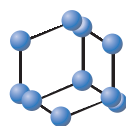


LETTER ARTICLE

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SCIENCE

Allergen Homologues, Pathogenesis-Related 1, Polygalacturonase, and Pectin Methyl Esterase from a Japanese Hop

Seok Woo Jang^{1,2}, Kyoung Yong Jeong^{1,3,*}, Ji Eun Yuk¹, Jongsun Lee¹, Kyung Hee Park¹ and Jung-Won Park^{1,3}¹Department of Internal Medicine, Institute of Allergy, Yonsei University College of Medicine, Seoul 03722, South Korea; ²Brain Korea 21 PLUS Project for Medicine Science, Yonsei University, Seoul 03722, South Korea; ³Prolagen Ltd., Seoul 03722, South Korea**Abstract: Background:** Japanese hop is an important cause of weed pollinosis in East Asia. Its pollen is abundant in autumn. This pollen is known to be the cause of many allergic diseases. However, molecular characteristics of its allergens have not been elucidated.**Objective:** In this study, we produced recombinant proteins of allergen homologues from Japanese hop by the analysis of expressed sequence tags (EST), and evaluated its allergenicity.**Methods:** cDNA library was constructed using as little as 50 ng of total RNA from Japanese hop pollen. Allergen homologues were identified by the initial screening of 963 EST clones. Recombinant proteins were overexpressed in the *E. coli* expression system and purified using Ni-nitrilotriacetic acid-agarose. Purified proteins were analyzed by ELISA.**Results and Discussion:** Japanese hop pathogenesis-related 1 protein (PR-1) shares 37.0 to 44.4% of amino acid sequence identity with Art v 2, Cuc m 3, and Cyn d 24. Pectin methyl esterase (PME) shows 23.2 to 50.2% of identities to Act d 7, Ole e 11, and Sal k 1. Polygalacturonase (PGs) shows 16.7 to 19.3% of identities to Phl p 13, Cry j 2, Cha o 2, Jun a 2, Pla a 2, and Pla o 2. IgE antibodies from Japanese hop allergy patients' sera recognized PR-1 (3.4%), PME (13.8%), PGs (3.7%), and profilin (13.8%), respectively.**Conclusion:** Novel allergenic components were identified, even though low IgE reactivity was displayed reflecting the low degree of cross-reactivity with other pollen allergens. We believe that these molecules have worth further studies.**Keywords:** Pathogenesis-related 1, pectin methyl esterase, polygalacturonase, pollinosis, allergic diseases, Japanese hop.

1. INTRODUCTION

Molecular biology and bioinformatics enabled us to identify and characterize allergens and more than 2,000 allergens are officially listed in WHO/IUIS allergen nomenclature database (www.allergen.org). Not surprisingly, allergenic molecules are distributed into a few protein families [1]. Various web-based databases have been developed for the better analysis and understanding of allergenic molecules [2, 3].

It is not easy to obtain enough amounts of pollen grains from the fields for molecular biological analysis and allergen characterization. PCR-based methods for cDNA synthesis kit, such as SMARTer PCR cDNA Synthesis Kit and In-Fusion SMARTer Directional cDNA library kit (Clontech Laboratories, Inc., Mountain View, CA, USA) allow us to construct a cDNA library from nanogram quantities of total RNA.

Japanese hop, *Humulus japonicus*, is an important cause of weed pollinosis in East Asia. It is wind-borne and has a flowering period. In Korea, its pollen is found to be abundant in the autumn whereas with oak pollen is abundant in spring [4-9]. Pollen is known to be the main cause of atopic dermatitis, as well as respiratory allergies such as asthma and allergic rhinitis [10, 11]. Many studies have been reported on the link between airborne and allergic diseases and respiratory diseases, and the extracts of the pollen are clinically used to skin test, serum-specific immunoglobulin E test, and for immunotherapy [12-14]. Skin positivity was also shown to be well-correlated with the pollen counts [15-18]. A 10-kDa protein was described as a major allergen of Japanese hop [19]. However, its molecular cloning has not been done. We produced recombinant proteins of allergen homologues from PR-1 [20, 21], PGs [22-24], and PME. Profilin is the only allergen characterized in molecular detail, although it was shown to be a minor allergen [25].

In this study, we tried to construct a Japanese hop pollen cDNA library by using a PCR-based cDNA synthesis kit. Allergen homologous molecules were identified by the

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Table 1. Clinical features of individuals analyzed.

Age	IgE to w22	Total IgE	Symptom*	Sensitization Profile**	Absorbance at 450 nm			
					IgE to PR-1	IgE to PME	IgE to PGs	IgE to Profilin
53	23.5	58.1	AR, BA	t2, t3, t7, t8, t17, t70, w1, w5, w7, w22,	0.105	0.091	ND	0.076
26	13.5	165	AR	d1, d2, e2,m6, w5, w7, w9, w22	0.089	ND	ND	0.096
59	4.51	181	CR	d1, d2, d72, e1, e2, g6, t2, t3, t5, t7, t10, w22	0.101	0.089	0.095	0.069
46	5.76	56.8	AR	f5, f9,, f11, f13, f25, f33, f47, f48, f95, f208, g1, g2, g6, g7, g201, i6, i206, t7, t12, w1, w4, w6, w8, w11, w22	0.101	0.093	0.097	0.080
23	32	83.1	AR, CV	m1, t1, t2, t3, t5, t7, t8, t10, t11, t70, w1, w5, w7, w8, w10, w12, w22	0.110	0.135	0.123	ND
42	16.5	101	AR, BA, CV	d1, d2, g2, g3, g5, g6, g8, g12, t1, t2, t3, t7, t8, t10, t11, t12, t15, t17, t70, w9, w10, w12, w16, w22	0.084	0.096	0.090	0.417
56	20.6	306	AR, BA	f6, f14, t1, t2, t3, t5, t7, t15,t70, w5, w7, w12, w22	0.119	0.122	0.120	0.092
45	17.5	ND	AR	d1, d2, f4, g15, t3, t7, w1, w5, w7, w10, w16, w22	0.100	0.101	0.100	0.086
64	6.06	3	CR	e71,i6, w22	0.093	0.113	0.103	0.111
32	42.2	ND	AR	d2, t3, t7, w22	0.098	ND	ND	ND
41	45.4	319	AR, BA	d1, d2, t17, w22	ND	0.093	0.098	0.083
51	66.6	ND	AR, CV	w22	0.107	0.111	0.095	0.093
58	11.8	ND	AR	w22	0.110	0.112	0.109	0.070
24	93	348	AR, CV	d1, d2, e1, e83, g2, g3, g5, g6, g8, g12, t8, t10, t12, t17, w1, w5, w7, w9, w10, w12, w22	0.109	0.181	0.111	0.073
49	>100	232	AR, CV	w1, w6, w10, w22	0.118	0.108	0.145	0.072
41	30.1	230	AR, CV	w1, w6, w8, w12,w22	0.113	0.313	0.113	0.088
24	5.3	ND	AR	w22	0.111	0.102	0.213	0.080
49	31.1	ND	AR, CV	w1, w22	0.098	0.102	0.107	0.146
64	2.55	183	AR, CV	f4, f12, f13, k84, w1, w22	0.097	0.101	0.100	0.064
18	33.3	ND	AR	d1,d2, t7, w22	0.109	0.112	0.099	0.064
28	71.5	ND	AR	w22	0.103	0.100	0.110	0.064
12	29.7	ND	AR	d2, e5, w22	0.124	0.203	0.101	0.072
54	>100	356	AR	d1, d2, w1, w6, w22	0.126	0.104	0.164	0.090
30	>100	ND	AR, AD	d1, d2, d72, g5, g6, m1, m2, m3, m5, t10, t70, w5, w10, w12, w22	0.115	0.103	0.115	0.096
57	5.96	410	AR, CV	d1, d2, g3, g5, g8, g12, m5,,t1, t2, t3, t4, t7, t8, t10, t11, t12, t15, t70, t205, w1, w5, w7, w9, w10, w12, w22	0.113	0.105	0.109	0.458
25	12.7	ND	AR, CV	d2, e2, g5, t1, t8, w1, w5, w7, w8, w12, w22	0.077	0.089	0.109	0.094
56	34.7	ND	AR	w6,w22	0.113	0.103	0.083	0.080
58	16.9	ND	AR	f11, t2, t3, t7, t15, w5, w7, w12, w22	0.111	0.105	0.108	0.079
70	33.1	108	AR	w22	0.106	0.104	0.108	0.063
23	4.56	86.5	AS	d1, d2, w1, w9	0.133	0.087	0.105	0.064
25	13.5	ND	AR	w22	ND	0.090	0.110	0.088

*Abbreviation: AD, Atopic dermatitis; AR, Allergic rhinitis; AS, Asthma; BA, Bronchial asthma; CR, Chronic rhinitis; CV, Conjunctivitis. **d1, *Dermatophagoides pteronyssinus*; d2, *Dermatophagoides farinae*; d72, *Tyrophagus putrescentiae*; e1, Cat dander; e2, Dog hair; e5, Dog dander; e71, Mouse epithelium; e83, Swine epithelium; f4, Wheat; f5, Rye; f6, Barley; f9, Rice; f11, Buckwheat; f12, Pea; f13, Peanut; f14, Soybean; f25, Tomato; f33, Orange; f47, Garlic; f48, Onion; f95, Peach; f208, Lemon; g1, Sweet vernal grass; g2, Bermuda grass; g3, cocksfoot; g5, Rye-grass; g6, Timothy grass; g7, Common reed; g8, Meadow grass; g12 Cultivated Rye; g15, Cultivated wheat; g201, Barley; i6, German cockroach; i206, American cockroach; k84, Sunflower seed; m1, *Penicillium chrysogenum*; m2, *Cladosporium herbarum*; m3, *Aspergillus fumigatus*; m5, *Candida albicans*; m6, *Alternaria*; t1, Acer; t2, Alder; t3, Birch; t4, Hazel; t5, Beech; t7, Oak; t8, Elm; t10, Walnut; t11, Platanus; t12, Willow; t15, White ash; t17, Japanese cedar; t70, Mulberry; t205, Elder; w1, Common ragweed; w4, False ragweed; w5, Wormwood; w6, Mugwort; w7, Chrysanthemum; w8, Dandelion; w9, Plantago; w10, Goosefoot; w11, Russian thistle; w12, Goldenrod; w16, Rough marshelder; w22, Japanese hop.

Table 2. Sequences of oligonucleotides used for the production of recombinant proteins.

Oligonucleotide Primers		Sequence
PR-1 (Pathogenesis-related 1)	Forward	ATGGCCAAACCCCATTTTCATAATG
	Reverse	TCAGTATGGTTTCTCACCAAA
PME (Pectin methyl esterase)	Forward	AGTGTAACGGCGGCTAAAGAC
	Reverse	TTACTGAACTTCGCTACGATTCTG
PGs (Polygalacturonase)	Forward	TAAGGCCTCTGTCTGAATGCTAGTGGTATTGCTCTTG
	Reverse	CAGAATTCGCAAGCTTCATGCTGTAAAACTGTTCTGTG

Table 3. Allergen homologues obtained from Japanese hop pollen EST analysis.

Biochemical Identity	Allergen	Species	Sequence Identity (%)	Clone Number	Clone
Pathogenesis-related protein 1	Art v 2	Mugwort	43.3	3	1011, 1025, 1756
	Cuc m 3	Muskmelon	42.5		
	Cyn d 24	Bermuda grass	49.3		
Polygalacturonase	Phl p 13	Timothy grass	19.3	3	0153, 0176, 0987
	Cry j 2	Japanese cedar	16.7		
	Cha o 2	Japanese cypress	17.3		
	Jun a 2	Mountain cedar	17.4		
	Pla a 2	London plane tree	19.1		
	Pla or 2	Oriental plane tree	19.5		
Pectin methyl esterase	Act d 7	Green kiwi	50.2	4	0025, 0411,1361, 1452
	Ole e 11	Common olive	24.2		
	Sal k 1	Russian thistle	23.2		

A total of 963 clones were analyzed for initial screening. *The full sequence clones were printed in bold.

analysis of expressed sequence tags (EST), and allergenicity of their recombinant proteins were examined by ELISA.

2. MATERIALS AND METHODS

2.1. Serum Samples

Sera were collected from patients at the Allergy-Asthma Center at Severance Hospital, Seoul, Korea. Diagnosis was based on case history and specific IgE by skin prick tests and/or by ImmunoCAP (ThermoFisher Scientific, Uppsala, Sweden). Blood samples were drawn for serum collection from patients. Thirty-one allergic patients' samples were used (Table 1). Consent from all patients was obtained before the collection of blood. This study was approved by the institutional review board of our institute (4-2017-1197).

2.2. Preparation of Japanese Hop Extract

Japanese hop pollen extract was obtained from Prolagen (Seoul, Korea). Briefly, pollen was collected from fields. Allergen was extracted in phosphate-buffered saline at pH 7.4 for 48 hours at 4°C after defatting with ethyl ether (1:5 w/v) 3 times. The extract was centrifuged at 13,000 g for 15 minutes at 4°C. The supernatant was then dialyzed extensively against distilled water (cutoff of 3.5 kDa, Spectrum). The dialyzed sample was filtered (0.22 µm, Millipore) to sterilize and eliminate insoluble matter. Protein concentration was determined by Bradford assay (Bio-Rad),

and the extract was then lyophilized and stored at -80°C until use.

2.3. Allergen Homologues

A cDNA library was constructed with 50 ng of total RNA isolated with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Double stranded RNA synthesized with In-Fusion SMARTer™ Directional cDNA library Construction kit (Clontech) was ligated into pCR4 TOPO vector (Invitrogen) after PCR amplification of double stranded cDNA. Insert sizes were checked by *EcoRI* digest, and DNA sequences of the clones with insert (>200 bp) were determined at Solgent (Daejeon, Korea), and NCBI BlastX search was performed. Thus, allergen homologues were identified by the initial screening of 963 EST clones.

2.4. Molecular Cloning

PCR was performed with specific primers designed on the basis of identified DNA sequences (Table 2). After 5 min of initial denaturation at 95°C, 35 cycles of thermal cycling (each of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) were performed and this was followed by 7 min at 72°C for a final extension. The PCR amplified DNA fragments were gel-purified and subcloned into pEXP5NT vectors for PR-1 and PME and pET6xHN-N vector for PGs. The subcloned DNA sequences were determined again at Solgent. As for profilin, profilin A was produced as described previously [8].

2.5. Expression and Purification of Recombinant Proteins

Recombinant proteins were overexpressed in the *E. coli* expression system. Cells were grown in one liter of LB broth. Cultured flasks were incubated at 37°C with shaking until absorbance at 600 nm reached 0.7. Expression of recombinant proteins were induced by adding 1 mM isopropyl-1-thio- β -galactopyranoside (IPTG). Cells were harvested 4 h after IPTG induction. Then recombinant proteins were purified under denaturing conditions using 6 M urea by using Ni-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA). Purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

2.6. IgE ELISA

Microtiter plates (Corning, USA) were coated with recombinant protein (2 μ g/mL in 50 mM sodium carbonate, pH 9.6) and incubated overnight at 4°C. After blocking with 3% skim milk in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST), serum samples (1:4 dilution in PBS containing 1% bovine serum albumin) were applied and incubated for one hour at room temperature. IgE antibodies were detected by incubating with biotinylated goat anti-human IgE (1:1000) (Vector, Burlingame, CA, USA) for one hour, followed by incubation with streptavidin peroxidase (1:1000) (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes. Color was developed using TMB Microwell Peroxidase Substrate (SeraCare Life Science, Inc., Milford, MA, USA). The absorbance at 450 nm was measured after the addition of 0.5 M H₂SO₄. All samples were duplicated. The mean absorbance plus double standard deviation of the negative controls was determined as a cutoff value.

3. RESULTS

3.1. Construction of cDNA Library and EST Analysis

cDNA library was successfully constructed with the minimum amount (50 ng) of RNA from pollen. We obtained allergen homologues from Japanese hop pollen with EST analysis (Table 3). PR-1 shares amino acid sequence identity of 43.3% with Alt v 2 (mugwort), 42.5% with Cuc m 3 (Muskmelon), and 49.3% with Cyn d 24 (Bermuda grass). PME shows 50.2% of identities to Act d 7 (Green kiwi), 54.2% to Ole e 11 (Common olive), and 23.2% to Sal k 1 (Russian thistle). PGs shows 19.3% of identities to Phl p 13 (Timothy grass), 16.7% to Cry j 2 (Japanese cedar), 17.3% to Cha o 2 (Japanese cypress), 17.4% to Jun a 2 (Mountain cedar), 19.1% to Pla a 2 (London plane tree), and 19.5% to Pla o 2 (Oriental plane tree). The cDNA sequences obtained in this study was deposited to the GenBank under accession No. MN971582 for PR-1, MN971584 for PME, and MN971583 for PGs, respectively (Figure 1).

3.2. Purification and IgE Reactivity of Recombinant Proteins

The purified PR-1 showed bands of about 20 kDa, PGs about 55 kDa, PME about 38 kDa, and profilin about 13 kDa on SDS-PAGE gel stained with Coomassie blue (Figure 2A-D).

The IgE reactivity of the recombinant proteins was measured by ELISA (Figure 2E). Twenty-nine serum samples from Japanese hop pollinosis patients were examined. Specific IgE to PR-1 was recognized by 3.49% (1/29) of the serum IgE, 13.8% (4/29) by PME, 3.7% (1/27) by PGs, and 13.8% (4/29) by profilin.

A)		→ Mature sequence	
J hop 1756	MAKP-HFIMLLLFILCTLQFSKTLSSSSTKDESSQFLEAHKRARAKKGLPPLAWDSKLA	59	
Art v 2	MGLGNFWLVLAISFMAILHLSHAHETYGEPTGDDYVHAHNCIRRVLMKPLCWD-EIG	59	
Cuc m 3	-----DFVDAHNAARAQVGVGVHWT--VD	23	
Cyn d 24	-----STQLPSDEPLNGLNDKAIQDILNEHNMFRKEHVPPLTWNTTLA	44	
	: : . * : * : * : *		
J hop 1756	KYAHDYAS-KRKADCQLKHSFEHGNFLGENIFWGS-GHDWTPDVTNNIWMEEKNYSYE	117	
Art v 2	KVAQAWAE-TRTPDCSLIHSRDC-----GEN--MAQGAINGSMA--VQLWLDERLDYDYN	109	
Cuc m 3	AYARQYAN-----DRNLVHSAT-----	40	
Cyn d 24	KFSQDYAESKLLKDKMVSHP-----YGENLMFGSGAISWKTT--VDTWSDEKKSYPHYG	98	
	: : : * : : *		
J hop 1756	TNSCVEGKMGHYTQIVWKDTKKVGCARVVCDDT-KDTFMTCNYPVGNFGEKPY	171	[171/171, 100.0%]
Art v 2	ENKCI-KMGHYTQIVWANSERVGCGRALCSNGWAYIIVCNYPGPNVVGQKPY	162	[60/162, 37.0%]
Cuc m 3	-----		[17/40, 42.5%]
Cyn d 24	SNTCDQGMKGHYTAVVWKDTTSVGCGRVLCDDKDDTMIMCSYWPNGNYENQKPY	153	[68/153, 44.4%]

Figure 1 contd....

B)

Hum j	0987	-----MLVVL L L L L L A L S N-----AVK I N G E E S G G R C N H N K L S L Q P R P H S V	40
Phl p	13	-----G K K . . K K E E K K E S G D A A S G A D G T Y	24
Cry j	2	M A M K L I A P M A F . A M Q . I I M . A A E D Q S A Q I M L D S V . E K Y L R S N R S L R K V E H . - R H D A I N I F	59
Cha o	2	M G M K F M A A V A F . A L Q . I V M . A A E D Q S A Q I M L D S D I E Q Y L R S N R S L K K L V H . - R H D A A T V F	59
Jun a	2	M S M K F M A A L A F . A L Q . I V M . A G E D Q S A Q I M L D S D T K Q Y H R S . R N L R K R V H H A R H D V A I V F	60
Pla a	2	-----R G V Q S S G-----S V F	10
Pla or	2	-----M R G V Q S S G-----G T F	11
Hum j	0987	S I S E F G A V G D G K T L N T I A F Q N A I F Y L K S F A D K G G A Q L Y V P P G R W L T G S F S L T S-----H L T L	97
Phl p	13	D . T K L . . K P D C . K E V E E .-----W A S A C G G T . K N T I V I . K . D F . . G P L N F . G P C K G D S V	82
Cry j	2	N V E K Y H D C . E . . S T .-----W Q A A C K N P S A M L . V P G S K K F V V N N L F F N G P C Q P-----H F	116
Cha o	2	N V E Q Y H D S . E . . A T T-----W N A A C K K A S A V L . V P A N K K F F V V N N L V F R G P C Q P-----H .	116
Jun a	2	N V E H Y H D S . D . E K T-----W N A A C N K L S A V F . V P A N K K F V V N N L V F Y G P C Q P-----H F	117
Pla a	2	N V N D Y . . K-----A G D I S Q . V M K .-----W K A A C . S Q . P S T V L I . K . N Y N M G E V A M Q G P C K G S K I	66
Pla or	2	N V N D Y . . K-----S G D I S Q . V M K .-----W E A A C . S P . P S T V L I . T . N Y I M G E V L . E G P C K G S K I	67
Hum j	0987	F L E K G A I I L G S Q N P S H W E V D P L P S Y G R G I E I P G S R Y R S L I N G Y N V K D V V I T G G N G T I D G	157
Phl p	13	T I K L D G N L . S . N D L A K Y-----K A N-----W I E I M .-----I . K L T . . .-----K . . L . .	122
Cry j	2	T F K V D G . . A A Y A S . K N N R I W L Q F A K-----L T G F T L M .-----K . V . .	158
Cha o	2	S F K V D G T . V A Q P D . A R . K N S K I W L Q F A Q-----L T . F N L M .-----T . V . .	158
Jun a	2	S F K V D G T . A A Y P D . A K . K N S K I W M H F A .-----L T . F N L M .-----T . V . .	159
Pla a	2	G F Q I D G V V K A P A D . . K F-----K S . G W V . F Y .-----I D G L T V S .-----T . . L . .	107
Pla or	2	G F Q L D G V V K A P A D V . A F-----K S E G W V V F N H-----D G L T V S .-----K . . F . .	108
Hum j	0987	Q G S V W W D W F N S H S L K-----Y S R P H L V E F V N S E S V V V S N L S F L N A P A Y S I H P V Y C S G V H	211
Phl p	13	. . K A V . G K N S C A K N-----Y N C K I L . N T L V L D F C D D A L I E G I T L . . . K F F H M N I Y E . K . T	178
Cry j	2	. . K Q . . A G Q C K W N G R E I C N D R D . . T A I K . D F . T G L I I Q G . K L M . S . E F H L V F G N . E . . K	218
Cha o	2	. . Q Q . . A G Q C K V V N G R T V C N D R N . . T A I K I D Y . K . . T . K E L T L M . S . E F H L V F G E . E . . K	218
Jun a	2	. . N R . . S D Q C K T I N G R T V C N D K G . . T A I K I D F . K . . T . K E L T L T . S . E F H L V F G E . D . . K	219
Pla a	2	. . Q T A . A K N . C D K N-----P N C K H A A M N L R . D F L K H A M . R D I T S . . S K M F H . N V L E . E D I T	163
Pla or	2	. . Q K A . A A N . C D K D-----E N C N R P . M N I R . N F L K N A . . R D I T S M . S K M F H . N V L E . D N I S	164
Hum j	0987	V H N I S V S T P L D S P Y T N G I V P D S S D D V C I E D C A I T S G Y D A I S L K S G W D E Y G I A Y G R P T K N V	271
Phl p	13	. . K D V T I T A . G N . D . . H I G D . S K . T . T . T T . G T . D . C . . I G P .-----S T G L	227
Cry j	2	I I G . . I T A . R N . D . . D I F A . K N F H L Q K N T . G T . D . C V A I G T .-----S S . I	267
Cha o	2	I Q G L K I K A . R N . D . . D I F A . K R F H . . K . V . G T . D . C . A I G T .-----S S . I	267
Jun a	2	I Q G . K I K A . R N . D . . D I F A . K R F E . . K . T . G T . D . C V A V G T .-----S S . I	268
Pla a	2	F Q H V T . T A . G T . I N . D . . H V G I . K G . T . T N T K . A T . D . C . . I G P .-----S Q . .	212
Pla or	2	F Q H V T I . A . G T . I N . D . . H I G L . R G . T . T . T N . A T . D . C V . I G P .-----S Q . .	213
Hum j	0987	H I R R V D L Q S S S G S S L A F G S E M S G-----G I S N V L V E Q V H L Y N S H G V E F R T T K G R G-----G Y I K E	327
Phl p	13	N . T G . T C G P G H . I . V G S L G R Y K D E K D V T D I T . K N C V . K K . T N . L R I K S Y E D A K S P L T A S K	287
Cry j	2	V . E D L I C G P G H . I . I G S L G R E N S R A E V . Y . H . N G A K F I D T Q N . L R I K . W Q . G S-----M A S H	325
Cha o	2	T . K D L I C G P G H . I . I G S L G R D N S R A E V . H . H . N R A K F I D T Q N . L R I K . W Q . G S-----L A S Y	325
Jun a	2	T . K D L T C G P G H . M . I G S L G K G N S R S E V . F . H L D G A K F I D T Q N . L R I K . W Q . G S-----L A S H	326
Pla a	2	T . T Q . N C G P G H . I . I G S L G R Y N N E K E V R G I T . K G C T F S G T M N . . R V K . W P N S P-----P . A A T D	271
Pla or	2	T V T K . N C G P G H . I . V G S L G K Y K D E K D V R G I T . T G C T F T G T S N . . R V K . W P D S P-----P . V A T D	272
Hum j	0987	I T A S D V V I K N V F V A F S A S G Q C G S H P D D K Y D P N-----A L P V L D H I S L Q N V V G K K I S V A G I F N G I	386
Phl p	13	L . Y E N . K M E D . G Y P I I I D Q K Y C P N K I C T S K G D S . R V T V K D V T F R . I T . T S S T P E A V S L L C	347
Cry j	2	. . Y E N . E M I . S E N P I L I N Q F Y C T S A S A C Q N Q R-----S A V Q I Q D V T Y K . I R . T S A T A . A . Q L K C	384
Cha o	2	. . Y E N . E M I . S E N P I L I N Q F Y C T S A S A C Q N Q R-----S A V Q I Q G V T Y K . I H . T S A T A . A . Q L M C	384
Jun a	2	. . Y E N . E M I . A E N P I L I N Q F Y C T S A A A C K N Q R-----S A V K I Q D V T F K . I H . T S A T . A . Q L M C	385
Pla a	2	L . F Q . L T M N . . Q N P V I L D Q E Y C P Y G Q C S R Q A P-----S R I K . S N . N F N . I R . T S T G K V A V V I A C	330
Pla or	2	M A F E . L T M K . . Q N P V I L D Q E Y C P Y G Q C S L K A P-----S R V K . S N . K F N . I R . T S S G P D A . V I A C	331
Hum j	0987	Q E S-----P F S S I C L Y N I S L S I N P G A P T T W E C S N V S G S S D S V F P E P C P E L Q S T N S N S P S A C F S L	445
Phl p	13	S D K Q . C N G V T M N D V K I E Y S G T N-----N K T M A V . T N A K V . A K G-----V S E A N T C	392
Cry j	2	S D . M . C K D . K . S D I . . K L T S . K I A S C L N D . A N . Y F S G H V I P A . K N . S P S A K R K E S K S H K H	444
Cha o	2	S D . V . C T G . Q . S . V . . K L T S . K . A S C V D K . A R . F Y S G R L I P T . K N . R P G P . P K E F E L Q Q Q	444
Jun a	2	S D . V . C . N . K . S . V F . K L T S . K V A . C V N K . A N . Y Y T N P L N P S . K S . H P G R T P K E L H Q K	445
Pla a	2	S H G M . C . N M K I G E . N . . Y R G A G-----G P A T S T . S N V K P . F . G K Q V P A I K C	376
Pla or	2	S H G F . C . N L E I G E . N . A L H A A G-----A P A N S T . T N A K P I F . G K Q V P A I K C	377
Hum j	0987	S T L D G K A S A L-----	455
Phl p	13	A A-----	394
Cry j	2	P K T V M V E N M R A Y D K G N R T R I L L G S R P P N C T N K C H G C S P C K A K L V I V H R I M P Q E Y Y P Q R W I	504
Cha o	2	P . T V M D E N K G A C A K G D S T C I S L S S P P N C K N K C K G C Q P C K P K L I I V H P N K P Q D Y Y P Q K W V	504
Jun a	2	P . T L L M D E K-----M G A S L N S S P P N C K N K C K G C Q P C K P K L I I V H P N Q P E D Y Y P Q R W V	497
Pla a	2	A-----	377
Pla or	2	A-----	378
Hum j	0987	-----	[455/455, 100%]
Phl p	13	-----	[76/394, 19.3%]
Cry j	2	C S C H G K I Y H P 514	[87/514, 16.9%]
Cha o	2	C S C H N K I Y N P 514	[89/514, 17.3%]
Jun a	2	C S C H N K I Y N P 507	[88/507, 17.4%]
Pla a	2	-----	[72/377, 19.1%]
Pla or	2	-----	[70/378, 18.5%]

Figure 1 contd....

C)	
J hop 0025	-----
Act d 7	-----TAV 3
Ole e 11	MSCIAVEAVLLGILLYIPIVLSDDRIPISNSAQLNSWFDGI IQPVAVRKATMDPALVTA 60
Sal k 1.02	-MEEHVSMLLVGFVL-INIAFTSIAQLIPPNPAAELESWFQGAVKPVSEQKGLEPSVVTQTE 58
:	
J hop 0025	-----SVTAAKDGTGDFRTINDALAAIPTKYEGRYVIYVKEGLYEETVTVTKKMANVTMY 55
Act d 7	TDIVPD.VV...S.N.T.VGA.V..AKDSSTA.F...I...A.F.Y.D.D..KT.LMFI 63
Ole e 11	EGQTKVIKLS..S...KS..E.IKS..DDNTK.VILSLAP.N.S.K.KIGMYKHYI.F. 120
Sal k 1.02	SGGVETIEVRQ..S.K.K..S..VKHKVGNTK.VI.TIGP.E.R.K.KIERLHPYI.L. 118
:	
J hop 0025	GDGSQKSIITGSKNFVDGVRTFQTATFVVLGGGFMGKAMGFGNTAGPEK-----HQAVAA 110
Act d 7	...IG.TW.K.NRSV...WT..RSS.VA.V.T..IARGIS.E.Y...S.-----...L 118
Ole e 11	.EDPNMP.LVFGGT.AEYG.VDS..LI.ESNY.SAVNLKIV.S.PRPDGKRVGA..A.L 180
Sal k 1.02	.IDPKNRPTITFAGT.AEFG.VDS..VI.ESDYSV.AHLIVT.S.PRPDGKRGGA..G.L 178
:	
J hop 0025	RVQADRAIFANCRFEGYQDTLYTQTHRQFYRSCVVAGTIDFIFGDAAVVFQNCMLVVRLP 170
Act d 7	.SG..FSA.YQ.S.V.....VHSL....SE.D.Y.....N..A.L.K.N.YA.K. 178
Ole e 11	.ISG.K.S.Y.VKIY.F....CDDKGKH..KD.YIE..V....SGKSI.L.TE.HAVP- 239
Sal k 1.02	.ISG...A.Y..K.T.F...VCDKGNH.FTD.YTE..V....E.RSLYL.TE.H.VP- 237
:	
J hop 0025	MANQKNIVTARGRVDKQQTGTGIVIHNCRIMADKKLEPEKARVKSFLGRPWKEYSRTIIME 230
Act d 7	NE....F..Q..D.PN.N...S.L..KVA.AAD.I.VLSSF.TY.....VFL 238
Ole e 11	-GD.PA.I..QA.KTDSER..YYFV...VTGGG-----A...S.MPAAKVVFAY 288
Sal k 1.02	-GDPMAMI..HA.KNADGVG.YSFVH.KVTGTGGT-----AL...A.FDAA.VVFSY 288
:	
J hop 0025	STIEDLIHPDGWLPWEGEFALKTLYYAEYNNKGPAGKVTTRVNWPYGSVIN-KKEAEKFT 289
Act d 7	.QM.S..D.A...E.S.D...T...R..K.T...SNT.A..T...A.TTNET.VIQ.. 298
Ole e 11	TEMV.A...E..I-LVKPEHES.VRFS.....NMEK.A--KFVKRLSDA..KQSI 344
Sal k 1.02	CNLS.AAK.E..SDNNKPEAQK.IILFG..K.T...APDK.A--P.TKQLTEAD.KT.. 345
:	
J hop 0025	VENFLQGNDWLNIGIPVRYTLILRANRSEVQ 321 [321/321, 100.0%]
Act d 7	.G..I..SQ..TSYN...YLNLT----- 321 [163/321, 50.8%]
Ole e 11	SLGSIEASK..LPPRVVGLP----- 364 [89/364, 24.5%]
Sal k 1.02	SLEYIEAAK..LPPPKV----- 362 [82/362, 22.7%]
:	
:	

Figure 1. Amino acid sequence alignments of allergens homologues from Japanese hop pollen with previously identified pollen allergens. **A**, pathogenesis-related-1; **B**, polygalacturonase; **C**, pectin methyl esterase; *, identical; :, highly conserved; ., less conserved. The percentage of sequence identity is given in square brackets at the end of each sequence. Art v 2 (*Artemisia vulgaris*, Mugwort, AM279693), Cuc m 3 (*Cucumis melo*, Muskmelon, P83834), Cyn d 24 (*Cynodon dactylon*, Bermuda grass, AY720896), Phl p 13 (*Phleum pratense*, Timothy, AJ238848), Cry j 2 (*Cryptomeria japonica*, Japanese cedar, D29772), Cha o 2 (*Chamaecyparis obtusa*, Japanese cypress, Q7M1E7), Jun a 2 (*Juniperus ashei*, Mountain cedar, AJ404653), Pla a 2 (*Platanus acerifolia*, London plane tree, AJ586898), Pla o 2 (*Platanus orientalis*, Oriental plane, EU296477), Act d 7 (*Actinidia deliciosa*, Green kiwi fruit, P85076), Ole e 11 (*Olea europaea*, common olive, ACZ57582), Sal k 1 (*Salsola kali*, Russian thistle/Saltwort, AAT99258).

4. DISCUSSION

Identification and characterization of the major allergens are necessary for the better development of allergy diagnostic and immunotherapeutic reagents [26-29]. Recently, component-resolved diagnosis, which utilized purified native or recombinant allergens, improves the allergy diagnosis. However, molecular characteristics of Japanese hop pollen allergens have not been elucidated yet [30]. In this study, we searched allergen homologous molecules from Japanese hop pollen by EST analysis.

Firstly, it is thought to be meaningful to identify the allergen-like molecules from only 50 ng of total RNA from pollen grain (about 10 mg) since it is not easy to collect enough amount of pollen grains of interest from field. Recently, progress in NGS and omics technologies have enabled analysis and interpretation of enormous data [31]. Therefore, application of these technologies will facilitate the allergens of regional importance [32].

Secondly, three novel allergenic molecules (PR-1, PME, and PGs) were identified from Japanese hop pollen. However, all of them showed a low degree of homology with the previously identified pollen allergens (Figure 1) and showed marginal IgE reactivities (Figure 2). All three

molecules identified in this study displayed a low degree of IgE reactivities. These results are in line with the previous study which showed a low degree of cross-reactivity between Japanese hop and common hop [33]. Misfolding of recombinant protein may partly explain the low IgE reactivity. However, it is necessary to further investigate the structure analysis of these molecules, since conformational epitopes appear to be of primary importance for inhalant allergens [34]. Possible contribution of glycan moiety to IgE reactivity also needs to be investigated. Furthermore, polymorphism analysis and cross-reactivity with homologous allergens are to be done for the better understanding of these novel allergenic molecules.

Sensitization prevalence to native Art v 1, PR-1 allergen from mugwort, was reported 63.2%. However, recombinant Art v 1, expressed in prokaryotic system, displayed low IgE reactivity probably due to improper folding or lack of glycan moiety [35]. PME was described as a major allergen of Russian thistle, Sal k 1 [36, 37]. Recombinant Sal k 1, expressed in prokaryotic system, was shown to have IgE reactivity equivalent to native Sal k 1. However, there are multiple isoforms with different IgE reactivity [38]. PGs is also known to be glycosylated potent allergen from Japanese cedar, Cry j 2 [39]. Therefore, these aspects (improper fo-

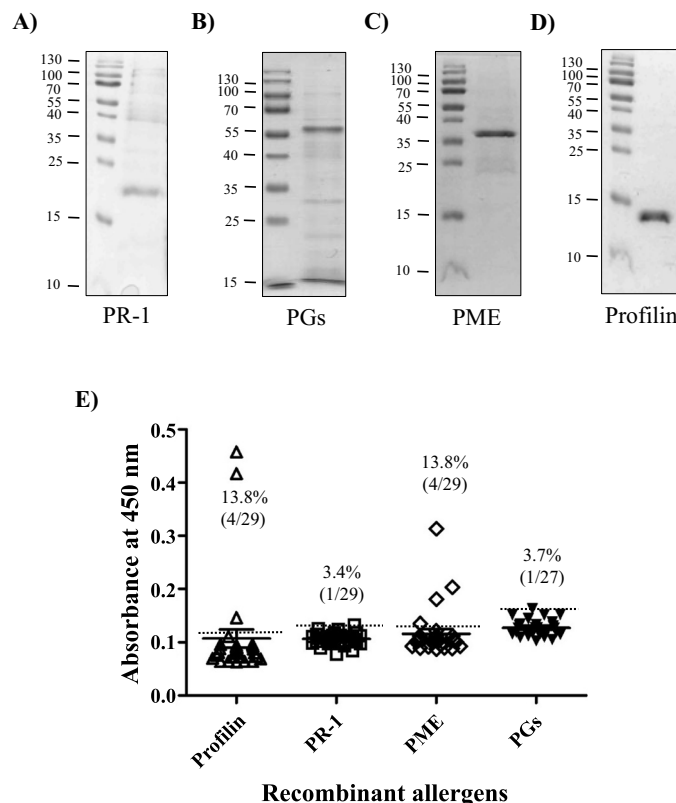


Figure 2. Purification and IgE reactivity of recombinant allergens from Japanese hop pollen. Proteins (20 µg) were separated to SDS-PAGE gel. (A), PR-1; (B), PGs; (C), PME; (D), Profilin. (E), IgE reactivity of recombinant allergens from Japanese hop pollen. The dotted lines indicate the cutoff value.

lding, lack of glycan moiety, and multiple isoforms with different IgE reactivity) of recombinant proteins should be in mind for the interpretation of data.

Finally, conventional biochemical approach seems to be necessary for the better characterization this elusive major allergen, a 10 kDa allergen, in case of Japanese hop.

CONCLUSION

Taken together, three allergens homologues were identified by means of a technology using 50 ng of total RNA. These molecules showed a limited degree of homology and IgE reactivities. Still, these molecules are believed to have worth further studies for minor allergens have shown to some values for the CRD in case of house dust mite allergy [40].

LIST OF ABBREVIATIONS

CRD	= Component-Resolved Diagnosis
ELISA	= Enzyme-Linked Immunosorbent Assay
EST	= Expressed Sequence Tags
IgE	= Immunoglobulin E
IPTG	= Isopropyl-1-thio- β -galactopyranoside
IUIS	= International Union of Immunological Society
NGS	= Next Generation Sequencing
PBS	= Phosphate Buffered Saline
PBST	= PBS containing 0.05% Tween 20
PCR	= Polymerase Chain Reaction
PGs	= Polygalacturonase
PME	= Pectin Methyl Esterase
PR-1	= Pathogenesis-Related 1
SDS-PAGE	= Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the institutional review board of our Institute of Allergy, Yonsei University College of Medicine, South Korea (Approval no. 4-2017-1197) for the collection and use of patients' sera.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. The reported experiments were performed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013 (<http://ethics.iit.edu/ecodes/node/3931>).

CONSENT FOR PUBLICATION

Consent from all patients was obtained before the collection of blood.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (Grant no. 2017R1D1A04028193). This study was also supported by a grant from the Korea Healthcare Technology R&D Project, Ministry of Health & Family Affairs, Republic of Korea (Grant no. HR14C0006).

CONFLICT OF INTEREST

KY Jeong, KH Park and JW Park have stocks in Prolagen. JW Park reports serving as an unpaid chief technology officer for Prolagen. KY Jeong receives a consulting fee from Prolagen. Other authors have no potential conflicts of interest to disclose.

ACKNOWLEDGEMENTS

The authors thank Medical Illustration & Design, part of the Medical Research Support Services of Yonsei University College of Medicine, for all artistic support related to this work.

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