

Prohevein from the rubber tree (*Hevea brasiliensis*) is a major latex allergen

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Summary

Background There is general agreement that proteins eluting from different natural rubber latex products can cause immediate type hypersensitivity reactions in latex-allergic patients. However, there is as yet no consensus as to what are the most important allergens in natural rubber latex.

Objective We wanted to purify and characterize at the primary structure level three natural latex proteins, suggested to represent significant allergens.

Methods Proteins were purified from ultracentrifuged bottom fraction of natural rubber latex using high performance liquid chromatography gel filtration and reversed phase chromatography. Purified proteins were subjected to tryptic cleavage, peptide separation and amino acid sequencing. Immunoblotting was used to demonstrate IgE antibodies to the purified proteins in sera from latex-allergic patients.

Results A 20 kDa protein was identified by amino acid sequencing as prohevein, a major protein in the rubber tree *Hevea brasiliensis*, and a 30 kDa natural rubber latex protein as hevamine, another essential rubber tree protein. A third, previously undescribed natural rubber latex protein, showed high homology to several plant endo-1,3- β -glucosidases. In immunoblotting, the purified prohevein bound IgE antibodies from 24/29 (83%) sera of latex-allergic patients including positive results in 4/6 latex-allergic children with spina bifida or other congenital anomalies. The purified prohevein elicited positive skin-prick test reactions in all six latex-allergic patients showing IgE to prohevein. The purified 36 kDa protein bound IgE from 6/29 (21%) latex-allergic sera, and the purified hevamine from only 1/29 patient sera.

Conclusion The observed high frequency of IgE antibodies to prohevein suggests that this protein is a major natural rubber latex allergen

Keywords: prohevein, latex proteins, latex allergy, amino acid sequencing

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Introduction

Proteins eluting from natural rubber latex (NRL) products, such as surgical and examination gloves and catheters, as well as from condoms, toy balloons and baby pacifiers, can cause immediate type I allergic reactions in sensitized patients. The symptoms range

from contact and systemic urticaria to asthma, anaphylaxis and even death [1]. In immunoblotting, latex-allergic patient sera show IgE antibodies binding to several NRL allergens, the most important of which appear to be proteins with approximate molecular weights of 14, 20, 27, 30, 36, 45 and 75 kDa [2–4].

Previous clinical studies have defined two major risk groups for latex allergy: first, children with spina bifida and other multioperated paediatric patients, most of

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Table 1. Clinical data and laboratory results of 29 latex-allergic patients

Patient	Sex (F/M)	Age (years)	Reactions to NRL*	Serum total IgE (kU/L)	Latex RAST (kU/L)	IgE-immunoblot reactions** against:		
						20 kDa	30 kDa	36 kDa
1	F	38	S	395	20.0	++	+	++
2	F	48	L	800	5.0	++	—	+
3	M	32	L	17200	2.2	++	—	++
4	F	40	A	640	3.3	++	—	+
5	F	33	L	1840	3.0	++	—	+
6	F	44	L	100	4.7	++	—	—
7	M	38	L	1890	<0.4	+	—	—
8	F	32	L	7050	18.0	++	—	—
9	F	31	A	2870	18.0	++	—	—
10	F	36	L	28	<0.4	+	—	—
11	F	34	L	260	8.3	++	—	—
12	F	34	L	47	<0.4	+	—	—
13	F	34	L	290	<0.4	+	—	—
14	F	43	L	1545	3.9	++	—	—
15	F	32	L	135	2.7	++	—	—
16	F	34	L	19	<0.4	—	—	—
17	F	45	L	11	<0.4	+	—	—
18	F	35	L	165	<0.4	—	—	—
19	F	45	L	6	<0.4	—	—	—
20	M	62	L	3335	2.5	++	—	—
21	M	15	S	110	2.2	++	—	—
22	F	9	L	39	1.6	+	—	—
23	F	12	L	98	2.2	—	—	—
24	F	8	L	285	70	++	—	—
25	F	12	L	190	<0.4	—	—	—
26	F	10	L	140	11.2	+	—	—
27	M	14	L	935	1	+	—	++
28	M	8	L	280	0.9	++	—	—
29	M	15	S	890	2.3	++	—	—

*L. Local reaction. S. Systemic reaction. A. Anaphylactic reaction. **Grading of IgE-immunoblot test results: —. Negative. +. Positive (weak but definitive reaction). ++. Positive (medium to strong reaction).

whom have IgE antibodies to 14 and 27 kDa NRL proteins [3, 5], and, second, healthcare workers and other persons using protective gloves, about 80% of whom have antibodies to a 20 kDa allergen [4]. In the present study we purified and partially sequenced three NRL allergens. The 20 kDa NRL allergen was identified as prohevein and the 30 kDa allergen as heveamine. In addition, we provided evidence that the 36 kDa allergen is an endo-1,3- β -glucosidase, a previously unrecognized protein in the rubber tree.

Materials and methods

Extraction and purification of NRL proteins

Fresh NRL of rubber tree (*Hevea brasiliensis*) was

centrifuged at $43\,000 \times g$ for 1 h and the bottom fraction was frozen and thawed several cycles to rupture its membrane bound organelles. After centrifugation, the supernatant was collected and stored at -70°C . Two hundred microlitres of the supernatant was dried in a vacuum centrifuge, redissolved in 200 μL of equilibration buffer (0.05 M phosphate, pH 7.0, 0.6 M NaCl) and filtered through 0.45 μm Millex-HV membrane (Millipore). The sample was then subjected to high performance liquid chromatography (HPLC) gel filtration on a Superdex 75 HR 10/30 column (Pharmacia) at flow rate of 0.5 mL/min and monitoring at 280 nm. Fractions were collected and analysed by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [4]. The fractions with most intense

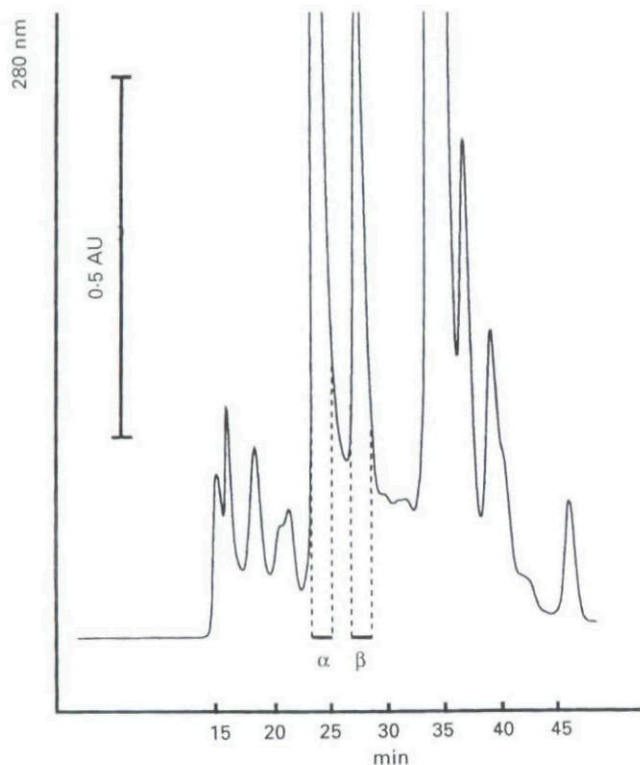


Fig. 1. HPLC chromatogram of a gel filtration of NRL on a Superdex 75 HR 10/30 column. Fractions with most intense bands in SDS-PAGE at 20, 30 and 36 kDa (indicated by α and β) were collected for further purification.

bands at 20, 30 and 36 kDa were collected and further purified by reversed phase chromatography on a ProRP 5/10 column (Pharmacia) using a linear gradient of acetonitrile (0–60% in 40 min) in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min. Elution was monitored at 280 nm and eluting peaks collected manually.

Patient and control sera

Sera were obtained from 20 adult latex-allergic patients (17 women and three men, aged from 31 to 62; mean 38.5 years; Table 1). Nine of the adults were healthcare workers and the majority of the subjects were considered as regular users of protective gloves. Nine patients were children (five girls and four boys, aged from 8 to 15 years) of whom six were children with spina bifida or other congenital anomalies and histories of multiple surgeries (patients numbered 21–26 in Table 1). Sera from six non-allergic adult subjects were used as controls. All patients had positive and controls negative NRL skin-prick test results. Latex-RAST (CAP method, Pharmacia) was positive (cut-off level 0.4 kU/L) in 20 patients (69%) and negative in all controls. Total IgE level was elevated in 14 adult patients and in five children.

Skin-prick testing with glove extract and purified prohevein

An extract of latex gloves (Exona, Semperit, Vienna,

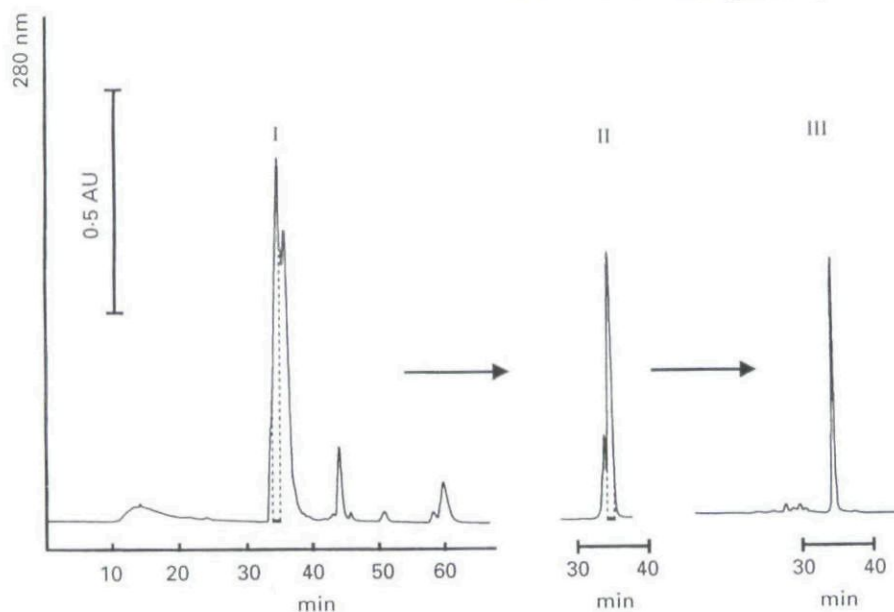


Fig. 2. Sequential HPLC chromatograms of the purification steps of the 20 kDa allergen (from fraction β , Fig. 1) by reversed phase chromatography on a ProRP 5/10 column. The fractions containing the 20 kDa protein in the two preparative steps (I and II) are indicated by a horizontal bar. The homogenous peak III consists of the purified 20 kDa protein.

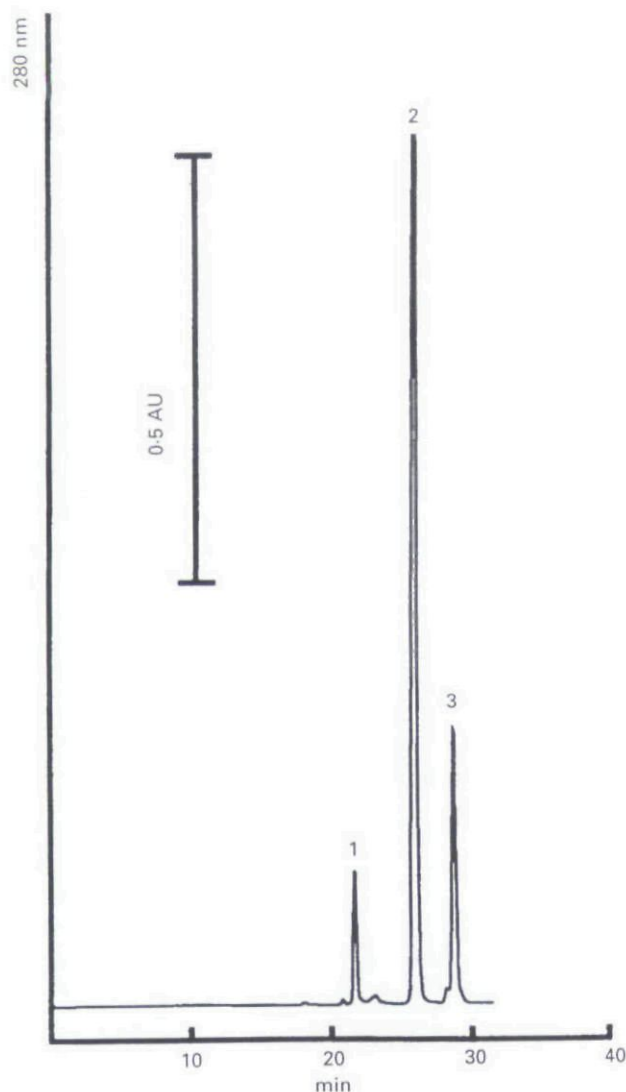


Fig. 3. HPLC chromatogram from the purification of 30 and 36 kDa proteins (from fraction a, Fig. 1) on a ProRP 5/10 column. Peak number 2 contains the purified 30 kDa protein and peak number 3 the purified 36 kDa protein. Peak number 1 represents a 25 kDa NRL protein which was shown not to bind anti-latex IgE antibodies.

Austria) was prepared and the skin-prick test performed as described earlier [6]. Skin-prick testing with purified prohevein (using concentrations of 0.1, 1, 10, and 50 $\mu\text{g}/\text{mL}$ in phosphate-buffered saline, pH 7.2) was performed in a similar way to seven latex-allergic patients.

Tryptic cleavage, peptide separation and amino acid sequencing

Approximately 20 μg of the purified proteins were dried in a vacuum centrifuge, dissolved in 100 μL 0.1M

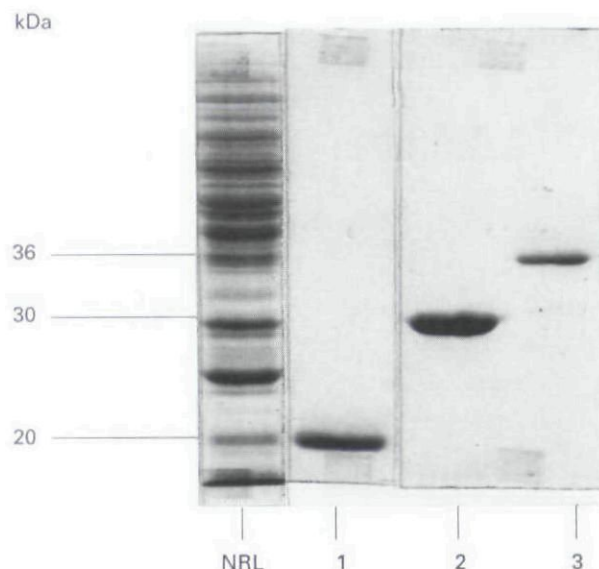


Fig. 4. Coomassie staining after electrophoresis (SDS-PAGE) of three purified NRL proteins. NRL = Natural rubber latex extract. Lane 1. Purified 20 kDa protein. Lane 2. Purified 30 kDa protein. Lane 3. Purified 36 kDa protein.

ammonium bicarbonate and subjected to tryptic cleavage (0.5 μg , Promega V 5111) at 37°C overnight. The tryptic peptides were separated on a 0.21 x 15 cm Rexchrom C8 (Regis Chemical Company) 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 $\mu\text{L}/\text{min}$ and peptide elution monitored at 214 nm. The eluted peptides were manually collected and concentrated to 30 μL in a vacuum centrifuge. Peptide sequencing was performed on a gas-pulsed-liquid-phase sequencer equipped with on-line phenyl-thiohydantoin-(PTH)-amino acid analysis [7].

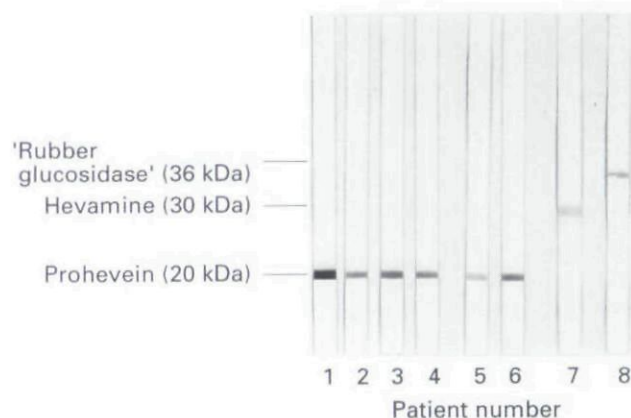


Fig. 5. Immunoblot analysis of binding of IgE-antibodies to three purified NRL proteins from selected eight latex-allergic patient sera.

	Peptide 1										Peptide 2										Peptide 3															
20 kDa	E	Q	X	G	R						L	X	P	N	N	L	X	X	S	Q	E	Q	X	G	R	Q	A	G	G	K	L	X	P	N	N	L
Heve_Hebr	E	Q	C	G	R	Q	A	G	G	K	L	C	P	N	N	L	C	C	S	Q	E	Q	C	G	R	Q	A	G	G	K	L	C	P	N	N	L
position	1				5						11								20	1															16	

	Peptide 4										Peptide 5										Peptide 6														
30 kDa	Y	L	A	A	Y	S	K				W	T	T	S	I	N	A	G	K		Y	G	G	V	M	L	W	S	K						
Chly_Hevbr	Y	L	S	A	Y	A	K				W	T	T	S	I	N	A	G	K		Y	G	G	V	M	L	W	S	K						
position	140						146				205							213		249														257	

	Peptide 7										Peptide 8																							
36 kDa	S	W	V	Q	K											Y	I	A	V	G	N	E	I	S	P	V	N	R						
E13b_Lyces	W	W	V	Q	K	N	V	R	D	F	W	P	H	V	K	I	K	Y	I	A	V	G	N	3	I	S	P	V	T	G				
position	97					101											114																	126

	Peptide 9									
36 kDa	S	Y	L	N	P	I	I	R		
E13b_Phavu	S	Y	L	D	P	I	I	G		
position	157							164		

Table 2. Sequences of the selected tryptic peptides from three purified NRL proteins and their comparison to amino sequences available in databases. X. No phenyl-thiohydantoin-amino acids could be identified (for details see text). Heve_Hevr. Hevein precursor c also known as prohevein or hevein preproprotein). Chly_Hevr. Hevamine A. E13b_Lyces. Glucan endo-1,3-beta-glucosidase B (Tomato). E13b_Phavu. Glucan endo-1,3-beta-glucosidase (Kidney bean).

Immunoblotting

Immunoblotting was performed as described earlier [4] to verify the ability of the purified proteins to bind relevant IgE. Appropriate concentrations for optimal resolution of the purified NRL proteins were first determined in SDS-PAGE experiments.

Results

Gel filtration of NRL extract revealed several clearly separated fractions which were collected and analysed by SDS-PAGE. The fractions containing the 20, 30 and 36 kDa proteins (Fractions α and β , Fig. 1) were purified into homogeneity by reversed phase chromatography. Only single, sharp peaks for each purified protein were seen (Figs 2 and 3). The purity and the electrophoretic mobility of the fractions were also confirmed by SDS-PAGE (Fig. 4).

Sequence analysis of the selected tryptic peptides and their comparison to amino acid sequences available in existing databases are shown in Table 2. The three partially overlapping tryptic peptides from the purified 20 kDa protein revealed 100% homology with prohevein except that no PTH-amino acids were identified at positions containing cysteins (this amino acid is the

only one which cannot be identified from the non-alkylated proteins in PTH-amino acid analysis). Three peptides derived from the purified 30 kDa protein showed 100% homology with hevamine. Three sequenced peptides from the purified 36 kDa protein showed no 100% homology to any known protein but all three peptides gave high homology (from 67% to 83%) to several endo-1,3- β -glucosidases of the plant kingdom.

In immunoblotting, 24/29 (83%) sera from latex-allergic patients showed IgE binding to the purified prohevein (Table 1). The overall frequencies were very similar for adults (85%) and for children (78%), including positive results in 4/6 children with spina bifida or other congenital anomalies and histories of multiple surgeries (children numbered 21–26, Table 1). There was an obvious correlation between the latex-RAST and the prohevein immunoblotting results: 19/24 (79%) immunoblot-positive sera were also RAST-positive. IgE antibodies binding to the purified 36 kDa protein were found in 6/29 (21%) latex-allergic patient sera (all latex-RAST-positive) and to the purified hevamine in 1/29 patient sera (Table 1). All six control sera gave negative results to the purified proteins (data not shown). Figure 5 shows typical IgE immunoblot reactions to the purified prohevein, hevamine and to the 36 kDa protein in eight selected patient sera.

The purified prohevein elicited positive skin prick test reactions in 6/7 consecutive latex-allergic patients (who had positive skin-prick test results with a standard NRL glove eluate) verifying that the purified prohevein had retained its capacity to provoke allergic reactions. In two patients positive skin-prick test reactions were inducible by purified prohevein at a concentration as low as 1 µg/mL, in three patients at 10 µg/mL and in one patient at 50 µg/mL. All these six patients recognized purified prohevein in IgE immunoblotting while the patient with negative skin-test result was also immunoblot-negative.

Discussion

In the present study, we identified a 20 kDa NRL allergen, previously shown to be the most significant NRL allergen for adult latex-allergic patients [4], as prohevein, a major protein in the rubber tree latex [8]. In addition, we purified a 30 kDa protein and identified it as heveamine, another rubber tree protein [9]. The third purified protein (36 kDa) showed high homology with endo-1,3-β-glucosidases of different plants but this protein has not been earlier identified in NRL.

The 20 kDa prohevein was recognized as a significant allergen for the majority of our latex-allergic patients. More than 80% of the patients, either adults or children, had IgE antibodies recognizing the 20 kDa molecule in immunoblotting and the immunoblot results correlated closely with a commercial latex-RAST, suggesting that prohevein is a significant allergen also in this particular diagnostic assay. To confirm that prohevein is an important allergen also *in vivo* we performed skin-prick tests with the purified 20 kDa protein and obtained positive results in the majority of latex-allergic patients tested. Why other investigators have not reported similar high frequencies of IgE antibodies to the 20 kDa prohevein is unclear. It is possible that patient materials have been different or that intact 20 kDa prohevein molecules have not been present in sufficient quantity in NRL source materials used. It is also possible that prohevein molecules have not been successfully bound to the solid-phases of the assays used.

Previous studies by us [4, 5] and others [2, 10] have suggested that NRL proteins with approximate molecular weights of 14 and 27 kDa are important allergens for latex-allergic children with spina bifida or other congenital anomalies. We recently purified and partially sequenced the 27 kDa NRL protein which turned out to be a previously undescribed protein at the primary structure level [11]. Czuppon *et al.* [12] have recently suggested that rubber elongation factor, a 14.6 kDa NRL protein, is the major allergen in NRL but this

view has been criticized [13]. Results of the present study strongly suggest that the 20 kDa prohevein is the most important NRL allergen in latex-allergic patients in general and an important allergen also in latex-allergic children with spina bifida. The 36 kDa protein appears to be a significant, previously uncharacterized allergen, whereas the 30 kDa heveamine seems to play only a minor role as a NRL allergen.

The information from the primary structures of significant allergens, such as prohevein in latex allergy, can be used for the identification and localization of allergenic epitopes. This knowledge also provides tools for the production of substantial amounts of relevant antigens for diagnostic purposes, which at present in latex allergy are not based on standardised allergens. Moreover, proteins of NRL and of several food allergens, present, in e.g. banana, avocado and chestnut, show crossreactivity and these foods are known to be able to cause hypersensitivity reactions in latex-allergic patients [14–18]. Further studies may settle if the crossreactive food allergens have primary structures similar to NRL allergens described in the present study.

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