificity of purified glove peptides

From the chromatograms in Figure 6, six peptides (numbers 1–6) were collected and analyzed by ELISA inhibition. Binding of prohevein-specific IgE to solid phase prohevein was significantly inhibited (>80%) by all six peptides. As an example, peptide 2 (Fig. 6A) inhibited the binding of IgE to prohevein by 50% even at an inhibitor concentration as low as 200 pg/ml (Fig. 7). No significant inhibition was seen when unrelated polypeptides (OVA, gastrin, and substance P) were used as inhibitors at protein concentrations as high as 1 mg/ml (data not shown). Binding of IgE to solid phase glove extract was inhibited 72% and that to NRL was inhibited 45% in a serum pool obtained from 10 patients allergic to NRL (Fig. 8A). Inhibition curves using the reference serum with apparently monospecific IgE to prohevein N-terminus are shown for comparison (Fig. 8B).

Molecular structure of purified glove peptides

Amino-terminal sequencing of all six peptides showed 100% identity to the N-terminal part of prohevein. In mass spectrometry, all purified peptides gave a molecular mass of 4719 ± 1.9 daltons as shown for one analyzed peptide in Figure 9.

Skin prick tests with a purified glove peptide

One purified glove peptide (peptide 2 in Fig. 6A) was chosen to study whether peptides isolated in this way can also cause allergic reactions in vivo. The four patients selected had previously shown positive SPT results with a standard NRL glove eluate and with purified prohevein (18). The examined peptide elicited strong pos-

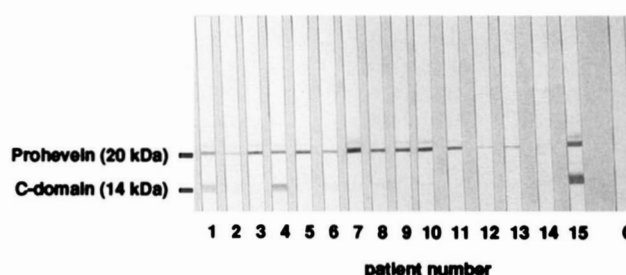


FIGURE 3. Immunoblot analysis of binding of IgE Abs to purified prohevein (20-kDa protein) and prohevein C-domain (14-kDa protein) from sera of 15 selected latex-allergic patients (lanes 1–15) and from one nonallergic control subject (lane C). All 15 patients reacted with prohevein (although reactions in lanes 2, 12, 13, and 14 are weak), but only three sera (lanes 1, 4, and 15) showed reactivity also with the prohevein C-domain.

itive SPT reactions in three of the four latex-allergic patients tested (Table I). In immunoblotting, all these three (patients 2–4, Table I) had IgE Abs to the N-terminal part of prohevein, whereas the patient with negative SPT (patient 1, Table I) showed strong IgE binding to prohevein C-domain. In one patient (patient 3, Table I), a positive SPT reaction was inducible by the purified glove peptide at a concentration as low as 0.7 μ g/ml, and in two other patients (patients 2 and 4 in Table I), a positive reaction was obtained at a purified glove peptide concentration of 7 μ g/ml. All 10 controls had negative SPT with the purified glove peptide.

Discussion

We have previously shown that prohevein is a significant allergen for the majority of latex-allergic patients. More than 80% of the patients, either adults or children, had IgE Abs binding to prohevein in immunoblotting (14). This finding was also confirmed by SPT with purified prohevein, which revealed positive results in the majority of latex-allergic patients tested. In the present study, 69 to 75% of the latex-allergic patients showed IgE binding to prohevein, whereas only 15 to 21% of the patients had IgE Abs to prohevein C-domain in immunoblotting and ELISA. These results indicate that the main IgE-binding epitope is located in prohevein N-terminus. This view is also in agreement with the finding that latex gloves contain IgE-binding peptides with 100% sequence identity to the N-terminus of prohevein, revealing a molecular mass (4719 ± 1.9 daltons) corresponding to that of hevein, a 43-amino acid N-terminal fragment of prohevein. When purified hevein was used as the solid phase Ag in the ELISA, 56% of the NRL-allergic patients showed anti-hevein IgE Abs.

To confirm that the isolated hevein is an important allergen in vivo, we performed SPT with it and obtained strong positive results in all three latex-allergic patients showing strong IgE binding to the N-terminus of prohevein. However, one patient showing IgE Abs to prohevein C-domain had negative results in SPT with hevein, but showed positive SPT reactions with prohevein. These results together indicate that although some of the IgE-binding epitopes are present in prohevein C-domain, the main allergenic epitope is located in the prohevein N-terminus between amino acid positions 18 to 60, and this fragment, known as hevein, is also present in latex gloves. The N-terminal sequence results together with those from mass spectrometry strongly suggest that immunologically active peptides extracted from latex gloves essentially represent the 43-amino acid N-terminal part of prohevein, known as hevein. At present we cannot explain the varying elution positions of these, on primary structure level identical, peptides in reverse phase chromatography, but conformational differences in

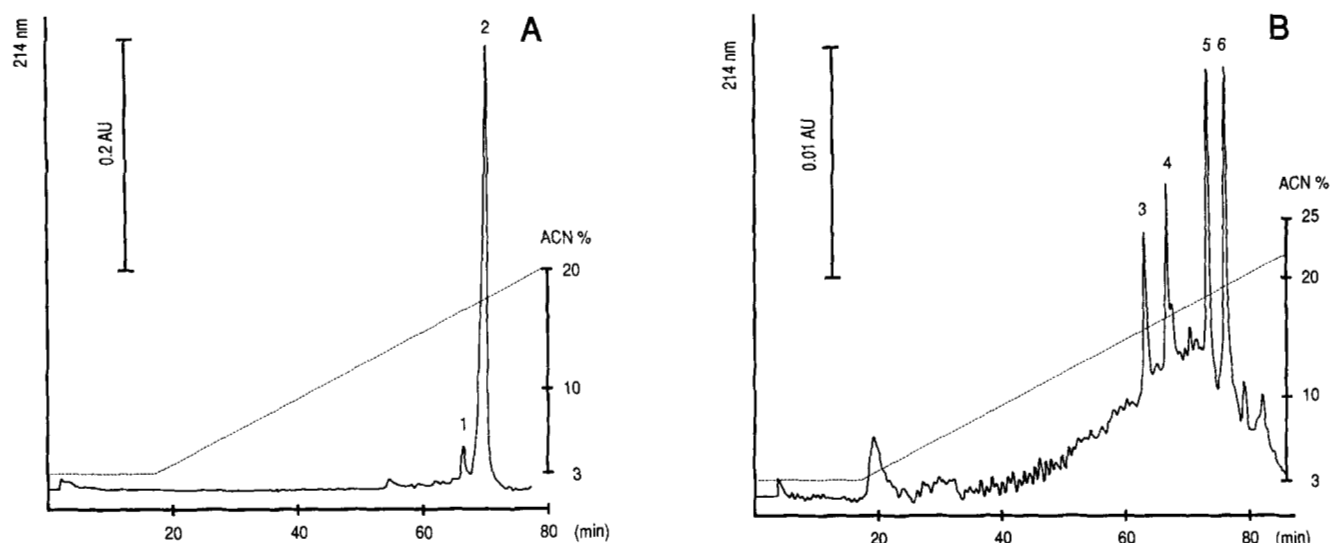
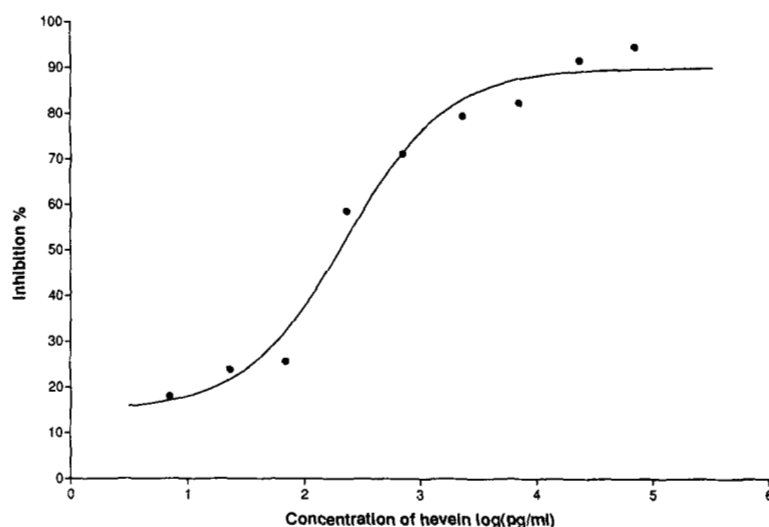


FIGURE 6. Reverse phase chromatograms of the material containing prohevein-specific immunologic activity originating from two different aliquots (indicated by the horizontal bar in Fig. 5B) on a PepRP 5/5 column using a linear gradient of ACN (3–40% in 160 min). Peptides 1 and 2 (A) and 3 to 6 (B) were collected and subjected to prohevein-specific ELISA inhibition, N-terminal amino acid sequencing, and mass spectrometric analysis. Note the different scales of sensitivity in A (0.2 absorbance units) and B (0.01 absorbance units).

FIGURE 7. Inhibition of binding of prohevein-specific IgE to solid phase prohevein by different concentrations of purified glove peptide (peptide 2 in Fig. 6A; mature hevein) in ELISA. A 50% inhibition occurred at a protein concentration of 200 pg/ml.



One might speculate that (pro)hevein could also bind in a non-immunologic manner to carbohydrate residues present in IgE molecules, due to lectin-like binding to *N*-acetyl-D-glucosamine, and thus provoke false positive reactions in *in vitro* and perhaps also in *in vivo* assays. Although experimental data to prove or reject this hypothesis are lacking, in our opinion it is unlikely that nonspecific binding phenomena would play an important biologic role in the allergic reactions caused by (pro)hevein. If lectin-like binding was important, IgE-class anti-(pro)hevein Abs in patient sera should closely correlate with elevated serum total IgE levels. However, no significant correlation was found between elevated total IgE levels (>100 kU/l) and hevein-specific IgE levels ($r = 0.32$, $p = 0.07$, $n = 33$, Pearson's linear correlation) and prohevein-specific IgE levels ($r = 0.27$, $p = 0.11$, $n = 37$, Pearson) measured by ELISA in sera from NRL-allergic patients (H. Alenius, K. Turjanmaa, and T. Palosuo, unpublished observations). All control subjects (including 12 atopic individuals, of whom one had a total IgE level of 17,000 kU/l) were negative in an ELISA and by immunoblotting,

and several patients showing highly elevated total IgE levels had low or moderate anti-prohevein IgE values and vice versa. In addition, as shown in Table I, all three latex-allergic patients with strong IgE binding to prohevein N-terminus by immunoblotting gave strong positive SPT reactions with purified hevein, while a patient with IgE Abs to the prohevein C-domain and all 10 atopic controls without latex allergy, but with elevated total IgE levels, were completely negative in the SPT. It thus appears that a non-immunologic mechanism cannot explain the specificity of the immunologic antihevein-hevein interactions here documented to occur both *in vitro* and *in vivo*.

Slater and Chhabra were not able to demonstrate inhibition of IgE binding by purified hevein to immobilized latex proteins in a radioallergo-sorbent test (RAST). This observation led the authors to suggest that hevein is not an important allergen (11). However, there are at least two significant differences in the assays used by these authors and by us that could explain the divergent results. Slater and Chhabra (11) used sera from five

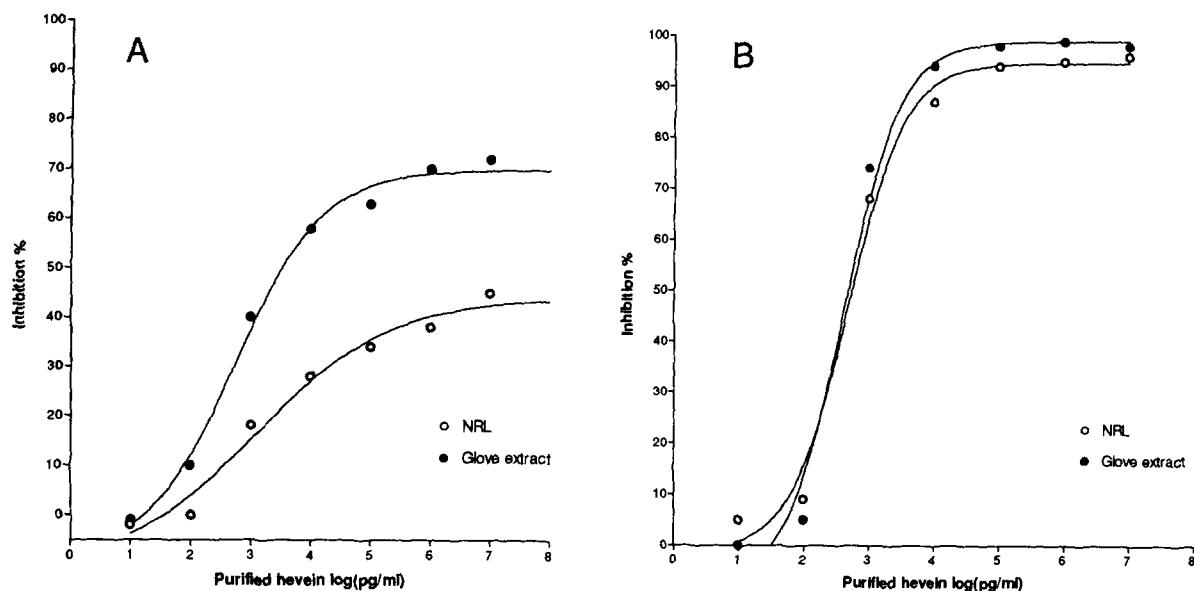


FIGURE 8. Inhibition of IgE binding to solid phase glove extract and NRL by different concentrations of purified glove peptide (peptide 2 in Fig. 6A; mature hevein) in ELISA. Hevein inhibited maximally 72% of the IgE binding of pooled serum IgE (obtained from 10 NRL-allergic patients) to glove extract and 45% to NRL (A). Inhibition curves using the reference serum with apparently monospecific IgE to prohevein N-terminus are shown for comparison (B).

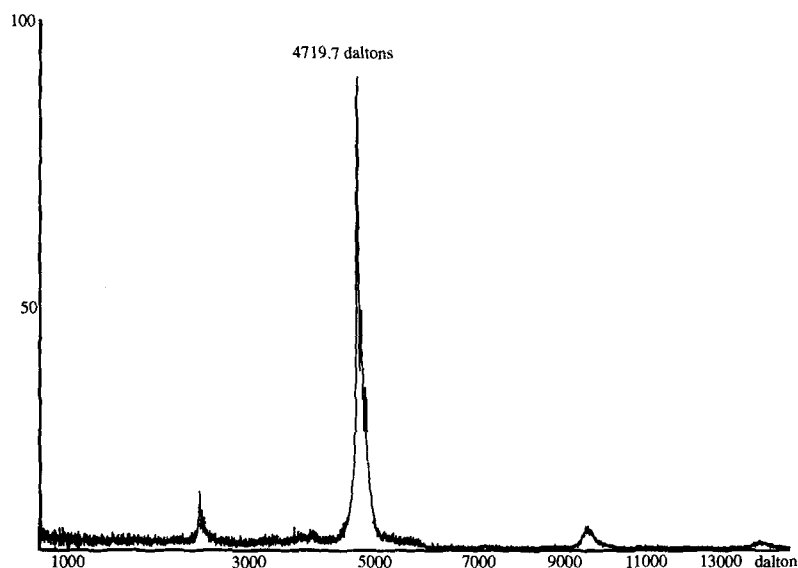


FIGURE 9. A matrix-assisted laser desorption/ionization time of flight mass spectrometry spectrum obtained from 1 pmol of purified glove peptide (peptide 2, Fig. 6A).

Table I. Skin prick test (SPT) and immunoblot results with purified NRL peptides (20 kDa prohevein, 14 kDa prohevein C-domain, and 4.7 kDa hevein) for four latex-allergic patients^a

Patient Number	Age/Sex	Skin Prick Test		Immunoblotting	
		Prohevein	Hevein	Prohevein	C-domain
1	23/F	3+	negative	+	+
2	33/F	2+	3+	+	—
3	64/M	3+	3+	+	—
4	35/F	2+	4+	+	—

^a A positive SPT reaction is denoted as $\geq 2+$ (for details, see Reference 18). A positive immunoblot reaction is denoted by a +.

latex-allergic spina bifida patients as the source of latex-specific IgE for the RAST inhibition. We have previously reported that IgE Abs in patients with spina bifida who have developed latex

hypersensitivity are directed primarily to 27- and 14-kDa NRL (12) allergens, while other latex-allergic patients most frequently show IgE Abs to prohevein (14). Second, they used ammoniated latex, which may not have contained sufficient amounts of prohevein or hevein, as the solid phase Ag in RAST inhibition. In the present study, ELISA inhibition tests were performed using purified prohevein, glove extract, and nonammoniated NRL extract as the solid phase Ags. In addition, the majority of the sera from NRL-allergic patients used in ELISA and ELISA inhibition employing purified hevein were not from patients with spina bifida.

In conclusion, we have localized the main IgE-binding epitope of prohevein to its N-terminus, between amino acids 18 and 60. The majority of prohevein's IgE binding ability resides in this 43-amino acid N-terminal fragment, known also as (mature) hevein. We showed that hydrophilic peptides, identified as hevein molecules by their immunologic specificity, N-terminal amino acid sequence, and

molecular mass, elute readily from allergenic latex gloves and carry most of the IgE binding ability of one glove extract examined. Purified hevein also caused strong SPT reactions in latex-allergic patients. These results strongly suggest that hevein molecules represent significant sensitizing allergens in latex gloves. Hevein is the most predominant protein in NRL and has chitin-binding properties. High structure homologies between hevein and other chitin-binding proteins, such as wheat germ agglutinin, as well as between prohevein C-terminus and wound-inducible proteins in various plants may open new avenues for the study of numerous food and plant allergies found in association with latex hypersensitivity.

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