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Plant Defense-related Proteins Eluting from Latex Gloves and Ammoniated Latex: Potential Latex Allergens

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Hypersensitivity reactions caused by latex products are ascribed to proteins eluting from the products, but the detailed properties of the responsible proteins have not been revealed yet. We have speculated that defense-related proteins biosynthesised in a rubber tree comprise the major part of the allergens. The aim of this study is to ascertain whether defense-related proteins are extracted from latex gloves and raw ammoniated latex. Characteristic hydrolytic-enzyme activities of defense-related proteins were used for their detection, and we verified the existence of esterase (EC 3.1 1 1), chitinase (EC 3.2.1 14), lysozyme (EC 3.2.1.17) and β-1,3-glucanase (EC 3.2.1 6) in all the latex extracts. The enzymes other than esterase were similar to reported latex allergens in heat stability and molecular weights These results indicate the elution of defense-related proteins from latex products and their potential participation in latex allergy.

There are many reports describing allergenic reactions to latex health care products[1,2]. This type-I allergy has proved not to be due to chemical additives, which are causative for type-IV allergy, but due to a number of proteins eluting from latex products. It is thought that these proteins originate from the rubber tree and remain in the products even after the manufacturing processes, but their detailed properties and biological roles in the rubber tree have not been clarified yet. Another notable point of latex allergy is the unaccountable cross-reaction between rubber latex and fruits or vegetables[3-8]. This fact gave us a clue to suspect defense-related proteins to be latex allergens.

It is well known that defense-related proteins are remarkably induced in a plant as a result of defense responses against stress conditions such as pathogen attack, phytohormone application and wounding[9-11].

Some defense-related proteins are also called 'pathogenesis-related proteins' because of their clear induction after pathogen attack, and others have characteristic hydrolytic-enzyme activities that should relate to the host-pathogen interactions[12]. Interestingly, serological relationships or amino acid sequence similarities have been found among the various defense-related proteins obtained from different plant species beyond the frame of classes[9-11]. Moreover, Breiteneder *et al.* reported a 55% homology of the amino acid sequence between a birch pollen allergen and a defense-related protein in a pea[13]. Provided that plant defense-related proteins are constructing the major part of the latex allergens, we can explain the curious cross-reactivity between rubber latex and various fruits, vegetables or pollens. Recently, Beezhold *et al.* reported that hevein preproteins were latex allergens[14]. Hevein preproteins are defense-related proteins of a

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rubber tree[15]. Therefore, their observation agrees with our hypothesis.

The object of this study is to verify the existence of defense-related proteins in raw ammoniated latex as well as commercially available surgical gloves and household gloves. For detecting the defense-related proteins in the sample extracts, we measured their characteristic hydrolytic-enzyme activities. We also examined the extracted defense-related proteins in heat stability and molecular weight.

MATERIALS AND METHODS

Ammoniated latex was kindly provided by Sumitomo Rubber Industries, Ltd., Japan. A commercially available brand of sterilised surgical gloves (*Triflex*, Baxter, USA) and non-sterilised household gloves (*DUNLOP Home Products*, Japan) were examined as representative latex gloves. Reagent grade chemicals were used in all experiments.

Extraction of Protein from Ammoniated Latex

One volume of ammoniated latex was diluted with one volume of water and made weakly acidic (*ca.* pH 6) by adding acetic acid. The separated rubbery solid was discarded, and the remaining solution was filtered. The protein in the filtrate was precipitated by addition of $(NH_4)_2SO_4$ to 70% saturation. After being allowed to stand overnight, the precipitate was collected and desalted by passing through a Sephadex G-25 column (3.0 × 40 cm). Finally, the desalted and lyophilised protein was dissolved in 10 mM phosphate buffer (pH 6.5) to obtain a sample solution (20 mg/ml); the total protein concentration of which was measured by the bicinchoninic acid (BCA) method[16]. For heat-denaturation of the extracted protein, the prepared sample solution was heated in a boiling water bath for 20 min.

Extraction of Protein from Latex Gloves

The surgical gloves or the household gloves were cut into small pieces and extracted with phosphate buffered saline (pH 7.4) for 2 h at room temperature. The extracted protein was precipitated, desalted and dissolved as described above to obtain a sample solution (20 mg/ml). The total protein amounts extracted from the latex gloves were as follows: surgical gloves, 0.55 mg/g (5.0 mg/glove); household gloves, 1.4 mg/g (11 mg/glove).

Detection of Enzyme Activities

The following enzyme activities in each extract were measured. These are the representative hydrolytic-enzyme activities of defense-related proteins[12]. All the detected enzymes were expressed by specific activity to compare the extracts from latex gloves with the extract from ammoniated latex.

Lysozyme (EC 3.2.1.17) activity was turbidimetrically detected following the method described by Shugar[17]. *Micrococcus lysodeikticus* cell walls were used as a substrate. One unit was defined as a 0.001 decrease in absorbance at 570 nm per 1 min. Chitinase (EC 3.2.1.14) activity was colorimetrically detected using the method reviewed by Boller and Mauch[18]. Colloidal chitin was used as a substrate. The amount of enzyme producing 1 mmol *N*-acetylglucosamine equivalent per 1 min was defined as 1 unit. β-1,3-Glucanase (EC 3.2.1.6) activity was detected as described by Kombrink *et al.*[19]. Laminarin was used as a substrate. One unit was defined as the enzyme activity catalysing the formation of 1 mmol glucose equivalent per 1 min. Esterase (carboxyesterase, EC 3.1.1.1) activity was detected according to the method described by Lynn and Clevette-Radford[20]. *p*-Nitrophenyl acetate was used as a substrate. One unit was defined as a 0.001 increase in absorbance at

410 nm per 1 min. α-amylase (EC 3.2.1.1)[21], cellulase (EC 3.2.1.4)[22] β-*N*-acetylglucosaminidase (EC 3.2.1.30)[23] and protease[20] activities were assayed using starch azure, cellulose azure, *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (*p*-nitrophenyl-GlcNAc) and azocasein as a substrate, respectively.

Gel-filtration Study

The proteins extracted from ammoniated latex were applied to a Sephadex G-75 column (2.6 × 77 cm) and eluted with 0.3 *M* aqueous $(NH_4)_2CO_3$. Every collected fraction (10 ml) was assayed for the enzyme activities and total protein concentration. The column was also calibrated with molecular weight standards: bovine serum albumin (66 kD), ovalbumin (45 kD), pepsin (35 kD), trypsinogen (24 kD) and β-lactoglobulin (18 kD).

RESULTS

Among the enzyme activities assayed, lysozyme, chitinase, β-1,3-glucanase and esterase activities were detected in all three extracts (*Table 1*). Specific lysozyme activities of the three extracts were similar. On the other hand, specific chitinase and β-1,3-glucanase activities were considerably different from sample to sample. Specific esterase activities differed a little among the extracts. The extract from the household gloves showed stronger specific chitinase and β-1,3-glucanase activities than the extract from the surgical gloves. Each extracted total enzyme activity was also different between the two latex gloves. The extract from ammoniated latex showed relatively stronger specific β-1,3-glucanase and esterase activities, although its specific chitinase activity was not so strong. Even if the proteins extracted from ammoniated latex were denaturated by heating (*100°C, 20 min*), lysozyme, chitinase and β-1,3-glucanase activities survived to measurable extent. This demonstrated the comparatively heat-stable feature of these enzymes. On the other hand, the esterase activity of the extract was completely lost after the heat-denaturation.

As long as the detection was tried with the methods mentioned in the experimental section, none of the β-*N*-acetylglucosaminidase, α-amylase, cellulase and protease activities were detected in any of the extracts.

In the Sephadex G-75 gel-filtration study, lysozyme, chitinase and β-1,3-glucanase activities were detected at almost the same elution volume, which corresponded to 25 kD – 40 kD (*Figure 1b–d*). The esterase activity was detected at a smaller elution volume corresponding over 60 kD (*Figure 1e*). The total protein elution profile (*Figure 1a*) was quite different from the elution profiles of the enzymes. The delayed elution of a large part of the protein indicated that most of the protein had an unexpectedly small molecular weight.

DISCUSSION

It was verified that the extracts from the latex gloves and the raw ammoniated latex possessed several hydrolytic-enzyme activities that are characteristic to defense-related proteins[12]. The amounts of these enzymes differed from sample to sample. This may be due to variations in the raw latex used and the manufacturing processes of the gloves. Though the sample number was small, these experimental results suggested the general contamination of defense-related proteins in a wide variety of latex products. Several defense-related proteins detected were comparatively heat-stable and had relatively low molecular weights (25kD – 40 kD). These features meaningfully meet the reported features of latex allergens[24,25]. More detailed biological and immunological studies have to be done to confirm that these defense-related proteins are latex allergens, while it was

TABLE 1. HYDROLYTIC-ENZYME ACTIVITIES OF PLANT DEFENSE-RELATED PROTEINS IN THE LATEX EXTRACTS

Enzyme	Substrate	Household gloves[d]	Surgical gloves[d]	Ammoniated latex	Ammoniated latex (100°C, 20 min)
Lysozyme[a] (EC 3.2.1.17)	*M lysodeikticus* cell walls	208±0	201±2	204±11	41±1
Chitinase[b] (EC 3.2.1.14)	Colloidal chitin	126±9	23±2	47±10	3±0
β-1,3-glucanase[b] (EC 3.2.1.6)	Laminarin	404±62	74±4	653±58	19±4
β-*N*-acetylglucosaminidase (EC 3.2.1.30)	*p*-nitrophenyl-GlcNAc	n.d.[c]	n.d.	n.d.	n.d.
α-amylase (EC 3.2.1.1)	Starch azure	n.d.	n.d.	n.d.	n.d.
Cellulase (EC 3.2.1.4)	Cellulose azure	n.d.	n.d.	n.d.	n.d.
Esterase[a] (EC 3.1.1.1)	*p*-nitrophenyl acetate	457±6	502±9	597±15	n.d.
Protease	Azocasein	n.d.	n.d.	n.d.	n.d.

[a]U/mg protein (mean ± S.D., n = 3)
[b]MU/mg protein (mean ± S.D., n = 3)
[c]Not detected
[d]Extracted total protein amounts: household gloves, 1.4 mg/g (11 mg/glove); surgical gloves, 0.55 mg/g (5.0 mg/glove)

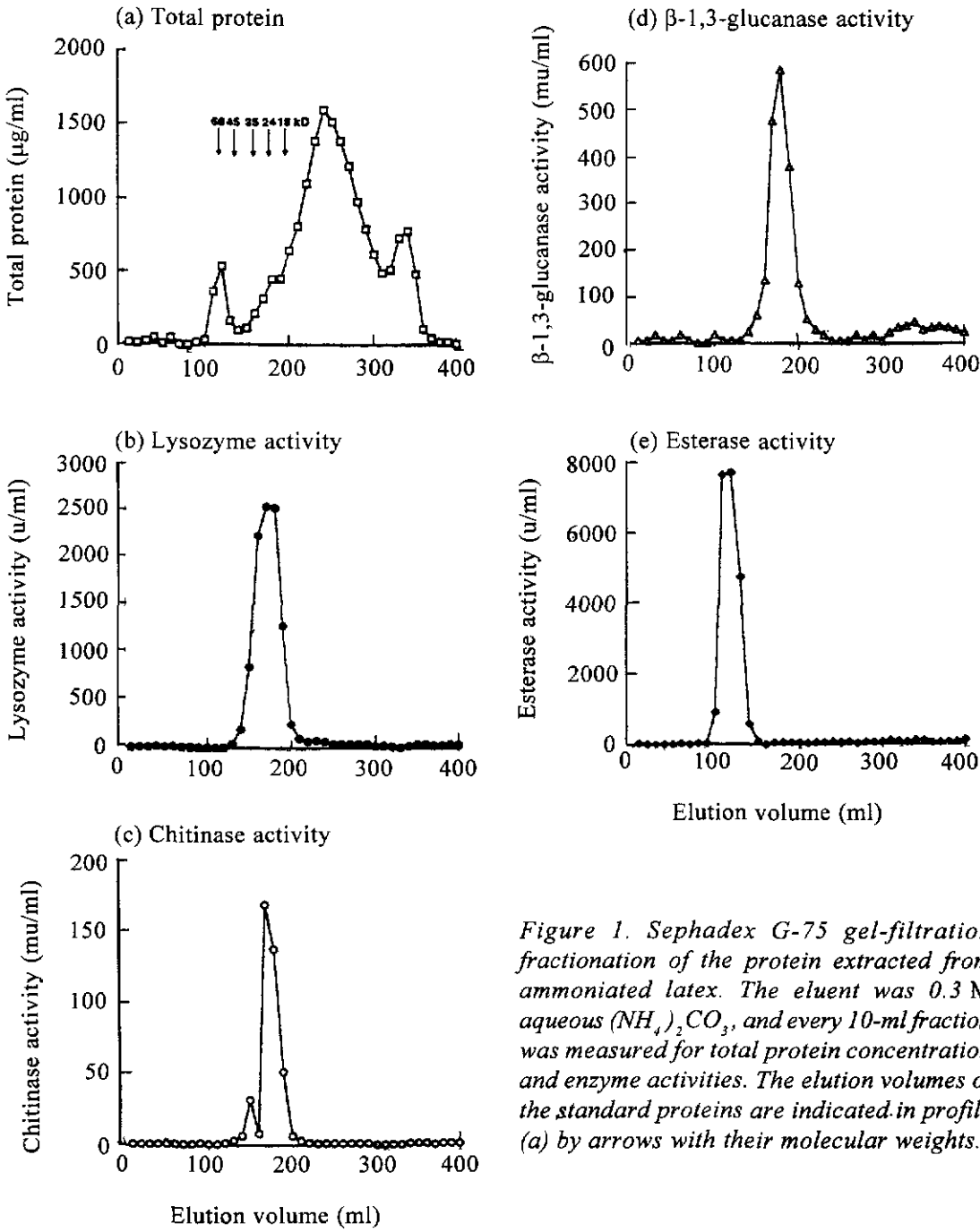

Figure 1. Sephadex G-75 gel-filtration fractionation of the protein extracted from ammoniated latex. The eluent was 0.3 M *aqueous* $(NH_4)_2CO_3$, *and every 10-ml fraction was measured for total protein concentration and enzyme activities. The elution volumes of the standard proteins are indicated in profile (a) by arrows with their molecular weights.*

previously proven that a 27kD lysozyme was a latex allergen[26]. Most of the proteins extracted from ammoniated latex had unexpectedly small molecular weight. This may be due to degradation of large proteins under alkaline conditions. We cannot neglect these small proteins as potential latex allergens, too.

We have speculated that the recent effective latex-production methodology would influence the transcription levels of defense-related genes in rubber tree cells and that the mass of the defense-related proteins thus induced may comprise the major part of the allergens eluting from latex products. Defense-related proteins function to strengthen the impaired plant cell wall, biosynthesising small anti-microbial molecules called phytoalexins and attacking plant pathogens where some plant pathogens contain chitin or peptidoglycan as constituents of their cell walls[9-12]. The expression of defense-related proteins is not confined to the impaired locality but systemic induction is observed following their localised expression[27]. Although very small amounts of several defense-related proteins are steadily expressed in a healthy plant, their marked induction is one of the proofs indicating that the plant is suffering from stress conditions. Rubber latex is sap oozing from the bark of a rubber tree. In plantations, rubber trees have been repeatedly cut and treated with phytohormones or phytohormone precursors for efficient latex production. Moreover, the rubber trees for planting have been genetically selected for mass production of the latex. All of these factors could result in further induction of defense-related proteins in the rubber trees[15,28,29]. In a comparative study, higher transcription levels of defense-related genes in rubber latex than in leaves were confirmed, and this also depended on phytohormone application and wounding[30]. It is likely that efficient latex production and induction of the defense-related proteins are synchronous phenomena. Possibly, latex oozing can be seen as one visible phenomenon of defense responses of a rubber tree. If these markedly induced defense-related proteins remain in latex products, they would construct potential latex allergens judging from the usage of products such as surgical gloves and catheters. Recent marked prevalence of latex allergy[31] could correlate partly to the recent efforts for economically effective latex production. This point should be more carefully investigated.

Measuring the characteristic enzyme activities of defense-related proteins may be applicable to safety tests of latex medical products[32], because detection of some enzyme activities is more specific and sensitive than the generally conducted total protein assays. Although the enzymatic assay alone cannot provide conclusive decision about the safety of a latex product, the combination of a total protein assay and an enzymatic assay would provide useful information.

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