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Characterization of Latex Allergenic Components by Capillary Zone Electrophoresis and N-Terminal Sequence Analysis

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Abstract

In a previous study, protein components purified from latex gloves that elicited allergenic reactions were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and yielded apparent molecular weights of 14, 22, 30, 34, 46, and 58 kD. These allergenic components were isolated for further characterization by capillary zone electrophoresis and N-terminal amino acid sequence analysis. These components all migrated at approximately 25 and 35 min on capillary zone electrophoresis. Diode array spectral analysis detected indistinguishable characteristics between these two protein peaks. In addition, complex formation of these components with patients' immunoglobulin was demonstrated by capillary zone electrophoresis. Analysis of components separated by SDS-PAGE on a polyvinylidene difluoride membrane showed that the first 13 residues were identical to the sequence of hevein. Based on the criteria of charge-to-mass ratio and N-terminall amino acid sequence, our results suggest that these components of latex proteins are similar in the primary structure.

Key Words
Latex
Capillary zone electrophoresis
Sequence
Hevein

Several reports [8, 18, 23] have indicated that molecular weight discrepancies among latex protein components might arise from using different sources of latex or from extraction procedures that cause degradation or polymerization of the protein. Previously, we reported that major immunoglobulin E (IgE) reactivity was detected in the 34-kD area by Western blot analysis and that other minor components (of 14, 22, 30, 46, and 58 kD) were detected in a less discrete pattern [9]. It has been proposed that several allergic components of latex isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) are actually aggregates of one protein, degraded to various degrees [22].

Capillary zone electrophoresis (CZE), which is based on the charge-to-mass ratio of the analyte for the separation [16], is probably the commonest mode of capillary electrophoresis [16]. The application of CZE in the critical separation of amino acids, peptides, and proteins is well documented [12, 15, 24]. The application of CZE in the separation of antibody-antigen complexes was scant [7, 19].

In the current study, these latex allergenic components were isolated from the gel and characterized as to their mobility by charge and size using CZE. The antigenicity of these components was also demonstrated by separation of the resulting antigen-antibody complexes using the

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CZE technique. Isolated protein components from polyvinylidene difluoride (PVDF) membranes were also analyzed by N-terminal amino acid sequence analysis of the first 15 amino acids, and the results were compared with those from CZE. We tested the hypothesis that the different allergenic components observed on SDS-PAGE were derived from one latex protein, which should reveal whether these proteins represent distinct allergens or isoallergens (for example, oligomers or fragments).

Methods

Preparation of Latex Extracts

Twenty-gram batches of latex gloves used in the laboratory were cut into approximately 1 cm^2 and stirred in a 0.02 *M* phosphate-buffered saline (PBS) solution containing 0.06 *M* NaCl and 0.04% NaN_3, pH 6 for 24 h at 4°C. The elutes were centrifuged at 2,000 *g* for 30 min. Supernatants were collected and filtered through No. 2 filter paper and then through Amicon® filter apparatus with a 3-kD molecular weight cut-off membrane. The retentate was aliquoted and lyophilized.

Preparative Gel Electrophoresis

Gel electrophoresis containing SDS was performed in a PAGE apparatus as described previously [9]. Gels were stained with 0.25% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid, and then destained by immersing in 25% methanol and 7% acetic acid in water.

After Coomassie blue staining, the stained gel bands corresponding to 12, 22, 30, 34, 46, or 58 kD became apparent and were excised, placed in 0.01 *M* PBS, and extracted by stirring overnight [13]. Samples were then passed through SM 2 gel (Bio-Rad, Hercules, Calif., USA) to remove the Coomassie blue stain, passed through AG 11 resins (Bio-Rad) to remove SDS, and lyophilized.

Capillary Zone Electrophoresis

CZE experiments were performed on a P/ACE 5510 (Beckman, Fullerton, Calif., USA) instrument with a built-in diode array detector using an uncoated, fused silica capillary, which was 57 cm long (50 cm to the detector) with a 75-μm inner diameter. The capillary was rinsed with 0.1 *M* NaOH, chromatographic-grade water, and run buffer containing 0.1 *M* sodium borate, pH 9.2, between injections. New capillaries were rinsed with 0.1 *M* NaOH, 0.1 *M* HCl, and water prior to use. In all experiments, the detector end of the capillary was negatively polarized relative to the injector end.

CZE separation was performed in a 0.1 *M* sodium borate buffer, pH 9.2, using a constant voltage of 19 kV and a current of about 150 μA. The samples were diluted with distilled water to 1 μg/μl and injected for 15 s. The proteins were detected at 280 nm, and electrophoresis was run at 23°C.

Purification of Latex Proteins by DEAE-Sephadex Chromatography

To study the immunoreactivity of IgG and IgE, anion-exchange chromatography and the enzyme-linked immunosorbent assay (ELISA) were used. Partial purification of the latex protein was performed on a DEAE-Sephadex column using a fast-performance liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology, Uppsala, Sweden). Preparative FPLC was carried out by applying 15 mg of the glove extracts to the column, and 500-μl fractions were collected. Each of these fractions was desalted and assayed by the Lowry method for protein concentration and by ELISA for immunoreactivity.

The FPLC unit was filtered with a DEAE-Sephadex column (1 × 30 cm) equilibrated with 0.01 *M* bis-Tris buffer, pH 6, and the unit was programmed to deliver a continuously increasing NaCl gradient ranging from 0 to 0.6 *M* with a flow rate of 0.2 ml/min. The absorbency was monitored at 280 nm.

Serum Collection

Sera were collected from 8 patients (age ranged from 8 to 17 years). Severn of them had spina bifida, and 1 had urinary tract anomalies. All of them had had at least 5 surgeries in the past and had moderate to severe symptoms of latex allergy (cutaneous and respiratory). Skin tests using latex extract from latex gloves were positive in all of them. Sera from 7 patients had been confirmed for the presence of IgE and IgG against latex allergens in a previous study [9], but one serum sample contained specific IgE without detectable specific IgG.

Immunoreactivity Measurement by ELISA

A sample from each fraction of anion-exchange chromatography was diluted to 2 μg/200 μl in a sodium carbonate-bicarbonate buffer (0.07 *M*, pH 9.6). ELISA plates were coated with 2 μg per well for each fraction in triplicate. The ELISA procedure described previously [9] was used. Eight serum samples from allergic individuals were used to test each fraction for immunoreactivity to IgE and IgG. The curve of the average optical density for serum samples versus fraction number was plotted using the Delta-Graph Pro program by Macintosh.

Protein G Affinity Purification of IgG from Patients

IgG was isolated from the sera of 5 latex-allergic patients by precipitation at 50% $(NH_4)_2SO_4$ saturation and purification by protein G affinity chromatography using 0.02 *M* PBS, pH 7.2, as the binding buffer and 0.1 *M* glycine, pH 2.7, as the elution buffer.

Detection of Antigenicity

The major latex allergenic component (34 kD) and IgG purified against latex were mixed at room temperature to initiate the antigen-antibody binding. The approximate molar ratios of antibody:antigen in the mixture used for CZE was 1:2. After 30 min of incubation, the mixture was applied to the CZE apparatus.

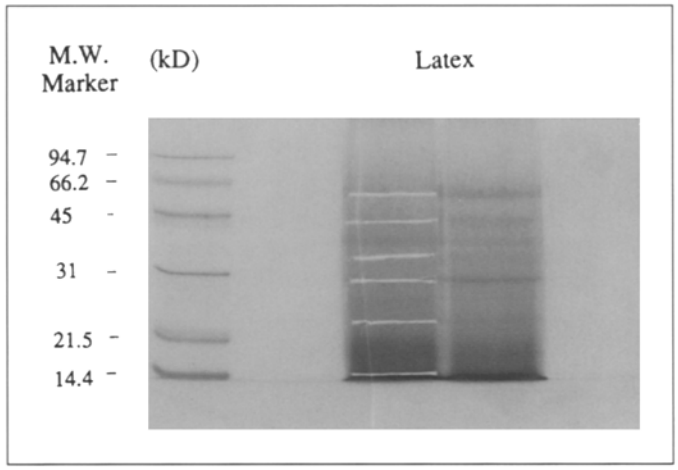
N-Terminal Sequence Analysis

Amino acid sequencing was performed on a model 494A Procise™ sequencer (Applied Biosystems, Foster City, Calif., USA). All chemicals were supplied by Applied Biosystems. Two-hundred micrograms of crude latex extracts were electrophoresed and electroblotted onto a PVDF membrane using 0.01 *M* 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11, as transfer buffer [17]. The PVDF-blotted bands of latex proteins were excised, cut into small pieces, and arranged in the upper cartridge block of the sequencer. Sequencing was carried out according to the manufacturer's instructions using a standard program.

Results

Separation of Latex Antigens by SDS-PAGE

Figure 1 shows the separation of latex proteinsin the 12% polyacrylamide gel. The stained gel revealed multiple protein bands with molecular weight ranging from 14 to 58 kD. Previously, 14-, 22-, 30-, 34-, 46- and 58-kD bands had been shown to be immunoreactive against sera of allergic patients. Those bands were excised from the gel and submitted for CZE studies as described below. In a parallel experiment, separated proteins without prior staining were transferred to a PVDF membrane for the N-terminal amino acid sequence analysis.



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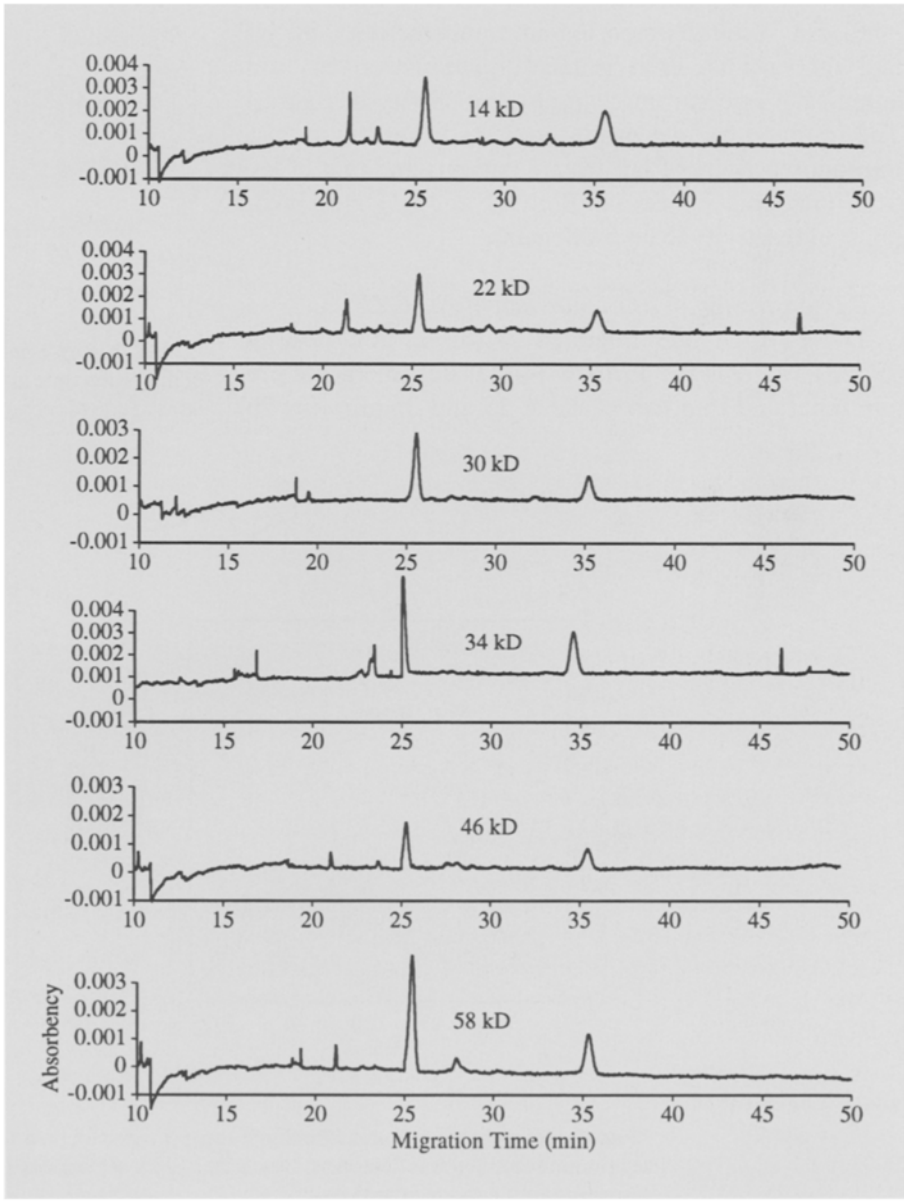

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Fig. 1. Separation of latex antigenic proteins by SDS-PAGE and identification of band cut for CZE.

Fig. 2. Electropherograms of the 14-, 22-, 30-, 34-, 46-, and 58-kD latex components isolated from SDS-PAGE gels and purified by AG 11 resin and SM 2 gel chromatography.

Analysis of Purified Latex Components by CZE

Results showed that there were 2 major peaks at 25 and 35 min in the electropherograms for the 14-, 22-, 30-, 34-, 46- and 58-kD components after purification. Migration times for these two peaks were matched in the electropherogram for each component (fig. 2).

Diode array spectral scanning of the peak fractions from 6 components (14, 22, 30, 34, 46 and 58 kD) of the same migrating time (25 min) showed a very similar spectrum with a peak at 280 nm. Figure 3 shows diode array spectra of three representative 14-, 22- and 34-kD components.

Immunoreactivity of Isolated Fractions by Ion-Exchange Chromatography

ELISA results comparing immunoreactivity by IgE and IgG against each isolated fraction derived from anion-exchange chromatography are shown in figure 4. The immunoreactive profile indicated that the average immunoreactivity of IgE from 8 patients and that of IgG from 7 patients was similar. Both IgE and IgG had a broad range of reactivity to latex allergens.

Demonstration of Immunoreactivity by CZE

The purified IgG migrated at the 12-min position (fig. 5a), whereas the purified 34-kD latex-allergenic protein separated into two peaks at 25 and 35 min (fig. 5b) under the same CZE conditions. However, the mixture consisting of IgG and the 34-kD latex-allergenic protein in a molar ratio of 1:2 emerged at a unique position at 23 min (fig. 5c). The 23-min peak presumably occurred as a result of the antigen-antibody complex.

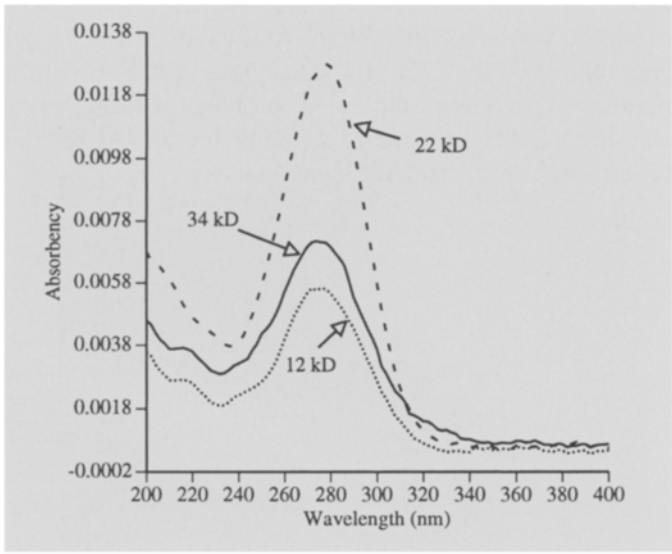

Fig. 3. Representative diode array analysis of the peak at 25 min of migration time in the electropherograms of the 14-, 22-, and 34-kD latex allergenic components.

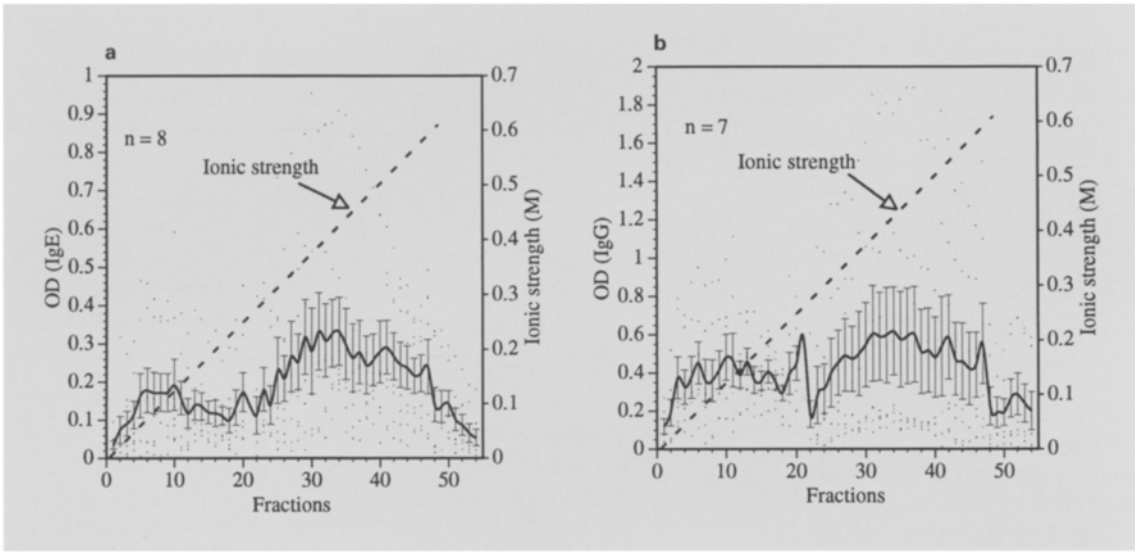

Fig. 4. Ion-exchange chromatography of the glove extract on a DEAE-Sephadex column. OD = Optical density. The immunoreactivity was determined using an ELISA. **a** Profile of IgE immunoreactivity. **b** Profile of IgG immunoreactivity against collected fractions.

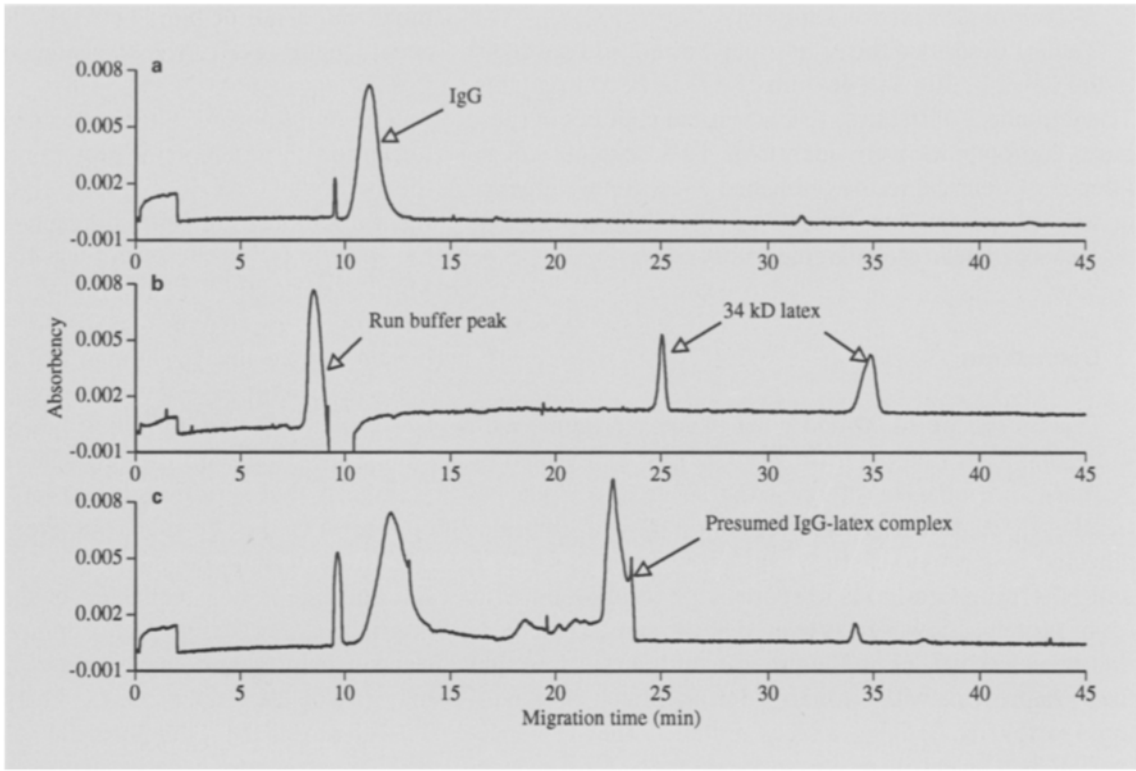

Fig. 5. Representative separation of IgG, latex components, and the IgG-latex complex by CZE. **a** Electropherogram of purified IgG from latex-allergic patients. **b** Electropherogram of the isolated 34-kD latex component. **c** Electropherogram of mixtures containing IgG and the 34-kD latex component. The experimental conditions were as described in Materials and Methods.

Table 1. The N-terminal amino acid sequence of the 14-, 22-, 30-, 34-, 46-, and 58-kD latex components isolated from PVDF membrane

Latex component	Residue number 1 6 11 16
14-kD	Glu-Gln-Cys-Gly-Arg-Gln-Ala-Gly- *[a]-Lys-Leu-Cys-Pro-Asp[b]-Asn
22-kD	Gly - * - * - Gly-Arg-Gln-Ala-Gly - * - Lys-Leu-Cys-Pro-Asp-Asn-Leu
30-kD	Glu-Gln-Cys-Gly-Arg-Gln-Ala-Gly-Gly-Lys-Leu-Cys-Pro-Asp-Asn-Leu-Cys-Cys
34-kD	Glu-Gln-Cys-Gly-Arg-Gln-Ala-Gly-Gly-Lys-Leu-Cys-Pro-Asp-Asn
46-kD	Glu-Gln-Cys-Gly-Arg-Gln-Ala-Gly-Gly-Lys-Leu-Cys-Pro-Asp-Asn
58-kD	Glu-Gln-Cys-Gly-Arg-Gln-Ala-Gly-Gly-Lys-Leu-Cys-Pro-Asp-Asn
Hevein[c]	Glu-Gln-Cys-Gly-Arg-Gln-Ala-Gly-Gly-Lys-Leu-Cys-Pro-Asn-Asn-Leu-Cys-Cys-Ser-Gln

[a] Asterisks (*) indicate that the amino acid residue is not determined (one or two other amino acids identified at a given position).
[b] Asp at residue 14 is different from Asn at the same position in hevein.
[c] The N-terminal 1- to 20-amino-acid sequence was published by Andersen et al. [3].

N-Terminal Sequence Analysis

Table 1 described the N-terminal amino acid sequence of the 14-, 22-, 30-, 34-, 46- and 58-kD latex components. The sequences of the first 13 N-terminal residues of these latex components were identical. This segment of sequence is identical to the published N-terminal sequence of hevein, except that residue number 14 was aspartic acid (Asp) instead of aspargine (Asn).

Discussion

Previously, we identified a set of latex proteins with molecular sizes ranging from 14 to 58 kD, as revealed by Western blot analysis of IgE from severely allergic patients [9]. A further characterization of the peptides detected in a previous study was carried out using CZE and N-terminal amino acid sequencing techniques. This set of proteins, however, migrated at the same velocity as that revealed by CZE in the present study indicating that they are proteins with similar, if not identical, charge-to-mass ratios (fig. 2). They also exhibited similar UV spectra (fig. 3). The nature of the two peaks in the CZE electropherogram was unknown since the CZE equipment used in these studies did not facilitate collection of fractions for peak characterization. N-terminal sequence analysis of the 14-, 22-, 30-, 34-, 46-, and 58-kD components of the latex proteins yielded a sequence, at least for the first 13-residue segments, that was identical to the sequence of hevein. Taken together, these results clearly suggest that the multiple components observed with SDS-PAGE were derived from a common protein, hevein. Our findings are consistent with results of a previous study by Todt and Ownby [22], who reported similar amino acid compositions for the 12.7-, 14.5-, 29- and 48-kD bands of the latex proteins on SDS-PAGE, and concluded that these proteins likely were derived from one latex protein, possibly the rubber elongation factor. Although the formation of multiple components from one protein is attributable to the different degrees of aggregation and subsequent degradation, the explanation for why the aggregation is maintained in the presence of SDS and sulfhydryl reagents [4, 14, 21] is still lacking. Furthermore, prohevein (molecular weight 19,087 D) and hevein (molecular weight 4,727 D) are rich in cysteine residues, each containing 14 and 8 residues, respectively. It is highly likely that the sulfhydryl agents used in SDS-PAGE of the latex proteins containing hevein and prohevein may not have been sufficient to reduce all of the disulfide bridges. Consequently, homologous and heterologous polymerization through the intermolecular disulfide bond between these two proteins may occur, yielding a range of aggregates as seen on SDS-PAGE.

The immunoreactivity was confirmed by the complex formation of the allergenic proteins and IgG of patients by the method of CZE. In this study, IgG was used instead of IgE to demonstrate immunoreactivity in CZE, because the specific IgE in the serum was too low to be purified. More importantly, IgG and IgE were shown to have similar immunoreactive profiles (fig. 4). Although epitopes of hevein recognized by human antibody have not been identified, in the case of rubber elongation factor (another latex allergen), the minimal IgE-binding epitope was almost identical with the IgG-binding epitope [10]. The possibility that specific IgE and IgG react with the same epitope(s) cannot be ruled out even though competitive inhibition of immunoreactivity to latex allergens between IgE and IgG is not available. As shown in figure 5, IgG migrated faster than the latex component due to the isoelectric point (pI) being between 4.0 and 6.5 [5] for a majority of the latex proteins. The pI of IgG was in the 7.2–8.0 range [19]. Therefore, the 34-kD latex component had a much greater negative charge than IgG molecules in the CZE separation buffer (0.1 *M* borate buffer, pH 9.2), resulting in faster migration of the IgG molecules toward the end of the negatively polarized detector of the capillary. Based on a comparison of migration times, the peak at 23 min showed a newly emerged entity, most likely due to the formation of IgG-latex complexes. A significant peak for IgG alone (at 12 min) can be expected in the electropherogram of the IgG-latex component mixture (fig. 5c), since the whole IgG was used and the majority of it was non-latex specific. Theoretically, two types of complexes [IgG-latex component and IgG-(latex component)$_2$] would be formed that correspond to a reaction at the antigen-binding sites of each antibody molecule.

IgG + latex component → IgG-latex component
IgG-latex component + latex component → IgG-(latex component)$_2$

Under the molecular ratio 1:2 for IgG:latex component, the 34-kD protein was in excess because only a portion of whole IgG was latex specific. It is highly possible that the small peak around the 35-min migration time represents a free latex component. The CZE peaks for the IgG and IgG-latex complex were relatively broad; charge heterogeneity of the IgG species and interaction of IgG with the wall may also cause such broadening.

The sequences we have now obtained for the 14-, 22-, 30-, 34-, 46-, and 58-kD allergenic components revealed unequivocally that they had identical N-terminal se-

quence at least in the first 15 residues. Most importantly, the first 13 residues of the N-terminal sequence were identical to those of hevein based on SWISS-PROT databank. Of the 15 amino acid residues determined, only residue number 14 was different from hevein, being Asp instead of Asn (table 1). Mutations between Asp and Asn are common in proteins. This difference might be due to phylogenetic variations between rubber tree species. Alternatively, deamidation may occur, resulting in the conversion of Asn to Asp or glutamine (Gln) to glutamic acid (Glu), since the latex has been processed by ammonification. If this is the case, it is intriguing that deamination occurred only at position 14 and not at positions 2 and 6.

Possible latex allergens, such as the rubber elongation factor [13], prohevein [1, 6] and hevamine [1, 6], were found to be immunoreactive to the patients' sera. The view that the rubber elongation factor is a single major allergen has been debated [20]. Hevamine seems to play only a minor role as a natural rubber latex allergen [1]. In recent reports [2, 11] the importance of hevein in latex allergy has been addressed.

The present results obtained from CZE and N-terminal sequence analysis clearly show that the latex allergens derive from hevein-related molecules: the allergenic components had similar charge-to-mass ratios and highly homologous N-terminal sequences as hevein, and the chance is only one in twenty to the thirteenth power that the allergenic components are not hevein related, but had identical N-terminal 1–13 amino acid sequences.

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