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Purification and Partial Amino Acid Sequencing of a 27-kD Natural Rubber Allergen Recognized by Latex-Allergic Children with Spina Bifida

Key Words

Latex Allergy
Latex proteins
Amino acid sequence
Rubber elongation factor
Spina bifida

Abstract

We purified from natural rubber latex (NRL) by means of high-performance liquid chromatography a 27-kD protein, recognized characteristically by IgE in sera from latex-allergic children with spina bifida or other congenital anomalies and histories of multiple surgeries. N-terminal sequence analysis of the purified 27-kD protein was unsuccessful suggesting that its N-terminus is blocked. To obtain internal sequence information from the protein it was digested with trypsin and the purified tryptic peptides were subjected to sequence analysis. Thirteen of the 14 sequenced peptides revealed no significant homology to any of the published protein sequences indicating that the 27-kD protein is previously undescribed at the primary structure level. However, one of the 14 sequenced peptides showed significant homology to the rubber elongation factor, a 14.6-kD NRL protein. For the time being, the 27-kD NRL protein is the first molecularly characterized NRL allergen associated with defined clinical manifestations of latex allergy.

Introduction

Immediate hypersensitivity reactions to natural rubber latex (NRL) are caused by polypeptides eluting from various rubber products, such as surgical and household gloves, catheters, condoms and toy balloons [1, 2]. Several IgE-binding proteins with a wide molecular weight range (5–200 kD) have recently been detected by immunoblotting in NRL [3–7]. A few rubber tree proteins, i.e., the rubber elongation factor (REF), hevein, and hevamine have earlier been characterized at molecular level and sequenced [8–10]. Of these molecularly characterized NRL proteins, REF has been suggested by one group to be the major latex allergen

[11] but this view has been criticized [12], and for the time being, the biological significance of various NRL proteins in latex allergy awaits to be established. We recently showed that IgE immune response towards a 27-kD NRL allergen was restricted to latex-allergic children with spina bifida and other congenital anomalies and histories of multiple surgeries [13]. Systemic allergic reactions after exposure to NRL products were especially frequent in this particular subgroup of latex-allergic patients suggesting a special pathogenetic role for the 27-kD allergen [14]. In the present study we purified the 27-kD NRL allergen and subjected its tryptic peptides to amino acid sequence analysis.

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Materials and Methods

Protein Purification

NRL from the rubber tree, *Hevea brasiliensis*, was collected in Malaysia, deep-frozen as such immediately, shipped to Finland in dry ice, and then stored at -70°C . For the extraction of soluble proteins, NRL was thawed, the coagulated solids were removed, and the liquid centrifuged at $100,000\text{ g}$ for 90 min, following which the supernatant was collected and stored at -70°C . Total protein concentration of the NRL extract was determined using Lowry's method with bovine γ -globulin as the standard (Bio-Rad) as earlier described [4].

High-performance liquid chromatography (HPLC) gel filtration was performed on a Superdex 75 HR 10/30 column (Pharmacia) equilibrated with 0.05 M phosphate, pH 7.0, 0.6 M NaCl. The column was calibrated with known molecular weight markers. Two hundred microliters of NRL extract prepared as described above was dried in a vacuum centrifuge, redissolved in $200\text{ }\mu\text{l}$ of equilibration buffer and filtered through a $0.45\text{-}\mu\text{m}$ Millex-HV membrane (Millipore). The NRL sample was then subjected to HPLC gel filtration at a flow rate of 0.5 ml/min and monitoring at 280 nm . Fractions were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions showing the most intense band at 27 kD were combined and further purified by reversed phase chromatography.

Reversed phase chromatography of the combined fractions from gel filtration was performed on a $0.46\times 4\text{ cm}$ TSK TMS250 (C1) (TosoHaas) column using a linear gradient of acetonitrile (20–60% in 100 min) in 0.1% trifluoroacetic acid at a flow rate of 0.25 ml/min . Elution was monitored at 214 nm and eluting peaks collected manually.

Polyacrylamide Gel Electrophoresis

A method described previously in detail [4] was used. Briefly, samples from gel filtration were diluted 1:3 into sample buffer containing 2% SDS and 5% 2-mercaptoethanol. Fractions from reversed phase chromatography were first evaporated in a vacuum centrifuge and then dissolved into the sample buffer. The samples were boiled for 2 min and then applied to a 12.5% polyacrylamide gel ($45\text{-}\mu\text{l}$ sample/cm of gel). Electrophoresis was carried out for 45 min at a constant voltage of 200 V (Mini Protean II Cell; Bio-Rad). After electrophoresis, gels were immediately analyzed by immunoblotting or stained with Coomassie brilliant blue.

Patient and Control Sera

Serum from a 13-year-old latex-allergic boy with spina bifida, who had experienced systemic reactions (generalized urticaria and asthma) after exposure to NRL products, was used as a source of IgE antibodies to the 27-kD allergen. In immunoblotting, this serum had earlier been shown to contain IgE antibodies combining with a 14-kD and the 27-kD protein [13]. Latex RAST (Pharmacia, Uppsala, Sweden; CAP method, cut-off point for positivity 0.4 kU/l) in this reference serum was 2.2 kU/l and total IgE 110 kU/l . Two other latex-allergic patients included in the study were health care workers, first, a 39-year-old female, who had experienced systemic reactions (generalized urticaria and asthma), and, second, a 32-year-old male, who had had local cutaneous reactions after exposure to NRL products. Their sera contained IgE antibodies in immunoblotting to 14- , 20- , 30- , 45 and 75-kD NRL proteins but not to the 27-kD protein. Latex RAST results for the two sera were 14.4 and 0.9 kU/l , and total IgE levels 396 and $15,000\text{ kU/l}$, respectively. Sera from 3 healthy nonallergic adults were used as further controls.

Immunoblotting

Immunoblotting was performed as described earlier [4]. Briefly, the proteins were transferred from the gel to a $0.45\text{-}\mu\text{m}$ pore size nitrocellulose filter (Trans-Blot; Bio-Rad) by electroblotting at 100 V for 1 h (Mini Trans-Blot Cell; Bio-Rad). 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 room temperature with the reference serum (diluted 1:5) containing IgE antibodies to the 27-kD allergen. The strips were then washed and incubated with biotinylated goat anti-human IgE antibody (Vector) diluted 1:2,000. An extra blocking was carried out for 2 min after washing and the membranes were then washed again. Streptavidin-conjugated alkaline phosphatase (Bio-Rad), diluted 1:5,000 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.

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis was performed as described earlier [14]. Briefly, tube gels were pre-electrofocused at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min (Mini Protean II Tube Cell; Bio-Rad) before adding the samples. Samples ($20\text{ }\mu\text{l}$ /tube gel) containing approximately $100\text{ }\mu\text{g}$ of unpurified NRL proteins or $10\text{ }\mu\text{g}$ of purified 27-kD protein were applied to the 4% acrylamide tube gels. Loaded samples were overlaid with 20 ml of sample overlay buffer and isoelectric focusing was performed at a constant voltage of 500 V for 10 min and thereafter at 750 V for 5 h. After isoelectric focusing, SDS-PAGE was performed in a 12% acrylamide slab gel, using a 4% stacking gel. The slab gels were then stained by Coomassie brilliant blue. Gels run similarly with appropriate standards (2-D SDS-PAGE standards; Bio-Rad) were used to determine isoelectric points and molecular weights of the proteins.

Tryptic Cleavage, Peptide Separation and Amino Acid Sequencing

Approximately $20\text{ }\mu\text{g}$ of the purified 27-kD protein was dried in a vacuum centrifuge and dissolved in $100\text{ }\mu\text{l}$ 0.1 M ammonium bicarbonate. The modified trypsin ($0.5\text{ }\mu\text{g}$) (Promega V 5111) was then added and the mixture incubated at 37°C overnight. The tryptic peptides were separated on a $0.21\times 15\text{ cm}$ Rexchrom C8 (Regis Chemical Co.) column using linear gradient of acetonitrile (3–40% in 160 min) in 0.1% trifluoroacetic acid. Chromatography was performed at a flow rate of $200\text{ }\mu\text{l/min}$ and peptide elution monitored at 214 nm . The peptides were manually collected and concentrated to $30\text{ }\mu\text{l}$ in a vacuum centrifuge. Protein and peptide sequencing was performed on a gas-pulsed liquid-phase sequencer equipped with on-line phenyl-thiohydantoin-amino acid analysis [15] or an Applied Biosystems ProciseTM sequencer.

Results

Gel filtration of NRL extract revealed several clearly separated fractions (fig. 1A) which were collected and further analyzed by SDS-PAGE for identification of the 27-kD protein. The early eluting fraction (indicated by the bar in

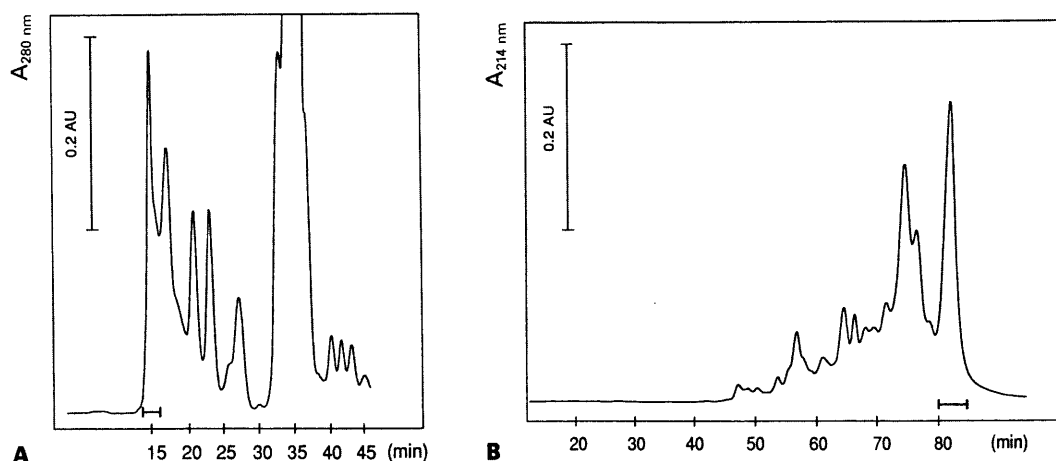


Fig. 1. HPLC chromatograms from the purification steps of the 27-kD allergen. Gel filtration (**A**) on a Superdex 75 HR 10/30 column. Reversed phase chromatography (**B**) on a TSK TMS250 column. The fractions containing the 27-kD allergen are indicated by horizontal bars.

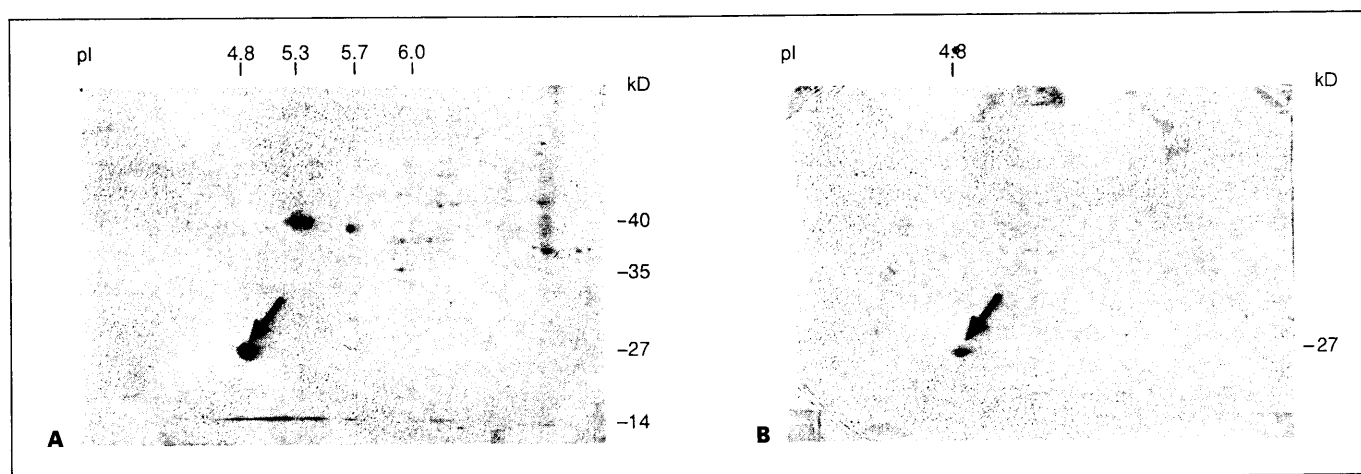


Fig. 2. Two-dimensional PAGE of (**A**) total proteins in NRL extract and (**B**) the purified 27-kD protein. The 27-kD protein is denoted by an arrow.

fig. 1A), containing the majority of the 27-kD protein, was collected and further purified by reversed phase chromatography (fig. 1B). The 27-kD protein was found to elute in the most hydrophobic fraction (indicated by the bar in fig. 1B) which showed a single band at 27 kD in SDS-PAGE. The purity of the fraction was further confirmed by two-dimensional electrophoresis that revealed only one spot with a molecular weight of 27 kD and an isoelectric point of pH 4.8 (fig. 2A, B). To verify the ability of the purified protein to bind relevant IgE antibodies, one-dimensional immunoblotting, using reference serum containing IgE antibodies to the 27-kD protein, was performed. IgE from the reference

serum detected one band at 27 kD (fig. 3). No IgE binding was observed when sera obtained from nonlatex-allergic controls (results not shown) or from latex-allergic health care workers with IgE antibodies to several NRL proteins but not to the 27-kD protein were tested (fig. 3).

In order to further characterize the purified 27-kD allergen it was subjected to N-terminal sequence analysis. However, no amino acid derivatives were released in 10 cycles of Edman degradation indicating that the N-terminus of the 27-kD protein is blocked. To obtain internal sequence information from the purified protein it was subjected to trypsin digestion and the formed peptides were separated by re-

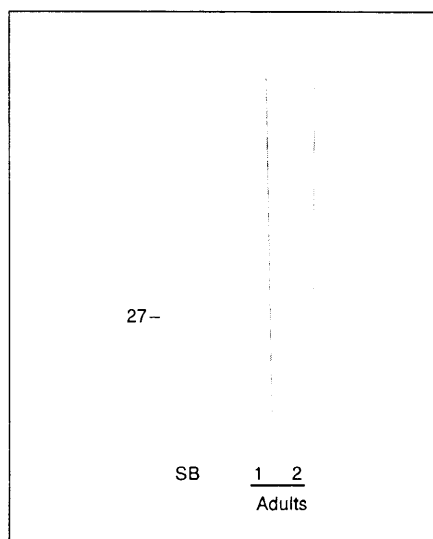


Fig. 3. Binding of IgE antibodies from reference serum containing anti-27-kD IgE antibodies (denoted as SB) and from 2 latex-allergic health care workers (strips 1 and 2) to purified 27-kD protein. Reference serum was from a latex-allergic child with spina bifida.

Table 1. Amino acid sequences of tryptic peptides derived from the purified 27-kD protein (peptide numbers refer to the corresponding peaks in figure 4)

Peptide No.	Amino acid sequence
1	G T T E Q G Y R
2	S V A Q D A P
3	G T V E Q G Y R
4	Q V S A Q T Y
5	Peak containing several peptides
6	Q V S A Q T Y S V A Q D A P
7	A L Y A N L E P K
8	Y L D F V R
9	A V D S F S T L Y
10	A E Q Y A V I T W R
11a ¹	D I S G P L K P G V D T I E N
11b ¹	V S S Y L P L L P G
12	I V L D V A S S V F N T G V Q E G A
13	T V D V S V T S L D G V V P
14	T V V T P V Y Y I P L E A V K

¹ Peptides were sequenced after separation by rechromatography using a modified acetonitrile gradient.

versed phase chromatography and collected (fig. 4). Sequence analysis of selected tryptic peptides gave results as shown in table 1. Comparison of the obtained amino acid sequences with existing databases revealed that 13 of the 14 sequenced peptides showed no significant homology to any of the available primary structures. One peptide (number

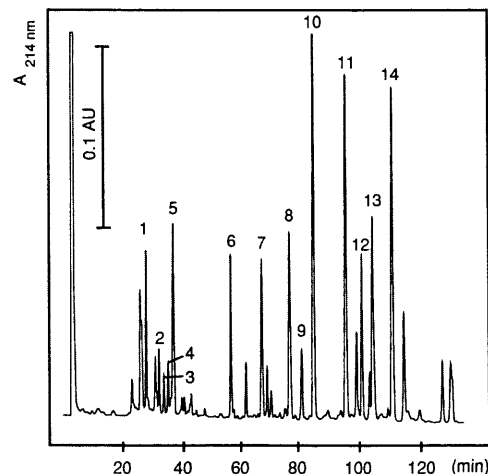


Fig. 4. Reversed phase chromatography of tryptic peptides from purified 27-kD protein on a Rexchrom C8 column. Peak numbers refer to the sequencing results shown in table 1.

Table 2. Sequence comparison of a tryptic peptide (11a) from the purified 27-kD NRL protein and of REF

Peptide 11a	D I S G P L K P G V D T I E N
REF	I I I I I I I I I I
Position	D K S G P L Q P G V D I I E G 54

Nonhomologous amino acids in peptide 11a are italicized.

11a, fig. 4, table 1) showed, however, significant homology to the sequence of REF (table 2).

Discussion

We have previously reported that IgE response to a 27-kD NRL protein seems to be restricted to latex-allergic children with spina bifida or other congenital anomalies and histories of multiple operations [13]. In the present study, we purified the 27-kD protein from NRL to homogeneity and determined its partial amino acid sequence. N-terminal sequence analysis of the purified 27-kD protein gave no result suggesting that its N-terminus is blocked. Thirteen of the 14 purified tryptic peptides sequenced revealed no significant homology to any of the published amino acid sequences indicating that the 27-kD NRL allergen is previously undescribed at a primary structure level. Interestingly, however,

one peptide from the purified 27-kD protein showed homology to REF, a previously sequenced NRL protein [8].

Slater and Trybul [16] have recently shown by immunoblotting binding of IgE from latex-allergic children with spina bifida to 14.3- and 27.5-kD NRL proteins. They also reported that a monoclonal antibody raised against ammoniated NRL detected 26- and 27-kD proteins and also several other polypeptides in NRL. One of these approximately 27-kD proteins could be the same protein purified by us in the present study, however, when the authors used the monoclonal antibody for immunoaffinity purification of NRL proteins, no 27-kD protein was obtained. It thus appears that this particular monoclonal antibody is not suitable for purification of the intact 27-kD protein purified in the present study.

The reason why IgE antibodies to the 27-kD NRL protein, occurring in sera from most latex-allergic spina bifida patients [13, 14], have been described only by few investigators, is unclear. One explanation could be that the 27-kD protein binds relatively loosely to the blotting membrane (unpubl. observation), and is therefore easily washed away if too extensive conditions are used during immunostaining. It could also be that the NRL antigens used in earlier immunoblot studies did not contain the relevant 27-kD protein due to different NRL source materials.

Among the various NRL allergens, the 27-kD protein is of considerable interest because of the peculiar association

of the corresponding IgE antibodies with severe manifestations of latex allergy. Purification methods described in the present study allow production of substantial amounts of the purified 27-kD protein to be used as in vivo (skin testing) and in vitro (solid-phase immunoassays) reagents to improve diagnosis. Detailed information about the structure of NRL allergens may provide useful tools for investigation of immune responses in latex allergy. Furthermore, by using the present purified allergen, production of specific monoclonal or polyclonal antibodies may be facilitated.

In conclusion, a 27-kD NRL allergen, recognized characteristically by sera from spina bifida patients with severe symptoms of latex allergy, was purified to homogeneity. Partial amino acid sequencing of the purified 27-kD protein showed that it has previously not been described at the primary structure level. For the time being, the 27-kD protein is the first molecularly characterized NRL allergen associated with defined clinical manifestations of latex allergy.

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