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Clinical Allergy

Occupational respiratory allergy to lettuce in lettuce farmers

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Abstract

Background: Lettuce-associated respiratory allergy has never been reported before. The aim of this study was to clarify the clinical condition of lettuce-associated respiratory allergy and to identify the lettuce antigen which induces allergic symptoms.

Methods: We distributed questionnaires to 1168 lettuce farmers and performed medical examinations in those who exhibited respiratory symptoms related to occupational exposure to lettuce. We analysed specific IgE-binding proteins in the sera of patients through immunoblotting analysis and determined molecular characterization of the IgE-binding bands using liquid chromatography-mass spectrometry.

Results: A total of 932 farmers (80%) responded to the questionnaire. Of those, 7% exhibited lettuce-associated respiratory symptoms, during harvesting and packaging. Thirteen patients were diagnosed with allergy to lettuce and agreed to undergo further examinations. The percentage of activated basophils in these patients was significantly higher compared with that reported in negative controls ($P < .05$). Lettuce-specific IgE (ImmunoCAP[®]) and skin prick testing was positive in 46% and 62% of patients, respectively. Notably, occupational lettuce-allergic asthma was detected in one patient through specific bronchial provocation testing. The IgE-binding bands recognized in the sera of >50% of patients were identified as epidermis-specific secreted glycoprotein EP1-like (51 kDa).

Conclusion: The present analysis identified a novel lettuce allergen. This allergen may have clinically useful applications, such as specific IgE testing and allergen-specific immunotherapy.

KEYWORDS

IgE, immunologic tests, lettuce, occupational allergies

1 | INTRODUCTION

Exposures to allergens increase the risk of occupational respiratory conditions, including asthma and asthma-like syndromes (ie eosinophilic bronchitis, aluminium pot room asthma, and irritable larynx

syndrome).¹⁻³ Occupational asthma can be classified into two types: immunologically induced asthma and irritant-induced asthma. The former type involves allergens that can be divided into two groups, according to their molecular weight. The high-molecular-weight agents (>10 kDa) include animal or plant allergens, and induce the

production of specific IgE antibodies and typical allergic responses. In contrast, the low-molecular-weight agents include occupational chemicals that induce asthma through unknown mechanisms.³

Although the means by which farmers become sensitized is unknown, grains and cereals (eg wheat, rye, and barley) are established plant-derived antigens.³ Although lettuce (*Lactuca sativa*) is extensively cultivated globally, occupational lettuce-induced respiratory allergy is rarely reported. Only a limited number of case reports have specifically reported respiratory symptoms in lettuce farmers in Japan.⁴ In contrast, both contact and food allergy to lettuce have recently been described.⁵⁻¹⁰ Indeed, previous studies using IgE antibodies identified several proteins as possible allergens in the sera of patients with lettuce anaphylaxis or oral allergy syndrome (OAS).⁷⁻¹² Therefore, the aim of this study was the following: (a) to clarify the clinical condition of lettuce-induced respiratory allergy, and (b) to identify new major allergens recognized by IgEs in the sera of patients with lettuce-induced respiratory symptoms.

2 | MATERIALS AND METHODS

2.1 | Questionnaire

In 2013, we distributed a questionnaire to all 1168 lettuce farmers in the Hyogo prefecture, Japan (Table S1). In 2017, this questionnaire was also distributed to lettuce farmers in the Kagawa prefecture, Japan.

2.2 | Patients and clinical examination

A total of 13 lettuce farmers with a clinical history of lettuce-induced respiratory symptoms during harvesting and packaging were enrolled into the study from the outpatient clinic of two general hospitals: Hyogo prefectural Awaji Medical Center and Kobe University Hospital, Japan. The patients underwent examinations in either of the two general hospitals. All patients provided written informed consent prior to their participation. Protocol approval was obtained from the institutional review board of both of the hospitals (permission number #280007). The general parameters of this study are openly available in UMIN at <https://www.umin.ac.jp/>, reference number UMIN000027002.

Following a detailed medical interview, skin prick tests (SPTs) were performed in all patients using lettuce leaves extracts, stem extracts, and latex with applied water (0.1 mg/mL). The skin was pricked with a drop of sample, and the responses were read after 15 minutes. Ten mg/mL histamine dihydrochloride (Torii, Tokyo, Japan) and phosphate-buffered saline (PBS) served as positive and negative controls, respectively. A positive reaction was defined as a wheal diameter of ≥ 3 mm in the absence of a reaction to the PBS and in the presence of a positive reaction to histamine dihydrochloride. Determination of the total IgE and lettuce-specific IgE was performed using the CAP System[®] (Thermo Fisher, Uppsala,

Sweden). Determination of the other allergen-specific IgE was performed using the CAP System[®] and fluorescence immunoassay (SRL, Tokyo, Japan). Basophil activation tests (BATs) were performed in all patients through the BAT system (BML, Tokyo, Japan) using lettuce stem extracts, as described in the Appendix S1. Three patients were excluded as non-responder, with the result of no difference of positive control (1 mg/mL anti-IgE antibody; Beckman Coulter) and negative control (PBS). The sera of seven non-allergic individuals were used as negative control. Specific inhalation challenges (SICs) were performed using lettuce stem extracts in the four patients who provided written informed consent. Placebo-controlled SICs were performed using normal saline 30 minutes prior to exposure to the lettuce stem extracts. Inhalation tests were performed with 5 mL lettuce stem extract (0.04 mg/mL, 0.4 mg/mL) and 5 mL PBS by ultrasonic nebulizer (Omron, Kyoto, Japan). Bronchial reaction was monitored using FEV1 (Forced expiratory volume in the first second). A positive SIC result was defined as $\geq 15\%$ decrease of FEV1 vs the pre-challenge value.¹³

2.3 | Preparation of the lettuce extract

Fresh lettuce (*L. sativa*, var. *capitate*, Figure S2A) leaves and stem were grated using a commercial food grater. The grated lettuce was filtered through three layers of gauze.

The fresh lettuce stem was cut using a sharp scalpel, and the white juice (latex) spread on the section was collected (Figure S2B). This lettuce latex was dissolved into distilled water to prevent hardening.

2.4 | ELISA

ELISAs were used to detect the binding of patients and control serum IgE to the lettuce latex, lettuce stem, and lettuce leaves extract. Fifty microlitres of lettuce latex, lettuce stem extract, and lettuce leaves extract (50 μ g/mL) in PBS was added to each well of the ELISA plates (Asahi Glass, Tokyo, Japan) and allowed to coat overnight at 4°C. After blocking the plates with 1% BSA in 10 mmol/L PBS containing 0.1% Tween-20 (PBS-T) for 1 hour at room temperature and three washes with PBS-T, 1:50 dilutions of patient or control sera in Can get Signal[®] Immunoreaction Enhancer Solution1 (Toyobo Co., Ltd., Osaka, Japan) were added to the wells and incubated for 1 hour at 37°C. After washing the wells with PBS-T 5 times, 1:3000 dilutions of HRP-conjugated affinity-purified goat anti-human IgE antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) in Can get Signal[®] Immunoreaction Enhancer Solution2 were added to the wells and incubated for 1 hour at 37°C. After five washes with PBS-T, the bound secondary antibodies were detected by reactions with 50 μ L of a TMB peroxidase substrate (Kirkegaard & Perry Laboratories). The reactions were stopped by the addition of 50 μ L of 1 mol/L phosphoric acid, which also amplified the signal. The absorbance of each well was measured at 450 nm using a plate reader

(Wallac ARVO SX 1420 multi-label counter; PerkinElmer, Waltham, MA, USA).

2.5 | SDS-PAGE

The lettuce protein (approximately 6–15 µg protein/lane) was separated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).¹⁴ Proteins on the gel were stained with Coomassie Brilliant Blue R-350 (GE Healthcare, Chicago, IL, USA) or Silver Staining kit ProteoSliver (Sigma-Aldrich, St. Louis, MO, USA) to detect the total protein patterns.

2.6 | Immunoblot analysis

The immunoblotting analysis was conducted by transferring the SDS-PAGE gel onto an Immobilon-P™ polyvinylidene difluoride membrane (Millipore, Burlington, MA, USA) using a semi-dry blotting method.¹⁵ This method has been described in detail elsewhere.¹⁶ The sera of two non-allergic individuals were used as negative controls. Following an incubation in PBS-T containing 5% skim milk for blocking, the membrane was incubated overnight with human sera (1:20 dilution) at 4°C. After three washes with PBS-T for 10 minutes, the membranes were incubated with a HRP-conjugated goat anti-human IgE antibody (1:3000 dilution) for 1 hour, after which the membranes were washed four times with PBS-T for 10 minutes. The bound secondary antibodies were detected using an Enhanced chemiluminescence Western blotting substrate (GE Healthcare, Chalfont St. Giles, UK). The resultant chemiluminescent signals were detected on X-ray films (GE Healthcare).

2.7 | Inhibition experiment

Fraction B10 (by a gel-filtration column chromatography) was used for inhibition experiment. The fraction B10 was separated through SDS-PAGE and transferred onto PVDF (polyvinylidene difluoride) membranes. Lettuce latex (0.1 mg/mL) or distilled water was pre-incubated for 1 hour at 4°C with a patient 003's sera (diluted 1:1), before exposure to membranes. After pre-incubation, patient 003's sera were incubated with the membranes. Bound IgE was detected by using ECL Plus (Thermo Fisher Scientific).

2.8 | Purification of lettuce latex allergen

Allergen was purified from lettuce latex by ion-exchange chromatography and gel-filtration chromatography using high-performance liquid chromatography, as described in the Appendix S1. After selecting the appropriate fractions, the effluents were studied through SDS-PAGE and immunoblotting.

2.9 | Mass spectrometry

Protein bands recognized in the sera of >50% of patients were selected for further study. The bands were extracted from the gel as previously described.¹⁶ LC-MS/MS analysis was performed a LCMS-IT-TOF instrument (Shimadzu, Kyoto, Japan) interfaced with a nano-reverse-phase liquid chromatography system (Shimadzu). MS/MS data were analysed using Mascot (version 2.3.01; Matrix Science, London, UK). The search parameters were as follows: enzyme, trypsin; variable modifications, carbamidomethyl (Cys), deamidated (Asn, Gln), and oxidation (Met); peptide mass tolerance, ±50 ppm; fragment mass tolerance, ±0.05 Da; and max missed cleavages, 1.

2.10 | Statistical analysis

All statistical analyses were performed with EZR version 1.37 (Saitama Medical Center Jichi Medical University; <http://www.jichi.ac.jp/saitama-sct/SaitamaHP/files/statmed.html>; Kanda, 2018), a graphical user interface for R (version 2.4.0; The R Foundation for Statistical Computing, Vienna, Austria).

Categorical data are presented as numbers and percentages. Numeric data presented as mean and standard deviation. Univariate analyses were performed using Fisher's exact test for categorical data. Parametric data analysed using student *t* test, and non-parametric data analysed Mann-Whitney *U* test. Logistic regression models were used to analyse correlation associated with lettuce-induced respiratory symptoms. The results are expressed as odds ratios with 95% confidence intervals. All *P*-values were two-sided, and values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Questionnaire

A total of 932 lettuce farmers in the Hyogo prefecture responded to the questionnaire, representing an overall return rate of 80%. Table 1 shows the baseline characteristics of these lettuce farmers. Table 2 shows the point prevalence and characteristics of lettuce-associated symptoms. "Respiratory symptoms" mean cough, sputum, or dyspnoea during any time of farming. "Respiratory symptoms associated with harvesting and packaging of lettuce" mean cough, sputum, or dyspnoea only during harvesting and packaging lettuce. Among the responders, 8% reported specific lettuce-associated symptoms. Lettuce-associated respiratory symptoms were observed in 7% of the respondents. Forty-nine farmers out of 63 farmers had respiratory symptoms during harvesting and packaging of lettuce. Of note, the mean time between the start of the occupation and onset of symptoms is 19 years, which seems to be relatively longer than other occupational allergic diseases.¹⁷ From Q6 of the questionnaire, only two lettuce farmers reported symptoms associated when eating lettuce. Multivariate analysis revealed that history of bronchial

asthma, allergic rhinitis, and pollinosis were significant factors related to lettuce-associated respiratory symptoms ($P < .05$) (Table S2). In 2017, the questionnaire was distributed to lettuce farmers in the Kagawa prefecture. Although the response rate in the Kagawa study was lower than that observed in the Hyogo study (34%), the analysis yielded almost identical results. Specifically, lettuce-associated respiratory symptoms were observed in 7% of respondents both in the Hyogo and Kagawa surveys, respectively (Table 2).

TABLE 1 Baseline characteristics of lettuce farmers

	Hyogo		Kagawa	
	N	%	N	%
Effective response rate	932/1168	80	368/1073	34
Mean age (y) ^a	65 ± 10		65 ± 12	
Sex (male/female)	455/477	49/51	225/143	61/39
Mean duration of farming (y) ^a	33 ± 16		29 ± 17	
Smoking status				
Current smoker	83	12	69	19
Ex-smoker	227	24	86	23
Never	622	64	213	58
Allergic history				
Paediatric asthma	6	0.6	5	1
Bronchial asthma	36	4	19	5
Allergic rhinitis	49	5	32	9
Pollinosis	79	9	54	15
Atopic dermatitis	8	0.9	11	3
Urticaria	29	3	27	7
Food allergy	14	2	12	3
Drug allergy	13	1	6	2

^aMean ± SD.

3.2 | Clinical features of patients with lettuce-induced respiratory symptoms

The characteristics of the patients included in the study are shown in Table 3. The median age of the patients was 67 years with a sex distribution of five males and eight females. Eleven farmers were specialized on growing vegetables. Two farmers kept Japanese cattle (patient 3, patient 11) but did not have respiratory symptom during caring animals. All patients exhibited respiratory symptoms during the harvesting or packaging of lettuce. Detailed history examination suggested that patients developed respiratory symptom during cutting lettuce from stem to harvest, packaging of lettuce, and wiping lettuce stem juice (latex) in small poorly ventilated room. However, none of the patients exhibited symptoms after ingestion of lettuce leaves. Three patients exhibited skin symptoms, following accidental skin contact with the lettuce latex, while three patients had lettuce-associated nasal symptoms. The SPT and lettuce-specific IgE were positive in eight patients (62%) and six patients (46%), respectively. The median value of total IgE was 445.6. Sensitization to pollen or plant foods was observed in five patients (38%).

Allergy to moulds was observed in four patients, and allergy to animals was observed in two patients (Table S4). The percentage of activated basophils in patients was significantly higher than that observed in the negative controls ($P < .05$, Figure S1). Moreover, when comparing the values of single patients with those of the negative controls, one can conclude that 8 of 11 patients were positive. The specific bronchial provocation test was performed in four patients. Notably, this test was positive in only one patient (patient 3) (Table S3). These results indicate that patients with lettuce-induced respiratory symptoms were allergic to lettuce, and respiratory symptoms were associated with the development of asthma in at least one patient. Patient 3 is the most valuable patient in the whole study.

	Hyogo		Kagawa	
	N	%	N	%
Symptoms specifically associated with lettuce farm work	71	8	40	11
Respiratory symptoms	63	7	24	7
Rhinitis	39	4	23	6
Skin symptoms	11	1	13	4
Conjunctivitis	26	3	11	3
Gastrointestinal tract symptoms	2	0.2	2	0.5
Symptoms associated with ingesting lettuce	0	0	2	0.5
Respiratory symptoms associated with harvesting and packing of lettuce	49	5	18	5
Mean years from start of harvesting lettuce to the onset of the symptoms*	19 ± 11		15 ± 13	

*Mean ± SD.

TABLE 2 Symptoms associated with lettuce farm work

TABLE 3 Clinical features of the patients who are allergic to lettuce

Patient no.	Age	Sex	Past history	Smoking	Other allergy	Lettuce reaction	SPT (leaves)	SPT (stem)	SPT (latex)	Total IgE (IU/mL)	ImmunoCAP lettuce-specific IgE (IU/mL)	Uncontrollable symptom with treatment
1	60	M	HL	-	Ce, Hi	R	ND	+	ND	413.4	<0.100	-
2	76	F	-	-	Ce	R	ND	+	ND	226.1	<0.100	-
3	73	M	AD	+	Mi	R, S	-	+	+	2784.3	5.65	+
4	70	F	HT, HL, GERD	-	-	R	ND	-	ND	46.8	<0.100	-
5	72	F	-	-	-	R	ND	±	ND	3.4	<0.100	-
6	60	M	-	+	-	R	ND	+	ND	126.3	0.439	-
7	81	M	HT	-	-	R, N	ND	-	ND	4.7	<0.100	-
8	63	F	HT, psoriasis	-	Ce	R, N, E	ND	+	ND	28.6	<0.100	-
9	77	F	HT, HL	-	EA	R, S	ND	-	ND	120.8	<0.100	-
10	75	F	-	-	Mi, HD, Co, Sh, Mo	R	ND	-	ND	179.7	0.388	-
11	56	F	-	-	Ti, Or, Ce, Hi, Ja, Ca, Wh, So, Ph, La, Se, Cr, Ki, Pc, To	R, N	-	+	+	1384.3	2.3	-
12	64	F	GIST	-	Mi, PM	R	ND	-	ND	200.2	<0.100	-
13	44	M	AD	-	Or, Ti, Ve	R, S	-	+	+	274.4	0.518	+

Abbreviations: AD, atopic dermatitis; Ap, apple; BAT, basophil activation test; Ca, cat; Ce, cedar pollen; Co, cockroach; Cr, crab; E, eyes symptoms EA, egg albumin; F, female; GERD, gastro-oesophageal reflux disease; GIST, gastrointestinal stromal tumour; HD, house dust; Hi, Hinoki cypress pollen; HL, hyperlipidaemia; HT, hypertension; Ja, Japanese white birch; La, latex; M, male; Mi, mite; Mo, moth; N, nasal symptoms; ND, no data; Or, orchard grass; Pc, peach; PM, pig meat; R, respiratory symptoms; S, skin symptoms; Sb, buckwheats; Se, sesame; Sh, shrimp; So, soybeans; SPT, skin prick test; Ti, timothy grass; To, tomato; Ve, vernal grass; Wh, wheat.

3.3 | Electrophoresis, immunoblotting, and chromatography

Considering that some patients exhibited skin symptoms following the accidental contact of their skin with the lettuce latex, we hypothesized that this substance may contain allergens. Therefore, initially, we investigated the differences between the extracts from lettuce leaves and the lettuce latex. Figure 1A shows the SDS-PAGE of the lettuce leaf extracts and the lettuce latex. The latter showed multiple protein bands with an apparent molecular weight ranging from 10 to 100 kDa. These bands were partially different from those observed in the lettuce leaf extract. In addition, immunoblotting analysis of lettuce leaf extract and the lettuce latex with the pooled sera (Pt 001, 002, 003, 007, 013) showed

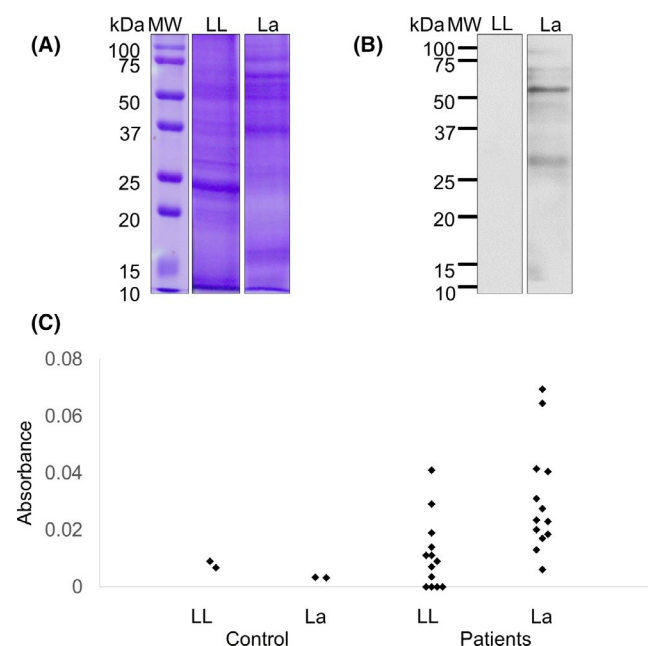


FIGURE 1 Coomassie Brilliant Blue staining, immunoblotting analysis, and ELISA of the lettuce leaf extract and the lettuce latex. A, Coomassie Brilliant Blue-stained SDS-PAGE gel of the lettuce leaf extract (LL) and the lettuce latex (La). Molecular weights are indicated on the left. MW, molecular weight (kDa). B, IgE immunoblots of LL and La probed with the pool of five patients' serum. C, ELISA analysis of negative controls of serum pool from two non-allergic individuals and 14 patients' serum with lettuce LL and La. Patients' sera show higher absorbance when reacted with lettuce latex than lettuce leaves extract

marked differences in the IgE-binding bands (Figure 1B). Several IgE-binding bands were observed in the lettuce latex with the sera of pooled sample, and main band was seen in 51 kDa. In contrast, there were no bands observed in the lettuce leaf extract with the sera of pooled two non-allergic individual's sample. The result of ELISA supported the tendency that patients' sera mark higher reactivity with lettuce latex than lettuce leaves extract, while negative control's sera react neither of them (Figure 1C). These findings suggested that allergens may be present only in the lettuce latex. Figure 2A shows the binding band of specific IgE from the sera of 13 patients with lettuce-associated respiratory symptoms. The most frequently recognized bands—detected in the sera of >50% of the patients—showed molecular weights of approximately 51 kDa. As shown in Figure 2A, the sera of 11 patients (ie patients 1-8, 10, 12, and 13) yielded a band at 51 kDa. In contrast, there were no IgE-binding bands were seen in the sera of control individuals.

By first purification step with ion-exchange chromatography, eight fractions of the lettuce latex were obtained (A1-A8, silver stain; Figure 3A, ELISA with pt 003's sera; Figure 3B). The result of immunoblot of fractions A5 and A6 contained protein band at 51 kDa with pt 003's sera (Figure 3C). Peak fractions of the result of ELISA (A5, A6) were combined. Secondly purification step through a gel-filtration column chromatography with HPLC system was performed with fractions A5 and A6.

Forty fractions were obtained by a gel-filtration column chromatography. The result of ELISA using the 40 fractions with the sera of patient 003 showed a peak in fraction B10 as in Figure 4A. The result of silver staining analysis of fraction B10 showed protein band at 51 kDa (Figure 4B, lane 1). The result of immunoblotting analysis with pt 002, 012, and 003 proved specific IgE-binding band at 51 kDa, compared with negative control (Figure 4B, lane 2-5). Inhibition experiment also proved that 51 kDa band disappears by inhibition with lettuce latex as in Figure 4B lane 6.

3.4 | Protein identification and characterization

The 51 kDa IgE-binding bands from the gel-filtration column chromatography fraction B10 were cut out from the gel and subjected to mass spectrometric analysis after in-gel tryptic digestion to determine the peptide sequences of the lettuce latex allergens. We performed nanoLC-MS/MS and obtained the sequence of several internal peptides

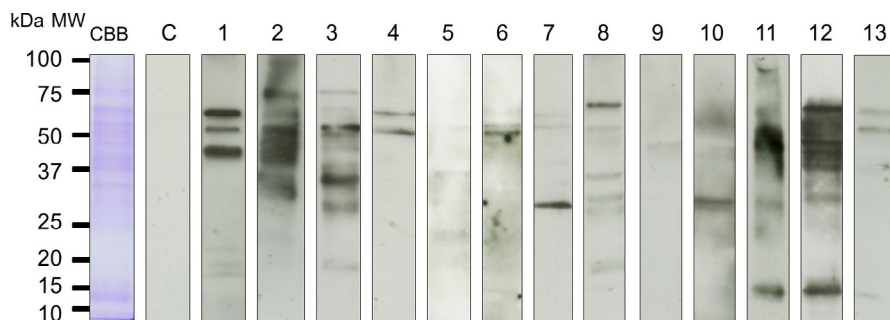


FIGURE 2 IgE-binding bands in the lettuce latex. A, Immunoblotting analysis shows IgE-binding bands. Lanes 1-13 represent the sera of the 13 patients. Lane C (Control) represents a negative control of serum pool from two non-allergic individuals. MW, molecular-weight marker (kDa); CBB, Coomassie Brilliant Blue-stained SDS-PAGE gel of the lettuce latex

(Table 4). Protein identification was performed through a search in the SwissProt database using the Mascot program (<http://www.matrixscience.com>). After excluding trypsin and keratin protein originates from experimenters, protein identification was reperformed through

a search in the genome database of *L. sativa* (<https://www.ncbi.nlm.nih.gov/genome?term=Lactuca%20sativa>) using the Mascot program (Table S4). This analysis led to the identification of the 51 kDa proteins as the epidermis-specific secreted glycoprotein EP1-like.

FIGURE 3 Silver staining, ELISA, and immunoblotting analysis of the fraction obtained through ion-exchange chromatography. A, Silver staining of the eight fractions obtained through ion-exchange chromatography for the lettuce latex. B, ELISA analysis of the eight fractions obtained through ion-exchange chromatography for the lettuce latex showed peak on fraction of A5 and A6 with pt 003's sera. Pt, patient 003; C, control. C, Immunoblotting analysis of the eight fractions obtained through ion-exchange chromatography for the lettuce latex with pt 003's sera. Fractions of A5 and A6 contain 51 kDa band

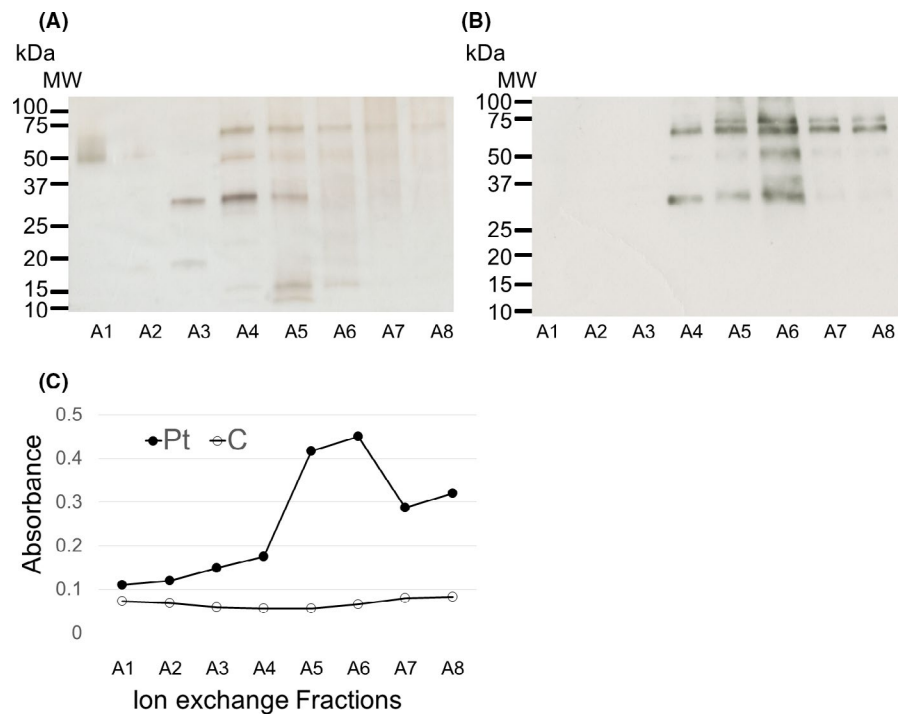


FIGURE 4 ELISA, immunoblotting, and inhibition analysis of the fraction obtained through gel-filtration chromatography. A, ELISA analysis of the 40 fractions obtained through gel-filtration chromatography of the peak fractions (A5 + A6) of the ion-exchange chromatography. Pt, patient 003; C, control. B, Immunoblotting analysis of specific IgE reactivity to the peak fraction (B10) obtained through gel-filtration chromatography of the peak fractions (A5 + A6) of the ion-exchange chromatography. Lane 1 marked silver stain of fraction B10. Lane 2 marked pooled serum from healthy individuals as negative control reacted with the fraction B10. Lanes 3-5, representatives of sera from lettuce allergy patients (Pt012, 002, 003) reacted with the fraction B10, show single protein bands at 51 kDa. Lane 6, the inhibition of the immunoblot with Patients 003's sera with lettuce latex

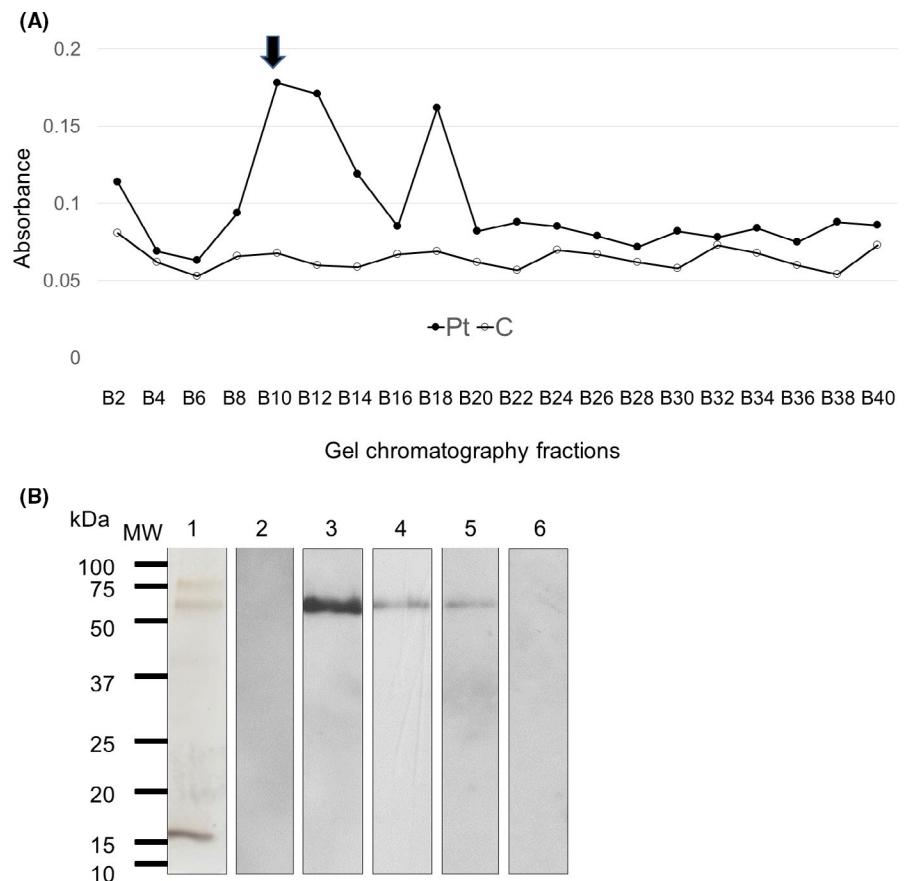


TABLE 4 Protein identification and characterization

Estimated molecular weight by SDS-PAGE	51
Molecular weight of identified protein	50 971
% recognition of patient	84.6% (11/13)
Taxonomy	<i>Lactuca sativa</i>
SPROT ID	XP_023765238.1
Match score	195
Identification	Epidermis-specific secreted glycoprotein EP1-like
% of matched peptide	11%
Peptides (matched peptides)	MTSPNSILVT ALLLLSFQL LYTISEAIVP SADTFSYVNE GDFGEYIVEY DADYRTLPPF SNPFQLCFYN TTPNTFTLAL RMGTVRSESL MRVWVEANRG NPVGENATLT FGTDGNLVLA DSDGRIAWQT NTANKGVVGL QVLP TGNMVL HDGTGTFIWQ SFDSPDTLL VGQSLRAGGA SNLVSRAAE NNIDGPYSLV MEPKRLALYY KSANSPYML YWTSVEWFTV DVGSVTNGSL INLTLSVPD TDEGFLYYLT FDYYITNPLS GWNRRNMAFSR YNNTLSYLR L GIDGNLKFYT YNPNVQGVSW ELVYTFLDNRN SIEGECQLPE RCEKFGLCED NQCVCAPTPF GLSGWSKDCE ASKVTSCQAS DFGYFKLDGV DHFMTKYTTG DWVSNQWDCE SKCTKDCNCM GYFYHTADSR CWIAYDLKTL TRVGNSTHFA YIKAPYNLSL NGIRL
Taxonomy: <i>Lactuca sativa</i> (45 242 sequences; 197 238 886 residues)	

4 | DISCUSSION

This is the first study to identify the allergen of lettuce using the sera from patients with lettuce-induced respiratory allergy. Past reports have described the development of occupational dermatitis by exposure to lettuce, or the occurrence of systemic adverse reactions after ingestion of lettuce.⁵⁻¹⁰ Based on the reported lettuce-associated OAS and anaphylaxis cases, several proteins have been identified as allergens in previous studies. Lac s 1 (9 kDa, lipid transfer protein),⁹⁻¹¹ 16 kDa,⁸ 26 kDa (Thaumatin-like protein family), and 35 and 45 kDa (aspartyl protease)¹² were identified in whole lettuce mainly leaves. However, a limited number of studies have investigated the respiratory symptoms induced by lettuce, and a causal allergen has not been identified thus far.

In the survey conducted in the Hyogo prefecture, 7% of the lettuce farmers reported the respiratory symptoms associated with lettuce farm work. The results of the questionnaire and patients' interviews suggested that most symptomatic farmers exhibited lettuce-associated respiratory symptoms during cutting of the lettuce stem, wiping the lettuce latex, and packaging lettuce in small poorly ventilated rooms. Of note, ingestion of lettuce leaves did not result in the occurrence of symptoms. These results suggested that the allergens are present in the lettuce stem, rather than the lettuce leaves.

In the current study, we identified the following novel allergen: epidermis-specific secreted glycoprotein EP1-like (51 kDa). Studies have shown that EP1 proteins are involved in cell elongation.¹⁸ A higher level of EP1 is found in the basal parts of seedling hypocotyls and roots vs that reported in the apical parts. EP1 gene expression was detected in cells located on the surface of the seedling, the epidermis of the root or root cap, and it is thought to be involved in the development of seeds.¹⁹

Although we were unable to detect the specific band, Lac s1 (9 kDa) firstly identified in allergy to lettuce.¹⁰ The difference of respiratory vs ingestive allergic phenotypes causes the difference patterns of allergen recognition. The present allergens may be closely related to the development of respiratory symptoms (eg asthma) although the previously identified allergen was related to OAS or anaphylaxis.^{8-10,12} Moreover, we focused on the differences between parts of lettuce to gain further insight into the cause of these differences in allergen. Previous studies used whole lettuce, which was mainly composed of leaves. In contrast, in the current study, we used the lettuce latex instead of leaves. In 2017, Reyes-Chin-Wo S reported all the sequencing of the lettuce genome.²⁰ Therefore, we can more accurately examine the lettuce vs previous studies.

Several sensitization pathways may be related to allergic airway inflammation.²¹ In particular, two sensitization pathways are thought to be important in provoking lettuce-associated respiratory symptoms: trans-airway sensitization and transdermal sensitization. All three patients who suffered from respiratory symptom even with treatment exhibited atopic dermatitis and lettuce-associated dermatologic symptoms. Moreover, lettuce latex contains the sesquiterpene lactone, which proved to be sensitizers and often causes non-IgE-mediated type IV allergic contact dermatitis.⁵ Therefore, we speculate that transdermal sensitization might be an important pathway involved in the development of lettuce-associated respiratory symptoms. These patients were ordinarily exposed to the lettuce latex and may have been sensitized through the skin. Indeed, atopic dermatitis can progress from a skin disease to asthma.²² The skin barrier in patients with atopic dermatitis has been linked to sensitization through aeroallergens.²³ Oral sensitization is the most established sensitization pathway. However, the pathway is not applicable to the present cases, because none of the patients exhibited OAS or anaphylaxis after ingestion of lettuce.

Although the result of ELISA and immunoblot of the patients' sera to lettuce latex supported that all the patients' sera contain much more lettuce-specific IgE compared to negative control's sera, we could not deny the possibility that some of the patients had type IV allergy caused by sesquiterpene lactones, which are present in many plants from the Asteraceae family, including lettuce.

Patient 3 is the most valuable patient in the whole study, and patient 3 was allergic to mould. We cannot deny that the inhalation provocation test was affected by mould, since we could not sterilize lettuce sample which denatures with heat. We think this is one of the limitations of our study.

The epidermis-specific secreted glycoprotein is most likely glycosylated, as its name implies and as has been shown for related proteins from other species. Hence, IgE binding to this protein may be caused by IgE specific to cross-reactive carbohydrate determinants. We think this is one of our limitations.

The results of the present study provide a basis for the development of in vivo and in vitro diagnostic tools, as well as allergen-specific immunotherapy for patients with respiratory allergy to lettuce.

5 | CONCLUSIONS

In conclusion, we surveyed lettuce-induced respiratory allergy and identified a causative novel allergen in the lettuce latex: epidermis-specific secreted glycoprotein EP-like.

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AUTHOR CONTRIBUTION

RS and TN conducted data collection, performed analysis, and wrote the manuscript. YN and HK conceived the research questions and designed the study. YK and KK helped with the writing of the manuscript. TK, HS, MK, KU, NK, SH, NHZ, and MY distributed the questionnaire to farmers. TM, EY, NHT, AF, and MS helped with data collection and contributed to statistical analyses. All authors contributed to data interpretation and critically revised the manuscript for important intellectual content. All authors approved the final version of the submitted manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in UMIN at <https://www.umin.ac.jp/>, reference number UMIN000027002.

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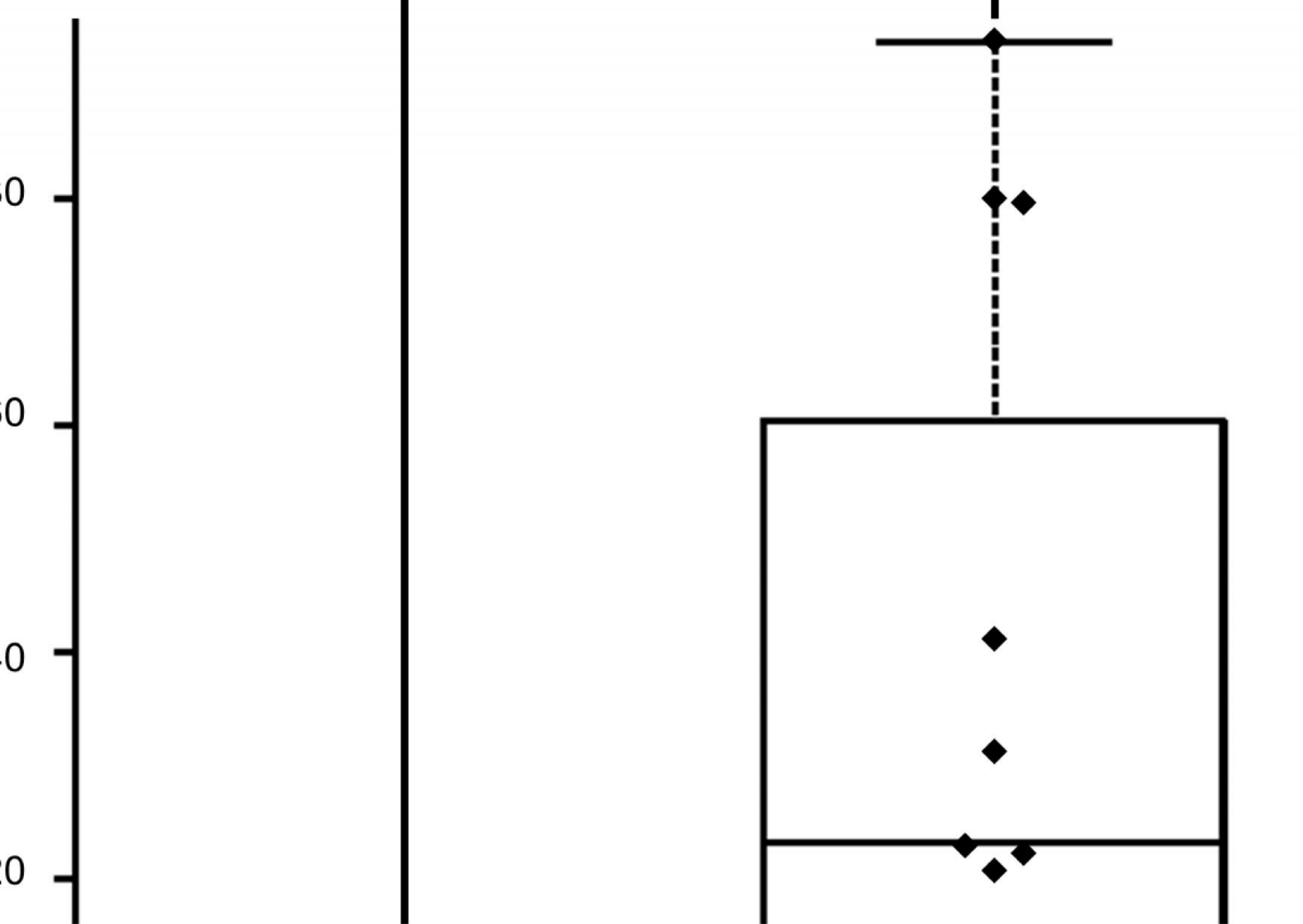
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Basophil Activation Test

CD203c expression on peripheral blood basophils was assessed by using the allergenicity Kit (Beckman Coulter, Fullerton, Calif, USA) on the day of sample collection. First, whole blood was drawn into plastic tubes containing EDTA 2Na (100 µl), 20 µl of antigen, 20 µl of tricolored antibodies (CRTJ-2 FITC, CD203c-PE and CD3-PC7) and 100 µl of activation buffer mixed in plastic tube. The mixture was then incubated at 37°C for 15 min. After incubation, 50 µl of stop reaction, as well as lysis-fixing solution, and the mixture was incubated for 10 min at room temperature. The sample was washed with phosphate-buffered saline (PBS), fixed with 0.1% formaldehyde, and then assayed by flow cytometry. The following antigens were used: **anti-IgE antibody (Beckman Coulter)(1 mg/ml) as positive control, lettuce stem extract (4 mg/ml, 0.4 mg/ml, and 0.04 mg/ml), and PBS as negative control.** Negative controls were sera of seven patients with no history of allergy. CD203c expression on basophils was assessed using negative control (PBS) and positive control (Anti-IgE antibody), in comparison of the amount of CD203c expression on basophils. The samples which have no difference of reactivity between positive and negative control were excluded(3 patients' samples were excluded).

Purification of the allergen(Ion exchange chromatography and gel chromatography)

Purification of lettuce latex was firstly performed by ion-exchange chromatography. Then we performed secondly purification from the fraction of the ion-exchange by gel chromatography.

At first, PD-10 column (GE healthcare, Chicago, USA) was used for **desalt the sample.**

Open column filled with anion resin(TOYOPEARL® SuperQ-650, TOSOH, Tokyo, Japan) was used for ion exchange chromatography. After loading the sample, the column was initially washed with 10 mM Tris-HCl buffer pH 8.0 for 5 min to separate any unbound protein. The bound protein was subsequently eluted using a gradient of 0.05, 0.1, 0, 0.2, 0.3, 0.4, 0.5 M and 1 M NaCl in 10 mM Tris-HCl buffer pH 8.0. Pointed fractions from ion-exchange columns were concentrated by ultrafiltration on the Ultracel 10K Amicon Ultra filters. The concentrated proteins were separated again through a high-performance liquid chromatography system (L-6200 Intelligent Pump, Hitachi, Tokyo, Japan. Column: TOSOH, TSK-Gel, G3000SW-XL (7.5 mm x 300 mm) into 40 fractions. Proteins were eluted by ~~a linear gradient mixture~~ of starting buffer (10 mM TrisHCl pH 8.0) supplemented with 0.15 M NaCl solution and 1mM EDTA. The fractionation was performed at a 0.5 ml/min flow rate and the fractions were collected in 0.5 ml volumes.