

Isolated Protein of *Astragalus membranaceus* Acts as an Allergen by Binding Human Immunoglobulin E on Human sera

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Abstract Allergy is nearly always triggered by protein molecules and the majority of individuals with documented immunologic reaction to foods or plants IgE reactions. However, proteins derived from herbal medicine act as an allergen is unknown. In this study, we aimed to confirm if herbal medicine, *Astragalus membranaceus*, at proteomic level may contain to trigger the immunoreactivity of IgE. If it does, what components of protein molecules in herbal medicine, *Astragalus membranaceus*, is react to a binding IgE. In order to resolve this hypothesis, we performed proteomic tools, SDS-PAGE stained coomassie blue and identified protein by LC-MS/MS. Also, we tested plasma IgE reactivity on an isolated protein of *Astragalus membranaceus* using western blot of human IgE and enzyme immunoassay panel, Allergy-Q test in 400 patients. Through the described performances, we could select four sera of positive candidate and identify *Astragalus membranaceus* IgE binding proteins. Our data suggested that herbal medicine, *Astragalus membranaceus* was possible to act an allergen by identify IgE binding protein with isolated protein of *Astragalus membranaceus*.

Keywords: Allergy, IgE, Allergy-Q, Herbal medicine, *Astragalus membranaceus*

Introduction

Plants have evolved to protect against toxic element or insecticidal properties by producing plant proteins and they have took advantage of produced protein to perform propagation and survivals of the species¹. However, this protein derived from the defense mechanism of plants sometimes affect harmful properties. Because allergens derived from plant proteins have found by molecular cloning and observed an immunoglobulin E (IgE)-mediated allergic reaction in human². The known causing factor of allergic reaction on plants was pollen, spores, fruit, plant food and so on^{2,3}.

IgE has classically been thought to be important in allergic diseases, which is increasing worldwide⁴. Because an allergic reaction is initiated when an antigen crosslinks IgE anti-bodies bound to their high-affinity Fc receptor on tissue mast cells or blood basophils⁵. Confirmation of culprit allergens in an individual patient is crucial for the diagnosis of IgE as an allergic diseases, such as asthma, rhinitis, conjunctivitis, and dermatitis. Proving the presence of allergen-specific immunoglobulin E (sIgE) in the serum is able to use as a surrogate method to detect and identify the culprit allergen⁶. For confirmation of sIgE concentration on sera, many commercially available immunochemical assays based on enzyme immunoassay (EIA) or fluorescent enzyme immunoassay (FEIA) are now widely used in clinical settings⁷. Among the EIA technique using nitrocellulose membrane as the solid-phase for allergen immobilization, Allergy-Q test, developed multiplex sIgE test, can detect 43 allergen sIgEs simultaneously. Also, this system has an internal calibration setup that can adjust the user of sIgE concentration in

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relation to measured total IgE level in patients' sera quantitatively⁸.

The use of medicinal plants, so called herbal medicines for the maintenance of good health has been widely observed in developing countries. After World Health Organization (WHO) established a guidelines for the assessment of herbal medicine in 1991⁹, the herbal extracts and herbal medicines were used to prescribe drugs for health care throughout all over the world including European Union¹⁰. Because, herbal medicines have reported that the phyto-therapeutic chemicals present in herbal medicine and have stood the test of time for their safety and pharmacological efficacy without side effects in clinical evaluation of oriental medicine area¹¹.

Among the tremendous herbal medicines, *Astragalus membranaceus* is one of the most used traditional herbal medicines¹². Because it reported therapeutic and pharmacologic effects on tremendous biological mode of action. In perspective of therapeutic effects, *Astragalus membranaceus* is used as immune-modulating activity¹³, hypoglycemia¹⁴, anti-inflammation¹⁵, and so on. Also, it is treat chronic phlegmatic disorders and general gastrointestinal disturbances in clinical trials¹⁶. In perspective of chemical constituents, *Astragalus membranaceus* has more than 100 compounds, such as flavonoids, saponins, polysaccharides, and amino acids have been identified^{17,18}. Although the advantages of the described therapeutic and pharmacological efficacy, many people should deliberate to prescribe and treat *Astragalus membranaceus*. Because, some of the *Astragalus* species synthesize aliphatic nitro compounds¹⁹. Then, synthesize aliphatic nitro compounds are transformed in the rumen to the toxic principles 3-nitro-1-propanol (3-NPOH) and 3-nitropropionic acid (3-NPA). According to these transformed compounds, β -glucosides and some glucose esters are derived from 3-NPOH (miserotoxin) and 3-NPA respectively²⁰. Even if herbal medicine researches of biological activity analysis and constituents-based efficacy are currently underway, their proteomic researches are insufficient. Here, our group observed protein profiling on natural *Astragalus membranaceus*. Also, *Astragalus membranaceus* of indirect IgE detection was measured by Allergy-Q test from among 400 Korean patients with known allergies for 21 individual allergens, including important inhalant, food, microorganism allergens, and isolated protein of *Astragalus membranaceus*. After this measurement of IgE detection, we selected 4 sera of positive candidate. The IgE binding protein was identified by LC-MS/MS. We suggested that what proteins were binding with human IgE on *Astragalus membranaceus* and identified proteins was

possible to act as an allergen.

Results and Discussion

Astragalus membranaceus is Possible to Act as an Allergen

Recent, many studies have reported that plant proteins exists and several plant proteomes may have on plant IgE binding proteins profiles². Also, dust particles causing allergy and chronic respiratory problem were researched by bio-analytical approaches for proteomic analysis²¹.

However, proteomic analysis of herbal medicine is poorly known. Here, our group firstly tested that whether the *Astragalus membranaceus* contains some proteins or not. We clearly observed the Coomassie blue-staining SDS-PAGE protein profiles on *Astragalus membranaceus* (Figure 2A). Also, whether the isolated proteins on herbal medicines act as an allergen or not, we measured the indirect IgE intensity on positive or negative sera for *Astragalus membranaceus* using Allergy-Q test (Figure 1). In this results, we observed that the *Astragalus membranaceus* contains plant proteins and this plant protein of *Astragalus membranaceus* is possible to act as an allergen. The Allergy-Q test detects 43 allergens of IgE concentration in human sera⁸. Then, the 400 sera of patients pool were firstly screened by Allergy-Q test. We found 2-fold intensity of indirect IgE about isolated protein of *Astragalus*

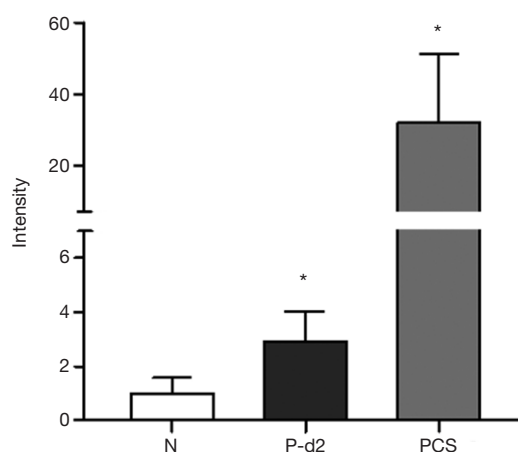


Fig. 1. The indirect IgE concentration on protein extracts of *Astragalus membranaceus* was measured by Allergy-Q test. The Allergy-Q test was analyzed using a paired t-test. The arrow indicates in-gel digestion. AM; *Astragalus membranaceus*, N; negative sera, n = 3, P-d2; positive sera of d2 allergen, triplicate, PCS; Positive candidate sera of *Astragalus membranaceus*, n = 4, * $p < 0.05$.

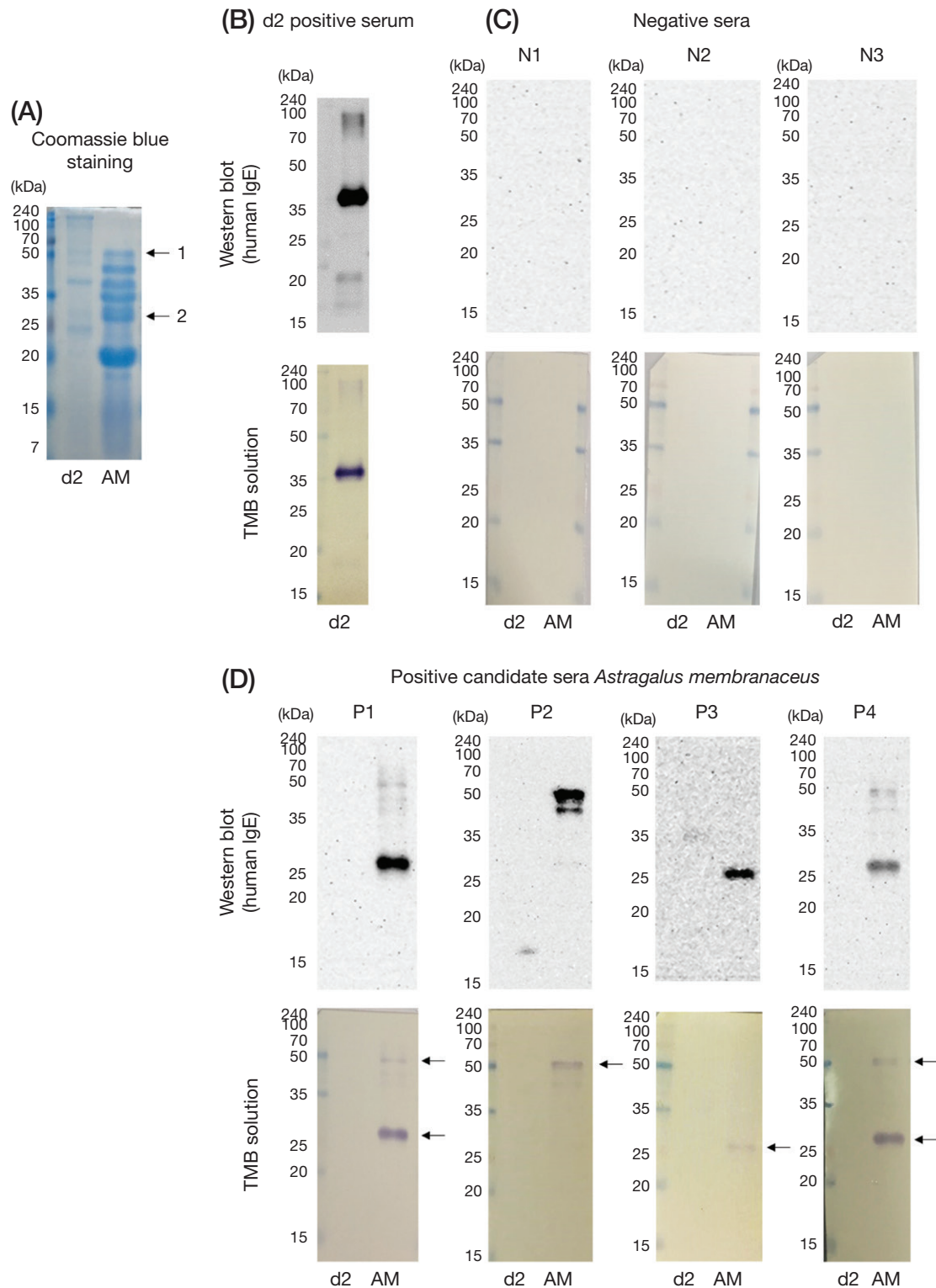


Fig. 2. (A) SDS-PAGE analysis on protein extracts of *Astragalus membranaceus*. (B, C, and D) Western-blot analysis and TMB solution on the human sera was detected on nitro-cellular membrane. (B) The human IgE reactivity was represented on the positive serum of d2 allergen. (C) The negative sera for d2 allergen and *Astragalus membranaceus* were represented. (D) The positive candidate sera for *Astragalus membranaceus* were indicated. The d2, 2 μ g and AM, 20 μ g were loaded on SDS-PAGE gel. The specific IgE-binding sites on the two point of nitro-cellular membrane (A and D, arrow) were identified by LC-MS/MS.

Table 1. Indirect IgE intensity was measured on Allergy-Q test.

			IgE concentration (IU/mL)							
			d2 Positive serum	Positive candidate sera				Negative sera		
No.	Allergen		P-d2	P1	P2	P3	P4	N1	N2	N3
1	<i>D. pteronyssinus</i>	d1	31.08	0.1	58.62	0.1	>100	0.1	0.1	0.1
2	<i>D. farinae</i>	d2	25.25	0.1	100	1.31	>100	0.1	0.1	0.1
3	Cat	e1	6.99	0.1	0.1	0.1	2.37	0.1	0.1	0.1
4	Dog dander	e5	10.58	0.1	0.1	0.1	0.1	0.1	0.1	0.1
5	Egg white	f1	4.26	0.1	0.1	0.1	0.1	0.1	0.1	0.1
6	Milk	f2	0.15	0.1	0.1	0.1	2.43	0.1	0.1	0.1
7	Cockroach	i6	24.58	0.61	2.59	5.13	0.85	0.1	0.1	0.1
8	Peanut	f13	22.12	0.1	0.1	0.1	0.1	0.1	0.1	0.1
9	Soy bean	f14	0.89	0.1	0.1	0.1	0.1	0.1	0.1	0.1
10	Wheat flour	f4	0.3	3.31	0.24	4.49	0.59	0.1	0.1	0.1
11	Birch-alder mix	t3	0.15	7.61	0.1	8.95	0.1	0.1	0.1	0.1
12	Oak white	t7	2.26	0.1	0.1	0.1	2.62	0.1	0.1	0.1
13	Ragweed, common	w1	0.87	25.01	2.9	37.03	6.89	0.1	0.1	0.1
14	Mugwort	w6	0.15	0.81	0.1	2.42	3.57	0.1	0.1	0.1
15	<i>A. alternata</i>	m6	2.23	0.18	0.87	0.1	0.54	0.1	0.1	0.1
16	Crab	f23	12.22	0.1	0.71	2.39	0.1	0.1	0.1	0.1
17	Shrimp	f24	23.3	0.1	0.1	0.1	0.27	0.1	0.1	0.1
18	Mackerel	f206	0.15	0.1	0.1	0.1	0.1	0.1	0.1	0.1
19	Rye	g12	0.15	23.71	1.45	18.89	7.69	0.54	0.34	0.1
20	Peach	f95	0.15	2.32	0.1	4.53	1.61	0.1	0.1	0.1
21	Apple	f49	0.15	1.35	0.1	2.12	1.61	0.1	0.1	0.1

D. pteronyssinus, *Dermatophagoides pteronyssinus*; *D. farinae*, *Dermatophagoides farinae*; *A. alternata*, *Alternaria alternata*

membranaceus on them and select the four sera of positive candidate on patient's pool. The selected-positive or negative sera of intensity about standard allergens were added on Table 1.

***Astragalus membranaceus* is React to Human IgE on the Sera of Positive Candidate**

Although the plant protein of *Astragalus membranaceus* significantly measured indirect IgE concentration on human sera using Allergy-Q test, we additionally confirmed the human IgE-binding intensity on nitro-cellulose membrane of separated herbal proteins using western blotting and reaction of TMB substrate (Figure 2B, C, and D). Here, we use to detect the human IgE-binding band on the four sera of positive candidate (Figure 2D) and, use the negative sera of the worst sensitivity about herbal proteins (Figure 2C). The negative sera are able to firstly distinguish un-specific binding of human IgE antibody using Allergy-Q test. Through the results of negative sera (Figure 2B), we can select the detection of human IgE binding protein without un-specific band on nitro cellulose membrane. However, any un-specific binding of human IgE reactivity was not detected on negative sera. The distinguished bands as a reactivity of human IgE were pointed with an arrow on positive candidate sera for *Astragalus mem-*

branaceus (Figure 2D). As this article of introductions described, *Astragalus membranaceus* reported various pharmacological mode of action in biological activity. But, this results of *Astragalus membranaceus* suggested dangerous possibility to induce allergic diseases by IgE. However, an interesting pattern was reported that pathogenesis of eosinophils on anti-apoptotic effects had different between actopic and non-actopic asthma depending on the presence of different house dust mite-specific IgE²². According to this pattern, researches about some of the *Astragalus* species should be necessary to confirm the IgE reactivity and be considered to prescribe or treat for safe treatment reflected these individual characters in allergic reactivity.

Identification of the Candidate Protein of *Astragalus membranaceus* with Human IgE Binding Protein

Furthermore, to identify the protein of *Astragalus membranaceus* with human IgE binding protein, we performed the in-gel digestion on indicated number detected human IgE anti-body (arrow of Figure 2A) and analyzed the human IgE detected-proteins of same location on digested SDS-PAGE gel by LC-MS/MS (Table 2). We have identified five different IgE binding proteins on NO.1 band (Table 2A). Two proteins in NCBI BLAST library, gil464734 and gil667671892,

Table 2. Identified *Astragalus membranaceus* IgE binding proteins.

A. No.1 band of an arrow on Fig. 2A.

No.	NCBI BLAST	Protein name	Score	Mass
1	gil464734	S-adenosyl-L-homocysteine hydrolase	166	53199
2	gil502135172	adenosylhomocysteinase [Cicer arietinum]	166	53284
3	gil667671892	S-adenosyl-L-homocysteine hydrolase [Populus tomentosa]	147	53414
4	gil470145062	enolase [Fragaria vesca subsp. vesca]	145	48170
5	gil658051505	adenosylhomocysteinase-like [Malus domestica]	126	32876

Ion score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event.

Individual ions scores > 59 indicate identity or extensive homology ($p < 0.05$).

Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

B. No.2 band of an arrow Fig. 2A.

No.	NCBI BLAST	Protein name	Score	Mass
1	gil596280094	hypothetical protein PRUPE_ppa001515mg [Prunus persica]	73	93693
2	gil545353644	Actin/actin-like protein [Coccomyxa subellipsoidea C-169]	66	41465
3	gil645260108	PREDICTED: legumin J-like [Prunus mume]	56	58188

Ion score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event.

Individual ions scores > 56 indicate identity or extensive homology ($p < 0.05$).

Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

indicate S-adenosyl-L-homocysteine (SAH) hydrolase. But, gil464734 is derived from *Catharanthus roseus* and gil667671892 is derived from *Populus tomentosa*. The hydrolysis of SAH to adenosine and L-homocysteine by SAM hydrolase is one of the process in the re-generation of S-adenosyl-L-Met²³. SAH hydrolase is associated with the maintenance of methylation potential in cells, and is able to further affect plant growth and development²⁴. Adenosylhomocysteinase is associated with pathway of L-homocysteine biosynthesis. Adenosylhomocysteinase is involved in step of the subpathway that synthesizes L-homocysteine from S-adenosyl-L-homocysteine. gil470145062 is enolase which is a highly expressed key enzyme of glycolysis and gluconeogenesis²⁵. Enolase, so called Hev b 9, was first identified as an allergen in *Saccharomyces cerevisiae*²⁶. However, the IgE crossreactivity was found between enolases of *Cladosporium herbarum* (Cla h 6), *Alternaria alternata* (Alt a 5), *Candida albicans*, *Aspergillus fumigatus*, and *Fusarium solani*^{27,28}. Secondly, No.2 band was significantly identified about three proteins by LC-MS/MS (Table 2B). Hypothetical proteins perform to several functional activities, which are a metal ion binding, a magnesium ion binding (GI: 595803746), zinc ion binding (GI: 595817415), and manganese ion binding (GI: 462397090). Even if, the function of hypothetical protein (GI: 596280094) on *Astragalus membranaceus* is unclear, several identified hypothetical proteins used to find allergen using consensus sequence of N-glycosylation in almond²⁹. This application and the mention to be considered as

suspicious allergen supported that *Astragalus membranaceus* act as an allergen³⁰. Legumin (Ara h 1) is one of the seed storage proteins³¹. The legumin of continuous IgE-binding epitopes have been characterized on Ara h 1 sequence. Also, the legumin was identified as the major peanut allergens³². A legumin on *Astragalus membranaceus* need to research specific IgE-binding epitopes through spot technique and structural assessment³³. Our results exhibited IgE reactivity to protein band on isolated protein of *Astragalus membranaceus* using 1D gel electrophoresis and western blotting. And the human IgE binding protein was identified by LC-MS/MS. Even if we identified IgE binding protein with human sera of positive candidate on isolated protein of *Astragalus membranaceus*, 2D gel electrophoresis and its spot analysis need to develop specific and exact evaluation of IgE binding protein.

Conclusions

In this study, our group suggested that the isolated protein of *Astragalus membranaceus* existed IgE binding protein with human sera. Through the identification of IgE binding protein of *Astragalus membranaceus*, our group found that S-adenosyl-L-homocysteine hydrolase, adenosyl-homocysteinase associated with maintenance of methylation potential in cells were binding with human IgE. Also, enolase, hypothetical protein, and legumin were detected by LC-MS/MS. Even if, this IgE reaction on *Astragalus membranaceus* was

few people in 400 human sera, prescription or treatment of herbal medicines including *Astragalus membranaceus* should consider the proteomic characters associated with allergenic molecules in potential patients.

Materials and Methods

Herbal Protein Isolation

Herbals were purchased from Omniherb (Yeoncheon, South Korea). The herbal tissue was shock frozen in liquid nitrogen, and then ground using a mortar and pestle until a smooth paste was achieved. Herbals were vortexed (Scientific Industries, USA) and soaked overnight in 20 mM Tris buffer (pH 8.5) at 4°C. After incubated at 4°C, the buffer were centrifuged on 13,000 rpm. Supernatant derived from the buffer was isolated and measured in protein quantitative analysis. Isolated supernatant concentration was then determined using Bradford reagent (Bio-Rad, USA) and a microplate reader (Bio-Rad, USA). All herbal extracts were stored at -80°C.

SDS-PAGE and Western Blot Analysis

Astragalus membranaceus were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The represented positive allergen about house dust mite, *Dermatophagoides farinae* was purchased by Diagnostische Systeme & Technologien GmbH (DST, Schwerin, Germany). DokDo-MARK Broad-Range Protein Ladder (Elpis bio, Daejeon, South of Korea) was used as molecular weight markers to estimate protein size. To staining the separated proteins on SDS-PAGE, Coomassie G-250 Stain (Bio rad) was used³⁴. The separated proteins were transferred from the gel to a nitrocellulose membrane (NC) using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio rad)³⁵. And the NC membranes were then blocked with 3% Bovine Serum Albumin (BSA) for 1 hour at room temperature, and then incubated overnight with the patient sera, which was diluted 1 : 5 in 3% BSA. After washing with PBS-Tween (PBS-T), the membrane was incubated with HRP-labeled goat IgG-anti-human IgE (Thermo Scientific, Rockford, IL 61105 USA), which was diluted 1 : 5,000 in 3% BSA, for 1 hour at room temperature. After rinsing with PBS-T, the membrane was incubated with Luminata Crescendo Western HRP substrate (EMD Millipore, Billerica, MA, USA) for 5 minutes at room temperature. In case of TMB substrate reaction, NC membranes detected secondary anti-body of HRP-labeled human IgE was

incubated on TMB solution (Sigma) during 5-15 minutes.

Study Participants

Between June and December of 2014, participants who volunteered the blood donation at Kyung Hee Medical Center for the diagnosis of their allergic diseases were enrolled in our study. All patients' sera sampling for Allergy-Q test was performed. Informed consent was obtained from all patients. The Institutional Review Board of the Kyung Hee University College of Korean Medicine approved this study (IRB no. KOMCIRB-140821-HR-003).

In vitro Allergen sIgE Measurements

Serum aliquots were sent to the laboratory without any patient identification. We performed allergen sIgE detection using 2 different systems simultaneously (PROTIA™ Allergy-Q; Proteometech, Seoul, South of Korea). The Allergy-Q test was operated according to the manufacturers' instructions.

In-gel Digestion with Trypsin and Extraction of Peptides

Protein bands from SDS-PAGE gels were excised and in-gel digested with trypsin according to established procedures³⁶. In brief, protein bands were excised from stained gels and cut into pieces. The gel pieces were washed for 1 h at room temperature in 25 mM ammonium bicarbonate buffer, pH 7.8, containing 50% (v/v) acetonitrile (ACN). Following the dehydration of gel pieces in a centrifugal vacuum concentrator (Biotron, Inc., Incheon, Korea) for 10 min, gel pieces were re-hydrated in 50 ng of sequencing grade trypsin solution (Promega, Madison, WI, USA). After incubation in 25 mM ammonium bicarbonate buffer, pH 7.8, at 37°C overnight, the tryptic peptides were extracted with 5 µL of 0.5% TFA containing 50% (v/v) ACN for 40 min with mild sonication. The extracted solution was concentrated using a centrifugal vacuum concentrator. Prior to mass spectrometric analysis, the peptides solution was subjected to a desalting process using a reversed-phase column³⁷. In brief, after an equilibration step with 10 µL of 5% (v/v) formic acid, the peptides solution was loaded on the column and washed with 10 µL of 5% (v/v) formic acid. The bound peptides were eluted with 5 µL of 70% ACN with 5% (v/v) formic acid.

Identification of Proteins by LC-MS/MS

After desalting, eluted tryptic peptides were separated

and analyzed using a nano ACQUITY UPLC (Waters, Milford, MA, USA) directly coupled to a Finnigan LCQ DECA ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA). In brief, the peptides were bound to the ACQUITYUPLC peptide BEH C18 column (1.7 μ m size, 130 Å in pore size, 100 μ m $\times$ 100 mm) with distilled water containing 0.1% (v/v) formic acid and the bound peptides were eluted with a 40 min gradient of 0-90% (v/v) acetonitrile containing 0.1% (v/v) formic acid at a flow rate of 0.4 μ L/min. For tandem mass spectrometry, a full mass scan range mode was m/z = 400-2000 Da. After determination of the charge states of an ion on zoom scans, product ion spectra were acquired in MS/MS mode with relative collision energy of 55%. The individual spectra from MS/MS were processed using the SEQUEST software (Thermo Quest, San Jose, CA, USA) and the generated peak lists were used to query NCBI database using the MASCOT program (Matrix Science Ltd., London, UK). We set the modifications of methionine and cysteine for MS analysis and tolerance of peptide mass was 2 Da. MS/MS ion mass tolerance was 1 Da, allowance of missed cleavage was 1, and charge states (+1, +2, and +3) were taken into account for data analysis. We took only significant hits as defined by MASCOT probability analysis.

Statistical Analysis

The significant data was analyzed using Prism 5 software with paired t-test. Bars exhibit SEMs of means for triplicate assays. Statistical significance of IgE detection using Allergy-Q was analyzed by paired t-test (P cutoff value = 0.05).

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