

One of the rubber latex allergens is a lysozyme

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Background: Type I hypersensitivity reactions caused by latex products are ascribed to proteins eluted from them, but little is known about the properties of these allergenic proteins. The reason for the cross-reaction between rubber latex and fruits is also not known. We have speculated that a series of defense-related proteins in plants is a cause of latex allergy and the cross-reaction.

Objective: To verify our hypothesis, we selected a lysozyme as a representative defense-related protein and examined its relationship to latex allergy.

Methods: Lysozymes eluted from latex gloves were detected with a cell-suspension clearing test. A chromatographically separated lysozyme was investigated for its physicochemical and enzymatic properties and allergenicity.

Results: Lysozyme activity was detected in extracts from ammoniated latex and latex gloves. We separated a lysozyme (27 kd; isoelectric point, 9.5) using cation-exchange and gel filtration chromatography. This lysozyme was enzymatically very similar to fruit lysozymes and was demonstrated to be an allergen.

Conclusions: One of the rubber latex allergens is a lysozyme that has similarities to fruit lysozymes. This suggests the relevance of defense-related proteins to latex allergy and the cross-reaction. (*J ALLERGY CLIN IMMUNOL* 1995;96:677-86.)

Key words: Lysozyme, rubber latex allergen, latex gloves, immunoblotting, cross-reaction, defense-related protein, defense response

A large number of articles on type I hypersensitivity reactions caused by natural rubber products have been published since the first report by Nutter in 1979.¹ Several proteins eluted from the products are considered to be responsible for this latex allergy.²⁻⁵ The molecular weights of these allergenic proteins have been determined by several groups, but their detailed properties have not been revealed yet. It is well known that patients who are sensitive to natural rubber products often

Abbreviations used

BCA:	Bicinchoninic acid
CMC:	Carboxymethyl cellulose
HPLC:	High-performance liquid chromatography
pI:	Isoelectric point
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

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cross-react to various fruits such as banana, avocado, fig, and passion fruit.⁶⁻⁹ This knowledge gave us some hints for further investigation of the allergens.

Natural rubber products, sometimes called latex products, are prepared from the latex of the rubber tree (*Hevea brasiliensis*). The latex is composed of the cytoplasmic fluid of specialized rubber tree cells called laticifers. When a rubber tree is wounded (e.g., by tapping), the cytoplasmic contents of its laticifers are expelled, and the wounded sites are sealed by coagulation of the expelled latex. This process is considered to involve two

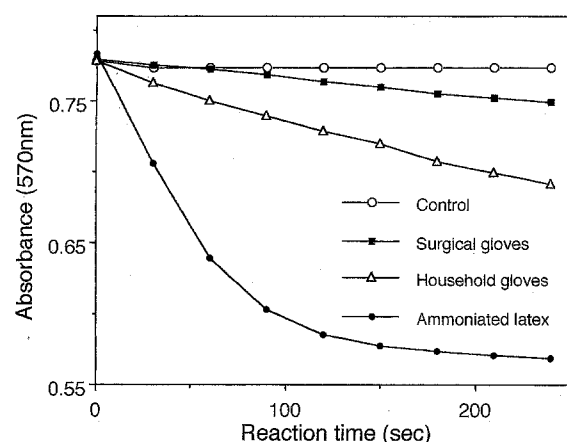


FIG. 1. Lysis of *M. lysodeikticus* cell walls by the three extracts. Each reaction contained 20 μ l of the extract, of which the total protein concentration was about 1.5 mg/ml, and 0.4 mg of the cell walls in 2.0 ml of 20 mmol/L acetate buffer (pH 4.6). All reactions were conducted at room temperature, and the absorbance at 570 nm was measured every 30 seconds. In the control experiment, 20 μ l of water was added to the reaction mixture instead of the extract.

successive events. One is bursting of luteoid bodies (vacuole origin) in the laticifers and release of their proteins. The other is interaction of the released cationic proteins with negatively charged rubber particles. It is known that repeated wounding increases the content of special proteins in the laticifers. Such a phenomenon is called a defense response, and the induced proteins are called defense-related proteins.¹⁰⁻¹² One of the main roles of defense-related proteins is protection of the plant from pathogen attacks. Hence, some defense-related proteins have antifungal, antimicrobial, and insecticidal activities; others catalyze the biosynthesis of small antimicrobial compounds called phytoalexins. Defense-related proteins are also induced by treating a plant with plant growth regulators or phytohormones such as auxin, gibberellin, cytokinin, and ethylene. It is reported that transcript levels of the genes involved in natural rubber biosynthesis and induced by wounding or ethylene treatment were significantly higher in laticifers than in leaves.¹³

We have speculated that defense-related proteins in rubber latex may form the major part of latex allergens. Some reports have described lysozyme activity of avocado,¹⁴ papaya,^{15, 16} fig,¹⁷ and *H. brasiliensis*.¹⁸ Lysozymes (mucopolysaccharide *N*-acetylmuramoylhydrolase, EC3.2.1.17) are considered to be defense-related proteins in higher plants.¹⁹ If similar plant lysozymes are allergens, we may be

able to explain the unaccountable cross-reaction between rubber latex and fruits. Accordingly, we noted the lysozyme activity of extracts from ammoniated latex, surgical gloves, and household gloves. All of the extracts exhibited strong lysozyme activity. Then we tried to separate the specific protein having lysozyme activity by means of cation-exchange and gel filtration chromatography. The separated lysozyme was examined for molecular weight, isoelectric point (pI) and allergenicity by using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing, and immunoblotting, respectively. The separated lysozyme was also compared with previously reported fruit lysozymes for enzymatic properties.

METHODS

Extract preparation

Surgical gloves (Triflex, Baxter Healthcare Corp., Pharmaseal Div., McGaw Park, Ill.) and household gloves (DUNLOP Home Products, Japan), which had caused type I hypersensitivity reactions in patients,²⁰ were cut into small pieces (approximately 1 $\times$ 1 cm) for protein extraction. Each glove sample was extracted with the proper amount of 20 mmol/L phosphate buffer (pH 7.4) at room temperature for 2 hours. After filtration, the dissolved protein was precipitated by addition of $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation. The precipitate was collected and re-dissolved in 3% acetic acid. This solution was applied to a Sephadex G-25 column (3.0 $\times$ 40 cm; Pharmacia, Uppsala, Sweden) to remove low molecular weight compounds. The fractions that corresponded to the void volume and constructed an intense peak when they were monitored by the Lowry method²¹ were combined. After lyophilization, the obtained powder was dissolved in 30 mmol/L phosphate buffer (pH 6.5) to produce a protein concentration of about 1.5 mg/ml. Thus prepared solutions were used as the extracts of the gloves for each analytic purpose. Proteins from ammoniated latex were extracted as previously described.²⁰ For the prick test, aqueous extracts had been prepared separately.²⁰

Subjects

The sera of two patients who were sensitive to latex products were used in immunoblotting. One subject was a 51-year-old housewife. She had experienced a generalized wheal after wearing household gloves and exhibited strong positive reactions, in the prick test, to the aqueous extracts from ammoniated latex and the two kinds of gloves.²⁰ The other subject was a 32-year-old surgeon. He had also exhibited positive reactions to the aqueous extracts in the prick test. The serum of a healthy person and commercially available normal plasma (George King Bio-Medical, Inc., Overland Park, Kan.) were used in the control experiment.

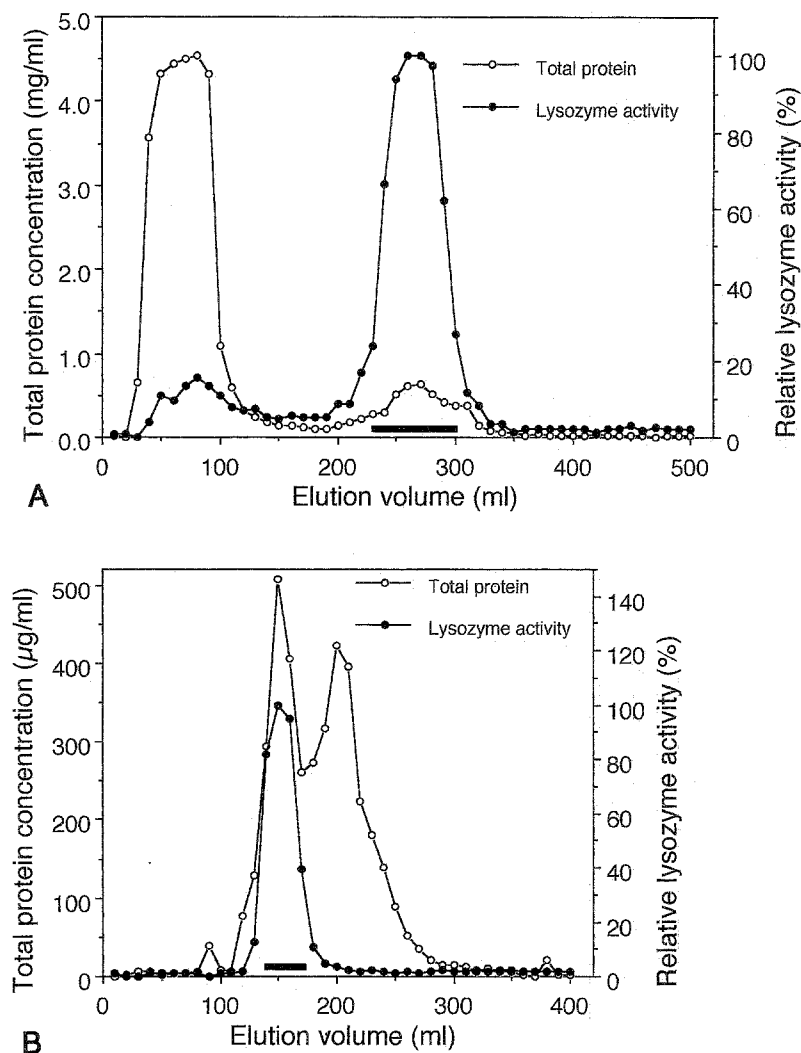


FIG. 2. **A**, Chromatography of the protein extracted from household gloves on a CMC column. The fractions indicated by the solid bar were combined, lyophilized, and separated further. **B**, Chromatography of the protein adsorbed on the CMC in a Sephadex G-50 column. The fractions indicated by the solid bar were combined and lyophilized to provide the separated lysozyme.

Total protein measurement

The total protein concentrations of the extracts were determined with the bicinchoninic acid (BCA) method according to the enhanced protocol.^{22,23} The Lowry method was used instead of the BCA method when the sample was a chromatographic fraction. This exchange did not produce any unfavorable results. In each method, bovine serum albumin was used as a standard protein. The eluted total protein amounts from the two kinds of gloves had previously been determined with the BCA method as follows: surgical gloves, 584 ± 12 $\mu\text{g/gm}$; household gloves, 1380 ± 30 $\mu\text{g/gm}$ (mean $\pm$ SD, $n = 3$).²³

Lysozyme activity test

The lysozyme activity of a sample was determined according to the method reported by Shugar²⁴ with some

modifications. Briefly, the proper amount of a sample solution was added to a buffer containing 0.2 mg/ml of *Micrococcus lysodeikticus* cell walls (Sigma Chemical Co., St. Louis, Mo.) as a substrate. After incubation at the temperature indicated in each case, the reaction was stopped by adding Na_2CO_3 to a final concentration of 17 mmol/L. Then the absorbance of the reaction mixture at 570 nm was measured to determine the decrease in turbidity. The lysozyme activity of a chromatographic fraction was expressed as the decrease in the absorbance relative to the maximum decrease.

Separation of a lysozyme

Fifty pairs of household gloves (about 800 gm) were cut into small pieces (approximately 3×3 cm) and extracted with 800 ml of 20 mmol/L phosphate buffer

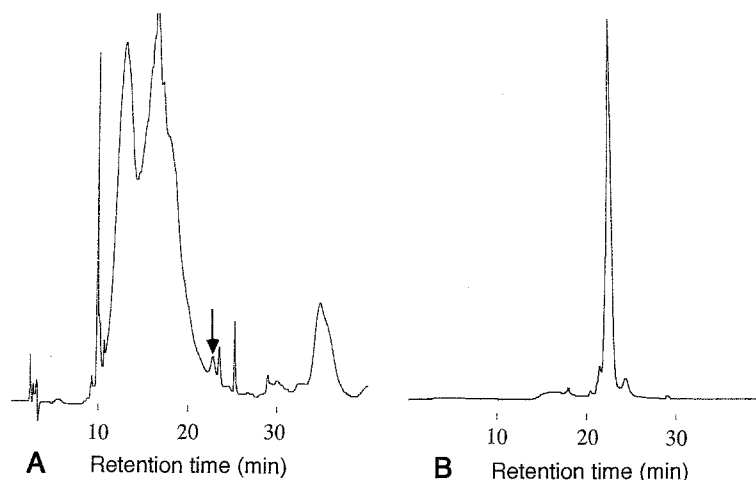


FIG. 3. HPLC patterns of the protein extracted from household gloves (**A**) and the separated lysozyme (**B**). The protein was eluted with a linear gradient of CH_3CN (10% to 90% during 40 minutes) in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min, and the absorbance at 280 nm was monitored. The vertical arrow in the pattern (**A**) indicates the peak corresponding to the lysozyme.

(pH 7.4) for 30 minutes at room temperature. After filtration, the dissolved protein was precipitated by addition of $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation, and the sample was allowed to stand overnight at room temperature. The precipitate was collected by filtration and re-dissolved in 50 ml of water. This solution was applied to a Sephadex G-25 column (3.0×40 cm) and eluted with 20 mmol/L acetate buffer (pH 5.0). Every 10 ml fraction was checked by measuring total protein concentration and lysozyme activity. The fractions (nos. 12 to 17) with lysozyme activity were collected, and the combined solution was applied to a carboxymethyl cellulose (CMC) column (1.6×25 cm), which was pre-equilibrated with 20 mmol/L acetate buffer (pH 5.0). The column was washed with 90 ml of the same buffer. The binding protein was then eluted with 350 ml of the same buffer by changing the NaCl concentration from 0 to 0.5 mol/L in an exponential gradient mode. Every 10 ml fraction was checked by measuring total protein concentration and lysozyme activity, and the fractions (nos. 23 to 30) in which lysozyme activity was concentrated were combined and lyophilized. The obtained, partially purified lysozyme was dissolved in 5 ml of 3% acetic acid and purified further by passing it through a Sephadex G-50 column (2.6×65 cm). The elution was carried out with 3% acetic acid, and the fractions (10 ml each, nos. 14 to 16) with lysozyme activity were combined. The solvent was removed by lyophilization to provide a fluffy powder of the separated lysozyme; yield was 5.2 mg.

Reverse-phase high-performance liquid chromatography

For high-performance liquid chromatography (HPLC) analysis of the extract from the household gloves and the separated lysozyme, a Wakosil-II 5C18 column ($4.6 \times$

150 mm; Wako Chemical, Osaka, Japan) was used in conjunction with an SP8800 HPLC pump (Spectra-Physics, San Jose, Calif.). The elution was performed with a linear gradient of CH_3CN in 0.1% trifluoroacetic acid from 10% to 90% during 40 minutes. The flow rate was 1 ml/min, and the eluate was monitored at 280 nm.

SDS-PAGE and immunoblotting

SDS-PAGE was performed with an ATTO AE-6450 type electrophoresis system (ATTO Corporation, Tokyo, Japan). By monitoring with HPLC, the extracts and solution of the separated lysozyme were properly concentrated to obtain similar strength of the visible protein bands in every lane after staining. The total protein concentrations of the applied sample solutions were as follows: separated lysozyme, 0.19 mg/ml; household gloves, 11 mg/ml; surgical gloves, 5.9 mg/ml; and ammoniated latex, 0.57 mg/ml. Each sample solution was diluted with an equal volume of electrophoresis sample buffer (pH 6.5) containing 2.5% SDS and 5% 2-mercaptoethanol or dithiothreitol. Reduction was carried out by keeping the diluted solution in a boiling water bath for 3 minutes. Thereafter, 10 μl of each solution was applied to two sheets of SDS-polyacrylamide gel (10% acrylamide, 3% stacking gel). Electrophoresis was performed at a constant voltage of 120 V. The separated proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Calif.) by using a semi-dry type ATTO horizontal-blot system (ATTO Corporation) with an electroblotting buffer (25 mmol/L Tris, 150 mmol/L glycine, and 20% methanol; pH 8.3). For total protein detection, one membrane was stained with Amido Black 10B. The other membrane was used for immunoblotting. It was washed three times for 5 minutes in 0.1% Tween-20 containing Tris-buffered saline (pH 7.6) and then incubated for 1 hour in blocking buffer. Thereafter,

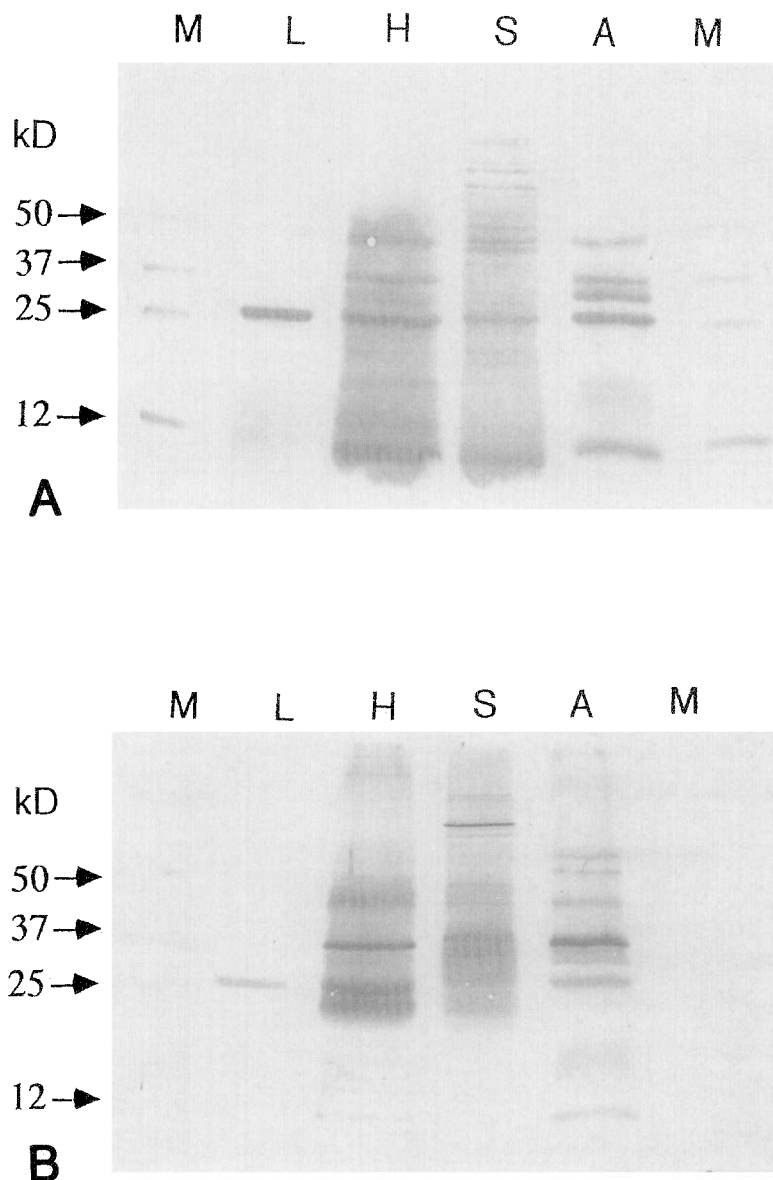


FIG. 4. **A**, SDS-PAGE of the three extracts and the separated lysozyme. *M*, Marker; *L*, separated lysozyme; *H*, household gloves; *S*, surgical gloves; *A*, ammoniated latex. All the samples were reduced in 2-mercaptoethanol. The separated protein was transferred onto a nitrocellulose membrane and stained with Amido Black 10 B for total protein detection. **B**, Immunoblotting of the three extracts and the separated lysozyme with a sensitized housewife's serum. The membrane was incubated with the patient's serum and then with mouse anti-human IgE. Membrane-binding mouse antibodies were visualized with enzyme immunoassay.

the membrane was incubated at room temperature overnight with the serum of a patient or a control subject, which had been diluted 5 times with the blocking buffer. It was further incubated at room temperature for 20 minutes with the ascites form of mouse anti-human IgE (Zymed Laboratories, San Francisco, Calif.), which had been diluted 2000-fold with the blocking buffer. The membrane-binding mouse antibodies were visualized by

using a blotting detection kit that utilizes biotin-streptavidin interaction (Amersham International Ltd., Little Chalfont, Bucks, U.K.).

Isoelectric focusing

The pI of the separated lysozyme was determined with pH 3-10 Bio-Lyte (Bio-Rad Laboratories) and Protein Test Mixture for pI determination (Serva Feinbio-

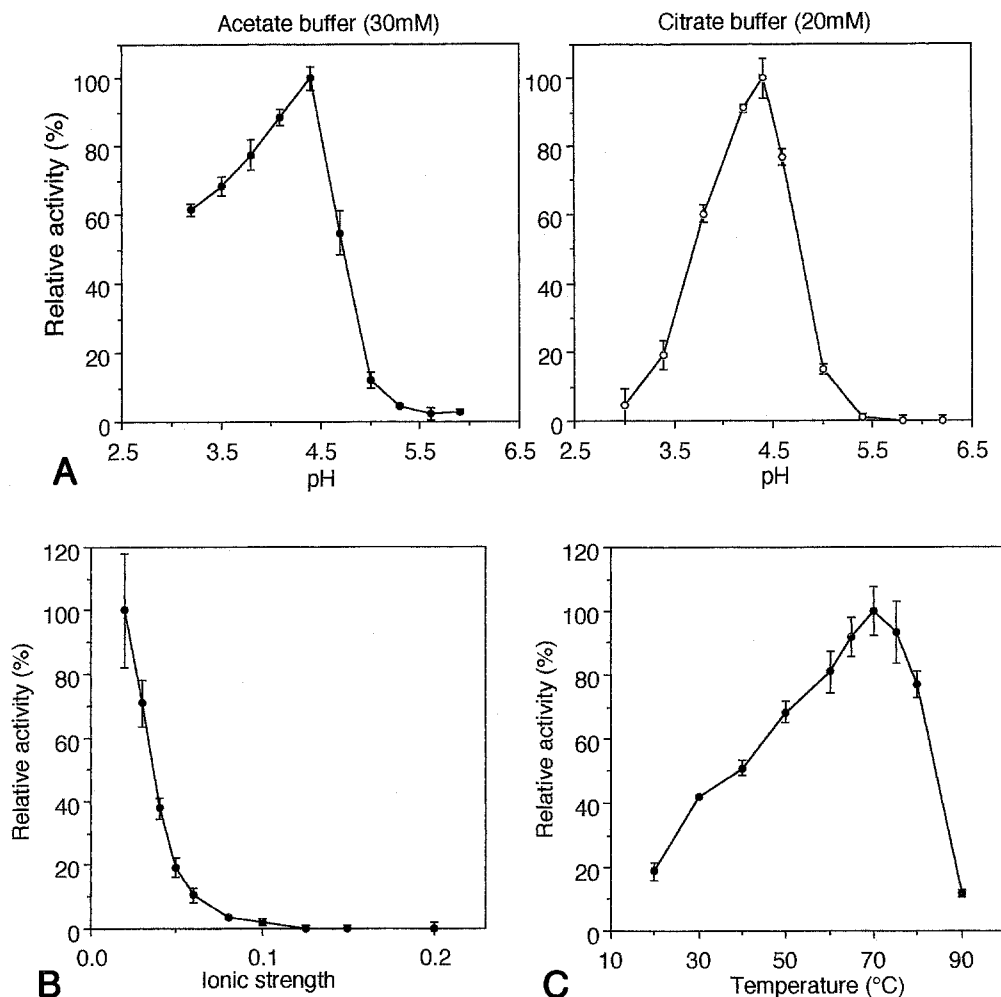


FIG. 5. **A,** pH activity profiles of the separated lysozyme against *M. lysodeikticus* cell walls. In 2.5 ml of 30 mmol/L acetate buffer, 0.2 μ g of the lysozyme was reacted with 0.5 mg of the cell walls; and in 2.5 ml of 20 mmol/L citrate buffer, 0.4 μ g of the lysozyme was reacted with 0.5 mg of the cell walls. All reactions were conducted at 60°C for 20 minutes. The decreases in absorbance at 570 nm were corrected by subtracting the control value and expressed as a percentage of the maximum decrease. **B,** Effect of ionic strength on activity of the separated lysozyme. In 2.5 ml of 20 mmol/L acetate buffer (pH 4.4), 0.2 μ g of the lysozyme was reacted with 0.5 mg of the cell walls. The ionic strength of the reaction mixture was adjusted by adding NaCl. The lysozyme was omitted to obtain the control values at the respective ionic strength. All reactions were conducted at 60°C for 20 minutes. **C,** Effect of temperature on activity of the separated lysozyme. In 2.5 ml of 30 mmol/L acetate buffer (pH 4.4), 0.2 μ g of the lysozyme was reacted with 0.5 mg of the cell walls for 20 minutes. All data represent means $\pm$ SD of three separate experiments.

chemica GmbH & Co., Heidelberg, Germany) with an AE-3230 type isoelectric focusing system (ATTO Corporation).

RESULTS

Lysozyme activity of extracts

The lysozyme activity of the extracts was examined under the condition in which the total protein

concentration of every extract was almost identical (approximately 1.5 mg/ml). All three extracts exhibited strong cell suspension clearing activity, that is, lysozyme activity. The rate of clearance was especially high when the extract from the examined lot of ammoniated latex was applied to the assay system (Fig. 1). The specific activities of the extracts were different from each other.

Separation of a lysozyme eluted from household gloves

A lysozyme eluted from household gloves was separated by measuring the cell suspension clearing activity. The protein in the extract from the household gloves was resolved into two major peaks when it was applied to a CMC cation-exchange column at pH 5.0 and eluted with an exponential gradient of NaCl (Fig. 2, A). The acidic protein that did not adsorb on the CMC stationary phase occupied about 90% of the total protein in the extract, but it exhibited only weak lysozyme activity. In contrast, the adsorbed protein was only 10% of the total protein, but it represented most of the lysozyme activity in the extract. The protein in the second peak was further resolved into two major peaks by passing it through a Sephadex G-50 column (Fig. 2, B). Although each peak contained an approximately equal amount of protein, all the lysozyme activity was detected in the first peak. The purity of the lysozyme separated in this way was checked with reverse-phase HPLC. Fig. 3 indicates how well the lysozyme was purified from the original extract, though it still contained a very small amount of impurities. The relatively long retention time of the lysozyme could represent its hydrophobic nature. The pI of the separated lysozyme was estimated to be 9.5 with isoelectric focusing.

SDS-PAGE of extracts and the separated lysozyme

All the samples applied to the SDS-PAGE shown in Fig. 4, A were reduced in 2-mercaptoethanol. Replacement of the reducing agent by dithiothreitol did not produce any appreciable changes regarding the patterns of protein bands in SDS-PAGE (data not shown). The proteins in the extracts from the surgical gloves and the household gloves emerged as strongly smeared patterns (Fig. 4, A). Presumably, these are due to numerous fragmented or damaged proteins formed during the manufacturing processes. Nevertheless, we could see strong common bands at 27 kd and around 12 kd in every lane to which an extract was applied. Besides these common bands, four clear bands (33, 34, 37, and 45 kd) were seen in the lane for the extract from ammoniated latex. These bands were also seen in the lane for the household gloves when a larger amount of the protein was applied to the gel. The separated lysozyme appeared as a single strong band at 27 kd. Strong lysozyme activity of the three extracts can be explained by this common 27 kd protein.

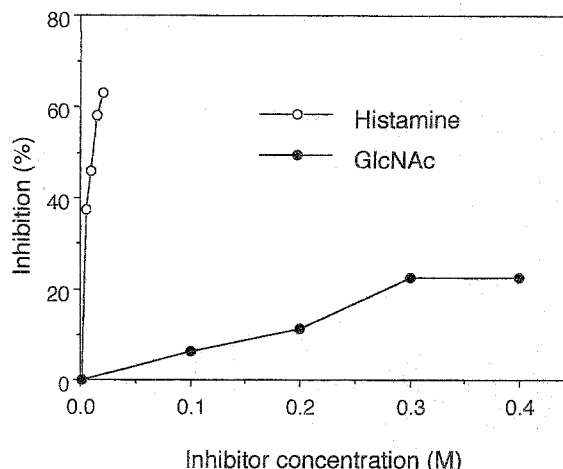


FIG. 6. Inhibitory effects of histamine and *N*-acetyl-D-glucosamine (GlcNAc) on activity of the separated lysozyme against *M. lysodeikticus* cell walls. In 3.0 ml of 30 mmol/L acetate buffer (pH 4.4), 0.5 μ g of the lysozyme was reacted with 0.6 mg of the cell walls (60°C, 20 minutes). Each reaction contained the respective amounts of histamine, and the ionic strength was adjusted by adding NaCl. In the case of GlcNAc, 0.2 μ g of the lysozyme was reacted with 0.4 mg of the cell walls in 30 mmol/L acetate buffer (2.0 ml, pH 4.4) where the respective amounts of GlcNAc were included. The decreases in absorbance at 570 nm were corrected by subtracting each control value. All the data are means of three separate experiments and are expressed as percentage of inhibition.

Immunoblotting of extracts and the separated lysozyme

The proteins separated by SDS-PAGE were examined for their allergenicity by immunoblotting. The IgE in the serum of a sensitized housewife bound to several proteins (12, 23, 27, 33, 34, 37, and 45 kd), and the separated lysozyme was also recognized by the IgE (Fig. 4, B). This fact indicated that multiple allergenic proteins existed in the extracts and that one of the rubber latex allergens was the 27 kd protein with lysozyme activity. Many of the allergenic protein bands were commonly observed in the lanes, but the densities of these bands were different from sample to sample. IgE antibodies in another patient's serum also recognized the 27 kd protein. None of the protein bands appeared when the control serum or the plasma was used instead of the patient's serum (data not shown).

Enzymatic properties of the separated lysozyme

The separated lysozyme showed its maximum activity at pH 4.4 and 70°C in acetate buffer of 0.03

TABLE I. Summary of the physical and enzymatic properties of lysozymes

	Separated lysozyme	Fig lysozyme*	Papaya lysozyme†	Hevamine A‡	Egg white lysozyme
Molecular weight (kd)	27	29	25	29	14
pI	9.5	>7.1	10.5	9.0	11.0
pH optimum for lysis of <i>M. lysodeikticus</i>	4.4	4.5	4.6	4.0	9.2
Ionic strength optimum for lysis of <i>M. lysodeikticus</i>	<0.02	0.015-0.025	0.04-0.07	0.15	0.10-0.30*
Temperature optimum for lysis of <i>M. lysodeikticus</i>	70° C		70-80° C	50° C	55° C‡
Percentage of inhibition of lysis of <i>M. lysodeikticus</i> in 10 mmol/L histamine	46	85	40		30*
Concentration of <i>N</i> -acetyl-D-glucosamine (mol/L) to produce 50% inhibition of lysis of <i>M. lysodeikticus</i>	>0.4 (23% inhibition at 0.4)	>0.4 (20% inhibition at 0.4)	>0.35 (40% inhibition at 0.35)		0.007*

*Data from Glazer et al.¹⁷†Data from Howard and Glazer.¹⁶‡Data from Tata et al.³²

ionic strength (Fig. 5, *A* and *C*). The lower the ionic strength of the reaction mixture was, the stronger was the activity (Fig. 5, *B*). Moreover, the activity was effectively inhibited in histamine (60% inhibition at 0.02 mol/L, Fig. 6) but not inhibited in *N*-acetyl-D-glucosamine (20% inhibition at 0.4 mol/L, Fig. 6). As shown in Table I, most of these enzymatic features coincided with those of fruit lysozymes and a lysozyme from *H. brasiliensis* (hevamine A). In contrast, these features were meaningfully different from those of egg white lysozyme.

DISCUSSION

We demonstrated that one of the rubber latex allergens was a 27 kd protein with lysozyme activity. All the examined samples (ammoniated latex, surgical gloves, and household gloves) contained the 27 kd protein. This result may indicate general contamination of latex products by the lysozyme. Many reports describe latex allergens of about 27 kd.²⁵⁻³⁰ These previously mentioned allergens could be identical to the lysozyme separated in this study, although none of the reports presented until now have described enzymatic activities of the allergenic proteins. As seen in the reverse-phase HPLC pattern (Fig. 3) and the temperature dependence of the lysozyme activity (Fig. 5, *C*), the relatively hydrophobic and heat-

resistant nature of this lysozyme could partly explain its persistent survival through the manufacturing processes of latex gloves.

Lysozymes from *H. brasiliensis* were first isolated by Archer³¹ and named hevamine A and B. Hevamines were studied in detail by Tata et al.,^{18, 32} and their primary structures were determined by Jekel et al.³³ They are basic proteins (pI, approximately 9.0) with a molecular weight of 29 kd and homologues to a pathogenesis-related chitinase from cucumber.³⁴ Besides the hevamines, six kinds of chitinases/lysozymes have been isolated from *H. brasiliensis* by Martin.³⁵ Precise structural analysis is necessary to ascertain whether the separated lysozyme corresponds to one of these previously isolated lysozymes.

The properties of the separated lysozyme were very similar to those of fig,¹⁷ papaya,^{15, 16} and other plant lysozymes^{14, 19, 36-39} (Table I). This finding might indicate their structural similarity. Provided that they have common antigenic determinants, lysozymes can be a cause of the cross-reaction⁶⁻⁹ between rubber latex and fruits.

The separated protein is most likely to be a defense-related protein biosynthesized in a rubber tree. It had characteristic bacteriolytic activity. It is known that the amount of defense-related proteins in laticifers markedly increases when a rubber tree

faces phytohormone application, wounding, and other biotic or abiotic stresses.^{13, 40, 41} For effective mass production of latex, genetically selected rubber trees have been repeatedly cut and treated with phytohormones. We can imagine that the latex produced in this way may contain a large amount of defense-related proteins, including the lysozyme, and that these proteins or their fragments would remain in the final latex products.

At the International Latex Conference held in Baltimore in 1992,⁴² Dr. Slater reviewed latex allergy and said that the total protein amounts of extracts from latex products might generally correlate with their allergen contents as determined by RAST or ELISA. This suggests that reducing the total extractable protein of the products should be effective in decreasing the incidence of latex allergy. However, Turjanmaa et al.⁴³ reported cases in which this idea was not applicable. Their patients strongly reacted to a product with low protein content rather than products with higher protein contents. This discrepancy may be due to the conditions that proteins eluted from individual latex products are not always of the same composition and that allergens for individual patients are not always the same proteins.⁴⁴ In such circumstances, the allergenicity of a latex product for a sensitized patient should not necessarily correlate with its total protein content. In his recent review article, Slater⁴⁵ also pointed out the ineffectiveness of the total protein measurements for allergenicity estimation compared with immunologic measurements. We estimate that every protein eluted from the latex products, which will have direct contact with the mucosa or body fluids, is a potential allergen and that total protein measurements are important for measuring such potential allergens. The main weak points of the standard total protein measurements are insensitivity and interference by nonprotein substances. We demonstrated the common presence of an allergenic 27 kD lysozyme in latex gloves. If the measurement were carried out under optimal conditions, detection of some enzyme activities would be more specific and sensitive than total protein measurements. Although the lysozyme activity test alone cannot provide a conclusive estimation of allergenic proteins, the combination of a total protein measurement and the lysozyme activity test would provide a more sensitive and reliable estimation than a total protein assay alone. This method does not require the reliable and representative patients' sera that are indispensable in immunologic methods. Other characteristic enzyme activities of defense-related

proteins¹¹ may also be able to be used in this method.

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