

Proteomic Analysis of Putative Latex Allergens

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Key Words

Proteomics · Allergenomics · Latex allergy · Allergen · Two-dimensional gel electrophoresis · Immunoblotting · In-gel digestion · Mass spectrometry · Database search

Abstract

Background: Extensive analysis of allergenic proteins is generally time-consuming and labor-intensive. Accordingly, a rapid and easy procedure for allergen identification is required. As sequence information on proteins and genes is accumulated in databases, it is becoming easier to identify a candidate protein using proteomic strategies, i.e. two-dimensional gel electrophoresis, site-specific fragmentation, mass spectrometry and then database search. In this study, we evaluated the usefulness of a proteomic strategy for identifying putative allergens through its application to latex proteins. **Methods:** Latex proteins were separated with two-dimensional gel electrophoresis, and putative allergens were visualized by IgE immunoblotting using pooled serum from latex-sensitive patients. The IgE-interactive proteins were cut out from the negatively stained two-dimensional gel and subjected to in-gel digestion by trypsin. Then the resulting peptides were analyzed with mass spectrometry. Based on the mass spectrometric data we obtained, the allergen candidates were assigned by a database search. **Results:** Five previously

reported allergens and five new allergen candidates were identified with the proteomic approach without isolating the individual proteins. Less than 1 mg of crude latex protein was sufficient for the entire protocol. Because plural proteins can be processed in parallel, analysis of about 50 IgE-interactive proteins was accomplished within 1 week. **Conclusions:** Analysis of putative allergens with proteomic strategies (allergenomics) is a promising avenue for rapid and exhaustive research. The high resolving power of two-dimensional gel electrophoresis is superior to conventional gel electrophoresis. Moreover, the notable sensitivity and speed of mass spectrometry have pronounced advantages over the N-terminal sequencing that has generally been used for protein identification.

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Introduction

Latex allergy has become a serious problem in medical settings since the late 1980s [1]. Intensive research on allergenic proteins responsible for this immediate-type hypersensitivity has revealed 13 officially registered latex allergens to date. It is notable that many of the latex allergens have something to do with the defense mechanisms of the rubber tree [2–6]. Defense-related proteins of higher plants are relatively conserved in the course of evolu-

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1018–2438/04/1351–0003\$21.00/0

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tion and therefore share the partial sequences indispensable for their function. Such a conserved sequence is sometimes called a functional domain. For example, hevein is one of the major latex allergens and contains a chitin-binding domain that is indispensable for its antifungal activity. A homologous chitin-binding domain has already been found in several antifungal proteins from various plant species. Class I chitinase is a representative containing a hevein-like domain. Because of this shared domain, class I chitinase is expected to be allergenic for hevein-sensitized patients [4–6]. Cross-reactivity between class I chitinase and hevein was indeed confirmed by several research groups [7, 8]. However, hevein cannot explain all of the cross-reactions of latex-allergic people to a wide range of fruits and vegetables (latex-fruit syndrome). Other undiscovered minor cross-reactive latex allergens must play a role in the latex-fruit syndrome. We need a powerful methodology in order to investigate such minor but significant allergens as well as major but hidden allergenic proteins.

As sequence data of proteins and genes are accumulated in databases, it is becoming easy to analyze candidate proteins rapidly and comprehensively using an effective separation technique coupled with mass spectrometry [9, 10]. This new strategy, aiming at comprehensive analysis of total proteins (proteome) in a cell or tissue, is now referred to as 'proteomics'. For the application of proteomic strategies to allergenic proteins in particular, the term 'allergenomics' has been proposed. In conventional approaches, we needed to isolate each allergenic protein to identify and fully characterize it. This process is usually complex, time-consuming and labor-intensive. However, in allergenomics, complex protein mixtures are effectively resolved with two-dimensional gel electrophoresis or the equivalent, and IgE-interactive proteins are detected by subsequent immunoblotting using pooled patients' serum. We can easily identify putative allergens through their site-specific degradation and the subsequent mass spectrometric analysis of the fragmented peptides and database search. The overall procedure is expected to be quicker and less labor-intensive than any procedures used to date. Moreover, we can analyze numerous IgE-interactive proteins in parallel. Semiautomatic analysis of allergen candidates is also possible. From these features, allergenomics is expected to be a key technique for fast, effective and comprehensive analysis of IgE-interactive proteins.

A few examples of the application of proteomic strategies to pollen allergens have been published in recent years [11, 12]. However, the effectiveness of allergenom-

ics has not been fully demonstrated yet. In this study, we evaluated the usefulness of allergenomics through the analysis of latex proteins. Latex allergens have been extensively studied using traditional biochemical methods [1]. Nevertheless, minor latex allergens that have been overlooked and new cross-reactive allergens pertinent to the latex-fruit syndrome remain to be scrutinized. The advantages of allergenomics in analyzing these troublesome allergens are demonstrated below.

Subjects and Methods

Subjects

Six latex-sensitive patients and four control volunteers provided their serum for this study. Latex-allergic patients (table 1) were diagnosed from their clinical history of allergy to natural rubber-containing products, positive results of a skin prick test (SPT) using crude extract of latex proteins [13] and scores from a latex-specific IgE radioallergosorbent test (CAP RAST, Pharmacia Diagnostics). When a subject with a convincing clinical history of latex allergy did not show positive reactions on the SPT, a gradual provocation test to a brand of highly allergenic latex gloves was carried out for definite diagnosis. Three of the control subjects were medical personnel who are exposed constantly to various kinds of products made from natural rubber. One control subject was a homemaker suffering from erythema nodosum but with no background of allergies. They were confirmed not to have a latex allergy from the diagnostic criteria mentioned above. Informed consent was obtained from all of the participants before collecting their blood.

Extraction of Latex Proteins

Latex proteins were extracted from nonammoniated latex (NAL) harvested from a rubber tree (*Hevea brasiliensis*, clone RRIM600) at the Rubber Research Institute of Malaysia. The NAL was immediately frozen at the site of collection and dispatched to Japan. By thawing and pressing the frozen latex, we obtained a brown liquid that oozed out from it. The liquid (about 100 ml) was supplemented with two tablets of protease inhibitor cocktail (Complete Mini, Roche Applied Science) and dialyzed against running water using Spectra/Por 3 membrane tubing (cutoff 3.5 kD; Spectrum Laboratories, Inc.) overnight. The dialyzate was then lyophilized and reconstituted in phosphate-buffered saline (pH 7.4). After condensation of the solution using Centriprep-10 (cutoff 10 kD; Millipore Co.), the concentrate was ultracentrifuged at 40,000 rpm for 24 h at 4°C (P70AT2 rotor and CP 65 β ultracentrifuge, Hitachi Koki Co., Ltd.). The protein content of the supernatant was determined with a BCA Protein Assay Kit (Pierce Chemical Co.) as 13.9 mg/ml. This supernatant was submitted to later study.

Two-Dimensional Gel Electrophoresis

Latex proteins (250 μ g) extracted from NAL were diluted in 250 μ l of Destreak Rehydration Solution (Amersham Biosciences) supplemented with IPG buffer (0.1%, pH 3–10, nonlinear; Amersham). This solution was used for overnight rehydration of an immobilized pH gradient gel strip (Immobiline DryStrip, Amersham; pH 3–10, nonlinear, 13 cm). Then the strip was submitted to isoelectric focusing on a Multiphor II Electrophoresis Unit (Amersham) at

Table 1. Summary of latex-allergic patients who provided their blood for IgE immunoblotting

Patient No.	Age years	Gender	Occupation	Total IgE IU/ml ¹	Latex-specific IgE IU/ml (score) ²	Latex SPT	Positive SPT	Other allergic symptoms
1	26	F	nurse	49.1	14.0 (3)	4+	Japanese horseradish	OAS to Japanese horseradish
2	34	F	housewife	1,110	4.50 (3)	4+	avocado, grapefruit	atopic dermatitis, bronchial asthma
3	22	F	dental hygienist	273	2.23 (2)	3+	tomato, spices	OAS, atopic dermatitis, pollinosis
4	25	M	medical doctor	15,500	82.0 (5)	3+	avocado	atopic dermatitis, atopic rhinitis
5	25	F	nurse	88.7	2.81 (2)	3+	–	OAS to melon
6	28	F	nurse	206	15.0 (3)	4+	–	OAS, atopic dermatitis, atopic rhinitis

OAS = Oral allergy syndrome.

¹ IgE radioimmunosorbent test (Pharmacia).

² CAP radioallergosorbent test (Pharmacia).

20 °C. Proteins were focused by applying programmed voltage as follows: 0–300 V in 1 min, 300–3,500 V in 1.5 h and 3,500 V for 7 h. Thereafter, the gel strip was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as the second dimension of electrophoresis. Before applying to SDS-PAGE, the gel strip was incubated at room temperature in 7.5 ml of equilibration buffer (Amersham) supplemented by dithiothreitol (1%) for 30 min, then in the same equilibration buffer containing iodoacetamide (1%) instead of dithiothreitol for 30 min. The gel strip was placed on top of a slab gel (10% acrylamide, 14 × 14 cm) and fixed with agarose (0.5%). The second electrophoresis was carried out by applying a constant current of 30 mA for 30 min then 70 mA for 5 h at 20 °C using an SE 600 Ruby Electrophoresis Unit (Amersham).

Immunoblotting

Two-dimensionally separated proteins were transferred onto a polyvinylidene fluoride membrane (14 × 14 cm; Immobilon-P, Millipore) using a semidry transfer cassette (ATTO Co.) by applying a constant current of 200 mA for 1.5 h. SDS (0.1%) was added to each of the discontinuous blotting buffers containing methanol (10%) for efficient transfer of proteins with high molecular weight or a basic isoelectric point. After washing with Tris-buffered saline (TBS; pH 7.4) supplemented with Tween-20 (TBS-T; 0.1%), the membrane was soaked in a chilled and degassed 10 mM NaIO₄-50 mM acetate buffer (pH 4.7) and incubated at 4 °C in the dark overnight. Then the membrane was repeatedly washed with TBS-T and incubated at room temperature in a blocking buffer (Block Ace, Dai-Nippon Pharmaceutical Co.) for 5 h. Following washing with TBS-T, the membrane was incubated overnight in the pooled patients' serum (20 ml) diluted 1/20 in TBS-T at room temperature. In the control experiment, the membrane was incubated in the pooled control serum (20 ml) diluted 1/20 in TBS-T. The next day, the membrane was washed again with TBS-T and incubated at room temperature in a solution of peroxidase-labeled affinity-purified antibodies to human IgE(ε) (Kirkegaard & Perry Laboratories, Inc.) diluted 1/10,000 in TBS-T for 1 h. After repeated washing with TBS-T, IgE-recognized protein spots were visualized using an ECL Western Blotting Detection System and Hyperfilm ECL (Amersham).

In-Gel Digestion

Two-dimensionally separated proteins on the gel were visualized by negative staining [14] using a Zinc Stain Kit (Bio-Rad Laboratories). Protein spots that were judged as IgE-interactive from the immunoblotting with pooled patients' serum were cut out from the gel (highlighted spots in fig. 1A). After treatment with Zinc Destain Solution (Bio-Rad) as instructed by the manufacturer's protocol, the protein spots were submitted to tryptic digestion using a Montage In-Gel Digest96 Kit (Millipore). Digestion proceeded at 30 °C overnight following the manufacturer's recommendations except that *n*-octyl-β-*D*-glucoside (Dojindo Laboratories) was added to the digestion mixture (final 0.1%) for efficient recovery of the fragmented peptides [15].

Mass Spectrometry and Database Search

Fragmented peptides were desalted by means of a reversed-phase microcolumn (ZipTip μC₁₈, Millipore) as instructed by the manufacturer, and eluted from the column with the help of α-cyano-4-hydroxy cinnamic acid solution on a sample plate for mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) was performed on a 4700 Proteomics Analyzer (Applied Biosystems Inc.). Positive-ion mass spectra of tryptic peptides were recorded on the reflectron mode of the instrument. The information from these spectra (peptide mass maps, fig. 2A) was used for a database search of the original proteins with the peptide mass fingerprinting (PMF) mode of a search engine (Mascot, Matrix Science). Further, tandem mass spectra (MS/MS spectra) of prominent peptide ions on the peptide mass map were recorded on the time-of-flight (TOF)-TOF mode of the instrument [16]. The information (peptide sequence tag; PST) from such tandem mass spectra (fig. 2B) was employed for a database search with the MS/MS Ion Search mode of Mascot for more precise identification of IgE-interactive proteins. National Center for Biotechnology Information (nonredundant) (NCBI nr) was selected as the first database to be searched using Mascot. When there was no candidate with enough certainty, SwissProt and Expressed Sequence Tags (EST) (*H. brasiliensis*) were employed as the second and third databases to be searched, respectively. The taxonomy of the database category was set to *Viridiplantae* (Green Plants), and carbamidomethyl cysteine was selected as a fixed modification of fragmented peptides. The number of allowable missed cleavages in tryptic digestion was set at

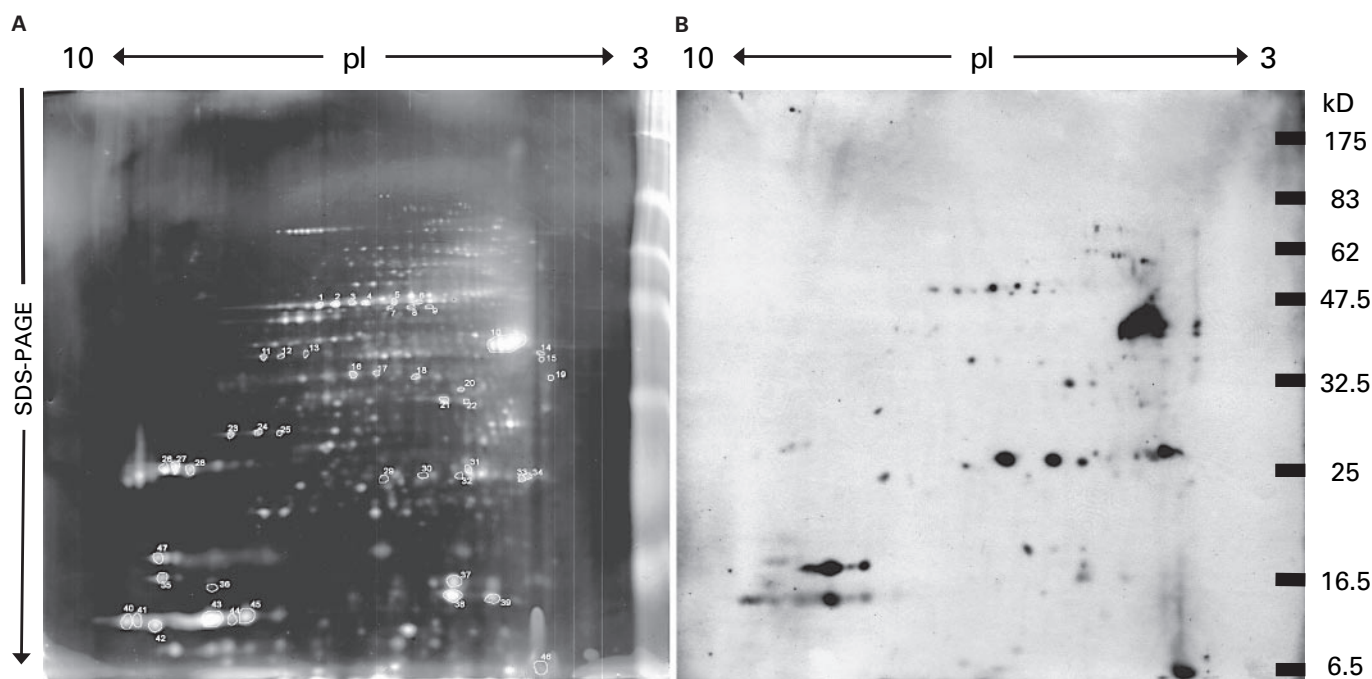


Fig. 1. Latex protein (**A**) and IgE-interactive protein (**B**) profiles on two-dimensional gels. Latex proteins extracted from NAL were first separated according to their isoelectric point (pI) on an immobilized pH gradient gel strip (pH 3–10, nonlinear). Second, the partially separated proteins were further resolved according to their molecular weight on a 10% acrylamide slab gel (SDS-PAGE). Two-dimensionally separated proteins were visualized by negative staining (**A**). IgE-interactive proteins submitted for subsequent in-gel digestion are highlighted and numbered (No. 1–47). For IgE immunoblotting (**B**),

two-dimensionally separated proteins were transferred onto a membrane and treated by NaIO_4 for degradation of the possible carbohydrate structures. After blocking the unoccupied sites, the membrane was incubated in the pooled serum of 6 latex-sensitive patients. Spots of proteins that interacted with the IgE antibodies were then visualized by reaction with peroxidase-labeled anti-human IgE antibodies. The enzyme activity was recorded on a film using a chemiluminescent detection system. The migrating position and molecular weight of each marker protein are shown on the right side of the gel.

1. Mass error tolerance was set to plus or minus 0.5 D for PMF. In the MS/MS Ion Search mode, the mass error tolerance of a precursor ion was set to plus or minus 1.0 D, and plus or minus 0.5 D for the product ions. All the peptide masses mentioned in this article are monoisotopic masses. Sequentially homologous proteins from other organisms were searched using the Basic Local Alignment Search Tool (BLAST) search engine-based overall sequence of the top candidate picked up from the EST (*H. brasiliensis*) database.

Results

Two-Dimensional Immunoblotting of Latex Proteins

Proteins extracted from NAL were separated with two-dimensional gel electrophoresis. Its high resolution was visualized by negative staining of the gel (fig. 1A). More than 300 distinct protein spots, each with a different isoelectric point or molecular weight, were detected. This resolving power is far superior to the conventional SDS-PAGE or isoelectric focusing where proteins are separated

one-dimensionally. IgE-interactive proteins were selectively detected by reaction with the pooled patients' serum following NaIO_4 treatment of a membrane binding the two-dimensionally separated proteins. NaIO_4 oxidatively degrades the carbohydrate structures of glycoproteins that tend to result in false-positive reactions of IgE antibodies by acting as monovalent antigens [17, 18]. Monovalent antigens cannot form a bridge-like structure of IgE antibodies attached to the specific receptor on a sensitized cell, and therefore they are believed not to provoke any allergic responses. We clearly detected more than 50 spots of proteins interacting with the IgE antibodies owing to the high resolving power of two-dimensional gel electrophoresis and the advanced sensitivity of the chemiluminescent detection system compared to conventional color-developing systems (fig. 1B). No significant spots of IgE-interactive proteins were detected when the pooled control serum was employed for immunoblotting (data not shown).

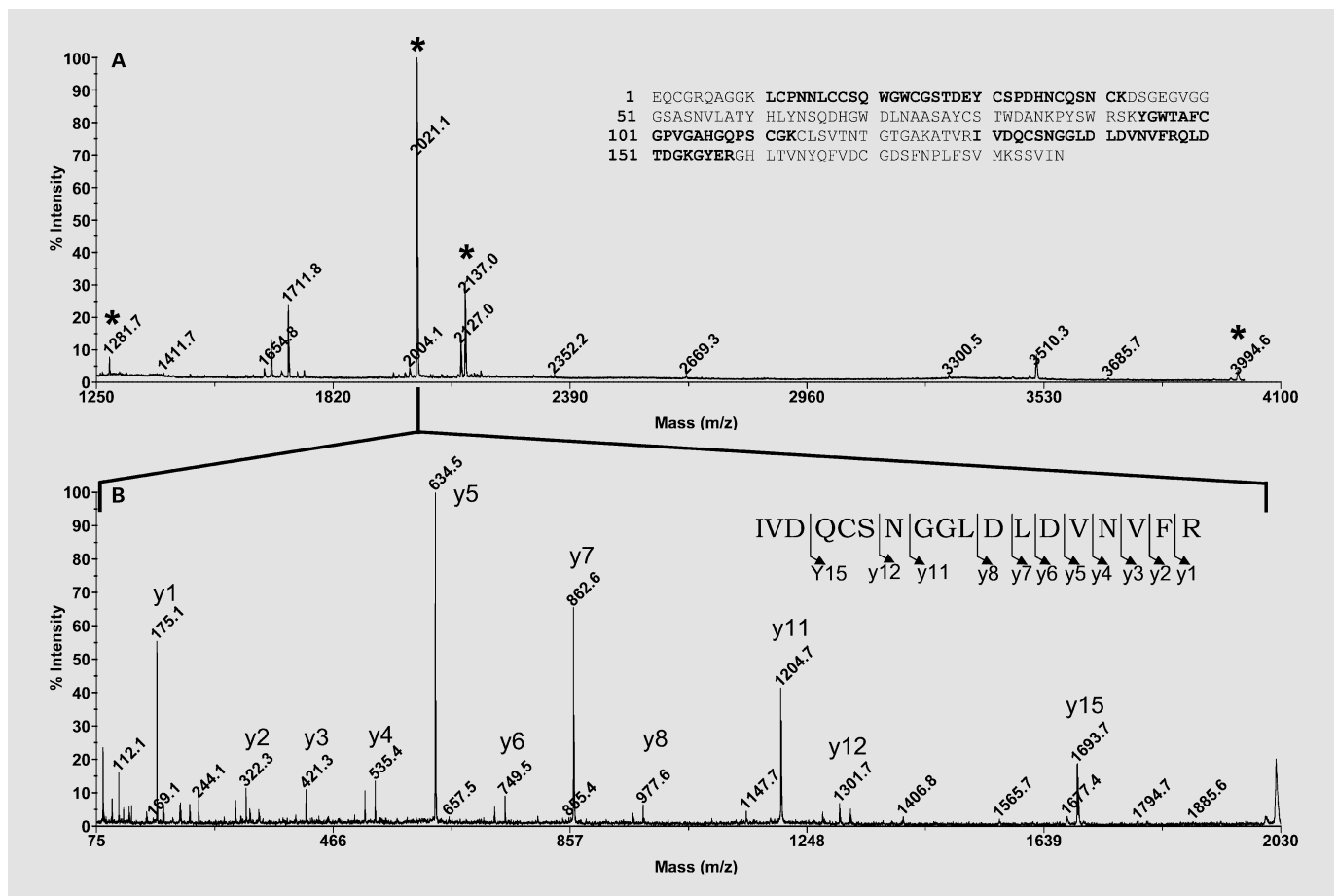


Fig. 2. Positive-ion reflectron MALDI-TOFMS spectrum (**A**) of tryptic peptides derived from an IgE-interactive protein (spot No. 38 in fig. 1A) and an MS/MS spectrum (**B**) of a prominently detected peptide ion (m/z 2021.1) on the peptide mass map. The spot of a putative allergen (No. 38) was cut out from a negatively stained two-dimensional gel (fig. 1A) and site-specifically cleaved by trypsin. The fragmented peptides were desalted using a reversed-phase microcolumn and spotted on a sample plate. α -Cyano-4-hydroxy cinnamic acid was used as a matrix for ionization. A peptide mass map (**A**) was used for PMF. This spectrum is a result of accumulation of 2,000 laser shots. The peptide ions assigned in the PMF search are labeled

by asterisks, and their position is shown in boldface in the entire sequence of the top candidate (insert). The MS/MS spectrum (**B**) was recorded on the TOF-TOF mode of the instrument (4700 Proteomics Analyzer) and used for reading the PST. This MS/MS spectrum shows the accumulation of 5,000 laser shots. A partial sequence read from this spectrum is depicted as an insert with the name of the fragment ions (y series) detected. Based on a series of mass spectra including the ones shown in this figure, the original antigen (spot No. 38 in fig. 1A) was identified as a prohevein-related protein by database search (table 2).

In-Gel Digestion of Putative Allergens and Mass Spectrometry of Tryptic Peptides

Forty-seven spots of latex proteins that interacted with IgE antibodies from patients' sera were cut out from a two-dimensional gel (fig. 1A). With the help of the microtiter plate-based format of an in-gel digestion kit (Montage In-Gel Digest96 Kit), the putative allergens in the gel pieces were simultaneously digested by trypsin with less risk of keratin contamination. By adding n -octyl- β -D-glucoside to the digestion buffer, we could significantly

improve the quality of mass spectra of fragmented peptides (fig. 2A). Desaltation of tryptic peptides using a reversed-phase microcolumn (ZipTip μ C₁₈) was also helpful for improving the signal-to-noise ratio of the mass spectra (fig. 2A). We used a MALDI-TOF-TOF instrument for mass spectrometric analysis of tryptic peptides. Though the quality of MS/MS spectra is generally inferior to the spectra obtained on an electrospray ionization-based tandem mass spectrometer, the operation is less labor-intensive and very quick. The sensitivity of the mass spectrometry

Table 2. Summary of IgE-interactive proteins identified by allergenomics with a Mascot search

Spot No.	Name of the top candidate	Method used	Database used	Total score	Sequence coverage, %	Accession No.	Calculated Mr, kD	Calculated pI	Observed Mr, kD	Observed pI
1	UDP-glucose pyrophosphorylase	PST	NCBIInr	46	2	gi 12585472	51.6	5.92	49.0	6.6
3	UDP-glucose pyrophosphorylase	PST	NCBIInr	24	2	gi 12585472	51.6	5.92	49.0	6.4
4	UDP-glucose pyrophosphorylase	PST	NCBIInr	58	6	gi 8099155	50.9	6.10	49.0	6.3
5	enolase 2 (Hev b 9)	PST	NCBIInr	32	5	gi 14423687	48.1	5.92	49.0	6.3
7	enolase 1 (Hev b 9)	PMF	NCBIInr	52	22	gi 14423688	48.0	5.57	47.5	6.0
	enolase 1 (Hev b 9)	PST	NCBIInr	38	5	gi 14423688	48.0	5.57		
10	patatin-like latex allergen 2 (Hev b 7)	PMF	NCBIInr	156	37	gi 7442023	43.0	5.00	40.0–43.5	4.6–5.5
	patatin-like latex allergen 1 (Hev b 7)									
		PST	NCBIInr	68	13	gi 7442022	43.1	5.12		
21	isoflavone reductase ¹	PST	EST(latex)	201	15	gi 29054556	27.0	5.32	32.5	5.6
22	isoflavone reductase ¹	PST	EST(latex)	69	15	gi 29054556	27.0	5.32	32.5	5.4
26	hevamine A	PMF	NCBIInr	151	54	gi 1311006	29.9	8.44	29.0	9.4
	hevamine A	PST	NCBIInr	156	19	gi 1311006	29.9	8.44		
27	hevamine A	PMF	NCBIInr	151	65	gi 1311006	29.9	8.44	29.0	9.3
	hevamine A	PST	NCBIInr	240	32	gi 1311006	29.9	8.44		
28	hevamine A	PMF	NCBIInr	137	54	gi 1311006	29.9	8.44	29.0	9.1
	hevamine A	PST	NCBIInr	113	13	gi 1311006	29.9	8.44		
33	class I chitinase ¹ (Hev b 11-related)	PST	EST(latex)	87	7	gi 29054149	23.5	8.28	26.5	4.2
34	class I chitinase ¹ (Hev b 11-related)									
		PST	EST(latex)	121	15	gi 29054149	23.5	8.28	26.5	4.0
36	hevein precursor (Hev b 6 -related)	PST	NCBIInr	36	8	gi 123062	22.7	5.63	16.5	8.6
37	prohevein (Hev b 6)	PST	NCBIInr	69	20	gi 2832430	20.9	5.64	17.0	5.6
38	prohevein (Hev b 6)	PMF	NCBIInr	62	43	gi 2832430	20.9	5.64	15.0	5.6
	prohevein (Hev b 6)	PST	NCBIInr	198	20	gi 2832430	20.9	5.64		
39	prohevein (Hev b 6)	PST	NCBIInr	127	20	gi 2832430	20.9	5.64	15.0	5.3
40	rotamase	PST	SwissProt	21	6	Q39613	18.5	8.36	11.0	9.8
41	rotamase	PMF	SwissProt	38	27	Q39613	18.5	8.36	11.0	9.6
	rotamase	PST	NCBIInr	50	6	gi 3334157	18.5	8.36		
42	rotamase	PST	NCBIInr	98	8	gi 118104	18.6	8.91	10.5	9.3
43	prohevein (Hev b 6)	PMF	NCBIInr	44	32	gi 2832430	20.9	5.64	11.0	8.7
	prohevein (Hev b 6)	PST	NCBIInr	201	26	gi 2832430	20.9	5.64		
44	pseudo-hevein (Hev b 6-related)	PMF	NCBIInr	49	33	gi 6562381	21.0	7.98	11.0	8.4
	prohevein (Hev b 6)	PST	NCBIInr	114	20	gi 2832430	20.9	5.64		
45	pseudo-hevein (Hev b 6-related)	PMF	NCBIInr	39	33	gi 6562381	21.0	7.98	11.0	8.0
	prohevein (Hev b 6)	PST	NCBIInr	196	20	gi 2832430	20.9	5.64		
46	thioredoxin h	PST	NCBIInr	48	13	gi 14485509	13.2	4.83	6.5	3.9
47	citrate-binding protein precursor	PST	NCBIInr	72	10	gi 32363139	27.5	9.09	17.0	9.4

Mr = Molecular weight; pI = isoelectric point.

¹ Sequentially homologous protein was searched using BLAST.

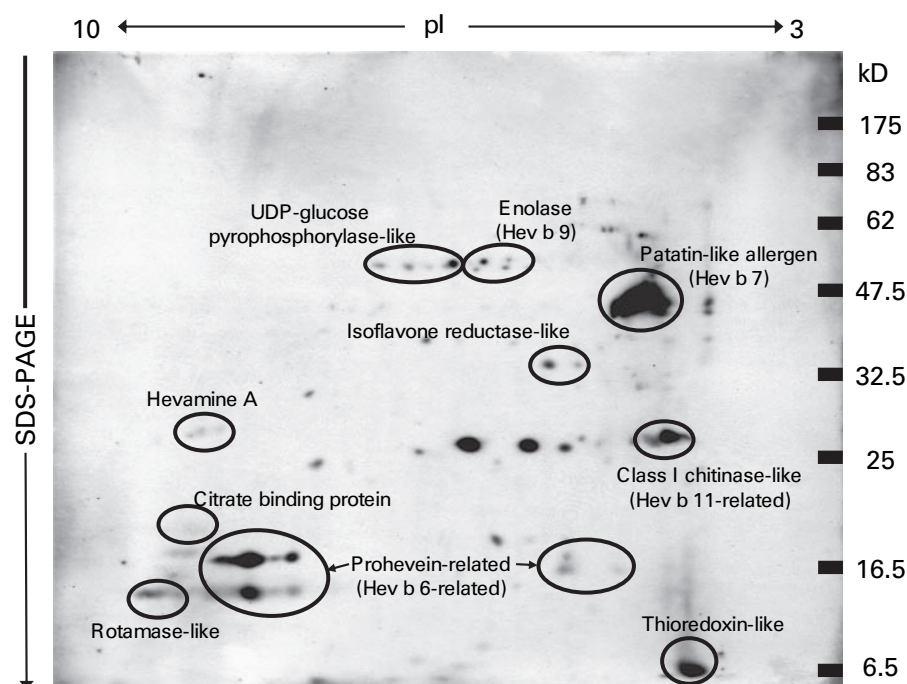
try is also notable. We could easily obtain positive-ion peptide mass maps (fig. 2A) and MS/MS spectra of prominent ions (fig. 2B) from as small as several hundred femtomoles of tryptic peptides on a sample plate.

Identification of Putative Allergens by Database Search

Based on a peptide mass map of the tryptic peptides and MS/MS spectra of the intense peptide ions, the proteins in question were identified by database search. The

identified putative allergens are listed in table 2, with the total score and sequence coverage rate showing the certainty of the protein candidate. Thanks to the high resolution of two-dimensional gel electrophoresis and the remarkable sensitivity of MALDI-TOFMS, we could identify five new allergen candidates (UDP-glucose pyrophosphorylase, isoflavone reductase, rotamase, thioredoxin and citrate-binding protein) and five previously reported latex allergens or their relatives (Hev b 9, Hev b 7, Hev b 11, Hev b 6 and hevamine) without isolating each protein

Fig. 3. Putative latex allergens identified by a proteomic strategy. The names of the top candidates are shown on an IgE immunoblot (same as in fig. 1B). Spots enclosed by an ellipse are IgE-interactive proteins assigned to the same or closely related entries (isoallergens) by database search. More information on each antigenic protein is presented in table 2.



(table 2, fig. 3). A few allergens were identified by database search with the PMF mode of Mascot, but the searching based on PST was much more powerful for protein identification. From the MS/MS spectra of tryptic peptides derived from a protein, we could obtain information on the partial sequences (fig. 2B) that would be valuable for designing the oligonucleotide probes as well as for the database search. The BLAST search was also effective in finding a protein candidate that is sequentially comparable to a DNA fragment registered in the EST database (*H. brasiliensis*). The entire protocol, from two-dimensional gel electrophoresis to the database search, was accomplished quickly. We were able to analyze about 50 IgE-interactive latex proteins within 1 week.

Discussion

In this study, we evaluated the usefulness of a proteomic strategy for analyzing putative allergens (allergenomics) through application to latex proteins. Latex proteins were first separated two-dimensionally according to their isoelectric point and molecular weight under denaturing conditions. Two-dimensional gel electrophoresis appeared to be remarkably superior to SDS-PAGE with regard to its high resolution. In conventional SDS-PAGE,

proteins were separated based solely on their molecular weight under a denaturing condition. Therefore, a seemingly single band on an SDS-PAGE gel frequently contains several protein entities. This poor resolution might bring about erroneous identification of IgE-interactive proteins. Additionally, faint structural differences of isoallergens are hardly reflected on SDS-PAGE gel and subsequent IgE immunoblot. In contrast, we can detect slight structural differences of isoallergens as distinct spots on a two-dimensional gel and subsequent IgE immunoblot. Hev b 7 isoallergens are one such example (fig. 3, table 2, spot No. 10). Historically, the reproducibility of two-dimensional gel electrophoresis has been a critical problem in applying this technique to research of complex protein mixtures. However, the appearance of commercially available immobilized pH gradient gels has solved a large part of this difficulty. The effectiveness of two-dimensional gel electrophoresis for analysis of allergenic proteins has already been demonstrated in previous studies [19–23]. Nevertheless, this technique has not attracted the attention of many researchers, due to the lack of a convenient identification method for the resolved proteins. N-terminal sequence analysis of a purified protein has been the first choice for identification [10, 20], but this technique is time-consuming and requires a relatively large quantity of purified sample. Putative allergens avail-

able only in minute amounts were therefore not readily amenable to this approach.

As the sequence data of proteins and genes accumulate in databases, the situation is changing. Candidate proteins are becoming easier to identify after site-specific fragmentation and mass spectrometric analysis of the component peptides [9, 10]. Mass spectrometry is so sensitive that we can analyze putative allergens on quantities as small as the nanogram or several hundred-femtomole level. Intensive research of such a small quantity of proteins was impossible with traditional Edman degradation-based N-terminal sequencing. The speed of mass spectrometric analysis is also advantageous. We can obtain thousands of mass spectra and MS/MS spectra within a day using a MALDI-TOFMS instrument. Semiautomated analysis of fragmented peptides from spotting on a sample plate to output of the mass spectra is also possible. By combining with high-resolution two-dimensional gel electrophoresis, the mass spectrometric approach will certainly become the most powerful means for fast and exhaustive analysis of IgE-interactive proteins [24].

The efficiency of allergenomics was substantiated in this study through the analysis of latex proteins. We were able to identify five new allergen candidates as well as five previously reported allergens without any difficulties (fig. 3, table 2). The required starting material was less than 1 mg. In addition, the entire analysis was completed within 1 week; two-dimensional gel electrophoresis and subsequent IgE immunoblotting usually takes not more than 5 days. Referring to the immunoblotting result with pooled patients' serum, IgE-interactive proteins were cut out from a two-dimensional gel and digested by trypsin overnight. A more rapid digestion protocol for proteins is also applicable [25]. The proteins in question were identified the next day from mass spectrometric analysis of the resulting fragments and subsequent database search. These analytical steps for each IgE-interactive protein proceed in parallel with the help of a microtiter plate-based tryptic digestion regime. In this way, allergenomics was concretely verified as a quick and effective strategy for exhaustive analysis of IgE-interactive proteins from various species.

We should point out at this stage that IgE-interactive proteins or antigens identified with allergenomics are just putative allergens or allergen candidates. IgE-binding activity of a protein is one of the prerequisites for it to be an allergen and does not necessarily indicate its actual allergenicity. Some IgE-interactive proteins are indeed asymptomatic but others are symptomatic [17, 18]. Therefore, the actual allergenicity of putative allergens must finally

be confirmed with other techniques like SPT or histamine release test. This situation is common to almost all the allergen identification procedures reported to date where allergen candidates are screened based on their IgE-binding activity.

Even though allergenomics is very promising, as already illustrated, some of the latex antigens were not identified by database search, as shown in figure 3, despite their clear detection in IgE immunoblotting. There are two possible reasons for this failure. One possibility is that the quantity of IgE-interactive protein was too small to gain meaningful data regardless of the high sensitivity of the mass spectrometry. Another possibility is that data on the sequence of the antigen in question had not been registered in the databases used. The genome of the rubber tree (*H. brasiliensis*) has not been sequenced thoroughly. Therefore, we cannot identify some of the latex proteins by the proteomic strategy in principle. If we analyze proteins from rice or *Arabidopsis*, this is not the case because the genome projects of these species have already been finished. Even when the sequence of an IgE-interactive protein is not included in databases, we can search sequentially homologous proteins from other species using the partial sequences of tryptic peptides or cDNA fragments. Moreover, the gene coding the putative allergen can be fully sequenced by cloning the corresponding cDNA using oligonucleotide probes designed from the revealed partial sequences. These prospects for further research also suggest the versatility of allergenomics.

Acknowledgement

This study was supported in part by a grant for Research on Advanced Medical Technology provided by the Ministry of Health, Labor and Welfare, Japan.

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